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Abstracts

FOCIS Abstract Supplement

**Regulatory T-Cells in Transplant & Autoimmunity**

10:30 AM–12:30 PM, 5/13/2005

**OR-01. Distinct Regulatory Functions Are Defined by HLA-DR Expression on Human CD4<sup>+</sup>CD25<sup>high</sup> Treg Cells.**

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Although the CD4<sup>+</sup>CD25<sup>high</sup> regulatory T cell population represents only 2–3% of all peripheral blood CD4 T cells, it contains over one third of all class II expressing T cells in the peripheral blood. Highly purified, FACS-sorted DR<sup>+</sup> (~30% DR<sup>+</sup>CD25<sup>high</sup>) and DR<sup>-</sup> (~70% DR<sup>-</sup>CD25<sup>high</sup>) CD4<sup>+</sup>CD62L<sup>high</sup>CD25<sup>high</sup> Treg cells demonstrate equivalent suppressive activity in an anti-CD3 driven, *in vitro* 'micro' co-culture system. Both types of CD25<sup>high</sup> Treg cells exhibit cell contact-dependent suppression, anergy, and expression of Foxp3 mRNA, at only slightly different levels.

Substantial differences in the inhibitory nature of the DR<sup>+</sup>CD25<sup>high</sup> and DR<sup>-</sup>CD25<sup>high</sup> populations are uncovered when the cells are provided with different strength costimulatory signals. Upon CD2 costimulation, DR<sup>+</sup>CD25<sup>high</sup> co-cultures exhibit a strong, early suppression of both proliferation and Th1/Th2 cytokine production, while DR<sup>-</sup>CD25<sup>high</sup> co-cultures exhibit a much delayed suppression (late) that is accompanied by a preferential inhibition of Th1 cytokines (IFN $\gamma$ ), and often an induction of IL-10 and IL-4. Importantly, IL-10 has contrasting effects on regulation by these two different types of Treg cells, underscoring another major difference between these two types of CD25<sup>high</sup> Treg cells. Unlike the usual effect of IL-10 in reducing the immune response, IL-10 actually inhibits the suppression by DR<sup>+</sup>CD25<sup>high</sup> and thus enhances co-culture responses. In contrast, IL-10 appears to be a component of the suppressive mechanism of the DR<sup>-</sup>CD25<sup>high</sup> cells. Possibly due to this differential involvement of IL-10, the DR<sup>+</sup>CD25<sup>high</sup> and DR<sup>-</sup>CD25<sup>high</sup> populations cross regulate each other *in vitro*, and may do so *in vivo* as well. Importantly, these differences in the kinetics of suppression, Th1/Th2 skewing, and involvement of IL-10 between the DR<sup>+</sup>CD25<sup>high</sup> and DR<sup>-</sup>CD25<sup>high</sup> populations are only seen when these two populations are studied as distinct populations. Thus it is apparent that the study of heterogeneous combined Treg populations would obscure possibly contrasting responses. It is possible that these different functional features may reflect a

temporal order to the utilization of different regulatory subsets *in vivo* as the immune response switches from innate to adaptive immunity.

**Dendritic Cells**

10:30 AM–12:30 PM, 5/13/2005

**OR-02. CNS Dendritic Cells Drive Naive T Cell Proliferation and Epitope Spreading in Relapsing Experimental Autoimmune Encephalomyelitis.**

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Chronic progression of relapsing experimental autoimmune encephalomyelitis (R-EAE) in the SJL mouse is dependent on the activation of T cells to endogenous myelin epitopes, i.e. epitope spreading which plays a major pathologic role in disease progression. Using transfer of naive CFSE-labeled TCR transgenic T cells and mixed bone marrow chimeras, we show that activation of naive PLP139-151-specific T cells in SJL mice undergoing PLP178-191-induced R-EAE occurs directly in the CNS and not in the cervical lymph nodes, spleen or other peripheral lymphoid organs. Flow cytometric and histologic examination of the CNS during R-EAE revealed the infiltration of significant numbers of CD11c<sup>+</sup> dendritic cells (DCs) (including myeloid, lymphoid and plasmacytoid subsets) which are not seen in the healthy CNS. Functional examination of the antigen presentation capacity of APC populations purified from the CNS of mice with established PLP178-191-induced R-EAE shows that only F4/80-CD11c<sup>+</sup>CD45<sup>hi</sup> DCs efficiently present endogenous antigen resulting in the activation of naive PLP139-151-specific Tg T cells. In contrast, DCs as well as F4/80<sup>+</sup>CD45<sup>hi</sup> macrophages and F4/80<sup>+</sup>CD45<sup>lo</sup> microglia have the capacity to activate memory PLP139-151-specific Th1 cells. The current results indicate that naive T cells can gain access to the inflamed CNS, bypassing the need for activation in peripheral lymphoid sites, and that epitope spreading initiates principally within the CNS target organ. Further, activation of naive T cells is involved in chronic R-EAE is mediated by CNS DCs, not infiltrating macrophages or resident microglia. Consequently, blocking the recruitment or differentiation of DCs may be a viable target for inhibiting relapse and disease progression in murine MS models and possibly MS patients themselves.

**Antibodies in Disease  
Pathogenesis and Therapy**  
10:30 AM–12:30 PM, 5/13/2005

**OR-03. A New Spontaneous Mouse Model for Human Devic's Disease.**

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Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) characterized by localized areas of inflammation and demyelination. MS can present itself as various clinical forms: a relapsing-remitting course, progressive course or unusual progression like in Devic's disease in which lesions are found only in the optic nerve and in the spinal cord but not the brain. In addition, the first clinical presentation in a significant proportion of MS patient is often isolated optic neuritis (ON). The underlying immunological basis for different forms of MS and its association with other diseases like optic neuritis is not well defined.

Experimental autoimmune encephalomyelitis (EAE) is a T cell mediated disease that shares many clinical and histological features with MS. However, to date, the development of ON has always been associated with EAE and there is no spontaneous animal model of Devic's disease. Although myelin-specific Th1 cells are able to induce EAE in unimmunized mice, studies in both MS and EAE suggest that B cells and antibodies may play a role in demyelination. We have developed a TCR transgenic mouse, 2D2, specific for a minor protein of the CNS myelin called myelin oligodendrocyte glycoprotein (MOG) and have previously reported that a large proportion (47%) of these TCR transgenic mice developed spontaneous isolated ON. We have now crossed our TCR transgenic 2D2 mice to an IgH knock-in mutant mouse (Th) in which all the B cells are specific for MOG and produce MOG-specific antibodies. The knock-in mice do not spontaneously develop EAE. However, over 60 % of 2D2xTh mice, which express both MOG-specific T and B cells, developed a very early and severe form of EAE. Histological examination of the central nervous system of these animals reveal a selective distribution of the inflammatory foci and lesions restricted to spinal cord and optic nerve, a lesion pattern that is typical of Devic's subform of MS. The importance of MOG specific T and B cells cooperation, and the role of antibodies in the development of Devic's disease will be discussed.

**Antibodies / B Cells**  
3:30 PM–5:30 PM, 5/13/2005

**OR-04. Elucidating the Mechanism of Anti-MOG Antibody-Mediated Demyelination.**

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Antibody-induced demyelination is an important component of the pathology in multiple sclerosis (MS). In particular, antibodies to myelin oligodendrocyte glycoprotein (MOG) are elevated in MS patients and have been implicated as mediators of demyelination. The aim of our studies is to elucidate the mechanism of anti-MOG mediated demyelination. We show that antibody cross-linking of MOG in oligodendrocytes results in the repartitioning of MOG into lipid rafts, leading to changes in the phosphorylation of specific proteins, and culminating in rapid morphological alterations. Using proteomic analyses, we have identified 10 target proteins whose phosphorylation state is altered upon anti-MOG treatment. These proteins fell into functional categories related to the regulation of signal transduction, leading to cellular stress response and cytoskeletal instability. These changes were specific for anti-MOG; although cross-linking myelin associated glycoprotein (MAG) also instigates signaling modifications, these are distinct from those observed with MOG and did not result in oligodendrocyte morphological alterations. We next applied our findings to EAE models, analyzing antibodies to MOG that develop after immunization of C57BL/6 mice with MOG from rat or human origin. Interestingly, although these regimens result in EAE with similar anti-MOG antibody titers as evaluated by ELISA, only human MOG requires B cells for disease induction. Further, IgG to human, but not rat MOG, bound unfixed rodent oligodendrocytes and induced repartitioning of MOG into lipid rafts and morphological alterations. These data suggest a novel mechanism for antibody pathogenicity in B cell mediated EAE, and provide in vitro tools to determine whether an autoimmune antibody is pathogenic, and may be useful for evaluating the pathogenicity of antibodies in MS patients as an adjunct to diagnosis and treatment. Supported by FG1423A (CM) and RG 2394 (NR) from the National MS Society, and NS10861 and NS41078 from NIH (SP).

**OR-05. Synovial Intracellular Citrullinated Proteins Colocalizing with Peptidyl Arginine Deiminase Are Pathophysiologically Relevant Antigenic Determinants of Rheumatoid Arthritis-Specific Humoral Autoimmunity.**

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*Objectives:* Based on previous demonstrations of citrullinated proteins in the synovial membrane, the present study addresses the ongoing debate of the specificity of synovial citrullinated proteins for rheumatoid arthritis (RA) and analyses in vivo their involvement in the induction or perpetuation of the highly RA-specific anti-citrullinated protein antibodies (ACPA).

*Materials and methods:* Synovial tissue samples of 19 RA and 19 non-RA controls were analyzed for the presence of citrullinated proteins by immunohistochemistry with two different anti-citrulline antibodies. Double immunofluorescence experiments were performed with antibodies against fibrinogen, vimentin, and the citrullinating enzyme peptidyl arginine deiminase type 2. Extending the RA cohort to 61 patients, ACPA levels were measured by

ELISA in serum and synovial fluid and related to the anti-citrulline stainings in synovium and the presence of HLA-DR shared epitope.

**Results:** Using different anti-citrulline antibodies, we confirm the RA-specific presence of synovial intracellular citrullinated proteins which are different from previously identified, non RA-specific deiminated proteins such as fibrin and vimentin. The RA-specificity is related to the distinct presence of the citrullinating peptidyl arginine deiminase type 2 enzyme in RA synovium. Additionally, the synovial intracellular citrullinated proteins detected in RA synovium determine directly the systemic ACPA levels as well as the local ACPA production in the joint. The relation between RA-specific intracellular citrullinated proteins and ACPA is dependent on the presence and load of the HLA-DR shared epitope.

**Conclusion:** These data identify the RA-specific synovial intracellular citrullinated proteins as primary antigenic targets of ACPA in vivo and provide a pathophysiological rationale for the specificity of this autoimmune process in human RA.

#### **OR-06. Antibodies to Citrulline-Modified Proteins Enhance Tissue Injury in Inflammatory Arthritis.**

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Antibodies to citrulline-modified proteins have been shown to be specific and predictive markers of Rheumatoid Arthritis (RA) although the pathologic relevance of these antibodies has been unclear. Similar to that which has been observed in RA, in the murine collagen-induced arthritis (CIA) model of RA we have identified serum antibody reactivity specific for citrulline-modified proteins that precedes clinically apparent disease. To understand the role of antibodies to citrulline-modified proteins in disease, we examined whether tolerance to these antigens could protect mice from CIA. Mice were tolerized by intravenous administration of 0.3 mg of a citrulline-modified peptide, a non-citrullinated control peptide, bovine type II collagen (CII), or ovalbumin for 3 days. Three and 24 days after the final dose of tolerogen, mice were challenged with CII in complete Freund's adjuvant. Mice tolerized with the citrulline-modified peptide demonstrated reduced disease severity compared to the control peptide and ovalbumin ( $2.4 \pm 0.8$ ,  $6.2 \pm 1.7$ ,  $6.4 \pm 1.3$ , respectively,  $P < 0.05$ ). These data suggest that immune responses to citrulline-modified proteins are important in the development of inflammatory arthritis. As a second demonstration of the pathogenic potential of antibodies to citrulline-modified proteins, we determined whether antibodies to these autoantigens could enhance disease. To accomplish this, we first identified an IgM monoclonal antibody, designated D513, specific for citrulline-modified fibrinogen. We then transferred D513 into mice alone and in combination with a submaximal dose of arthritis-inducing anti-CII monoclonal antibodies. In combination with anti-CII antibodies, D513 substantially enhanced disease severity ( $9.4 \pm 0.8$  with D513 vs.  $3.7 \pm 1.1$  without,  $P < 0.001$ ). Also, we created two additional monoclonal antibodies of the IgG class specific for citrullinated fibrinogen which also substantially enhanced disease severity in combination with anti-CII antibodies ( $8.3 \pm 1.2$  and  $8.3 \pm 0.7$ ,  $P < 0.05$  compared to anti-CII antibodies alone). These results demonstrate that antibodies specific to citrulline-modified proteins are pathogenic and likely play an important role in joint destruction in human RA.

#### **OR-07. T to B Epitope Spreading Is Responsible for the Diversification of Autoantibody Responses within the Small Nuclear Ribonucleoprotein Complex.**

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Autoantibodies reactive against different polypeptides within the small nuclear ribonucleoprotein complex (snRNP) are often present in patients with systemic lupus erythematosus. Although the mechanisms for the initiation of anti-snRNP autoantibody responses are not known, it is well accepted that the complexity in this response is achieved through intra and intermolecular epitope spreading. We have established a model for intermolecular epitope spreading within the small nuclear ribonucleoprotein (snRNP) complex using the recombinant SmD protein. We have previously demonstrated that the initiating antigen and the interaction between the MHC and non MHC genes influences intermolecular epitope spreading. To elucidate the mechanisms for intermolecular epitope spreading within the snRNP complex, we immunized mice with synthetic peptides containing T cell epitopes on SmD. All peptides induced T cells reactive with the immunogens. In the A/J strain of mice peptides SmD<sub>31-45</sub> and SmD<sub>52-66</sub> consistently induced intermolecular epitope spreading to A-RNP. Consistent epitope spreading was not observed in mice immunized with peptide SmD<sub>91-110</sub>. Although, all groups of mice had high titer of antibodies reactive with the peptide immunogens, antibodies capable of immunoprecipitating SmD antigen were minimally present. These data are in contrast to those obtained with whole protein immunization, wherein immunoprecipitating anti-SmD antibodies were readily generated. These data suggest that in SmD peptide immunized mice, a T to B cell epitope spreading was responsible for the generation of anti-A-RNP antibodies. Thus, if molecular mimicry was responsible for the initiation of anti-snRNP autoantibodies, our data indicate that a T cell mimic is sufficient to generate a diversified antibody response within the snRNP complex.

#### **OR-08. In Sjogren's Syndrome Serum Autoantibodies Recognize Tear Lipocalin and alpha-fodrin.**

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Sjogren's syndrome (SS) is a chronic autoimmune disease characterized by lymphocytic infiltration and tissue damage mainly confined to the salivary and lacrimal glands, which results in dryness of the mouth (xerostomia) and eyes (keratoconjunctivitis sicca). Patients with SS often show a wide spectrum of autoimmune related disorders, including pancreatitis, sclerosing cholangitis, interstitial nephritis, and interstitial pneumonitis. In these target organs, different epithelial cells in exocrine and non exocrine tissues are primarily affected. These clinical observations have led to the assumption that autoimmune reactions against antigens commonly expressed in epithelial cells may play a pathogenic role in this disease. Characterization of novel autoantigens associated with the systemic involvement in SS would provide useful information to better understand its pathogenesis and to develop new diagnostic and therapeutic strategies.

We used pooled IgG immunoglobulins derived from 12 patients with primary Sjogren's syndrome to screen a random peptide library to identify disease relevant autoantigen peptides. Among the identified peptides, one was recognized by 27/38 (72%) patients' sera in an Elisa assay employing the solid phase peptide. This peptide was not recognized by sera of normal donors and of patients affected by other autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis and systemic sclerosis. Therefore the reactivity against this peptide appears to be confined within primary Sjogren's syndrome patients.

The identified peptide showed homology with the EBV encoded early antigen protein D. EBV infection has been associated with Sjogren's syndrome. Our findings suggest that EBV infection may be involved in the disease pathogenesis as putative inciting stimulus. The same peptide shares similarity with the tear lipocalin, a protein highly expressed in tears and saliva, and with alpha-fodrin, a cytoskeleton protein considered an important autoantigen target in Sjogren's syndrome.

Anti-peptide antibodies affinity purified from patients' sera recognize tear lipocalin in western blot. Moreover the same antibodies are able to bind alpha-fodrin, which is known to be cleaved in several fragments and to be exposed on the cell surface during apoptosis. Our findings suggest that tear lipocalin may be considered a novel and yet unidentified autoantigen in Sjogren's syndrome. We are now investigating the functional role played by this autoantibody population in the pathogenesis of the disease.

#### **OR-09. Microarray Profiling of Anti-Lipid Antibodies in MS.**

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Recent studies suggest that increased T cell and autoantibody reactivity to lipids may be present in multiple sclerosis (MS) patients as compared to controls. We have created a 100-feature lipid ordered array containing duplicate spots of 50 brain, myelin, and microbial lipids and glycolipids that represent potential targets of the autoimmune response in MS. Using our lipid arrays, we tested cerebrospinal fluid from MS patients and other neurological disease (OND) controls for the presence of anti-lipid antibodies. Lipid array reactivity was quantified, and Significance Analysis of Microarrays (SAM) applied to identify lipids with statistically-significant differences in array reactivity between MS and control samples. Lipids with significant differences in array reactivity were ordered using a hierarchical cluster algorithm and displayed as heat maps using TreeView. Lipid arrays demonstrated increased autoantibody reactivity to lipids including sulfatides, 3 $\beta$ -hydroxy-5 $\alpha$ -cholestan-15-one (an oxidized form of cholesterol), two separate forms of oxidized phosphatidyl-choline, lysophosphatidyl-ethanolamine, and sphingomyelin in MS patient CSF. Based on the array-determined anti-lipid antibody profiles, the patients' samples clustered into groups of MS and OND controls. Our results suggest that lipids are autoantigen targets in MS and that anti-lipid antibodies could have diagnostic and predictive value in MS. The arrays demonstrated increased antibodies against brain polar lipid extract, oxidized lipids, and sulfatides in mice with acute EAE.

We are currently exploring the role of these lipids on demyelinating disease *in vivo* in mice.

#### **OR-10. High-Throughput Analysis of Autoantibodies Recognizing Myelin Antigens in Acute Disseminated Encephalomyelitis.**

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Acute disseminated encephalitis (ADEM) and multiple sclerosis (MS) are inflammatory demyelinating disorders of the CNS that share clinical and pathological characteristics. The role of autoantibodies as related to disease pathophysiology and diagnosis is unknown. To further characterize myelin autoantibodies in these diseases, we studied the humoral response to a panel of candidate autoantigens using protein arrays then confirmed these data using novel solid and solution phase antibody binding assays. Serum and CSF samples from patients with ADEM revealed robust binding to a wide range of autoantigens, but only a minor subset of samples from MS, encephalitis and normal controls demonstrated binding distinguishable from background. To confirm these data, two myelin antigens, myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), were further evaluated using traditional solid and novel fluid phase assays. Solid-phase binding to these antigens was observed in a minor subset of patients with MS and encephalitis, but solution-phase binding, a characteristic of higher affinity antibodies, was absent. Matched serum and CSF from patients with ADEM contained autoantibodies directed toward MBP and MOG. In contrast to their counterparts found in MS, these autoantibodies demonstrated robust solution phase binding. These data highlight a fundamental difference in the humoral response of these clinically similar diseases and reflects underlying differences in the pathogenesis of ADEM and MS.

#### **OR-11. Role of CD38 in Human B Cells: Evidence of a Functional and Physical Association with CD19.**

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Human CD38 is a 45 kD surface glycoprotein endowed with ectoenzymatic and receptorial activities. The study of CD38 functions in T and NK cells indicated that the molecule bypasses its intrinsic structural inability to transduce signals through physical and functional associations with the TCR and CD16.

Here we show that membrane localization is critical for CD38 signaling in human B cells. Membrane fractioning indicates that a relevant fraction of CD38 molecules (60–85%) is constitutively present in rafts in normal (tonsil) and neoplastic (Nalm-6, Raji, Daudi, Namalwa, Ramos, RPMI-8226 and U266) cells. Upon cross-linking all CD38 molecules translocate into the rafts, together with a fraction of CD19 but not of CD79a and b, suggesting that CD38 becomes physically associated with CD19.

Lateral associations between the two receptors were confirmed in live cells using co-capping and biochemically with co-immunoprecipitation experiments.

CD38-CD19 association also has a functional nature. Indeed, CD38 ligation transduces signals only in cells where CD19 is present and active. This is witnessed by the lack of CD38-mediated Ca<sup>2+</sup> fluxes in RPMI-8226 and U266, both CD19<sup>-</sup>. A formal proof of a functional interplay between CD38 and CD19 was obtained by the loss of CD38-mediated signals upon CD19 gene silencing.

Lastly, all these events are independent of CD38 enzymatic activities which are present in all the cell lines analyzed and are unmodified by CD19 silencing.

Together, these data further stress the notion that i) CD38 is a unique type of receptor working in synergy with the CD19 in B cells, and that ii) the enzyme and receptor functions of CD38 are distinct and independent.

### Innate Immune System Regulating Disease

3:30 PM–5:30 PM, 5/13/2005

#### OR-12. Toll-Like Receptor 2 Regulates Cellular Responses to Axonal Degeneration in the Central Nervous System.

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Toll-like receptors (TLRs) are evolutionarily conserved pattern-recognition receptors that are central to innate immunity. TLRs initiate innate responses to infection through selective recognition of conserved pathogen-associated motifs, which signal for cytokine induction. We have addressed whether endogenous signaling via TLRs directs the central nervous system (CNS) response to axonal injury. Stereotactic lesioning of axons in the entorhinal cortex causes axonal degeneration and rapid activation of CNS-resident cells (microglia and astrocytes) in specific regions of the hippocampus. We found that TLR2 was constitutively expressed in the hippocampus and was specifically upregulated by microglia in denervated zones prior to leukocyte recruitment. Detection of MyD88 and I $\kappa$ B $\alpha$  mRNA suggested engagement of downstream signaling pathways. TLR2-deficiency reduced cytokine (TNF $\alpha$ ) and chemokine (CCL3, CCL4, CXCL2 and CXCL10) levels, causing a delay in T cell infiltration. CCL2-driven macrophage infiltration was TLR2-independent, suggesting that the pathways which direct macrophage and T cell entry are differentially regulated by TLR2. Later expansion of the microglial population required TLR2 signaling, whereas astrocyte activation was unaffected by TLR2-deficiency. No responses were altered in TLR4-defective mice, arguing against endotoxin effects. Our findings suggest that TLR2 signaling directs the endogenous response to CNS axonal injury through selective regulation of early cytokine/chemokine expression that drives later cellular responses implicated in regeneration and repair.

This work was funded by the Multiple Sclerosis Society of Canada.

#### OR-13. Linking Innate Immunity to Autoimmunity through the Role of TLRs and the MyD-88 Signaling Pathway.

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There are frequent associations between microbial infections and autoimmune diseases in humans. An infection often exacerbates an ongoing autoimmune response leading to chronic inflammation, tissue destruction and degeneration of the corresponding target organ. The innate immune system is the first line of defense against pathogenic insult. Through Toll-like receptors (TLRs), the innate immune system has the ability to recognize pathogens or pathogen-derived products and to initiate signaling cascades that trigger macrophages and dendritic cells to produce proinflammatory cytokines. This leads to the stimulation of the adaptive immune response. All known TLR ligands activate through the MyD-88 intracellular signaling pathway leading to the nuclear translocation of the rel-type transcription factor NF- $\kappa$ B and the activation of MAP kinases to induce gene expression of proinflammatory cytokines. We hypothesized that blockade of the innate immune system through TLRs would be a tangible method of attenuating the inflammatory cascade due to the inability of signaling through the MyD88-dependent signaling pathway. In this study, we have investigated the involvement of TLR4 in the progression of experimental autoimmune encephalomyelitis (EAE), a prototypic model of multiple sclerosis. We found that mice deficient in TLR4 expression are resistant to MOG 35–55 induced EAE disease. To bypass the need for TLR4 binding, we administered CpGs, the ligand for TLR9, to activate the MyD-88 signaling pathway. We show here that TLR4-deficient mice receiving a single dose of CpGs at the time of disease induction had an overall mean disease severity similar to wildtype mice. Therefore, manipulating the innate immune system through TLRs and the MyD-88 signaling pathway offers a unique opportunity to suppress destructive inflammatory responses and may provide a novel approach for the treatment of Th1-mediated autoimmune diseases.

#### OR-14. Transcription Factor T-bet Regulates Inflammatory Arthritis through Linking the Innate and Adaptive Immunity.

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The transcription factor T-bet (*T-box* expressed in *T* cells) plays a major role in adaptive immunity. T-bet controls the development of both mouse and human Type 1 (Th1) T helper lymphocytes. However, the role of T-bet in the innate immune system has been largely unexplored. Here we demonstrate an essential function for T-bet in dendritic cells (DCs) in controlling inflammatory arthritis. We describe that collagen antibody-induced arthritis (CAIA) is a bipartite disease characterized by an early component, intact in Rag2<sup>-/-</sup> mice, mediated through the innate immune system and a later phase influenced by the adaptive immune system. T<sup>-/-</sup> mice had markedly reduced joint inflammation at both early and late time points and Rag2<sup>-/-</sup> /T-bet<sup>-/-</sup> double knockout mice were essen-

tially resistant to disease. Remarkably, adoptive transfer of T-bet expressing DCs singlehandedly reconstituted inflammation in T-bet<sup>-/-</sup> mice. Furthermore, we demonstrate that T-bet regulates the production of novel target genes, cytokine IL-1alpha, and inducible proinflammatory chemokine MIP-1alpha and TARC by DCs. Further, DCs from T-bet<sup>-/-</sup> mice display impaired antigen capture capability, and hence suboptimal priming of antigen-specific T cells. We conclude that T-bet plays a vital function in DCs that links innate and adaptive immunity. Thus, T-bet provides an attractive new target for the development of novel therapeutics for inflammatory arthritis.

**OR-15. Three Different TLR9 CpG Stimulants Exhibit Diverse Effects in Murine Graft-Versus Host Disease (GVHD).**

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The injection of parental strain T cells into unirradiated F1 mice results in an anti-host response that is either predominately cell mediated (acute GVHD) or antibody mediated (chronic GVHD). The lymphocytopenic phenotype of acute GVHD at day 14 is mediated by anti-host CTL which eliminate host lymphocytes whereas the lymphoproliferative, lupus-like phenotype of chronic GVHD is mediated by donor CD4+ driven polyclonal B cell hyperactivity. The DBA-into-F1 model of chronic GVHD results from a relative defect in donor CD8+ T cell maturation into effector CTL and administration of IL-12 in vivo converts the phenotype from chronic GVHD to an acute GVHD. Thus, the model is useful for screening agents with immunomodulatory or CTL promoting potential. In this study, we tested the immunomodulatory activity in vivo of three different TLR9 stimulants: 1) 2336, an A-Class CpG which targets APC and promotes Type I interferon; 2) 2006, a B-Class CpG which promotes B cell activity and is optimized for human TLR9, and 3) 1826, a B-Class CpG optimized for mouse TLR9. B6D2F1 mice received 80 million DBA splenocytes and 100 ug of CpG i.p. on days 0 and 2 after parental cell transfer. At 14 days after cell transfer, mice were assessed by flow cytometry for splenic lymphocyte subsets. Surprisingly, the results following treatment with TLR9 ligands varied with the preparation used. The B-Class CpG, 2006 exhibited significant immunomodulatory potential and converted chronic GVHD to acute GVHD as measured by elimination of host B cells, up-regulation of FasL on donor CD4+/CD8+ T cells and upregulation of Fas on host B cells. In contrast, the A-Class CpG 2336 exacerbated chronic GVHD as measured by increased donor CD4+ T cells engraftment, host B cells numbers and anti-ssDNA antibody levels and no increased donor CD8+ T cell engraftment. The B-class CpG optimized for mouse TLR9 (1826) exhibited no significant effect either way. These studies demonstrate that despite targeting of a single TLR in vivo, depending on the preparation used, there can be opposing effects on disease outcome.

**OR-16. HSP60 Inhibits Th1-Mediated Hepatitis Model Via Innate Regulation of Th1/Th2 Transcription Factors and Cytokines.**

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Extra-cellular heat shock protein (HSP)-60 has been considered a pro-inflammatory “danger signal”. Yet, HSP60 can also down-regulate experimental immune arthritis and diabetes models by specific inhibition of Th1-like responses. We now report that HSP60 in vitro differentially modulates the expression of Th1/Th2 transcription factors in human T cells: HSP60 down-regulates T-bet, NF-κB, and NFATp, and up-regulates GATA-3, leading to decreased secretion of TNFα and IFNγ and enhanced secretion of IL-10. These effects depended on TLR2-signaling, and could not be attributed to LPS or to other contaminants. In BALB/c mice, HSP60 in vivo inhibited the clinical, histological, and serological manifestations of ConA-induced hepatitis, associated with up-regulated T-cell expression of SOCS3 and GATA-3 and down-regulated T-bet expression. These results provide a molecular explanation for the effects of HSP60 treatment on T-cell inflammation via innate regulation of the inflammatory response.

**OR-17. Toll Receptor Ligands Potently and Broadly Enhance the Immune Response of Immunodepressed Cutaneous T-Cell Lymphoma Patients.**

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Patients with advanced cutaneous T-cell lymphoma (CTCL) exhibit profound defects in cell mediated immunity partially resulting from marked deficiencies in the numbers of peripheral blood dendritic cells (DCs) and in their capacity to make DC derived cytokines (IL-12, IL-15 and IFN-α). Because host immune function appears to play an integral role in mediating disease-controlling responses in CTCL, we investigated the effects of synthetic imidazoquinolines which have been recognized as immune stimulatory by virtue of activation of DCs following binding to Toll like receptor (TLR) 7 and 8. TLR 7, 8 and 7/8 binding compounds were cultured with freshly isolated peripheral blood mononuclear cells (PBMC) from patients with advanced CTCL (erythroderma with circulating malignant T-cells) and normal volunteers and a broad panel of immune functions assessed. All three TLR ligands significantly induced high levels of IFN-α, while the TLR 8 and 7/8 ligands induced IL-12, IL-15 and IFN-γ. The cytokine inducing effects were associated with marked activation of NK and CD8 T-cells assessed by CD69 expression and cytolytic activity. Furthermore, striking up-regulation of CD80 expression on DCs and monocytes also occurred. Thus, imidazoquinolines exhibit the ability to potently and broadly enhance the immune response of patients with advanced CTCL. Such pre-clinical findings have typically been associated with significant clinical improvement when put into clinical practice and therefore have important implications for the potential enhancement of anti-tumor immunity among patients with advanced CTCL.

**OR-18. Beta-Adrenergic and Toll-Like Receptor 2 Signalling in Epidermal Keratinocytes Drive the Skin Immune Response to a Soluble Protein.**

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Disorders of the skin immune activity are implicated in the pathogenesis of cutaneous infections, skin malignancies and acquired inflammatory skin disorders, including psoriasis, atopic

dermatitis, contact hypersensitivity, lichen planus, alopecia areata and vitiligo. Here we show that preconditioning of the skin by  $\beta$ -adrenergic antagonist and the Toll-like receptor 2 (TLR2) agonist S. Aureus peptidoglycan (PGN) results in increased local expression of the interleukin (IL)-1a (IL-1a) and IL-12 genes, which in turn instructs a T-helper 1 (Th1) adaptive response to a soluble protein antigen. On the contrary, when the TLR4 agonist E. Coli lipopolysaccharide (LPS) was used, the presence of the  $\beta$ -adrenergic antagonist was not effective. These effects were consonant with the pattern of TLRs expression shown by epidermal keratinocytes but not by skin dendritic cells. As  $\beta$ -adrenergic signalling defects together with S.Aureus infections are thought to serve as initiation and/or persistence factors for numerous Th 1-sustained inflammatory skin diseases, we might have disclosed at least part of the relevant pathogenetic mechanism.

## T Regs and Regulation of Immune Response

3:30 PM–5:30 PM, 5/13/2005

### OR-19. Regulatory Function Is Deficient in CD4<sup>+</sup>CD25<sup>+</sup> T Cells from Patients with Systemic Lupus Erythematosus.

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During the last decade, the role of active suppression has gained an important place in the operational models of the immune response. Regulatory T (Treg) cells are now considered an essential part of the normal immune response and their absence or malfunction has been causally linked to disease in both animal models and clinical scenarios. Recently, our group and others, have reported that Treg cells are numerically deficient in patients with Systemic Lupus Erythematosus (SLE), a systemic autoimmune disease. Treg cells have been found to be functionally abnormal in other autoimmune diseases, however their functional competence has not been explored in patients with SLE. Thus, the aim of this work was to isolate Treg cells from patients with SLE in order to investigate if their suppressive capacity is normal. **Patients and Methods.** Fifteen patients with SLE (according to ACR criteria) and 15 age- and sex-matched controls were included. At the moment of the study, patients were not receiving corticosteroids nor immunosuppressive drugs; 4 patients had active disease. Treg cells (CD4<sup>+</sup>CD25<sup>+</sup>) were quantified by flow cytometry. Results are expressed as the percentage of cells within total lymphocyte population. Intracellular IL-2 and IL-10 were quantified in CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells. CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells were isolated by magnetic separation. Suppressive capacity was quantified in 96 hour co-cultures: Treg cells were added to CD4<sup>+</sup>CD25<sup>+</sup> cell cultures stimulated with plate-bound anti-CD3 and soluble anti-CD28. Cell proliferation was calculated according to CFSE dilution. Proliferation results are expressed as an index that considers the number of cell divisions per cell (the cell proliferation method is the issue of another abstract). **Results.** Treg cells were quantitatively lower in patients than in controls (active SLE  $1.1 \pm 0.7$ ; inactive SLE  $0.71 \pm 0.8$ ; controls  $2.6 \pm 0.8$ ,  $P < 0.01$ ). As expected, intracellular IL-2 was exclusively observed in CD4<sup>+</sup>CD25<sup>-</sup> cells. Conversely, intracellular IL-10 was regularly observed in CD4<sup>+</sup>CD25<sup>+</sup> cells. Treg cells obtained

from healthy individuals did not proliferate neither in basal nor in stimulated conditions. Conversely, Treg cells from SLE patients proliferated when stimulated ( $P < 0.05$ ). When compared to cells obtained from normal controls, CD4<sup>+</sup>CD25<sup>-</sup> cells from SLE patients proliferated more spontaneously, but less after stimulation ( $P < 0.05$ ). When cocultured, Treg cells from 11 out of 15 healthy controls inhibited  $\geq 50\%$  the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> cells; on the other hand, inhibition was only observed in cells from 3/15 patients with SLE ( $P = 0.003$ ). **Conclusion.** Treg suppressive function is deficient in patients with SLE.

### OR-20. IL-2 Mediates Expansion but Not Maintenance of TGF $\beta$ Induced Tregs in Inflammation: A Novel Role of IL-10.

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Regulatory T cells have been implicated in the maintenance of self tolerance in many animal models and human diseases. These cells have been subdivided in acquired and natural regulatory cells on the base of their origin. The first group includes regulatory cells generated in periphery as the results of antigen specific tolerization protocols, while the second is represented by naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells, generating in the thymus during the first days after birth. This last group of cells are characterized by the expression of the Winged Helix transcription factor FoxP3, which has been implicated in the direction of the genetic program determining the regulatory potential of naturally occurring CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. We have recently shown that FoxP3 and a regulatory phenotype can also be induced in CD4<sup>+</sup>CD25<sup>-</sup> naive cells upon TGF $\beta$  stimulation. While the suppressive capacity of TGF $\beta$  induced Tregs (Ti-Tregs) has been extensively described in vitro, their role in vivo has been poorly investigated. In order to shed light on the in vivo physiology of Ti-Tregs we analyzed the expansion and phenotype stability of these cells in vivo. In brief, Ti-Tregs were adoptively transferred in SCID mice alone or together with colitogenic CD4<sup>+</sup>CD62L<sup>+</sup> naive cells in order to provide an inflammatory environment. Results obtained from these series of experiments indicate that Ti-Tregs depend for their survival and expansion on the presence of an ongoing inflammatory response as indicated by the loss of FoxP3 expression and regulatory capacity in Ti-Tregs transferred in SCID mice in absence of colitogenic cells. Further investigations of in vivo Ti-Treg requirements lead to the identification of exogenous IL-2 provided by the ongoing inflammatory response as the main responsible for Ti-Treg expansion and suppressive activity limited at the site of inflammation. Moreover, we have shown that IL-2 alone is not sufficient to maintain FoxP3 expression and the regulatory phenotype in Ti-Treg cells but that this requires the presence of IL-10. Therefore we propose TGF $\beta$  induced regulatory T cells (Ti-Tregs) as a new class of acquired regulatory cells, highly inflammation dependent for their expansion and survival. This makes their action limited to the space where the immune response occurs and limited to the time this response lasts. The properties shown by Ti-Treg cells not only offer a new insight on how normally occurring immune responses can be physiologically controlled, but also offer the possibility to generate regulatory cells in vitro as a therapeutical tool circumventing the problem related to a prolonged and generalized state of immunodepression.



**OR. 21. Identification of Important Functional Domains of FOXP3 by Analysis of Mutations Present in Patients with IPEX Syndrome.**

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FoxP3 is a member of the forkhead / winged-helix family of transcriptional regulators that has been shown to play a key role in the development and function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells in mice. In humans, defects in the *FOXP3* gene lead to a disease of systemic autoimmunity that is characterized by severe autoimmune enteropathy, endocrinopathy, and skin disease known as IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked). To define regions or domains of FOXP3 that may be functionally important, we have sequenced the *FOXP3* gene in more than 60 patients with a clinical phenotype suggestive of IPEX syndrome. In this cohort, we have identified 21 unique mutations of *FOXP3* in 60% of the patients. The remaining patients had normal *FOXP3* sequences at the genomic DNA level but half of these had low *FOXP3* mRNA expression. Clinically, patients with mutations in FOXP3 had more severe disease with a triad of enteropathy, dermatitis, and endocrinopathy often combined with other autoimmune phenomena whereas patients with normal FOXP3 sequences tended to have milder disease. There does not appear to be a genotype/phenotype correlation between mutations in a particular region of the gene and a specific complex of symptoms. The mutations identified thus far, cluster in one of three regions: the carboxy-terminal forkhead domain, the leucine zipper, and the amino-terminal proline-rich domain, particularly near the 3' end of exon 1. Interestingly, no mutations have been identified in the zinc finger domain. We have introduced the mutations identified in patients into the *FOXP3* cDNA and expressed them exogenously in cells to assess their effects on FOXP3 nuclear import, dimerization, DNA-binding and transcriptional control. Using this approach, we have identified regions in the carboxy-terminal portion of the protein that are required for nuclear import and DNA binding, regions in the central portion required for oligomerization, and regions important for the control of transcription.

**OR-22. GRAIL Expression Is Associated with the Biological Activity of CD25+ T Regulatory Cells.**

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CD25<sup>+</sup> T regulatory cells (CD25<sup>+</sup> Treg) are a subset of T cells with an anergic phenotype that suppress immune responses in an antigen-specific fashion by a poorly understood mechanism. To date the only specific gene marker for this subset of T cells is the transcription factor, Foxp3. We observe a link between CD25<sup>+</sup> Treg cells and GRAIL, an E3 ubiquitin ligase necessary for the development of CD4<sup>+</sup> T cell anergy in vivo. We hypothesize that GRAIL is important for the development and function of naturally occurring and induced CD25<sup>+</sup> Treg cells. Several lines of evidence support this hypothesis: 1) GRAIL is expressed in naturally occurring CD25<sup>+</sup> Treg cells at levels 10-fold greater than CD25<sup>-</sup> CD4<sup>+</sup> T cells, 2) a tolerizing immunization in vivo leads to the induction of long-lived CD25<sup>+</sup> expressing antigen-specific tolerized T cells. Gene expression analysis of these cells reveals that GRAIL mRNA is upregulated (700-fold increase vs. CD25<sup>-</sup> tolerized cells; 100-fold increase vs. naturally occurring CD25<sup>+</sup> Treg cells). Moreover,

GRAIL expression is linked with Foxp3 expression strongly suggesting a suppressor phenotype in this induced tolerized population. As an initial step to understanding the role of GRAIL in CD25<sup>+</sup> Treg cell suppressor function, we demonstrate that enforced expression of GRAIL in an antigen-specific T cell line is sufficient to convey a suppressor phenotype in vitro. These data link GRAIL expression to the biological activity of CD25<sup>+</sup> Treg cells. Mechanistic studies are ongoing and will be discussed further.

**OR-23. Induction of CD4<sup>+</sup>CD25<sup>+</sup>Treg by Tolerance-Inducing Antigen-Presenting Cells Derived from Bone Marrow Cultures Initiated with Anti-CD200R2/3.**

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**Objective:** The relatively ubiquitously expressed cell surface molecule CD200 delivers immunoregulatory signals following engagement of its receptor, CD200R. A family of CD200Rs has now been described by several groups, with the isoforms designated CD200R1-4. Tissue-restricted distribution of these isoforms has been described, with splenic tissue expressing predominantly CD200R1, while bone marrow is the tissue showing the most predominant expression of CD200R2/R3. Little is known to date concerning the functional activity of CD200Rs expressed on cells in different tissues. We have used a number of isoform-specific anti-CD200R agonist mAbs to investigate the effect of signalling via CD200Rs expressed on splenic cells vs bone marrow dendritic cell (DC) precursors.

**Materials and Methods:** We investigated the effect of agonist anti-CD200R mAbs in three independent functional assays: on the generation of alloimmunity in primary MLCs; on the generation of allostimulatory dendritic cells (DCs) from bone marrow cells cultured *in vitro* in the presence of (IL-4+GM-CSF); and on induction of Treg in ConA activated thymocytes in vitro.

Primary MLC cultures were set up with C3H stimulator spleen cells, and mitomycin-c treated C57BL/6 stimulator cells. Some cultures contained anti-CD200R mAbs. Cytokines were assayed in supernatants by ELISA at 40hrs, and CTL were assayed at day 5. In the second assay, bone marrow cells were cultured for 8 days with GMCSF and IL-4 with/without anti-CD200Rs, to generate DCs. DC maturation was induced overnight by LPS (1µg/ml), and the mitomycin-c treated DCs were used to activate fresh splenocytes in MLC, or to induce Treg in splenocytes cultured for 48hrs with these DCs. T reg were also induced in ConA activated thymocytes cultured with anti-CD200R mAbs. Treg were assayed by FACS (for CD4<sup>+</sup>CD25<sup>+</sup> cells), using real-time PCR for FoxP3 expression, and by their ability to suppress CTL and cytokine production in a fresh primary MLC culture.

**Results:** Anti-CD200R1 mAb (but not anti-CD200R2-4) suppressed cytokine production and generation of CTL directly in fresh MLC cultures. In contrast, addition of anti-CD200R1 caused no significant perturbation of development of allostimulatory DCs from bone marrow cultures with (IL-4+GMCSF). However, in the presence of anti-CD200R2/3 mAbs, no functional allostimulatory DCs developed from bone marrow cultures. Instead in these cultures a population of DCs developed which induced CD4<sup>+</sup>CD25<sup>+</sup>Treg in splenocytes. A similar Treg population was induced in thymocytes activated by ConA in the presence of anti-CD200R2/3. Both populations of Treg were FoxP3<sup>+</sup> and inhibited the antigen-specific

MLC response of fresh C3H responder cells on stimulation with C57BL/6 cells. Treg also suppressed skin graft rejection in vivo. Long-term culture of Treg in IL-2 was achieved (>4months), with preservation of suppressive function in vitro/in vivo.

**Summary:** Our data suggest that both CD200R1 and CD200R2/3 can signal immunosuppressive events, though they do so by different mechanisms.

#### **OR-24. Induction of TH1-Like Regulatory Cells That Express Foxp3 and Protect Against Airway Hyperreactivity.**

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The range of natural and adaptive regulatory T cell (T<sub>Reg</sub>) types that control the magnitude and extent of immune responses is poorly understood. We describe a unique population of regulatory T cells (T<sub>Reg</sub>) that developed in vivo from naive CD4+CD25- T cells during a TH1 polarized immune response, distinct from naturally occurring CD25+ T<sub>Reg</sub> cells. These antigen-specific T<sub>Reg</sub> cells were induced by CD8 $\alpha$ + DCs, produced both IL-10 and IFN- $\gamma$ , and potentially inhibited the development of allergen-induced airway hyperreactivity. These T<sub>Reg</sub> cells expressed Foxp3, as well as T-bet. The expression of IFN- $\gamma$  and T-bet, and their induction by CD8 $\alpha$ + DCs indicate that these T<sub>Reg</sub> cells are related to TH1 cells. Thus, adaptive T<sub>Reg</sub> cells are heterogeneous, and include TH1-like T<sub>Reg</sub> cells (TH1<sub>Reg</sub>), as well as previously described TH2-like T<sub>Reg</sub> cells (TH2<sub>Reg</sub>), which have been shown to suppress airway hyperreactivity and were induced by CD8 $\alpha$ - DCs.

#### **OR-25. Regulatory T Cell Dysfunction and a Unique Autoreactive T Cell Population May Be Associated with the Development of Colitis in WASP-Deficient Mice.**

V. Cotta-de-Almeida,<sup>1,2</sup> F. Takeshima,<sup>1,3</sup> M. Maillard,<sup>1,4</sup> P. Michetti,<sup>4</sup> S. B. Snapper.<sup>1</sup> <sup>1</sup>Department of Medicine, Massachusetts General Hospital/Harvard Medical School, Boston, MA, USA; <sup>2</sup>Department of Cell Biology, Oswaldo Cruz Foundation, Rio de Janeiro, RJ, Brazil; <sup>3</sup>Nagasaki University School of Medicine, Nagasaki, Japan; <sup>4</sup>Lausanne University Hospital, Lausanne, Switzerland.

**Background:** Wiskott-Aldrich syndrome (WAS) is a severe immunodeficiency associated with autoimmunity, with up to 10% of patients developing inflammatory bowel disease (IBD). The majority of WASP-deficient (WKO) mice also manifest severe colitis. **Objective:** We aimed to determine whether IBD resulted from aberrant regulatory T cell function. **Methods:** Lymphoid organs obtained from WT and WKO mice were analyzed by flow cytometry and the number of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) was determined. To directly assess the role of Tregs in colitis development, we adoptively transferred WT and WKO CD45RB<sup>hi</sup> (RB<sup>hi</sup>) and CD45RB<sup>lo</sup> (RB<sup>lo</sup>) CD4<sup>+</sup> T cells alone or in combination into SCID recipients. Clinical scoring and histopathological analyses were performed. **Results:** WKO mice had reduced numbers of Tregs in the peripheral lymphoid organs. This finding was already evident before the onset of clinical colitis. Interestingly, we also demonstrated decreased numbers of Tregs in the thymus. As expected, the adoptive transfer approach demonstrated that WT CD4<sup>+</sup>RB<sup>hi</sup> cells induced colitis, which was blocked by the

concomitant transfer of WT CD4<sup>+</sup>RB<sup>lo</sup> cells. However, WT CD4<sup>+</sup>RB<sup>hi</sup> cells transferred disease even in the presence of WKO CD4<sup>+</sup>RB<sup>lo</sup> cells. We also found that WKO CD4<sup>+</sup>RB<sup>hi</sup> cells are colitogenic, but they cause disease with late incidence and lower severity. Surprisingly, the co-transfer of WKO CD4<sup>+</sup>RB<sup>hi</sup> and WKO CD4<sup>+</sup>RB<sup>lo</sup> cells resulted in the development of more severe disease. Furthermore, WKO CD4<sup>+</sup>RB<sup>lo</sup> cells alone, but not WT CD4<sup>+</sup>RB<sup>lo</sup> cells, were able to induce colitis. **Conclusions:** Our data indicate aberrant development and function of Tregs in WKO mice. Furthermore, our transfer studies suggest the presence of unique colitogenic effector cells within the WKO CD4<sup>+</sup>RB<sup>lo</sup> population. Aberrant Treg function and this unique autoreactive effector cell population may account for autoimmunity and IBD in WAS patients and WASP-deficient mice.

#### **OR-26. MHC Class II Controls CD4+, CD25+ Regulatory Tolerance to Allogeneic Transplants.**

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MHC class II gene function has been closely associated with regulation of T cell immunity although the mechanism of their control remains unknown. We have previously demonstrated that the transfer of donor-type MHC class II genes in the bone marrow of miniature swine, recipients of subsequent renal allografts, induced immune tolerance which was not due to deletion of alloreactive T cells. In order to assess whether such tolerance mechanism would implicate the regulatory pathway, and notably CD4+, CD25+ regulatory T cells (T-regs), we examined the effect of somatic transgenesis of donor class II Iab genes in the bone marrow of CBA mice (H-2k), on the survival of subsequent C57BL/6 (H-2b) heart grafts. In this model, donor-specific tolerance as well as indefinite survival of fully allogeneic grafts were achieved, without the use of immunosuppression. Class II-mediated tolerance spread to T cell responses to all graft antigens. Seventy seven percents of long-term accepted hearts, analyzed 160 days after transplantation, presented no signs of chronic allograft vasculopathy. In addition, a single injection of 160-day-tolerant T-regs in naive immunocompetent CBA mice, prolonged the survival of C57BL/6 grafts. These results indicate that class II-mediated tolerance affects the regulatory T-reg pathway likely through the emergence of class II-specific T-regs for suppression of the whole alloresponse, thereby achieving graft survival. They also provide a mechanism by which class II expression may control the regulation of T cell immunity to transplants.

#### **New Immunotherapy Pathways Regulation**

3:30 PM–5:30 PM, 5/13/2005

#### **OR-27. Requirement for the Adipocyte Fatty Acid Binding Protein aP2 in Allergic Airway Inflammation.**

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The adipocyte fatty acid binding protein (aP2) is expressed in adipocytes and macrophages and regulates systemic glucose and

lipid metabolism, and inflammatory responses. Using Affymetrix oligonucleotide arrays we demonstrate that aP2 is also expressed in human bronchial epithelial cells (HBE), and shows a striking upregulation following stimulation of epithelial cells with the T helper 2 (TH2) cytokines IL-4 and IL-13. In HBE, aP2 was significantly down-regulated by the Th1 cytokine IFN- $\gamma$ . Upregulation by Th2-, and downregulation by Th1-cytokines strongly implicates aP2 as a participant in allergic inflammation. Consistent with this hypothesis, aP2 expression was markedly enhanced in bronchial epithelial cells from the lungs of mice undergoing allergic inflammation. Strong aP2 staining was also identified in the respiratory epithelium of human inferior turbinate biopsies. aP2 deficient mice were tested in the model of allergic airway inflammation and were found to be strongly protected, with reductions in airway eosinophilia, peribronchovascular inflammation and pulmonary Th2 cytokine production. Thus, aP2 is a critical regulator of allergic airway inflammation, and we are currently investigating its mechanism of action. Finally, our data suggests a molecular mechanism explaining the relationship between fatty acid metabolism, diet, obesity and asthma.

#### **OR-28. Transcriptional Regulation of Autoimmune Diseases by Tumor Suppressor p53.**

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The tumor suppressor p53 regulates apoptosis, cell cycle, and oncogenesis. To explore the roles of p53 in autoimmune diseases, we studied autoimmune inflammation and innate immune function using C57BL/6 mice deficient in p53. We found that p53-deficient mice were more susceptible to experimental autoimmune encephalomyelitis (EAE) and streptozotocin-induced type I diabetes than control mice, and produced higher levels of IL-1, IL-6 and IL-12. Upon activation in vitro, p53<sup>-/-</sup> T cells and macrophages produced significantly elevated levels of inflammatory cytokines. The innate immune response of p53<sup>-/-</sup> macrophages to lipopolysaccharides and interferon- $\gamma$  was also significantly enhanced, which was accompanied with increased levels of total and phosphorylated signal transducer and activator of transcription (STAT)-1. These results indicate that p53 inhibits autoimmune inflammation and innate immunity through downregulating STAT-1 and pro-inflammatory cytokines.

#### **OR-29. The Sjögren's Syndrome Associated Autoantigen Ro52 Is a RING-Dependent E3 Ligase That Suppresses Cellular Proliferation.**

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Objective: To determine the function of Ro52. Background: E3 ligases are a group of proteins mediating post-translational modification with ubiquitin. E3 ligases have been implicated in many cellular processes, including apoptosis, proliferation and signaling. Some E3 ligases, e.g. Cbl-b, GRAIL and Itch, are regulators of important signaling pathways in immune cells. There is indirect evidence that there are other uncharacterized E3 ligases involved in regulating immune responses. Ro52 (Ro/SSA, Trim21, SSA1) is the main autoantigenic target in the autoimmune

rheumatic disease Sjögren's syndrome. The function of Ro52 is not known, but based on sequence homology we hypothesized that this protein is an E3 ligase. Methods and results: To investigate if Ro52 is an E3 ligase, ubiquitination assays were performed in vivo and in vitro. FLAG-Ro52, or FLAG-Ro52 lacking the RING domain (FLAG-Ro52 $\Delta$ RING), was co-expressed with 6xHistidine-tagged ubiquitin in HEK293 cells. Ubiquitinated proteins were purified with Ni-NTA resin and ubiquitinated Ro52 was visualized by immunoblotting with anti-FLAG antibody. FLAG-Ro52, but not FLAG-Ro52 $\Delta$ RING, was polyubiquitinated suggesting that Ro52 is auto-ubiquitinated in a RING dependent manner. In vitro, Ro52 mediated polyubiquitination together with several ubiquitin conjugating enzymes, but most notably with UbcH6. Functional consequences of Ro52-mediated ubiquitination were investigated in a stably Ro52-transfected A20 B cell lymphoma cell line. Expression of Ro52-GFP in A20 B lymphoma cells resulted in a decreased proliferation in five independent clones compared to five GFP expressing A20 clones and the parental A20 cell line. Furthermore, when Ro52-GFP expressing A20 cells were activated with anti-CD40 antibody, there was an increased cell death in the Ro52 expressing A20 cells compared to the GFP expressing and parental cells. Conclusion: These data demonstrate that Ro52 is a novel RING-finger dependent E3 ligase, which inhibits proliferation and promotes activation induced cell death.

#### **OR-30. Graft-Versus-Leukemia Target Antigens in Chronic Myelogenous Leukemia (CML) Are Expressed on Myeloid Progenitor Cells.**

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Donor lymphocyte infusion (DLI) reliably induces durable remission in 75–80% of patients with relapsed CML following allogeneic bone marrow transplantation. To identify immunologic targets of the graft-versus-leukemia effect of DLI, we used CML post-DLI responder sera to screen a CML cDNA expression library. Two of the identified targets were derived from genes encoding proteins of 66 kD (CML66) and 28 kD (CML28). The development of high titer specific IgG responses against both antigens in DLI responders correlated with immune-induced remission. CML66 has no known homologies, and its coding region contains no known functional motifs. CML28 is identical to hRrp46p, a component of the human exosome, which is involved in the 3' processing of RNA. The human exosome contains known auto-antigens, such as PM/ScI-100, an autoantibody target of patients with polymyositis or scleroderma. The present studies were undertaken to characterize the expression of CML28 and CML66 in primary normal and malignant hematopoietic tissues. Northern blots showed high-level expression in a variety of leukemia cell lines, but not in normal tissue, except for testis. Specific monoclonal antibodies to CML28 and CML66 were developed and utilized to detect antigen expression in leukemia cell lines and primary leukemia tissue on western blot and immunohistochemistry. Expression patterns were confirmed by antigen-specific real-time PCR. Both CML28 and CML66 were highly expressed in leukemic blasts from patients with AML and CML blast crisis, but barely detectable in normal bone marrow, normal peripheral blood, or leukemic cells from patients with stable phase CML. In contrast purified CD34+ progenitors from normal

individuals and patients with stable phase CML expressed high levels of CML28 and CML66 transcript and protein. Immunohistochemical staining for CML66 confirmed rare staining of myeloid precursors in normal marrow and diffuse staining of myeloblastic cells in AML and blast crisis CML marrows. The expression patterns of CML28 and CML66 are similar to some other leukemia associated antigens, including the Wilms Tumor gene (WT1), and suggest that abundant expression in the malignant myeloid progenitor cell may be common to antigens that are targeted by the humoral immune response following DLI. The striking similarity between the expression patterns of CML28 and CML66 supports the notion that DLI exerts its curative effect by targeting antigens present in self-renewing malignant progenitor populations in CML.

### OR-31. BAFF-but Not LT-Dependent B Cell Expansion Contributes to the Suppression of Lethal Intestinal Inflammation.

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Expansion and accumulation of B cells are commonly observed in the inflamed intestine of IBD patients as well as experimental colitis models. Interestingly, the expanded B cells have been shown to play a regulatory role in certain intestinal inflammatory conditions (Immunity 2002,16:219; Gastroenterology 2004,126:115). However, the molecular events leading to the inflammation-associated expansion of regulatory B cells have not been elucidated yet. Therefore, this study was designed to define the molecular mechanism using an acute colitis model in which colitis is induced by addition of 4% dextran sodium sulfate (DSS) in drinking water for 4 days. Interestingly, flow cytometric and immunohistochemical analyses showed a marked expansion of B cells in the colonic lamina propria (LP) during the recovery phase (day 8: four days after the cessation of DSS intake) but not in the acute phase (day 4) of DSS colitis. The expanded B cells represented a pre-mature phenotype with similarity to splenic transitional type 1 (T1) or T2 B cell subset. In addition, analysis of IgH<sub>μ</sub> chain diversities showed that the B cells are polyclonally expanded. Surprisingly, lymphotoxin (LT) pathway, that is necessary for the development of GALT formation under normal conditions, was not required for the inflammation-induced B cell expansion as indicated by a marked expansion of B cells in the colon of DSS-treated LT<sub>α</sub> knockout (KO) mice. Therefore, to identify the factors involved in the B cell expansion, real-time PCR of purified B cells using a series of primer sets that can detect broad ranges of B cell-associated signaling molecules (237 molecules) was performed. Significant upregulations of BAFF-R (B cell-activating factor belonging to the TNF family-receptor), Btk (Bruton's tyrosine kinase), Rac2 and CD40 were observed in colonic B cells at Day 8 compared to Day 0 and Day 4. The functional roles of these molecules in the inflammation-induced B cell expansion were then examined using the specific KO mice treated with DSS. During the recovery phase from DSS-induced acute colitis, expansion of B cells in the colon was not observed in BAFF-R deficient mice and reduced in Btk and CD40 KO mice. In contrast, absence of Rac2 did not affect the B cell expansion. BAFF-mediated activation of NF<sub>κ</sub>B p52 has been shown to enhance the survival of B cells. Indeed, Western blot analysis showed an activation of NF<sub>κ</sub>B p52 from precursor NF<sub>κ</sub>B p100 in the Day 8 B cells. In addition, a marked increase in apoptosis of colonic B cells was observed in BAFF-R deficient mice

compared to WT mice. Finally, to examine the functional role of B cells in DSS induced colitis, the colitis was induced in B cell-deficient mice. Of note, over 60% of B cell-deficient mice died of the colitis until day 10, whereas over 90% of WT mice survived with a sign of recovery from the acute colitis. These studies indicate that intestinal inflammation-induced B cell expansion through BAFF but not LT pathway contributes to the suppression of acute lethal colitis.

### OR-32. Increased Constitutive Activation of NF-κB in Patients with Multiple Sclerosis.

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Multiple sclerosis (MS) is characterized by inflammation and demyelination in the central nervous system (CNS). Current evidence suggests that MS results from autoimmune responses mediated by lymphocytes, which are activated in the peripheral lymphoid organs and migrate into the CNS. Recent studies suggest that the transcription factor NF-κB, might play an important role in the development of chronic autoimmune attack in diseases such as MS. NF-κB is normally found in the cytoplasm of the cell, but under inflammatory conditions it moves into the nucleus to initiate gene expression and produce more pro-inflammatory molecules. The activation of NF-κB is regulated by a group of inhibitor molecules, known as IκB. Several of the IκB family members have polymorphisms that have been associated with susceptibility to MS in Caucasians. In immune cells from people with rheumatoid arthritis or asthma, NF-κB has been found with aberrant, constitutively nuclear localization and enhanced transcription of pro-inflammatory genes. It has been observed that NF-κB and IκB<sub>α</sub> are co-localized in macrophage nuclei in active MS lesions. However, it is unknown if NF-κB is constitutively activated in the immune cells of MS patients. The aim of the current study was to investigate this question.

Peripheral blood mononuclear cells (PBMC) were collected from 19 untreated patients with MS and 27 healthy controls. Some of the cells were fixed, pelleted, embedded in paraffin and sectioned for immunohistochemistry using antibodies specific for T cells, B cells or macrophages, and for the NF-κB p65 (RelA) subunit. The remainder of the cells were lysed, and cytoplasmic and nuclear fractions were purified from the lysate. These fractions were then analysed by polyacrylamide gel electrophoresis and immunoblotting using antibodies specific for the NF-κB p65 subunit and for IκB<sub>α</sub>. The percentage of translocated NF-κB p65, defined as the relative amount of NF-κB p65 found in the nucleus divided by the total amount present in the whole cell, was determined for each sample.

Sixty-eight percent of samples from untreated MS patients had increased NF-κB p65/RelA translocation to the nucleus, compared to 22% of PBMC from healthy controls ( $P < 0.002$ ). Immunohistochemical studies confirmed the translocation of NF-κB p65 to the nucleus, and showed that, in most patient samples, the increased NF-κB activity was in T cells and monocytes rather than in B cells. In addition, the level of IκB<sub>α</sub> protein was reduced in the cytoplasm of untreated MS patients compare to healthy controls, although not to a statistically significant degree.

The results indicate that T cells and macrophages from untreated MS patients show higher levels of constitutively activated NF-κB than do healthy controls. Such activity may lead to enhanced transcription of pro-inflammatory genes and relate to the chronic nature of MS.

### OR-33. Control of Macrophage Activation Via Myeloid CD200R Signaling during Experimental Autoimmune Uveoretinitis.

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Myeloid activation is partly controlled by inhibitory signals through CD200receptor (CD200R) expressed on these cells. We wished to determine the effects of the absence of CD200R signaling, or of its induction by DX109, an agonist mAb, in Experimental Autoimmune Uveoretinitis (EAU) a model of inflammatory eye disease.

EAU was induced in CD200<sup>-/-</sup> and WT (C57BL/6) mice by immunisation with (interphotoreceptor retinoid binding protein) IRBP<sub>1-20</sub>, and also in B10.RIII mice which were immunised with IRBP<sub>161-180</sub>, and treated at various time points with i.v. injections of DX109 mAb. Eyes were enucleated and spleens dissected, for histological disease score and functional assessment, respectively. For macrophage activation studies, bone-marrow derived-macrophages (BM-MΦ) were generated in Teflon bags containing M-CSF media and these cells were then stimulated *in vitro* with TGFβ, TNFα and/or IFNγ.

As previously reported in CD200<sup>-/-</sup> mice, disease onset occurred earlier than in wild type mice, with histologically increased structural damage, concomitant with increased numbers of macrophages infiltrating the retina. Whilst microglial-derived nitrite production was constitutively greater in CD200<sup>-/-</sup> mice, TGFβ-mediated suppression of nitrite was not seen in CD200<sup>-/-</sup> tissue-derived macrophages (taken from both spleen and retina). CD200R signalling was induced via an agonist binding mAb, DX109 *in vitro*. Incubation of macrophages with DX109 abolished IFNγ stimulated TNFα and IL-6 production, but not nitrite secretion. *In vivo*, the efficacy of systemic treatment of EAU in B10.RIII mice with DX109 was variable, but suppression of histological disease was observed compared with mice treated with an isotype control.

In summary DX109, an agonist antibody targeted at the CD200R, prevented full expression of classical IFNγ induced macrophage activation. The variable suppression of EAU may be because local tissue penetration of mAb is required to generate a significant therapeutic effect.

### OR-34. Role of Fgl2 Prothrombinase/Fibroleukin in Experimental and Human Allograft Rejection.

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**Objective:** Immune coagulation, microthrombus and fibrin deposition within the microvasculature are major contributors to the pathogenesis of xenograft rejection, viral induced hepatocellular injury and cytokine induced fetal loss syndrome. Here we

investigated the contribution of the novel gene product, fibrinogen like protein 2 (fgl2) prothrombinase, in mediating immune injury in experimental and human acute allograft rejection.

**Method and Result:** Using a mouse heterotopic cardiac transplant model, mouse fgl2 (mfgl2)/fibroleukin mRNA transcripts and protein were highly expressed in macrophages, CD4 and CD8 positive T lymphocytes and endothelial cells in rejecting cardiac allografts in association with deposits of fibrin. While mfgl2 deficient mice rejected allografts at similar rates to littermate controls, survival of grafts from mfgl2 deficient mice were markedly prolonged and largely devoid of intravascular fibrin. Furthermore, treatment of wild type mice with a neutralizing anti-fgl2 polyclonal antibody ameliorated histological evidence for allojection and intravascular fibrin deposition, and resulted in an increase in graft survival compared to graft survival from untreated mice or mice injected with an irrelevant antibody. To address further the relevance of human fgl2 (hfgl2)/fibroleukin in acute allograft rejection, we examined kidney biopsies from patients who had undergone renal transplantation. hfgl2 mRNA transcripts and protein were markedly expressed mainly in renal tubule cells, infiltrating lymphoid cells including macrophages, CD8<sup>+</sup> T cells, mature B cells (plasma cells) and endothelial cells. Dual staining showed fibrin deposition was localized mainly to blood vessels, in the glomerulus and interstitium and the lumen of tubules, and occurred in association with hfgl2 expression.

**Conclusion:** These data collectively suggest that fgl2 accounts for the fibrin deposition seen in both experimental and human allograft rejection and provide a rationale for targeting fgl2 as adjunctive therapy to treat allograft rejection. This work was supported by the National Science Fund for Distinguished Young Investigators (No. 30225040 for Dr. Q. Ning, No. 30123019 for Dr. XP Luo), from the Natural Science Foundation of China (NSFC), NSFC operating fund 30100171, and 30170846, and the Canadian Institutes for Health Research (CIHR) Grant #FRN33780.

## NK & NK T Cells

3:30 PM–5:30 PM, 5/13/2005

### OR-35. Glycolipid Mediated Activation of iNKT Cells Is Sufficient To Induce Airway Hyperreactivity Independent of Conventional CD4 T Cells.

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We recently showed that the development of allergen-induced airway hyperreactivity (AHR), a cardinal feature of asthma, requires the presence of Vα14 invariant T cell receptor<sup>+</sup>, CD1d restricted natural killer T cells (iNKT). Here we demonstrate that activation of iNKT cells is not only required, but is sufficient for the development of AHR, independent of conventional CD4 T cells, B cells and eosinophils. Respiratory administration of glycolipids that specifically activate iNKT cells (α-GalactosylCeramide [α-GalCer] or sphingomonous glycolipid antigen [PS30]) rapidly induces marked AHR. The induction of AHR with α-GalCer fails to develop in iNKT cell deficient (CD1d<sup>-/-</sup> and Ja18<sup>-/-</sup>) mice or in IL-4/IL-13 deficient mice, demonstrating the

dependence of AHR on *i*NKT cells producing IL-4 and IL-13. Eosinophils, B cells or IgE are not required for  $\alpha$ -GalCer/PS30 induced AHR, since AHR develops in B cell deficient *JHD* mice and in mice treated with anti-IL-5 mAb to eliminate eosinophils. Moreover, MHC class II deficient mice, which lack conventional CD4 T cells but which have *i*NKT cells, show exaggerated glycolipid induced AHR, clearly demonstrating that conventional CD4 T cells are not required for AHR. Therefore, activation of pulmonary *i*NKT is necessary and sufficient to induce AHR in the complete absence of conventional CD4 T cells. We suggest that because *i*NKT cells are central and critical to the pathogenesis of AHR, therapies that target *i*NKT cells may be clinically effective in limiting the development of AHR and asthma.

### **OR-36. The Involvement of CD1-Restricted NKT Cells in the Pathogenesis of Collagen-Induced and Antibody-Induced Arthritis.**

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Natural killer (NK) T cells are unique subset of T lymphocytes that express T cell receptor and a various NK receptors and produce a large amount of cytokines including IFN- $\gamma$  and IL-4 after stimulation with a glycolipid ligand such as  $\alpha$ -galactosylceramide ( $\alpha$ GC). We have previously shown that administration of OCH, synthetic analogue of  $\alpha$ GC, prevent collagen-induced arthritis (CIA) by preferentially inducing IL-4 production by NKT cells. However, the role of NKT cells in the natural course of arthritis models remains unclear. In the present study, we investigated the role of NKT cells in collagen-induced and antibody-induced arthritis. To induce collagen-induced arthritis, mice were immunized intradermally at the base of the tail with 100  $\mu$ g of bovine or chicken type II collagen (CII) emulsified with an equal volume of CFA. Mice were boosted by intradermal injection with the same antigen preparation on day 21. Arthritis development was monitored by inspection three times a week. Starting from day 21, mice were injected intraperitoneally twice per week with anti-CD1 antibody To induce antibody-induced arthritis, mice were injected either with the mixture of anti-CII monoclonal antibodies (mAbs) (Arthrogen-CIA mAb [Chondrex. LLC. Seattle, USA]) followed by LPS injection, or with serum from KRN T cell receptor transgenic mice crossed with non-obese diabetic mice (K/BxN). To detect NKT cells, cells were stained with  $\alpha$ -galactosylceramide-loaded CD1 dimer and were analyzed using flowcytometry. Anti-CII antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Histopathological examination was performed to evaluate arthritis. The number of NKT cells increased in the liver at the peak of the clinical course of CIA and antibody-induced arthritis. Administration of anti-CD1 mAb inhibited the development of CIA induced in DBA1J mice. Next we induced in C57BL/6 (B6) mice and NKT cell deficient (J $\alpha$ 281 knockout) mice. The severity of arthritis was significantly reduced in NKT deficient mice compared to wild type B6 mice. The level of anti-collagen antibody was not different between these groups. The IgG1/IgG2a ratio of anti-CII mAb was elevated and IFN- $\gamma$  production from draining lymph node cells were reduced in NKT cell-deficient mice. To elucidate the role of NKT cells in the effector phase of arthritis, we next examined antibody-induced arthritis in NKT deficient mice and wild type B6 mice. The clinical arthritis score and pathological examination revealed that the severity of arthritis was significantly lower in NKT deficient (J $\alpha$ 281 knockout or CD1d knockout mice) compared to wild type

B6 mice. CD1-restricted NKT cells play an important role in the pathogenesis of arthritis, particularly in the effector phase of arthritis. Considering the fact that Th2 skewing glycolipid ligand such as OCH inhibited the development of arthritis, NKT cells could be a new target for the treatment of rheumatoid arthritis.

### **OR-37. Effects of Natural Killer (NK) Cells on Allogeneic Bone Marrow Transplantation.**

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**Specific objectives of the study:** In this study, we wanted to define the role of the NK cells in myeloablation in MHC mismatch condition. NK cells are capable of receptor mediated lysis of target cells that lack self class I MHC molecules. Thus it can be used effectively in an MHC I mismatched allogeneic bone-marrow transplant to create myeloablation instead of the T lymphocyte. The advantage of using NK cells is that it can achieve the creation of space without developing GVHD. **Material, methods and results:** Preliminary experiments were done to assess the myeloablative property of NK cells, where C57BL/6 (B6) adult NK cells were injected i.p. to 2 days old Balb/c newborns and bone marrow was harvested and set up for in-vitro assays. This study showed that allogeneic NK cells destroys both erythroid and myeloid stem cells. In the next set of experiments we injected NK cells  $\pm$  bone-marrow from C57BL/6 (B6) into 14 D old Balb/c fetuses. However due to toxicity we had to move to restrict the usage of NK cells postnatal only. Seven out of ten recipients of lin- BM and allogeneic NK cells had multi-lineage engraftment 8 weeks post transplantation. The engraftment was inhibited by co-administration of anti NK 1.1 mAb or anti- TGF beta antibody. This indicate a) transplanted NK cells are playing important role in the engraftment and b) engraftment is occurring mostly through secretion of TGF-beta as the result of the addition of NK cells in transplanted cell mass. We used an immunodeficient T-B-NK+ scida -/- model to study the repopulation of the marrow following transplant with NK cells. We found significant repopulation of the T cells but significantly lower amount of B cells in blood. The genotyping PCR with thymocytes showed the successful reconstitution of thymus and T cells following NK cell + wt BM transplant in adult animals. The engrafted cells showed normal TCR rearrangement a feature lacking in the knockout animals. The bone-marrow analysis showed significant engraftment of B cells and normal IgG heavy chain rearrangement post-transplant unlike the scida where the development of B cells are halted at a very early stage. **Conclusion:** The NK cells can be used effectively as a cytotherapeutic myeloablative agent under immunosuppressed conditions.

This work was supported by a grant from the NIH NIAID RO1 HL58842.

### **OR-38. NK-Mediated Protection in CFA-Injected NOD Mice Is Dependent on IFN- $\gamma$ .**

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Autoimmune diabetes in non-obese diabetic (NOD) mice may be prevented by a single injection of complete Freund's adjuvant (CFA), but the mechanism of protection needs further elucidation. We previously showed that NOD mice immunized with CFA have a markedly reduced incidence of diabetes that is associated with a

significant decrease in the number of  $\beta$ -cell specific, autoreactive cytotoxic T lymphocytes (CTL) and that the preventive effect of CFA is mediated by natural killer (NK) cells. In the present study, to understand better the mechanism by which NK cells mediate CFA effect in diabetes, we adoptively co-transferred diabetogenic NOD spleen cells along with NK cells from either IFN- $\gamma$  deficient mice or standard NOD mice to NOD/SCID recipients that were treated with CFA. NOD/SCID recipients that received NK cells from IFN- $\gamma$  deficient mice developed diabetes rapidly after adoptive transfer despite CFA treatment; however, recipients that received NK cells from standard NOD mice either did not develop diabetes or had delayed onset of disease. In addition, NK cells derived from CFA-treated mice showed increased cytotoxicity against autologous dendritic cells. These results indicate that NK cells mediate the protective effects of CFA through secretion of IFN- $\gamma$  and raise the possibility that autologous cytotoxicity of antigen presenting cells may play a role.

**OR-39. Microsomal Triglyceride Transfer Protein Regulation of CD1d-Mediated Glycolipid Antigen Presentation.**

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Microsomal triglyceride transfer protein (MTP), an ER chaperone which loads triglycerides, cholesterol and phospholipids onto apolipoprotein B, also regulates CD1d presentation of glycolipid antigens in liver and intestine. We now show that MTP transcripts and functional protein are present in mouse and human professional antigen presenting cells. Using a novel lipid transfer assay, we demonstrate that purified MTP can transfer phospholipid to recombinant CD1d *in vitro*. Inhibition of MTP by gene silencing, chemical inhibition, or gene deletion results in decreased CD1d surface expression and a decrease in CD1d-restricted presentation of endogenous and exogenous antigens. By comparison, MHC II restricted presentation of ovalbumin is unaffected by loss of MTP indicating that MTP plays a specific role in CD1d presentation. Recent work has shown that lysosomal saposins edit CD1d-bound lipids. We propose that MTP acts upstream of the saposins and functions as a chaperone by loading endogenous lipids into the nascent CD1d groove. Our studies also suggest that a small molecule inhibitor could be used to modulate the activity of NKT cells.

**OR-40. Exogenous IL-2 Promotes IL-5 Production by Human CD4<sup>+</sup> NKT Cell Clones: The Role of IL-2 in the Immune Regulation.**

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The ability of CD1d-restricted NKT cells to produce Th1 and Th2 cytokines has been well described. However, the potential of human NKT cells to produce cytokines under various immune responses needs yet to be delineated. We have analyzed the cytokine production of CD4<sup>+</sup> NKT cell clones derived from human

PBMC of healthy subjects and multiple sclerosis patients, and found that exogenous IL-2 would promote their IL-5 production. *Methods:* The NKT cell clones were generated by initially stimulating fresh PBMC with  $\alpha$ galactosylceramide ( $\alpha$ GC) or its analogue OCH. CD4<sup>+</sup> NKT cells were then sorted based on the reactivity to anti-V $\alpha$ 24, V $\beta$ 11, CD4, and CD8 mAbs. To maintain the clones, V $\alpha$ 24<sup>+</sup> V $\beta$ 11<sup>+</sup> cells were sorted and then stimulated with PHA on monthly basis. The NKT cells were stimulated with  $\alpha$ GC or OCH loaded on monocyte-derived immature dendritic cells, with or without exogenous IL-2. The supernatant was evaluated 48 hours later by cytometric beads array (CBA). *Results:* Most clones produced IL-5, and the amount was higher than IL-4 in some cases. Exogenous IL-2 enhanced the cytokine production in response to the glycolipids in all the clones. In the presence of IL-2, a few clones produced a remarkable amount of IL-5 even in the absence of  $\alpha$ GC or OCH. *Conclusion:* Our data imply that, in the presence of IL-2, human CD4<sup>+</sup> NKT cells could exhibit the potential to produce abundance of IL-5 in response to weak endogenous antigen stimulation, showing the interesting connection of IL-2 and the NKT cell-mediated immune regulation.

**OR-41. Specificity of NKT Cells Against GD3 Ganglioside.**

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We have shown previously that GD3, a ganglioside expressed on melanoma, can be presented by CD1<sup>+</sup> antigen-presenting cells and that immunization with GD3 induces GD3-specific NKT cells. In the current study, we evaluated the specificity of the GD3-reactive NKT cells against other gangliosides that differ in the carbohydrate components that interact with the NKT cell T-cell receptor (TCR). By ELISPOT, we have found that a subset of NKT cells from GD3-immunized mice also recognize GM3 but not GM2, GD2, or lactosylceramide. We interpret these results to mean that the presence of N-acetylgalactosamine (GalNc) inhibits interaction with the TCR since the NKT cells recognize GD3 (Glucose-galactose-[sialic acid]<sub>2</sub>) but not GD2 (GalNc-glucose-galactose-[sialic acid]<sub>2</sub>) or GM2 (GalNc-glucose-galactose-sialic acid). We also found that lactosylceramide (glucose-galactose) was not recognized by the GD3-reactive NKT cells suggesting that at least one sialic acid molecule is necessary for TCR binding. In conclusion, we have begun to characterize the epitope recognized by GD3-reactive NKT cells and find that the TCR of these NKT require glucose-galactose linked to at least one sialic acid for recognition, but that GalNc blocks TCR binding. (Supported by NCI RO1 CA97041).

**OR-42. CD4<sup>+</sup> Invariant T Cell Receptor<sup>+</sup> NKT Cells and the Development of Bronchial Asthma in Humans.**

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NKT cells comprise a population of T cells that express cell surface markers characteristic of both NK cells and conventional T cells that have been shown to exert strong regulatory activity in autoimmune diseases, such as diabetes mellitus, EAE, oxazolone induced colitis and in tumor immunity. We previously showed in a mouse model of asthma that NKT cells producing IL-4 and IL-13 are

required for the development of airway hyperreactivity (AHR), a cardinal feature of asthma. We now show that in human subjects with asthma, the majority of cells in bronchoalveolar lavage (BAL) fluid are not conventional CD4<sup>+</sup> T cells, but rather CD4<sup>+</sup> NKT cells expressing an invariant T cell receptor (*i*NKT cells). In the BAL fluid of the 11 asthmatic subjects, 55–85% of the CD3<sup>+</sup> cells were *i*NKT cells, whereas in patients with sarcoidosis, another pulmonary inflammatory disease, <3% were *i*NKT cells. Similar results were obtained using immuno-fluorescence and confocal laser scanning microscopy of endobronchial biopsy specimens from patients with asthma and sarcoidosis. Like *i*NKT cells in mouse models of asthma, the *i*NKT in the lungs of patients with asthma produced both IL-4 and IL-13, but very little IFN- $\gamma$ . In contrast, *i*NKT cells in the peripheral blood of all of our subjects (asthmatic, sarcoidosis and normal individuals) produced all three cytokines, suggesting the compartmentalization of Th2 subset of *i*NKT cells in the lungs of patients with bronchial asthma.

Taken together, our studies demonstrate that the lungs of patients with asthma but not sarcoidosis contain predominantly CD4<sup>+</sup> *i*NKT cells rather than conventional CD4<sup>+</sup> T cells. These pulmonary CD4<sup>+</sup> *i*NKT cells produce a cytokine profile known to amplify the inflammatory response in asthma strongly suggesting that *i*NKT cells play a central role in the pathogenesis of human asthma.

### Genetics of Immune-mediated Diseases & Transplantation

10:30 AM–12:30 PM, 5/14/2005

#### OR-43. TIM-1 Induces T Cell Activation and Inhibits the Development of Peripheral Tolerance.

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Tim1 was initially identified as an atopy susceptibility gene through positional cloning of a congenic mouse model of asthma. In the mouse, distinct genetic variants of Tim1 were found to be associated with development of both Th2-biased immune responses and allergen-induced airway hyperreactivity. In humans, Tim1 was also found to be a significant atopy susceptibility gene, with specific alleles associated with protection against atopy. Here we examined the immunological function of TIM-1 using a specific monoclonal antibody (mAb), and demonstrated TIM-1 to be a potent T cell costimulatory molecule with a critical role in regulation of the immune system. TIM-1 is expressed on CD4 T cells early after activation, and expression is sustained preferentially in Th2 but not in Th1 cells. In vitro stimulation of CD4 T cells with a TIM-1 specific mAb in combination with T cell receptor activation markedly increased T cell proliferation, indicating that TIM-1 signaling provides a robust positive costimulatory signal to CD4 T cells. Administration of anti-TIM-1 mAb in vivo in combination with antigen strikingly enhanced production of cytokines, prevented the development of respiratory tolerance, and increased pulmonary inflammation. Our studies indicate that TIM-1 is a novel and significant costimulatory molecule, and altering TIM-1 function

during the immune response could provide potent immunomodulatory therapies for a variety of immunological disorders.

### TLR, NK, Innate Immunity 10:30 AM–12:30 PM, 5/14/2005

#### OR-44. In Vivo Homeostatic Proliferation of Naive CD4<sup>+</sup> T-Cells Restrains the TCR Repertoire in Healthy Human Adults.

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Objective: Human CD31<sup>-</sup> central naive CD4<sup>+</sup> T-cells have previously been shown to have post-thymically proliferated and to constitute the majority of naive CD4<sup>+</sup> T-cells in the elderly. By taking advantage of this new phenotypic marker we wanted to analyse possible consequences of postthymic proliferation for the naive Th-cell pool and elucidate the driving force of this process in humans.

Methods: The absolute numbers of peripheral blood CD31<sup>+</sup> and CD31<sup>-</sup> naive CD4<sup>+</sup> T-cells were determined in 25 donors of different age. Additionally, CD31<sup>+</sup> and CD31<sup>-</sup> naive CD4<sup>+</sup> T-cells of 9 healthy donors were highly purified and subjected to a detailed repertoire analysis by spectratyping. Finally RNA was isolated from purified CD31<sup>+</sup> and CD31<sup>-</sup> naive CD4<sup>+</sup> T-cells, cDNA prepared and subsequently Bfl/A1 expression analysed by RT-PCR.

Results: We show here that absolute numbers of CD31<sup>-</sup> central naive CD4<sup>+</sup> T-cells remain fairly stable in adult peripheral blood, excluding a thymus dependent regulation of the CD31<sup>-</sup> CD4<sup>+</sup> central naive T-cell pool. On the contrary CD31<sup>+</sup> thymic naive CD4<sup>+</sup> T-cells decrease during ageing, implying a dependence on thymic function. Most importantly we demonstrate that CD31<sup>-</sup> central naive CD4<sup>+</sup> T-cells isolated from healthy adults are characterised by a highly restricted oligoclonal T-cell receptor repertoire. In order to elucidate the driving force of this post-thymic naive CD4<sup>+</sup> T-cell expansion, we evaluated signatures of recent TCR engagement in purified CD31<sup>+</sup> thymic naive and CD31<sup>-</sup> central naive CD4<sup>+</sup> T-cells. Ex vivo RT-PCR analysis revealed upregulation of Bfl1/A1 among CD31<sup>-</sup> central naive CD4<sup>+</sup> T-cells, a gene which has been shown to be expressed exclusively upon TCR.

Conclusion: Our results demonstrate for the first time that presumably TCR driven peripheral homeostatic proliferation of naive CD4<sup>+</sup> T-cells in healthy human individuals causes a significant contraction of the peripheral TCR repertoire. Given the importance of a highly diverse repertoire for the ability to mount efficient immune responses, this age-dependent deterioration of CD4<sup>+</sup> T-cell immunity could entail ageing-associated increased susceptibility to infection or cancer and decreased efficiency of vaccination. Moreover preferential expansion of self-reactive naive T cells could contribute to autoimmunity.

### Immunodysregulation and Immunoreconstitution 10:30 AM–12:30 PM, 5/14/2005

#### OR-45. Intra-graft Foxp3 Expression Is Associated with Rejection and Is Suppressed by CsA.

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Foxp3 is a transcription factor and its expression has been described as a unique marker for regulatory T cells (Treg). TGF- $\beta$  seems to play a crucial role in the induction of Foxp3 expression. We have recently described that intragraft expression of latent TGF- $\beta$  is associated with stable kidney allograft function in monkeys no longer receiving immunosuppression. Thus we were interested if TGF- $\beta$  expression and stable graft function correlated with intragraft Foxp3 expression. We stained kidney biopsies and kidney tissue at the time of graft rejection obtained from monkeys treated with antibodies specific for CD40 and CD86 and from animals treated with ATG, CsA or with a combination of these drugs for Foxp3. A polyclonal goat-anti-human Foxp3 antibody was used. In addition, these tissues were stained for latent and active TGF- $\beta$ . All animals with clinical rejection (serum creatinin of 200 $\mu$ Mol or higher) and that showed significant graft infiltrates expressed Foxp3 in the nucleus in 5 to 20% of lymphocytic cells. There was one exception: one animal that rejected while still on CsA treatment did not show nuclear Foxp3 expression. In early biopsies (day 21–42 post transplantation) of animals treated with anti-CD40 and anti-CD40+CD86, only approximately 50% of the animals showed nuclear Foxp3 expression (5–10% of graft infiltrating cells). Most of these animals showed significant interstitial infiltrates without loss of graft function. Five animals were treated with ATG at the time of transplantation followed by anti-CD40+86 treatment. None of these animals showed nuclear Foxp3 expression on day 21. Only later when these animals also showed clinical graft rejection, Foxp3 could be found in the infiltrating cells. Animals ( $n = 6$ ) that were on delayed CsA treatment (combined with anti-CD40+86) did not show expression of Foxp3, while Foxp3 expression was evident prior to the CsA treatment. Three animals that had stable graft function for more than 2 years after all immunosuppression was withdrawn showed low levels of nuclear Foxp3 expression or no nuclear Foxp3 expression at all. TGF- $\beta$  was found in almost all tissue samples examined. There was no direct correlation between the amount of either latent TGF- $\beta$  or active TGF- $\beta$  present in the grafts and the presence of nuclear Foxp3. We conclude that nuclear intragraft Foxp3 expression is not unique for tolerated grafts. Rather, Foxp3 positive cells (Treg) must be considered as part of the normal immune response during graft rejection. The nuclear expression of Foxp3 is inhibited by CsA and ATG treatment and this may indicate that these drugs prevent Treg development.

### Disease Regulation: Role of Antigens, Cytokines, Chemokines

10:30 AM–12:30 PM, 5/14/2005

#### OR-46. Insulin with Native B:9-23 Sequence Is an Essential Autoantigen in the NOD Mouse.

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A fundamental question is whether there are primary autoantigens for specific autoimmune disorders. To determine

if insulin is essential for development of autoimmune diabetes, we created NOD mice without native insulin 1 and 2 genes but with a mutated insulin B:9-23 sequence.

The mutated insulin replaced tyrosine with alanine at B chain amino acid 16 (B16:Ala insulin). This mutation abrogates the autoreactivity of multiple insulin B:9-23-reacting T cell clones (e.g. BDC12.4.1) with preservation of hormonal activity. Four founder strains of B16:Ala insulin-transgenic mice were established directly in NOD mice and combined with insulin 1 and 2 knockout NOD mice.

NOD mice lacking insulin 1 and 2 genes with the mutated insulin transgene did not develop anti-insulin autoantibodies, whereas 80% of mice bearing at least one copy of the native insulin 1 gene without the insulin 2 gene developed insulin autoantibodies ( $P < 0.0001$ ). Remarkably, the initial two double insulin knockout mice sacrificed at 26 (Strain B) and 23 (F) weeks of age show no insulinitis. However sialitis was observed, suggesting that the protection from autoimmunity by deleting native insulin is organ-specific. Almost all mice with any native insulin gene sacrificed after 10 weeks of age had intra-islet insulinitis with or without the B:16Ala transgene ( $n = 40/41$ ,  $P < 0.01$ ). Consistent with lack of insulinitis, none of native insulin null NOD mice developed diabetes. All founder strains of B16:Ala-transgenic NOD mice developed diabetes and insulinitis when a native insulin gene was also present, suggesting the preventive effect is dependent on the absence of the native insulin. Splenocytes from the protected transgenic native insulin null NOD mice showed delayed transfer of diabetes into NOD.SCID mice (50% transfer: 13.5 weeks), compared with standard NOD splenocytes transfer (50% transfer: 6.4 weeks,  $P < 0.02$ ). The NOD.SCID mice have native insulin genes. Splenocytes from the diabetic NOD.SCID recipients showed immunologic memory and transferred diabetes into a second NOD.SCID mouse and this mouse developed diabetes at 5 weeks post splenocyte transfer.

Our observation that native insulin null NOD mice with a mutation of insulin B:9-23 sequence abrogates anti-islet autoimmunity suggests that insulin is an essential autoantigen for type 1 diabetes of NOD mice, and insulin peptide B:9-23 is likely a critical determinant. Ability to transfer disease with splenocytes from protected mice is likely due to expression of native insulin B:9-23 sequence of the recipient and indicates that splenocytes from protected native insulin null mice are competent to rapidly transfer diabetes into host with appropriate target.

### Genomics and Genetics

3:30 PM–5:30 PM, 5/14/2005

#### OR-47. Differential Transcript Profiling Identifies Candidate Genes in the New Zealand Model of Systemic Lupus Erythematosus, Including the B-Cell Transcription Factor, *Bach2*.

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Systemic Lupus Erythematosus (SLE) is an autoimmune disease with multiple target organs, an unknown etiology and a complex pathogenesis, with genetic, environmental and other, as

yet undetermined factors. The New Zealand (NZ) mouse model, comprising the New Zealand Black (NZB) and New Zealand White (NZW) strains, spontaneously develops a systemic autoimmunity, and is considered to be an excellent model of SLE. Numerous SLE-linked loci have been identified throughout the NZ genome- the challenge now is to determine the genes that underlie these linkage regions and their role in SLE pathogenesis. To this end, we have assayed, utilising the Affymetrix MOE430 GeneChip system, global gene expression in both splenic CD19<sup>+</sup> B-cells and CD4<sup>+</sup> T-cells. Samples from lupus-prone NZB and non-autoimmune BALB/c mice were investigated, along with two congenic mouse strains that carry NZB-derived disease-linked regions on the BALB/c genome, namely distal chromosome 1 (*Nba2*) and proximal chromosome 4 (*Nbwa2*).

Differentially expressed genes between NZB and BALB/c were identified by combining the results of two methods- a fold-change analysis and a significance analysis of microarrays (SAM). Genes that were observed as a result of both analyses were considered to be differentially expressed. Based on thresholds of  $\pm 2\times$  (compared to BALB/c) in the fold change analysis and 5% cut-off values for the SAM analysis, 559 genes and transcripts were differentially expressed between NZB and BALB/c B-cells and 758 genes and transcripts were differentially expressed between NZB and BALB/c T-cells.

In an attempt to define disease susceptibility genes within the congenic intervals, the transcript profiles of genes that mapped within the congenic intervals were compared with those of BALB/c. We successfully identified a number of differentially expressed genes specific to the congenic interval. For example, the interferon-inducible (*Iffi*) gene cluster, sited on distal chromosome 1, showed a considerable degree of differential expression in both NZB and the *Nba2* congenic strain compared to BALB/c. This replicates the findings seen in an investigation of gene expression in splenic tissue from NZB and C57BL/6.

In relation to the *Nbwa2* locus on proximal chromosome 4, upregulation of the BTB and CNC homology 2 (*Bach2*) gene was present in B-cells from the congenic strain when compared with BALB/c. *Bach2* is a transcription factor that has key roles in B-cell class switching and the somatic hypermutation evident in the humoral immune response. We are defining sequence variants across the *Bach2* gene in order to characterise its haplotype structure in inbred mouse strains.

#### OR-48. Impact of the Lupus Susceptibility Locus, *Sle1* on B Cell Tolerance.

K. Raman,<sup>1</sup> L. Li,<sup>1</sup> M. Bhaskarabhatla,<sup>1</sup> R. Samudrala,<sup>1</sup> K. Hsu,<sup>1</sup> C. Mohan.<sup>1</sup> <sup>1</sup>Internal Medicine-Rheumatology, UT Southwestern Medical Center, Dallas, TX, USA.

**Purpose:** Whereas B6 mice are autoantibody free; B6 mice rendered congenic for the NZM2410/NZW allele of the *Sle1* lupus susceptibility interval develop high titres of anti-nucleosome antibodies. Hence *Sle1* tips the balance from tolerance towards autoimmunity. These studies were designed to understand how *Sle1* might breach B cell tolerance.

**Methods:** B6.*Sle1* mice were bred to HEL-Ig.mHEL or HEL-Ig.sHEL transgenic mice. These mice were then examined for breach in B cell tolerance using various methods including flow cytometry, serology, calcium flux analysis and in vitro cultures.

**Results:** The presence of the HEL-Ig transgene "cured" the *Sle1* associated autoimmune phenotypes including splenomegaly

(B6.*Sle1*.HEL-Ig 100  $\pm$  17 mg vs B6.*Sle1* 235  $\pm$  86 mg  $p < 0.001$ ,  $n = 8-22$ , aged 8-14 months), B (mfi I-A<sup>b</sup> B6.*Sle1* 866.6  $\pm$  142.4 units vs B6.*Sle1*.HEL-Ig 419.1  $\pm$  80.38 units,  $n = 5-11$ , aged 3-7 months) and T cell activation (number of CD4<sup>+</sup>CD69<sup>+</sup> T cells: 9.187  $\pm$  3.736 $\times 10^6$  vs 4.222  $\pm$  1.264  $\times 10^6$   $n = 4-11$ ), anti-nuclear antibody production (anti-DNA-histone IgG: B6.*Sle1* 144.1  $\pm$  46.21 U/ml vs B6.*Sle1*.HEL-Ig 18.62  $\pm$  2.820 U/ml,  $n = 3-11$ , aged 4-7 months,  $P = .0002$ ) and glomerulonephritis.

To study the impact on central deletion, *Sle1* was bred to HEL-Ig.mHEL mice. B6.HEL-Ig.mHEL and B6.*Sle1*.HEL-Ig.mHEL ( $n = 6$ , each) mice had comparable diminution of splenic IgM<sup>a</sup> HEL<sup>+ve</sup> transgenic B cells and serum anti-HEL antibodies, indicating that *Sle1* did not abrogate central deletion of high avidity anti-self B cells.

To study the impact of *Sle1* on clonal anergy, *Sle1* was bred to HEL-Ig.sHEL mice. In this model, although self-reactive B cells are allowed to escape to the periphery they are functionally anergised. Importantly, *Sle1* breached tolerance in this model since B6.*Sle1*.HEL-Ig.sHEL mice had an increased number of B cells (18.66  $\pm$  3.791  $\times 10^6$  vs 10.24  $\pm$  2.829  $\times 10^6$ ,  $p < 0.0006$ ,  $n = 8-9$ , aged 3-6 months) and also had significantly higher levels of anti-HEL antibodies (39.70  $\pm$  5.643 U/ml vs 17.33  $\pm$  1.691 U/ml,  $n = 16-19$  aged 3-7 months,  $P = 0.0013$ ). Interestingly, some of the B6.*Sle1*.HEL-Ig.sHEL mice (3/10, aged 8-14 months) also produced anti-ssDNA IgM antibodies. *Ex vivo* overnight cultures of whole splenocytes demonstrated spontaneous activation of B6.*Sle1*.HEL-Ig.sHEL B cells in the absence of any stimulus and this activation was further enhanced in the presence of anti-IgM ( $n = 4$ , aged 3.5 months). Proliferation assays using CFDA-SE dilution revealed increased response of B6.*Sle1*.HEL-Ig.sHEL B cells to anti-IgM but not to sHEL(%undivided B cells: 28.33 B6.*Sle1*.HEL-Ig.sHEL vs 50.13% for B6.HEL-Ig.sHEL,  $n = 2$  aged 3-6 months). To examine if presence of *Sle1* rescues deletion of HEL-Ig B cells in sHEL mice, 25 $\times 10^6$  B6.HEL-Ig or B6.*Sle1*.HEL-Ig B cells were adoptively transferred into sHEL mice. However there was comparative deletion of either B cells.

**Conclusions:** These findings support the conclusion that though *Sle1* may not have the capacity to thwart central deletion of high avidity anti-self B cells, it certainly abrogates effective 'anergization' of low-avidity anti-self B cells. The molecular bases for these differences are currently being examined.

#### OR-49. Insulin-VNTR Modulate Functional Phenotypes of T-Cell Responses to Proinsulin in HLA-DRB1\*04 Positive Subjects with and without Type 1 Diabetes.

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Insulin (pro-insulin, P-Ins) is considered a central beta-cell autoantigen in young patients of Caucasian ancestry with the high risk HLA-DRB1\*04, DQ8 haplotype (DR4-haplotype). Genetic predisposition to type 1 diabetes (T1D) is, apart from HLA genes also determined by complex interactions of a number of other genetic loci including the INS-VNTR (IDDM2) locus. INS-VNTR determines diabetes susceptibility by modulating levels of P-Ins expression in the thymus, higher levels of thymic P-Ins expression are associated with VNTR class III alleles which facilitate tolerance induction and protection from T1D, whereas class I alleles predispose to T1D.

The aim of this study was to determine whether INS-VNTR polymorphisms modulate functional phenotype of the T cell response to P-Ins in subjects with high risk DR4-haplotype, with (T1D patients and Ab+ subjects) or without (control subjects) beta-cell autoimmunity. All subjects were typed for INS-VNTR class I and class III alleles. Peripheral blood lymphocytes and CD4+ T cell subsets (CD45RA+, naive and recently primed and CD45RA-, memory) were stimulated with immunodominant P-Ins73-90 epitope, and cytokine secretion (Th1:IFN $\gamma$ , TNF $\alpha$ , IL-2, and Th2:IL-4, IL-5, IL-10) was determined. Our analysis reveal the predominance of CD4+CD45RA+IL-10hi cells in subjects with protective VNTR class III alleles, but not in subjects with VNTR class I alleles. CD4+CD45RA+IL-10hi T cell phenotype has been associated with regulatory function in subjects with T1D, and in experimental models of autoimmunity.

Our analysis show, for the first time that transcriptional effects of VNTR genes in subjects with high risk DR4-haplotype affect the selection of P-Ins specific T lymphocytes in the periphery and influence predisposition to T1D.

#### **OR-50. A Toll Receptor Pathway Polymorphism Affects Susceptibility to Inflammatory Bowel Disease.**

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Inflammatory bowel disease (IBD) is a complex genetic disorders caused by a combination of genetic and environmental factors. Recent evidence has implicated a component of the innate immune system in the pathogenesis of IBD; mutations in NOD2/CARD15 in humans have been associated with susceptibility to IBD. These data prompted us to undertake a thorough investigation of one innate immunity pathway involved in extracellular pathogen-associated molecular pattern recognition: the Toll-like receptor pathway. We selected 18 genes (the TLR gene family as well as components of downstream signalling pathways) and performed a haplotype-based association analysis for each gene. This assessment of the common genetic variations found in the TLR pathway reveals that there is suggestive evidence for association of polymorphisms in IRAK2, TIRAP, TLR3, and TLR4 to IBD in a screening Canadian population of 161 IBD trios and 114 cases and 68 controls. However, these results were not replicated in an independent Belgian sample collection of 104 IBD trios and 610 cases and 383 controls. A second replication effort consisting of 1000 IBD trios and 400 IBD tetrads will be concluded shortly and should allow us to make definitive conclusions regarding the observed associations. Two polymorphisms previously associated with IBD (Asp299Gly in TLR4 and an NF-kB promoter indel) were also assessed using meta-analyses of the published studies and our study populations. The NF-kB polymorphism failed to show definitive association in our meta-analysis; however, we definitively show that the 299Gly allele of

TLR4 is associated with susceptibility to IBD and explore the phenotypic associations of this allele.

#### **OR-51. Finally Found: Human BAFF-R Deficiency Causes Hypogammaglobulinemia.**

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Introduction: BAFF-R is a member of the TNF-R superfamily and is expressed mainly on mature B cells. Both BAFF-R<sup>-/-</sup> (synonym: Blys) and BAFF-R<sup>-/-</sup> mice exhibit a severe defect in peripheral B cell homeostasis indicating a prominent role of BAFF-R/BAFF interaction in the peripheral B cell development of the mouse. Hypogammaglobulinemia, disturbed germinal center formation and impaired antibody responses in BAFF-deficient mice rendered BAFF and its receptor clear candidate genes in the search for the etiology of CVID. Here we report the first patient with a homozygous mutation of the BAFF-R causing the clinical phenotype of CVID.

Methods: 50 Patients were screened for surface expression of BAFF-R by flowcytometry. A suspected defect was confirmed by genomic DNA sequence, RT-PCR and Western blot analysis. Extensive immunologic phenotyping of peripheral blood cells was performed. Patient-derived B cells were compared with normal controls by in vitro activation via BCR  $\pm$  BAFF.

Results: A 60 yr old patient with hypogammaglobulinemia (IgG 0,6g/l, IgA 5,3 g/l, IgM 0,6 g/l) and no family history of immunodeficiency was identified by FACS analysis to express no BAFF-R on B cells. This was due to a genomic homozygous 24bp deletion in the transmembrane region of exon 2. The immune phenotype was distinct and may permit to screen for BAFF/BAFF-R deficiency. Functional assays are still ongoing. Besides recurrent pneumonias he suffered from an unusual fungal infection of his upper respiratory tract.

Conclusion: The first patient with a genomic BAFF-R defect confirms the role of BAFF as a master regulator of peripheral B cell homeostasis also in humans. The immune phenotype of this patient may allow the identification of patients with related defects.

Supported by DFG grants: a) SFB620 TPC1; b) SFB620 TPC2; c) SFB620 TPA2. d) GR1617/3.

#### **OR-52. Polymorphisms in CTLA4 and CD28 Are Associated with SLE.**

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**Purpose:** CTLA4 (Cytotoxic T Lymphocyte Associated Protein 4) may have widespread significance in the aetiology of several autoimmune diseases (AID). It is a good candidate gene for SLE because it is part of a suggestive linkage interval to lupus at 2q33, in two genome-wide linkage scans. Linkage to this region, and associations with different polymorphisms in CTLA4, have also been reported for type I diabetes (T1D), Graves' disease and coeliac disease. Functionally, CTLA4 is important in the inhibition of CD28-mediated T cell activation, through co-operation with two T cell co-stimulatory molecules, CD28 and ICOS. These key regulators of T cell activation are encoded in a 300kb region homologous to the *Bxs1* lupus linkage interval on mouse chromosome 1 in BXS mice. There are reported associations in several AID with polymorphisms in the 3' UTR, exon 1 and 3' flanking region of the gene. However, these results in both SLE and other AID, are inconsistent, largely being small

scale case-control studies in diverse populations and genotyping limited polymorphisms. To clarify whether *CTLA4* polymorphism contributes to the genetic predisposition to lupus, we undertook a family-based association study across the *CTLA4* locus in SLE. **Materials:** The study cohort consists of 474 SLE European Caucasian families, from a total collection of 630 SLE families. All probands conformed to the ACR criteria for SLE. The 20 genotyped variants were selected from a series of haplotype-tagging SNPs provided by John Todd. Additional polymorphisms were chosen from the SNP databases and the literature. The SNPs were typed by MALDI-TOF mass spectrometry. **Methods:** An initial haplotype map in our SLE trios was constructed in Haploview. TDT analysis, on both single SNPs and on haplotypes, was performed using GENEHUNTER on trios and using TRANSMIT to include single parent families. **Results:** *CD28* and *ICOS* are encompassed within regions of limited haplotype diversity; two haplotype blocks span *CTLA4*, with the first (5') block including the promoter and the second (3') block extending across the 3' flanking region. A single SNP in the promoter of *CD28* ( $p = 0.003$ ) was associated with SLE. In *CTLA4* there were 4 associated SNPs in the haplotype covering the 3' flanking region of *CTLA4*: SNP 12 ( $p = 7 \times 10^{-4}$ ), SNP 13 ( $p = 0.019$ ), SNP 14 ( $p = 0.006$ ) and SNP 16 ( $p = 0.019$ ). Furthermore, the same 3' flanking region of *CTLA4* is associated with T1D and Graves' disease, but there are additional associations in these disorders from SNPs in the 5' haplotype block. **Summary:** Our haplotype map delineates discrete haplotype blocks across *CD28*, *CTLA4* and *ICOS*. The two regions of association are in the *CD28* promoter and the 3' flanking region of *CTLA4*. Comparison of the *CTLA4* associations in SLE with those in T1D and Graves' disease, confirm the importance of the 3' flanking region of the gene in AID, although the SLE-associated SNPs differ from those identified in T1D. Thus, there may be disease-specific factors in the function and/or expression of *CTLA4* operating in SLE. We are currently fine-mapping the associated haplotypes in both *CD28* and *CTLA4*.

#### OR-53. Building an Admixture Map for Disease Gene Discovery in African Americans.

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Admixture mapping (also known as MALD, "Mapping by Admixture Linkage Disequilibrium") is a promising new tool for discovering genes that contribute to complex traits in admixed populations such as African Americans. Gene flow from ancestral populations into an admixed population generates associations of the disease with states of ancestry. MALD requires far fewer markers to search the genome for disease genes than a whole-genome haplotype mapping study, but has similar statistical power to find disease genes for markers that differ strikingly in frequency across populations. We have assembled a genomewide panel of markers for admixture mapping based on genotyping in African and European populations. Initially we screened several databases and identified 450,000 single nucleotide polymorphisms (SNPs) with known frequencies in Africans and Europeans. We selected 3,583 markers for experimental validation based on large enough

frequencies between Africans and Europeans to warrant potential inclusion in our map. Experimental validation involved genotyping new population samples by the 5' nuclease assay (TaqMan Assays-on-Demand) or by primer-oligo base extension assay resolved by MALDI\_TOF mass spectrometry on a chip (MassARRAY Sequenom). Markers were chosen if they were genotyped successfully in at least 20 West Africans and 20 European Americans, were in Hardy-Weinberg equilibrium in the parental populations, had a minimal level of ancestry informativeness (Shannon Information Content (SIC)>0.035 out of a maximum of 0.709) and were similar in frequency within continents. We are currently using a subset of the markers in a validated map (1,536) to screen for risk factors for disease in 742 patients with multiple sclerosis and 996 with prostate cancer.

#### Immunodiagnosis

3:30 PM–5:30 PM, 5/14/2005

#### OR-54. Detection of Allergen-Antibody Complexes on Murine B Cells Using Flow Cytometry.

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The hallmark of atopic allergy is production of allergen-specific IgE, which in turn requires the cytokines interleukin (IL)-4 and IL-13, products of allergen-specific Th2 type cells. In addition to being responsible for activation and release of inflammatory mediators by mast cells and basophils, allergen-specific IgE is able to form complexes with the allergen, which can be efficiently processed and presented by IgE-receptor expressing antigen presenting cells (APC). Capture and presentation of the allergen via this mechanism has been shown to result in 100-fold reduction in the concentration of allergen required to trigger T-cell activation. Binding of allergen-IgE complexes to the surface of human B lymphocytes has been demonstrated previously by flow cytometry using fluorescently labeled anti-IgE antibodies. We have now investigated the binding of allergen-IgE and allergen-IgG1 complexes to the surface of splenic B-cells isolated from BALB/c mice.

**Methods:** CD19<sup>+</sup> B-cells were isolated from the spleen of naive BALB/c mice using magnetic beads (Miltenyi). Serial dilutions of sera from ovalbumin-sensitized mice (immunized by intraperitoneal injection), or from naive (untreated) controls, were incubated alone or in the presence of 3 or 5 µg of ovalbumin (OVA) for 1 hour at 37 °C. Subsequently, B-cells were added at  $5 \times 10^5$ /sample and incubated for 1 hour at 4 °C. Following incubation with sera/allergen, B-cells were washed twice with PBS containing 1% bovine serum albumin (BSA) and stained on ice for 45 minutes with monoclonal fluorescently-labeled anti-IgE and anti-IgG1 antibodies. In additional experiments fluorescently-labeled OVA was used to reveal binding of OVA/antibody complexes. Cells were analyzed after washing using a FACScalibur flow cytometer. For each sample a minimum of 5000 cells was analyzed.

**Results:** In the absence of serum or allergen, a small percentage of B-cells was IgE and IgG1 positive. Incubation of B-cells with allergen (OVA) and sera from OVA sensitized mice at higher serum concentrations (40–80%) resulted in a marked increase in IgE<sup>+</sup> B-cells (up to 40% increase) and IgG1<sup>+</sup> B-cells, which was already detectable at low serum concentrations (<1%). Binding of both IgG1 and IgE was dependent upon the presence of both

allergen and allergen-specific antibodies since incubation with sera from OVA-sensitized mice and an irrelevant allergen, or from naive mice and OVA did not result in a similar increase in antibody positive B-cells. Furthermore, the binding of OVA-IgE, but not OVA-IgG1, complexes was shown to be inhibited markedly by treatment with anti-CD23 (FcεRII) antibody *in vitro*.

**Conclusion:** These data demonstrate that mouse splenic B-cells bind allergen-specific IgE through the low affinity IgE receptor (FcεRII), with efficient binding only occurring in the presence of allergen-IgE complexes. This may form the basis of an approach for the characterization and identification of IgE containing sera.

#### **OR-55. Novel Candidate Markers for Multiple Sclerosis Using Phage cDNA Display.**

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Multiple sclerosis (MS) is a chronic, inflammatory disease of the central nervous system, characterized by the presence of focal lesions resulting from myelin breakdown. In the past few years, an important contribution of B cells and autoreactive antibodies has been demonstrated in the pathogenesis of MS. To fully explore the complex information present within the antibody repertoire of patients, we have developed a novel and powerful molecular approach 'Serological antigen selection', which involves the display of a cDNA expression library on filamentous phage and subsequent selection on patient IgG. The aim of this study was to apply the SAS technology to identify antigens that are specifically recognized by antibodies (IgG) present in the cerebrospinal fluid (CSF) of MS patients. First, we constructed a cDNA display library by cloning a normalized cDNA library prepared from active chronic MS plaques, with varying degrees of demyelination and inflammatory activity (Soares et al, 1994) for expression as a fusion protein with a filamentous phage minor coat protein, pVI. Parallel selections were then performed on 2 pools of CSF ( $n = 10$ ) from relapsing-remitting MS patients. Affinity selections revealed a panel of 9 different clones reactive with the first CSF pool. A detailed serological analysis of the 9 different antigens on a large panel ( $n = 100$ ) of individual patient and control CSF showed exclusive reactivity to MS patient CSF for seven different antigens. Sequence analysis revealed that these clones have never been associated with MS. Antigenic cDNAs from the second pool of CSF are currently under investigation. In conclusion, our findings demonstrate that this novel molecular approach is useful to identify novel candidate antigens in MS that can be used as diagnostic markers, and can be used to study the humoral immune response in MS.

#### **OR-56. Phenotypic and Functionnal Ex Vivo Profiling of Human CMV Specific CD8+ T-Cell Responses after Allo-HSCT.**

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Recognition of viral antigen by the immune system induces a coordinate number of changes in lymphocyte subsets. This

involves changes in the expression of cell surface molecules, in lymphocyte migratory properties and in the ability to proliferate and exert T-cell mediated cytotoxicity. Primary infection with human Cytomegalovirus (hCMV) is followed by lifelong persistence with viral latency in cells of the myeloid lineage. CMV specific CD8-T cells are maintained at a very high frequency in healthy CMV carriers, reflecting a permanent control of CMV reactivation at a subclinical level by the hosts immune response. Because primary CMV infection is usually clinically silent, little is known about the longitudinal evolution of specific T cells during the course of antigenic challenge. Due to the latency of the immune reconstitution, patients with allogeneic haematopoietic stem cell transplantation (allo-HSCT) are at high risk of CMV reactivation during the first three months. We have designed a strategy to follow the CMV reactivation in this context, combining weekly determination of the viral load with the quantification of CMV specific immune responses. We took advantage of such monitoring to explore the complex host-pathogen relation during the course of infection and latency. We focused on the immune dominant response against the tegument protein pp65 and characterized the CD8+ T cell response using a combination of phenotypic (HLA-class I tetramers) and functional (ELISPOT) assays. Twelve patients were selected for their ability to mount a CMV response of more than 2% of total CD8+ T cells. Specific T cells were detectable at the time of reactivation as early as 34 days after allo-HSCT and underwent a phase of expansion with stabilization of the response depending on the intensity of antigenic challenge. In recipient from a seronegative donor, CD27+CD28+ and CD27+CD28- early/intermediate phenotypes predominated during the first wave of reactivation. This was rapidly followed by a progression though to CD27-CD28- late phenotype. Although the CD45RA molecule was observed on most late phenotypes, CD45RA and CD45RO expression did not correlate with the 3 stages defined by CD27 and CD28 expression. In patients who reactivated twice or more, an enrichment of CD8+T cells with different phenotypes was observed, consistent with different stages of differentiation. All CD8+T cells were perforin positive whereas granzyme expression seemed more restricted in the tetramer positive T-cell compartment. In conclusion this study showed that a specific and effective anti-CMV response can be mounted very early after allo-HSCT, even if the donor was seronegative, and gave an insight into the evolution of CMV-specific T cell responses in human, from the onset of reactivation to the stage of chronic infection.

#### **OR-57. A Compliant Solution for Monitoring Proteins Using a Lab-on-a-Chip Instrument.**

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Lab-on-a-Chip (LoaC) technology has had a major impact on the automatization of protein analysis. Traditionally, SDS-PAGE gels are run for sizing and quantitation of proteins. Microfluidic protein analysis is now beginning to replace this traditional method.

In August 2002, the Food and Drug Administration (FDA) announced a significant new initiative, Pharmaceutical Current Good Manufacturing Practices (CGMPs) for the 21st Century, to enhance and modernize the regulation of pharmaceutical manufacturing and product quality. With the increasing focus on proteins as pharmaceutical drugs, there is a strong demand for standardized

and reproducible protein analysis methods that comply with the GLP, GMP and 21CFR Part 11 requirements.

Here, LoaC technology can offer a benefit since for protein analysis it integrates sample handling, separation, staining, destaining, detection and digital data analysis. In addition, due to the integration of several individual procedures an increase in throughput and reproducibility can be achieved.

We have compared chip-based protein analysis with regard to sensitivity, sizing accuracy and reproducibility to SDS-PAGE. Data were comparable to that obtained from Coomassie-stained PAGE gels. The benefits of working on the microfluidic scale include speed of analysis, sample size and fully automated data evaluation. Ten samples can be run in thirty minutes. Electropherograms plotting fluorescence intensity against separation time are generated for each sample. Data for individual constituents of a complex mixture are shown along with calculations of concentration and percent total for each protein in the trace.

Analysis of 10 samples takes thirty minutes using only four microliters of sample. The data analysis is automatically performed in real-time and is stored and archived in digital format. IQ and OQ/PV tools and services as well as 21CFR Part 11 compliant software tailor the instrument for use in regulated environments.

#### **OR-58. Correlation of Clinical Outcome with Immunophenotype in Islet Transplant Recipients.**

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Insulin independence can be achieved via allogeneic islet transplantation in patients with type 1 diabetes. Our aim was to determine the immunophenotype in islet transplant recipients and to determine if alterations in the immune profile (IP) correlated with graft status. Thirteen islet allograft recipients with long-term (> 5 years) type 1 diabetes treated with steroid free immune suppression (rapamycin, FK506 and induction therapy with Zenapax) were monitored serially for changes in IP. Twelve of the 13 patients received 2 or more islet infusions and all patients received islets from 2 or more donors. All patients experienced insulin independence, with 8/13 eventually returning to reduced dosages of exogenous insulin. Peripheral blood (PB) samples were collected pre and post transplant for 4 color staining and multiparametric analysis of lymphocyte subsets (T, B, NK cell) and activation markers (CD25, CD69, HLA DR). Similar to our observations for cytotoxic lymphocyte gene (CLG) expression in PB, variable changes in IP occurred in the early post-transplant period (with each infusion) which initially did not appear to correlate with graft status. Differences, however, were subsequently observed for both overall white blood cell count (WBC) and IP between stable recipients vs. patients with partial islet allograft loss. Previously, we have shown that elevated CLG is predictive of islet rejection. In this study, we compared all IP to both CLG data and to results from anti-donor mixed lymphocyte reaction (MLR). Data show WBC decreased to less than 1/2 of baseline in 3/4 stable patients and remained relatively low, but for 7/8 patients that experienced rejection, WBC increased subsequent to elevation of CLG and remained higher. In 3/4 stable recipients, CD3/45 T cells dropped to less than 1/2 baseline and remained at or below this level. Similar to WBC, all T cell counts (including CD3/4 and CD3/8 T cell subsets) dropped initially and then increased after evidence of rejection (indicated by CLG elevation, or onset of hyperglycemia, or initiation of

exogenous insulin) in 6/8 patients. Despite anti-IL2R (Zenapax) therapy, 7/8 patients with partial graft loss showed elevation of CD4/25 cells after apparent rejection and 7/8 showed elevation of CD4/69 T cells; 2/4 stable patients showed clear and stable decreases from baseline and the other 2 experienced gradual increases over time. Regarding NK and B cell subsets, trends toward counts that remained below baseline were again seen for stable patients and increases after evidence of rejection were observed in patients with partial graft loss. This suggests that changes in post transplant IP are indicative of alterations in the recipient's immune response to transplanted islets, as supported by CLG, MLR, and clinical data in stable vs. rejecting patients. We are working to establish flow based methods to assess the functional status of recipient cell in response to both non-specific and donor cell stimulation to ultimately tailor patient therapy.

#### **OR-59. Autoimmunity in Children with Atypical Type 1 and Type 2 Diabetes.**

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*Aims:* The incidence of both type 1 (T1D) and type 2 diabetes mellitus (T2D) is increasing in children. Although T1D and T2D are classically thought to have separate pathogeneses and presentations, often an admixture of T1D and T2D features are present at diagnosis. Our aim was to examine the relationship between autoimmune measures, HLA, and clinical course. *Methods:* 28 subjects with atypical T1D (presenting with features such as obesity, acanthosis nigricans, absence of DKA and/or absence of weight loss), and 15 subjects with a clinical diagnosis of T2D were studied at time of diagnosis. Diabetes-associated autoantibodies (DAA) including islet cell, GAD65, IA-2, and insulin autoantibodies were measured. 23 subjects underwent HLA genotyping and were classified as having a high risk (DR4/DQ3; DR3/DQ2), protective (DR15/DQ6), or low risk (other HLA) haplotypes. Subjects were 8–18 years of age at diagnosis, and clinical course was followed in 84% of subjects for a mean period of 47.9 ± 18.7 months. *Results: Atypical T1D:* 25/28 (89%) atypical T1D subjects were positive for at least one DAA. 14/16 (88%) with HLA typing had one or more high risk HLA alleles. 24/28 (86%) were prescribed insulin at diagnosis and remained on insulin throughout the follow-up period (3 insulin-requiring subjects were lost to follow-up). All 24 insulin-requiring subjects were DAA positive at diagnosis. 12/13 HLA-typed insulin-requiring subjects had a high risk HLA genotype, 1 had a low risk HLA genotype. 2/4 initially non-insulin-requiring subjects remained on oral agents. Both of these were DAA negative, and the one typed subject was HLA low risk. The other 2 subjects initially treated with oral agents subsequently required insulin for glucose control. Both of these subjects had high risk HLA, and one was DAA positive. *T2D:* 5/15 (33%) T2D subjects were DAA positive at diagnosis. 1/7 HLA-typed subjects had a high risk HLA genotype, 2 had a combination of high risk and protective alleles, and the remaining 4 had low risk or protective alleles. At diagnosis, 14 subjects were treated with oral agents and one with insulin. Follow-up information was obtained on 11 subjects. The subject initially treated with insulin remained on insulin. This individual was DAA negative, but was not HLA typed. 7/10 subjects initially treated with oral agents remained on oral agents during follow-up. Three were DAA positive, and one had high risk HLA, but no subject in this group was positive for both

DAA and high risk HLA. Two of the three subjects initially treated with oral agents who subsequently required insulin for glucose control were DAA positive, and both had high risk HLA. **Conclusions:** Children clinically classified with T2D or with an atypical presentation for T1D have a high frequency of autoimmune markers and T1D-associated HLA haplotypes. In addition, these autoimmune markers in children with clinical features of T2D appear to be indicators of a more aggressive diabetes disease process, as has been previously shown in children with typical T1D.

#### **OR-60. Development of a Clinical Assay Evaluating Toll-Like Receptor Function.**

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Toll-like receptors (TLR) are transmembrane pattern recognition proteins that participate in innate immune responses. A number of genetic defects influencing the function of these receptors have been identified and are associated with recurrent and/or severe infection. Our goal was to develop a reproducible assay of TLR response for evaluation of TLR function in patients with recurrent infection. Peripheral blood mononuclear cells (PBMC) were isolated and incubated with ligands for TLRs 1/2, 2/6, 3, 4, 5, 6, 7 and 9. Tumor necrosis factor (TNF) in cell supernatant was then evaluated by enzyme-linked immunosorbent assay (ELISA) as a measure of immune response. Optimal concentrations of, and mean responses to, individual ligands were established in healthy adult control donors. A number of variables were assessed that could affect the assay, including blood anticoagulant, blood storage time, PBMC cryopreservation, assay media, and incubation period. The assay was most reproducible in media containing fetal bovine serum; neither serum-free nor human serum-containing media could be effectively substituted. Cryopreserved PBMCs resulted in a considerably higher TNF production in response to most ligands than freshly isolated cells (31% mean increase amongst all ligands tested). Using optimized assay conditions, three patients with a mutation in the *IKBKKG* gene encoding the NF-kappaB essential modulator (NEMO) protein were ultimately studied as disease controls. TNF responses in patients with *IKBKKG* mutations predicting C417Y, L153R, and exon 9 deletion alterations of NEMO were  $\geq$  6.0%, 12.0% and 8.0% of their corresponding controls, respectively. Although a number of variables influence TNF TLR responses this assay can be optimized for clinical use in screening for patients affected by primary immune deficiencies influencing TLR function.

#### **Tolerance**

3:30 PM–5:30 PM, 5/14/2005

#### **OR-61. Tolerance to Inhaled Proteins Does Not Develop in the Presence of Proteinase-Activated Receptor-2 (PAR-2) Activation.**

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**Aim of Study:** Inhalation of innocuous proteins induces the development of tolerance, an immune response characterized by the development of T<sub>reg</sub> cells and absence of airway hyper-responsiveness (AHR) or airway inflammation. The reason why some proteins promote allergic sensitization instead of tolerance

has been studied extensively but is still controversial. Many allergens possess serine proteinase activity. Some of these allergens/serine proteinases, such as those from house dust mites and cockroaches, can mediate their effects through the Proteinase-Activated Receptor-2 (PAR-2). We have previously shown that exogenous PAR-2 activation during allergen challenge enhances allergen-mediated AHR and airway inflammation. Our current hypothesis is that PAR-2 activation at the time of encounter with an inhaled protein may mediate allergic sensitization by preventing the development of tolerogenic responses. **Methodology:** We used a Balb/c mouse model of tolerance to intranasal (i.n.) administered ovalbumin (OVA). Balb/c mice were administered OVA (100 $\mu$ g) i.n. on 3 consecutive days. Other mice were administered OVA with the PAR-2 activating peptide (PAR-2AP) SLIGRL-NH<sub>2</sub> to mimic inhaled allergens with PAR-2 activating potential. Control mice received OVA with the PAR-2CP (control peptide) LSIIGRL-NH<sub>2</sub> or were administered saline alone. All mice subsequently received an interperitoneal (i.p.) immunization with OVA and Al(OH)<sub>3</sub> ten days after the last i.n. administration. Five days following this immunization, mice were euthanized and T cells were isolated from the spleen and cultured *in vitro* with antigen presenting cells, from a naive mouse, and OVA for 4 days and proliferation assessed. Other groups of mice were challenged twice with OVA on alternate days, 10 days after the i.p. immunization, and assessed for AHR and eosinophilic inflammation in the lung the day after the last challenge. **Results:** T cells isolated from mice treated initially with OVA alone or with PAR-2CP proliferated poorly to OVA *in vitro*, indicating the development of tolerance to OVA while T cells from mice treated with saline alone or OVA and PAR-2AP proliferated vigorously. Furthermore, mice treated initially with saline alone or OVA and PAR-2AP developed AHR and eosinophilic inflammation following OVA challenge while mice treated with OVA alone or with PAR-2CP showed no signs of either. **Conclusions:** PAR-2 activation in the airways prevents the development of tolerogenic responses towards i.n. administered OVA. These observations indicate that inhaled allergens/serine proteinases may induce allergic sensitization through the activation of PAR-2 in the airways.

#### **OR-62. Nasal Vaccination with a Proteasome-Based Adjuvant and Glatiramer Acetate Clears Alzheimer's $\beta$ -Amyloid in an Antibody-Independent Fashion.**

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Alzheimer's disease (AD) is the most common form of senile dementia, affecting more than 18 million people worldwide. Compact senile plaques comprised of  $\beta$ -amyloid ( $\beta$ ) fibrils are associated with pathological changes in the surrounding brain neurons, leading to their death. Immune therapy in mouse models of Alzheimer's disease (AD) via Amyloid  $\beta$  ( $\beta$ ) immunization or passive administration of  $\beta$  antibodies markedly reduces  $\beta$  levels and reverses behavioral impairment. However, a human trial of  $\beta$  immunization led to meningoencephalitis in some patients and was discontinued. Here we show that nasal vaccination with a proteasome-based adjuvant (IVX-908) that is well tolerated in humans mixed with glatiramer acetate (GA), a synthetic copolymer used to treat multiple sclerosis, potently decreases  $\beta$  plaques in an AD mouse model. Nasal administration of glatiramer acetate with

IVX-908 resulted in an 84% reduction of thioflavin S -positive fibrillar amyloid in the hippocampus ( $p < 0.001$ ) and 73% reduction of total brain A $\beta$  levels ( $p < 0.001$ ). vs. the non treated mice. This effect did not require antibody, as it was observed in B-cell deficient mice. Vaccinated animals developed activated microglia (CD11b+ cells) that co-localized with A $\beta$  fibrils, and the extent of microglial activation correlated strongly with the decrease in A $\beta$  fibrils. We also noticed a strong correlation between CD11b+ cells and IFN- $\gamma$  secreting cells and increased numbers of T cells ( $r = 0.9$ ), which may play a role in promoting microglial activation. Our results define an antibody-independent therapeutic approach for the treatment of Alzheimer's disease, utilizing compounds that have been safely tested or are currently in use in humans.

#### **OR-63. Collaboration between Central Tolerance and Peripheral Regulation To Control Autoimmunity.**

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A variety of mechanisms have been proposed for immunological tolerance of self tissue. Prominent roles have been attributed to central tolerance, illuminated by the role of aire in thymic deletion of autoreactive T lymphocytes, and peripheral regulation, exemplified by the function of Foxp3 in generating CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells to suppress pathogenic effectors. Here we report fulminant autoimmunity in very early life and a gravely shortened life-span in mice deficient in both aire and Foxp3 vis-à-vis animals lack either aire or Foxp3. The exacerbated inflammatory damage in the aire and Foxp3 double-deficient animals is particularly prominent in the lung and liver, and also involves several other organs but, despite massive lymphoproliferation, little or no inflammatory infiltration occurs in many other organs, possibly protected by aire- and Foxp3-independent mechanism(s). This study highlights the critical importance of both central tolerance and peripheral regulation in maintaining self-tolerance and suggests that there is still more to learn.

#### **OR-64. Mechanisms of Retinal Immune Privilege.**

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The eye is an immune privileged tissue that constitutively produces neuropeptides, cytokines, and factors that actively suppress inflammation mediated by innate and adaptive immunity. The neural retina (NR) and retinal pigmented epithelial (RPE) cells are documented to support immune privilege; however, most of our understanding of the mechanisms of ocular immunity has been from analyzing the anterior chamber of the eye. Therefore, to begin to identify factors important in regulating immunity in the retina, we investigated the effects of secreted factors from the NR and the RPE on the activity of resting and activated macrophages. The NR from healthy C57BL/6J mice was dissected and placed in culture for 24 hours. Culture media was placed into the resulting posterior eye cup containing RPE and incubated for 24 hours. These conditioned media (CM) was used to treat resting or endotoxin-stimulated macrophages (J774A.1 cell line). After 48 hours, the macrophage supernatants were assayed by multiplex analysis for IL-1beta, IL-6, IL-10, TNF-alpha, GM-CSF. In addition, the RPE-CM and NR-CM were assayed by multiplex analysis. We found IL-6, GM-CSF in RPE-CM, and IL-6 in the NR-CM. The NR-CM stimulated GM-CSF production by resting macrophages, but was

suppressed in endotoxin-stimulated macrophages. Both the RPE-CM and NR-CM suppressed IL-1beta and TNF-alpha production by the endotoxin-stimulated macrophages, while they induced significant levels of IL-10 production by the macrophages. While the NR may support macrophage differentiation through GM-CSF, both the NR and the RPE suppress the production of inflammatory cytokines (IL-1beta and TNF-alpha) by endotoxin-stimulated macrophages, while causing the same macrophages to produce the anti-inflammatory cytokine, IL-10. Such results suggest that the mechanisms of immunosuppression by the retina may involve factors that suppress classical activation while promoting alternative activation of macrophages. Therefore, both the neuroretina and RPE contribute to the mechanisms of retinal immune privilege.

Supported by grants from the USAMRMC.

#### **OR-65. Treatment with a Donor-Specific Transfusion and Anti-CD154 mAb Induces Non-Responsiveness in a Population of T Cells That Recognize Alloantigen Via Indirect Antigen Presentation.**

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Treatment with a donor-specific transfusion (DST) and anti-CD154 monoclonal antibody (mAb) induces prolonged allograft survival in mice, in part by deleting host CD8<sup>+</sup> T cells that recognize alloantigen by direct antigen presentation. The fate of host T cells that recognize alloantigen via indirect antigen presentation in mice treated with costimulation blockade is unclear. In this study, we investigated the fate of Tg361 TCR transgenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells that recognize alloantigen via indirect antigen presentation. Using CFSE-labeling, we first document that both Tg361 transgenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells proliferate *in vitro* and *in vivo* in response to allo-stimulation. Treatment of mice circulating Tg361 T cells with DST plus anti-CD154 mAb, however, fails to delete these CD4<sup>+</sup> and CD8<sup>+</sup> alloreactive T cells, and instead renders them non-responsive to re-challenge with alloantigen. Mice circulating these non-responsive alloreactive T cells also fail to reject skin allografts. The non-responsive state of Tg361 T cells is not reversed by the addition of IL-2, anti-CD28 mAb, or an agonistic anti-CD134 mAb in the presence of antigen, protocols that have been successful in reversing both the clonal and adaptive anergic states of tolerated CD4<sup>+</sup> cells. The non-responsive CD4<sup>+</sup> and CD8<sup>+</sup> alloreactive T cells are capable of activation, however, as evidenced by their robust *in vitro* proliferation in response to anti-CD3 mAb in the presence of costimulation. These data document a non-deletional mechanism by which costimulation blockade can block host alloreactive T cell responses and prolong graft survival.

#### **OR-66. Successful Induction of Mixed Chimerism and Tolerance with Non-Myeloablative Conditioning in Mice Presensitized with Fully Mismatched Skin Grafts.**

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Presensitization to donor antigens that may arise from prior transplants, blood transfusions or pregnancy excludes many patients from solid organ transplants. Inducing donor-specific tolerance in presensitized recipients would therefore represent a significant clinical advance. In presensitized patients, anti-donor antibodies could potentially be removed by plasmapheresis prior to transplantation; however this is not practical in mice. Therefore, we



have used B cell deficient mice to evaluate the ability to tolerize presensitized T cells via mixed chimerism induction through bone marrow transplantation (BMT). B cell deficient B6- $\mu$ MT and wild-type B6 (H-2<sup>b</sup>) mice, presensitized with B10.A (H-2<sup>b</sup>) tail skin, received non-myeloablative conditioning (anti-CD4, CD8, NK, CD40L & OX40L monoclonal antibodies and 3 Gy total body irradiation) before BMT with  $80 \times 10^6$  B10.A bone marrow cells (BMC). Twelve weeks after rejection, the presence of anti-donor IgG in the serum of the presensitized B6 mice was confirmed by indirect staining and flow cytometry. Multi-lineage chimerism was monitored at 2, 4, 6, and 12 weeks post-BMT by flow cytometry. All of the presensitized wild-type B6 mice rejected the donor BMC by 2 weeks. Thus, anti-donor IgG antibodies present in wild-type mice after presensitization posed a strong barrier to donor marrow engraftment. In contrast, 80% of the  $\mu$ MT mice showed engraftment of donor BMC with stable long-term multi-lineage donor chimerism. Importantly, chimeric  $\mu$ MT mice accepted donor grafts long-term, while third party grafts were rejected by 2 weeks. Thus our results show that pre-existing T cell immunity to BMC donor antigens may be overcome by non-myeloablative mixed chimerism induction in presensitized mice.

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#### OR-67. Dendritic Cell Response to Necrosis Is Defective in Atopy.

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We have previously shown that cellular necrosis augments CD40-mediated interleukin-12 secretion by human monocyte-derived dendritic cells. In the present study, we compare the dendritic cell response to cellular necrosis in atopic individuals with non-atopic control subjects. Using an *in vitro* culture system, monocyte-derived dendritic cells were stimulated with either necrotic K562 cells or a combination of TNF-alpha and IL-1beta. We demonstrate that dendritic cells from atopic individuals secreted significantly less interleukin-12p40 in response to necrotic cell products compared with dendritic cells from non-atopic subjects. Upon stimulation with necrotic cells and CD40 cross-linking, dendritic cells from atopic subjects secreted significantly less interleukin-12p70. Furthermore, CD80, but not CD86, was upregulated by necrosis significantly less on dendritic cells of atopic individuals compared with normal subjects. In contrast, the response of dendritic cells from atopic subjects to TNF-alpha and IL-1beta was not significantly different from normal individuals. We conclude that atopy is associated with a defective response of dendritic cells to necrotic cell death, which may play a role in the mechanism of atopic sensitisation.

#### OR-68. CD150 (SLAM) Modulates TLR and CD40 Pathways in Monocyte-Derived Dendritic Cells.

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SLAM (CD150, Signaling Lymphocyte Activation Molecule) is a self-ligand receptor on the surface of activated T- and B-lymphocytes, macrophages and dendritic cells (DC). Its importance at the interface of adaptive and innate immune responses is underscored by SLAM being a receptor for Measles virus, which induces immunosuppression. Moreover, recent reports indicated that high expression levels of SLAM in T-lymphocytes of patients infected with *M. tuberculosis* or *M. leprae* were associated with milder pathology.

As the function of SLAM on the surface of human dendritic cells is poorly understood, we examined the effect of SLAM/SLAM interactions on activation signals in human peripheral blood monocyte-derived dendritic cells. The effect of SLAM on CD40L-induced DC activation was analyzed in co-cultures of DC with L929 cells expressing CD40L alone or in combination with SLAM. CD40L-induced IL-12 production was strongly inhibited by SLAM engagement and resulted in a DC phenotype that was less potent to induce differentiation of naive T lymphocytes into IFN-g producing Th1 effector cells. Interestingly, the ability of these "SLAM-educated" DC to support the proliferation of naive T cells was significantly increased. To determine the effect of SLAM on different TLR-induced DC activation, L929 cells or L929 cells expressing SLAM were co-cultured with immature dendritic cells in the presence of LPS or poly-IC. Unlike CD40L-induced IL-12 production, TLR induced IL-12 secretion was augmented by SLAM engagement. In DC activated by CD40L and LPS, SLAM engagement reduced IL-12 production to the level of cultures activated by LPS and SLAM, indicating that SLAM modulates these pathways independently.

Thus, our findings suggest a dual function for SLAM in monocyte-derived DC that allows SLAM to exert opposing effects on IL-12-dependent functions, based on the received activation signals.

#### OR-69. Activation of Human NK Cells by Plasmacytoid DC and Its Modulation by CD4<sup>+</sup> T Cells and CD25<sup>hi</sup> T Regulatory Cells.

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**Background:** Plasmacytoid dendritic cells (pDC) represent a specialized cell population that produce type I interferon in response to virus. However, although pDC-derived type I interferon is a potent modulator of NK cell functions and NK cells are essential for antiviral immunity, the role of pDCs in coordinating NK cell functions has not yet been elucidated in detail, especially in humans.

**Objective of the study:** to investigate the interplay between human pDC and NK cells and to evaluate how CD4<sup>+</sup> Th and CD25<sup>hi</sup> T regulatory (Treg) cells can modulate these interactions.

**Methods:** Highly purified FACS-sorted human pDC, NK cells, CD4<sup>+</sup> CD25<sup>hi</sup> Treg cells and CD4<sup>+</sup> CD25<sup>neg</sup> T cells were co-cultured and NK cells were analysed for CD69 expression, for proliferation after staining with CFSE, for cytokine production using Bioplex and for cytotoxicity in 4 h-<sup>51</sup>Cr release assay. pDC were activated with IL-3 and CpG-A ODN2216 before co-culture with NK cells.

**Results:** pDC, following engagement of TLR9, can activate autologous NK cells, as indicated by the induction of surface CD69 expression. Under these conditions, pDC can also enhance NK cell effector functions, including cytotoxicity and cytokine production. Moreover, they can induce proliferation of CD56<sup>bright</sup> CD16<sup>-</sup>, but not of CD56<sup>dim</sup> CD16<sup>+</sup> NK cells. This activity can be highly up-regulated in an IL-2-dependent fashion by autologous CD4<sup>+</sup> CD25<sup>-</sup> T cells. Strikingly, CD4<sup>+</sup> CD25<sup>hi</sup> Treg cells can inhibit proliferation of NK cells induced by the interplay of pDC and T helper cell, while they do not influence NK cell activation or proliferation induced by pDC alone.

**Conclusions:** This is the first demonstration in humans that pDC can activate NK cells, enhance their effector functions and induce proliferation. In addition, it is the first demonstration that CD4<sup>+</sup> Th and CD25<sup>hi</sup> Treg cells can modulate NK cell proliferation, implying a direct role of adaptive immune response in amplifying or inhibiting innate immunity.

### **OR-70. Hepatitis C Virus Core Protein Induced, Monocyte-Mediated Mechanisms of Reduced IFN $\alpha$ and Plasmacytoid Dendritic Cells Loss in Patients with Chronic HCV Infection.**

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Immune responses to acute hepatitis C virus infection are insufficient in most individuals leading to chronic infection in the majority of infected individuals. Current successful therapies of HCV infection are based on use of IFN $\alpha$ . Type I interferons (IFN), including IFN-alpha (IFN $\alpha$ ), inhibit viral replication and promote antiviral immune responses. Limited and controversial information is available related to the capacity HCV-infected patients to produce endogenous IFN $\alpha$ .

The purpose of this study was to investigate the functional capacity of plasmacytoid dendritic cells (PDCs), the main producers of IFN $\alpha$ , in patients with chronic HCV infections compared to controls. We found significantly decreased production of IFN $\alpha$ , determined by ELISA, in PBMCs of HCV patients upon stimulation with PDC-specific TLR9 ligands, CpG-A DNA ( $p < 0.003$ ) and HSV KOS ( $p < 0.004$ ). This correlated with a decreased frequency of circulating PDCs (determined by flow cytometry as lineage-/CD4<sup>+</sup> or BDCA2+/CD123<sup>+</sup>) in HCV patients compared to controls (HCV 0.11  $\pm$  0.09%, control 0.25  $\pm$  0.15%;  $p < 0.003$ ). PDCs purified from HCV patients produced lower levels of IFN $\alpha$  compared to controls ( $p < 0.009$ ) and were apoptotic, as determined by staining with Annexin V and DNA laddering. In vitro stimulation with CpG-A DNA or HSV KOS lead to increased cell death in PDCs from HCV patients compared to controls ( $p < 0.01$ ). There was no correlation between the loss of PDCs and HCV viral count, genotype, or liver functions. Importantly, there was no reduction in PDC frequency or IFN $\alpha$  production in patients with sustained virological response after HCV elimination therapy suggesting a role for viral derived mechanisms for the DC defects. We found that recombinant HCV core protein did not directly affect PDC functions, but it significantly reduced TLR9-triggered IFN $\alpha$  production in PBMCs ( $p < 0.001$ ). We determined that HCV core protein activated monocytes to produce IL-10 and TNF $\alpha$ . In vitro both IL-10 and TNF $\alpha$  induced PDC apoptosis and inhibited IFN $\alpha$  production in normal PDCs. Anti-IL-10 and anti-TNF $\alpha$  neutralizing antibodies were additive in restoration of IFN $\alpha$  production in TLR9+HCV

core stimulated PBMCs. Depletion of monocytes from PBMCs abolished IL-10 and TNF $\alpha$  production and prevented HCV core-induced inhibition of PDC IFN $\alpha$  production.

Our results show that HCV core protein modulates host's ability to produce IFN $\alpha$  by indirectly interfering with PDC function via monocyte-derived cytokines. We reveal that the viral-induced mechanisms of PDC loss and IFN $\alpha$  production defects are likely to contribute to chronic viral persistence and may provide mechanistic explanation for the therapeutic benefits of IFN $\alpha$  therapy in HCV infection.

### **OR-71. Delayed IL-10 Induced Human Tolerogenic DC Inhibit Naive T Cell Proliferation by Mechanisms Other Than Their Exaggerated PD-L1/2 Induction.**

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Previous data indicated that IL-10 treated human monocytes (MO) differentiate to macrophage (Mac) expressing elevated Mac markers like MRP8/4 while addition of IL-10 to monocytes after their partial differentiation to dendritic cells (MO IL-4 & GM-CSF 3 days then IL-10 added for additional 2 days) induces a tolerogenic dendritic cell (tol DC) which can diminish T cell activation. The mechanisms for tolerogenic DC inhibition of T cells is still undefined, but is suggested as related to upregulation of inhibitory costimulation ligand-receptor combinations. Here, tol DC were generated from 7 control donors' MO by adding IL-10 after 3 days of culture of MO with IL-4 & GM-CSF and then inducing for an additional 2 days. Addition of IL-10 to the DC differentiation cultures did not significantly decrease DC CD1a levels (68  $\pm$  15% positive) versus those of IL-4+GM-CSF classic DC controls (76  $\pm$  14% positive), or their CD209 levels (97  $\pm$  1 versus 98  $\pm$  1% positive) nor increase Mac characteristics (CD14 5  $\pm$  2 versus 3  $\pm$  4% positive, or MRP8/14 <1% positive expression). However, tol DC expression of the inhibitory costimulatory ligand PD-L1 but not PD-L2 was significantly ( $< 0.0001$ ) increased from the 13  $\pm$  5% of classic DC to 36  $\pm$  9% positive. PD-L1 but not PD-L2 mean fluorescence intensity (MFI) was also significantly increased on tol DC. We have previously shown that tol DC are unable to act as adequate antigen presenting cells (APC). To assess the inhibitory activity of these tol DC, they were added to autologous T cell cultures in the presence of immobilized anti CD3 plus CD28 (iCD3+CD28). Since iCD3+CD28 stimulate T cell proliferation in the absence of any APC, any diminished proliferation in the presence of tol DC would indicate a T cell inhibitory rather diminished APC effect. The addition of 2  $\times$  10<sup>4</sup> tol DC significantly ( $P = 0.035$ ) reduced T cell proliferation to anti iCD3+CD28 as compared to T cell cultures with classic or no DC. However, there was no correlation between the degree of increased tol DC PD-L1 expression and then mediation of decreased T cells proliferation. Tol DC with the highest increases in PD-L1 did not exhibit the highest inhibitory activity. These data suggest that the tol DC function of reducing autologous naive T cell proliferation to T cell receptor stimulation resulted from induction of other inhibitory costimulatory receptors (ILT3/4) rather than the induction of PD-L1 or PD-L2. Nevertheless, the IL-10 induced augmentation of DC PD-L1 expression may still contribute to T cell inhibition and anergy induction when tol DC interact with previously activated T cells whose upregulated PD-1 levels can be triggered by exaggerated tol DC PD-L1 levels.

### OR-72. Induction of Heme Oxygenase-1 (HO-1) Inhibits Dendritic Cell Differentiation and Adaptive Immunity.

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The strong immunosuppressive potency of the stress protein HO-1 has been proven in several models of autoimmunity and transplantation. The underlying immune mechanisms, however, are poorly characterized. In our study, the potent HO-1 inducer Cobalt Protoporphyrin IX (Co-PPIX) strongly suppressed T cell proliferation in mixed lymphocyte reaction (MLR). As possible mechanism we demonstrated a selective Co-PPIX induced increase of HO-1 expression in monocytes associated with depression of accessory molecule expression and stimulatory cytokine secretion. The likewise induction of HO-1 in monocyte-derived dendritic cells (MDDC) by Co-PPIX was associated with an almost complete inhibition of the differentiation, maturation, and function of MDDC. So, a strong decrease of the expression of DC markers (CD1a, CD83) and accessory molecules (HLA-DR, CD86) was observed. Whereas IL-12 secretion was inhibited, IL-10 production increased. The antigen-presenting capacity of Co-PPIX treated MDDC was strongly diminished in lymphocyte transformation assay and MLR. The specificity of these effects was demonstrated by HO-1 transduction in immature MDDC. Together these changes indicated a switch of the DCs to an immature, non-stimulatory phenotype. *In vivo*, Co-PPIX treatment before challenge dose-dependently depressed ear inflammation in DNFB (Type 1) and TMA (Type 2) induced contact dermatitis in mice. Remarkably, Co-PPIX even more strongly inhibited T-cell-dependent inflammation when applied around sensitization. We hypothesize that the inhibition of DC differentiation, maturation, and function is a crucial mechanism for the suppression of adaptive immunity by HO-1 induction *in vitro* and *in vivo*.

### OR-73. Donor-Specific Allograft Tolerance by Administration of Recipient-Derived Immature Dendritic Cells and Suboptimal Immunosuppression.

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**Introduction.** The benefits of allogeneic immature bone marrow-derived dendritic cells (iBMDCs) on allograft survival have been reported in several studies. However, in contrast to protocols based on the injection of donor-derived DCs, the administration of recipient-derived DCs would be much more applicable to cadaveric organ transplantation. We recently showed that injection of recipient-type iBMDCs the day before transplantation induced a significant prolongation of allograft survival. In the present study, we aimed at improving this protocol to induce allograft tolerance.

**Methods.** iBMDCs were generated from LEW.1A rat bone marrow precursors with low-dose GM-CSF and IL-4. After 8 days of culture, adherent cells displayed immature phenotype, characterized by low MHCII and CD86 expression. Various amounts of iBMDCs (3 to 15 × 10<sup>6</sup> cells) were administered i.v. to syngeneic LEW.1A rats before and after transplantation of an allogeneic LEW.1W heart, with or without additional suboptimal immunosuppression, consisting of Rapamycin (0.4mg/kg/day, d0-d14, orally) or LF15-0195, a new deoxyspergulin analog (1.5mg/kg/day, d0-d9, i.p.).

**Results.** Allograft survival was not improved by repeated injections of syngeneic iBMDCs, or by additional treatment with low dose Rapamycin. Interestingly, combining injection of

iBMDCs and LF15-0195 had striking synergistic effect, and induced definitive allograft acceptance in 92% of recipients. Tolerant iBMDC-LF15-0195 treated recipients accepted donor-type, but not third party-type skin grafts, demonstrating that tolerance was donor-specific. We hypothesized that under LF15-0195 treatment, iBMDCs could maintain their immature phenotype and function. Indeed, we showed that, *in vitro*, LF15-0195 decreased MHCII and CD86 expression in rat BMDCs. The effects of LF15-0195 treatment on the *in vivo* maturation of the administered iBMDCs are currently under investigation.

**Conclusions.** Thus, we demonstrated that donor-specific allograft tolerance can be induced by a single injection of syngeneic iBMDCs one day prior to transplantation, and a suboptimal immunosuppressive treatment with LF15-0195. The reported findings may contribute to the development of new therapeutic strategies to induce transplantation tolerance in clinical settings.

### OR-74. Impact of Plasmacytoid Dendritic Cell (PDC) Recovery on Outcome after Allogeneic Stem Cell Transplantation (allo-SCT).

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Among DC subsets, the reconstitution of the natural type I-interferon-producing PDCs has been proposed to play a major role in establishing immune competence. Therefore, we investigated the impact of circulating PDCs measured at the 3rd month after reduced intensity conditioning (RIC, fludarabine-based conditioning) allo-SCT, in 54 patients with hematological and non-hematological malignancies who received a RIC-allo-SCT from an HLA-identical sibling, in order to determine whether this could provide an indicator for long term outcome. The median absolute count of PDCs measured at 3 months was 0.725/μL (range, 0–23.2). In a multiple logistic regression analysis including all relevant parameters (demographic and graft characteristics, RIC regimens, CMV infections, and acute GVHD), only the absence of grade II-IV acute GVHD was associated with an improved PDC recovery at 3 months ( $P = 0.003$ ; OR=6.4; 95%CI, 1.9–22). Being the major type I IFN-secreting cells, we also investigated whether PDCs recovered after allo-SCT are functional in response to viral stimulation. Patients experiencing grade 0-I aGVHD could secrete significantly higher amounts of IFN-alpha as compared to patients with grade II-IV aGVHD (mean, 91 vs. 0 pg/ml respectively;  $P = 0.002$ ), likely highlighting the deleterious impact of corticosteroids therapy on PDC function. The CD34+ stem cell dose and other lymphoid subsets infused with the allograft did not affect PDC recovery. Though PDC count could not predict death from progression or relapse, patients with a “high” PDC recovery profile had an improved overall survival (OS;  $P = 0.03$ ), in contrast to patients with a “low” PDC recovery profile who had an increased incidence of late transplant-related mortality (GVHD, infections) ( $P = 0.03$ ). In addition, the overall incidence of late infections (viral, fungal and bacterial) was significantly higher in the “low” PDC recovery group as compared to the “high” PDC recovery group (59% vs. 19%;  $P = 0.002$ ), illustrating the importance of PDCs in anti-infectious immune responses. In a multivariate analysis, only a “high” PDC count was significantly predictive of a decreased risk of death ( $P = 0.04$ ; RR=0.34; 95%CI, 0.12–0.96). The role of rare immune effector cells would tend to be more evident in truly RIC and less toxic regimens. In this study, we could show that monitoring of PDCs may be useful for patients’ management

(closer surveillance, infection prophylaxis...), and may have a significant impact on the probability of a favorable outcome in the context of RIC-allo-SCT.

## Immunodeficiency

3:30 PM–5:30 PM, 5/14/2005

### OR-75. Functional Interaction of Common Gamma Chain and Growth Hormone Receptor Signaling Apparatus.

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Severe combined immunodeficiency syndromes (SCIDs) are characterised by absent T- and B-lymphocytes function. SCIDs are usually fatal early in infancy without successful immune reconstitution. We previously reported on a patient affected with an X-linked SCID, who received a late Bone Marrow Transplantation at 5,2 years of age. During the follow-up a short stature due to peripheral Growth Hormone (GH) hyporesponsiveness and abnormalities of the protein phosphorylation events following GH receptor (GHR) stimulation were observed. Mutational screening and expressional analysis failed to reveal any molecular alteration of GHR, JAK2 and STAT5a/b genes. Since we hypothesized a role for the  $\gamma$  chain in GHR signaling, in this study we evaluate GHR/ $\gamma$  element functional interaction in EBV transformed lymphocytes (BCLs) from X-SCID PTs and CTRs. The functional response to GH, the pattern of GHR induced P<sub>Tyr</sub> and STAT5 nucleus translocation were studied. GH enhanced proliferation of CTRs BCLs in a dose-dependent fashion, with a maximal effect at 200 ng/ml. In contrast, PTs cells did not proliferate at all. Cytofluorimetric analysis did not reveal any difference in GHR expression. In PTs' cells, GH stimulation failed to induce phosphorylation of proteins of 90–92 kDa corresponding to STATs molecules in contrast to what observed in CTR, in which a peak of STAT5 phosphorylation between 5 and 15 min was observed. In all cell lines examined, STAT5a and b protein expression was comparable. In addition, in CTR cells GH induced nuclear translocation of STAT5 evaluated through confocal microscopy; in contrast, in PTs cells no efficient translocation occurred after GH stimulation. Here we report a previously unappreciated functional interaction between  $\gamma$ c and GHR, which eventually leads to the activation and intranuclear translocation of STAT5 protein.

### OR-76. Novel Humoral Immunodeficiency in Humans Associated with Deleterious Homozygous Mutation in CD19.

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In B cells, CD19 is found in a complex with the complement receptor CD21, the tetraspan membrane protein CD81, and CD225, and is critical both to balance antigen-induced BCR-

mediated signaling thresholds in B cells and to functionally link CD21 with the BCR following co-recognition of C3d-bearing Ag. CD19 is a 95 kd transmembrane protein with two extracellular Ig domains and a cytoplasmic tail containing several tyrosine residues that become phosphorylated after cross-linking of the BCR, allowing the interaction with SH2-containing cytoplasmic proteins and linking CD19 to downstream signaling cascades. In mice, mutations in CD19 lead to hypogammaglobulinemia, impaired B-cell memory, low CD5<sup>+</sup>/B1 B cells and decreased germinal center formation. In humans, a selective defect in CD19 expression has not yet been described. Here we present three adult siblings (one male and two females) affected with recurrent respiratory and gastrointestinal tract infections since childhood and low serum IgG, IgA and IgM. Peripheral blood CD20<sup>+</sup>CD22<sup>+</sup> B cells were within normal ranges by FACS, but showed profoundly reduced surface expression of CD19 in all three patients. DNA sequencing of CD19 revealed a homozygous deletion of two nucleotides in exon 11 (c.1428delAG), leading to a frameshift and a premature STOP codon in all patients. Six relatives in the family were heterozygous. B-cell subpopulations in PBL showed significant decreases in isotype-switched memory cells (CD27<sup>+</sup>IgD<sup>-</sup>) and low CD5<sup>+</sup> cells in all patients. Isohemagglutinins were decreased while soluble CD21 levels were slightly increased in all patients. Tonsil's morphology and cellularity in one CD19-deficient patient were normal (CD3, CD79a, CD20, CD5, Bcl-2, and Ki67) with highly active lymphoid follicles. These results show that mutations in CD19 lead to a novel humoral immunodeficiency in humans, affecting immunoglobulin production and leading to a increased susceptibility to recurrent infections.

Support from Colciencias, CODI, and the Deutsche Forschungsgemeinschaft (DFG).

### OR-77. Mutations in TACI Are Associated with Immunodeficient Phenotypes in Humans.

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Background: Transmembrane activator and CAML interactor (TACI) is a TNFR superfamily member, functionally related to BCMA and BAFFR as evidenced by the sharing the ligands BAFF and APRIL. Mice deficient in TACI exhibit a fatal autoimmune disease and lymphoproliferation, which contrasts the simultaneous inability of TACI <sup>-/-</sup> mice to mount robust T cell independent antibody responses. This coincidence of autoimmunity, lymphoproliferation and signs of immunodeficiency is well recognized in patients with common variable immunodeficiency (CVID), thus leading us to investigate TACI as a functional candidate gene in CVID.

Methods/Results: In a cohort of 16 families displaying autosomal recessive inherited CVID we found two distinct homozygous genetic defects in two unrelated families with three

affected individuals. The mutation in the first family affected a highly conserved cysteine residue (C104R) in the extracellular domain of TACI. The mutation observed in the second family was a nonsense mutation at position 144 (S144X) leading to a putative truncated TACI protein consisting only of its extracellular domain. We then extended our screening to patients with sporadic CVID and found 11 out of 139 patients, who carried a heterozygous mutation. Two of these affected the conserved cysteine residue (C104R), seven were located within the transmembrane region (A181E) and two were in the intracellular part of the protein (S194X and R202H). FACS staining of patients' B cells with heterozygous mutations revealed normal TACI surface staining. After stimulation with common mitogens (IgM, IL-2, CD40, IL-4) B cells from patients with mutations in TACI proliferated normally. A tonsil biopsy in one of the patients revealed prominent enlarged germinal centers with hypercellularity of B cells. Clinically the patients presented with hypogammaglobulinemia, especially low IgM, and displayed signs of lymphoproliferation and autoimmunity at a very high frequency.

**Conclusions:** In our evaluation of TACI as a candidate gene in patients with CVID we found both homozygous and heterozygous mutations in familial and sporadic CVID cases. Three mutations lead to substitution of highly conserved amino acids and two are nonsense mutations. The human TACI-deficient phenotype consists primarily of a humoral immunodeficiency and thus differs from the murine model, however, signs of autoimmunity and lymphoproliferation are also evident.

#### **OR-78. Expansion of Maternally Derived Monoclonal T Cells with a CD3 + CD8 + TCR $\gamma\delta$ Phenotype in Two Children with Severe Combined Immunodeficiency.**

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Maternal engrafted T cells occur in patients with SCID to a variable extent often with absence of clinical signs of GVHD. In this report we describe maternally engrafted TCR $\gamma\delta$  cells in two children (RK & JR) with Artemis and common chain deficiencies respectively. Both children had no clinical symptoms of GVHD. The purpose of this study was to characterise these populations in detail. Peripheral blood from both children was phenotyped and functionally characterised by response to mitogens. Both children were delivered at term after a normal pregnancy and were vaccinated according to protocol; in addition RK received BCG at birth. Both presented at 5 months with PCP, low serum immunoglobulins, lymphopenia and failure of lymphocytes to respond to mitogens. RK presented with a T lymphocyte count of  $1.1 \times 10^9/l$  positive for TCR $\gamma\delta$ , CD3, CD8 and CD45 R0. No B cells were found. JR presented with a T lymphocyte count of  $1.1 \times 10^9/l$  positive for TCR $\gamma\delta$ , CD3, CD4, CD8, CD45R0 and CD45RA. B cells numbered  $0.6 \times 10^9/l$ . DNA was isolated from peripheral blood cells and amplified with Biomed primers to TCR $\gamma$  and TCR $\delta$ . In both children clonal TCR $\gamma$  and TCR $\delta$  rearrangements were found compatible with TCR $\gamma\delta$  cells utilising TCR V $\gamma$ -J $\gamma$ 1.3/2.3 and TCR V $\delta$ 1-J $\delta$ 1, TCR V $\gamma$ -J $\gamma$ 1.2 and TCR V $\delta$  genes respectively. We present evidence in two children with significantly raised TCR $\gamma\delta$  populations of maternal origin in blood. While neither of the children had clinical evidence for GVHD, RK who received BCG at birth experienced BCG lymphadenitis, skin

and gut biopsies from this child were found to have infiltrates of TCR $\gamma\delta$  cells that were shown to be clonally identical to the TCR $\gamma\delta$  cells in blood. In this case it is possible that the clone arose in direct response to BCG vaccination at birth, however, cells were not investigated from mother for their response to BCG. These data confirm a previous report where clonal TCR $\gamma\delta$  cells were found to cross the placenta but failed to initiate GVHD in the neonate. In this child with SCID clonal TCR $\gamma\delta$  cells were also shown to be present in the mother. In this abstract the origin of these cells was not resolved.

#### **OR-79. Semi-Nested Degenerate rtPCR Allows Rapid Identification of TCR-beta Rearrangement on the Single Cell Level.**

*Dun Zhou,<sup>1</sup> Rajneesh Srivastava,<sup>1</sup> Verena Grummel,<sup>1</sup> Sabine Cepok,<sup>1</sup> Hans-Peter Hartung,<sup>1</sup> Bernhard Hemmer.<sup>1</sup>  
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T cell receptor variable beta chain (TCRBV) PCR is frequently used to investigate TCRBV usage in autoimmune diseases, infection and cancer. Because the TCRBV locus contains more than 50 variable regions, a large number of forward primers has to be used to cover all TCRBV segments. Previous studies simplified the TCRBV analysis by performing a multiplex PCR with 26 primers divided into 5 groups. While this approach has been worked out for single cell rtPCR, it is very time consuming and costly and can hardly be applied to large scale screening. To further simplify TCRBV analysis, we established a new semi-nested rtPCR method with two sets of degenerate primers covering 85% and 15% of the TCRBV genes respectively. For single cell analysis, we extended the rtPCR by designing a nested primers located in the TCRBV constant region. The specificity of the primers was confirmed by screening cDNAs from more than 200 T cell clones which were previously defined for their TCRBV usage by flow cytometry and conventional TCRBV rtPCR. We retrieved all TCRBV gene segments comprised in the sample with our primer sets. High sensitivity was demonstrated by successful amplification of rearranged TCRBV genes of single T cells sorted from body fluids or dissected from tissue. This new approach allows fast and cost-effective high throughput analysis of T cell receptor rearrangement at the single cell level facilitating studies on T cell responses in human diseases.

#### **In Vivo Immune Responses and Imaging**

10:30 AM–12:30 PM, 5/15/2005

#### **OR-80. Expanded T Cells from Human Type 1 Diabetic Pancreatic Draining Lymph Nodes React with Insulin A Chain 1–15.**

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A central question in Type 1 diabetes is the identification of autoantigens that trigger pathogenic T lymphocytes associated with specific destruction of insulin producing beta islet cells.

Single T cells were directly cloned with mitogen into 96 well plates from pancreatic draining lymph nodes (PLN) from human subjects with Type 1 diabetes and control subjects, with the generation of over 515 independent T cells clones. We sequenced the T cell receptor alpha and beta chains from these clones: modest or no expansion of TCR chains was seen in PLN from non-diseased subjects, including a subject with Type 2 diabetes, while a high degree of T cell expansion was observed in long-term diabetics, but not a recent onset diabetic with CD4+ T cells infiltrating the islets. Using a candidate antigen screening approach and EBV transformed B cells as antigen presenting cells, clonally expanded T cell clones from PLN from both long-term diabetics responded to insulin A chain 1–15 in the context of DRB1\*0401 and not in the context of DRB1\*0301 or to other insulin, GAD65, or MBP85-99 peptides and was specifically blocked by anti-DR antibody. T cell clones from the PLN of a DR4+ normal, from the cerebrospinal fluid of a DR4+ multiple sclerosis patient, a MBP85-99 reactive T cell clone from the periphery of a multiple sclerosis patient, and non-expanded T cells from one of the long term diabetic DR4+ PLN did not recognize the insulin peptide in the context of DRB1\*0401. Our results are the first to establish T cell clonal expansion in human pancreatic draining lymph nodes from subjects with Type 1 diabetes with the identification of a cognate autoantigen. These experiments demonstrate the feasibility of non-biased T cell cloning from the draining lymph node of an organ targeted in an autoimmune disease to identify a putative autoantigen. Moreover, these results confirm in humans results in the NOD mouse model identifying insulin as a critical autoantigen in diabetes.

### Costimulation and Tolerance

10:30 AM–12:30 PM, 5/15/2005

#### OR-81. Anti-CD28 Antibodies-Induced Transplant Tolerance Involving TCR<sup>+</sup> Class II<sup>-</sup> CD80/86<sup>+</sup> Regulatory Cells and Tryptophan Degradation.

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B7/CTLA-4 interactions negatively regulate T cell responses and are necessary for transplant tolerance induction. Tolerance induction may therefore be facilitated by selectively inhibiting the B7/CD28 pathway without blocking that of B7/CTLA-4. In this study, we selectively inhibited CD28/B7 interactions using the JJ319 modulating anti-CD28 monoclonal antibody in the LEW.1W to LEW.1A rat model of acute kidney graft rejection. An induction treatment (4mg/kg/day from day 0 to 7) abrogated rejection. On the long term (>100 to 300 days), kidney graft function was normal and stable in tolerant recipients with an absence of histological lesions of chronic rejection. Tolerant recipients developed alloantibodies of the Th2-type against donor MHC class II molecules, unlike untreated rejecting controls that developed Th1-type antibodies against MHC class I and II molecules. PBMC and spleen cells from tolerant animals did not proliferate against donor cells in MLR but proliferated against third party cells. However, purified T cells were fully reactive suggesting a regulation of T cells by a non-T cell population. The depletion from PBMC of either CD80 or CD86-positive, non-T cells, fully restored this reactivity whereas the depletion of B cells, CD11b/c<sup>+</sup>, MHC II<sup>+</sup> and CD8<sup>+</sup> cells had no effect. Over represented NK cells expressing CD80/86 were

found partially responsible for this suppressive effect. Anti-donor reactivity could be restored *in vitro* by blocking indoleamine 2,3-dioxygenase (IDO) and iNOS. *In vivo*, pharmacologic inhibition of these enzymes led to the rejection of the otherwise tolerated transplants. This study demonstrates that an initial selective blockade of CD28 generates B7<sup>+</sup> non-T regulatory cells and a kidney transplant tolerance sustained by the activity of IDO and iNOS.

### Living With the Bugs: Good or Bad

10:30 AM–12:30 PM, 5/15/2005

#### OR-82. Immature Myeloid Dendritic Cells Induce Intestinal Granulomas under Certain Environmental Conditions.

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Granulomatous inflammation is a characteristic feature of Crohn's disease. The factors involved in the development of intestinal granulomatous inflammation have not been fully defined yet. T cell receptor a knockout (TCR $\alpha$  KO) mice develop Th2-mediated colitis resembling human ulcerative colitis. Interestingly, the absence of both B cells and IL-4 led to the development of a distinct form of colitis showing transmural granulomatous inflammation (reminiscent of Crohn's disease) in TCR $\alpha$  KO mice (TCR $\alpha$   $\times$  B cell  $\times$  IL-4 triple knockout mice:  $\alpha\mu$ IL4 TKO mice). In contrast, granulomas were not detectable in IL-4-deficient TCR $\alpha$  double knockout ( $\alpha$ IL4 DKO) and B cell-deficient TCR $\alpha$  ( $\alpha\mu$ ) DKO mice. Colitis in TCR $\alpha$  KO mice is characterized by a marked increase of IL-4 production, whereas  $\alpha\mu$ IL4 TKO mice showed significant increase in IL-12p40 and IFN- $\gamma$  production. Interestingly, the development of granulomatous colitis was associated with an increase of immature myeloid dendritic cells (DCs) as indicated by the presence of DCs with CD86-CD11b<sup>+</sup>CD11c<sup>+</sup> phenotype. The colonic LP DCs produced large amounts of IL-12 p40 and p19 but not p35. This was associated with a marked increase in IL-17 expression by CD4<sup>+</sup> T cells in  $\alpha\mu$ IL4 TKO mice, compared to other KO mice. The IL-12p40/p19 production by colonic LP DCs was further upregulated by a toll-like receptor 9 ligand (CpG) and significantly downregulated by IL-4 as well as IgG (Fc fraction). *In vivo* neutralization of IL-12p40 activity by the administration by specific mAbs suppressed the development of granulomas in  $\alpha\mu$ IL4 TKO mice. To test the ability of immature myeloid DCs to induce granulomas, purified immature myeloid DCs isolated from the granulomas of  $\alpha\mu$ IL4 TKO mice (6 months of age) or bone marrow-derived immature or mature myeloid DCs from WT mice were directly injected into the ileocecal valve of recipient young  $\alpha\mu$ IL4 TKO mice (9 weeks of age) following laparotomy. Importantly, the transfer of colonic myeloid DCs as well as also bone marrow-derived WT immature myeloid DCs led to the development of granulomas in the recipient  $\alpha\mu$ IL4 TKO mice. In contrast, the transfer of mature myeloid DCs failed to do so. These findings indicate that immature myeloid DCs have the ability to induce granulomas under specific intestinal inflammatory conditions characterized by Th1 dominated immune responses (or absence of Th2 environment) and impaired B cell functions.

## New Animal Models: Defining Antigens Recognition by Animal Models

3:30 PM–5:30 PM, 5/15/2005

### OR-83. HLA-A11/KboDbo Transgenic Mice Are Susceptible to PLP<sub>41–60</sub> Induced Experimental Autoimmune Encephalomyelitis (EAE).

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Multiple sclerosis (MS) is a demyelinating disease of central nervous system (CNS) characterized by plaques of infiltrating CD4+ and CD8+ T cells. Although EAE, an animal model for MS, is considered a CD4+ T cell mediated disease, recent reports have suggested that myelin-specific CD8+ T cells can also cause EAE in susceptible strains of mice. Among all the genetic factors linked with MS strongest associations have been found with HLA class I and class II gene. However role of HLA class I in pathogenesis of MS is not well defined due to lack of proper animal model. In this study, we have generated HLA class I transgenic mice to investigate the function of these molecules in disease pathogenesis of EAE. Transgene (HLA-A11) were introduced into class I deficient mice by breeding to eliminate the effects of endogenous class I molecules. Using a software program ProPred I, we selected HLA-A11 binding epitope of myelin proteolipid protein (PLP) 41–60 and immunized transgenic mice expressing HLA-A11 and control mice with this peptide PLP<sub>41–60</sub>. T cells from the A11 tg mice responded to PLP<sub>41–60</sub> peptide in a dose dependent manner but no response was seen in KboDbo mice expressing same mouse class II. Further, using in vitro antibody blocking experiments, the T cell response in tg mice was shown to be mediated by both CD4+ T cells as well as CD8+ T cells and restricted by the HLA class I transgene molecule. PLP<sub>41–60</sub> peptide also induced pronounced neurological disease in A11 tg mice characterized by brain ataxia, spinning, spastic reflexes and head tilt. These mice also showed CNS pathology consisting of heavy inflammation in meningeal and cerebellum region of brain. This is the first animal model describing a encephalitogenic role of HLA class I-restricted CD8+ T cells. Further study is underway to understand role of HLA class I molecule in predisposition and onset of EAE.

### OR-84. Fibrinogen-Induced Arthritis in Mice as a Novel Model for Rheumatoid Arthritis.

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The pathogenesis of rheumatoid arthritis (RA) involves the deposition and excessive local generation of fibrin in the inflamed joint. It is believed that the imbalance of fibrinolysis and coagulation within the rheumatoid joint differentiates RA from other joint diseases. Recent studies have identified deiminated fibrin as a candidate autoantigen in human RA. We have developed a fibrinogen-induced arthritis mouse model for RA using human fibrinogen as the immunizing antigen. In this model we demonstrate strong T cell reactivity to fibrinogen

with no cross-reactivity to collagen type II. Using proteome microarrays containing proteins and peptides representing the putative autoantigens in RA, we also find strong B cell reactivity to fibrinogen, and robust autoreactive B cell spreading to collagen types I, II and V, human cartilage glycoprotein 39, and citrulline-substituted peptides derived from filaggrin. We also show that arthritis can be adoptively transferred to naive mice with either sera or whole splenocytes from diseased mice. Clinical symptoms from both immunized and adoptively transferred mice include erythema and mild swelling that encompass the ankle, foot, and digits. Histopathological analysis of H&E stained joint sections indicate a mononuclear infiltrate within the inflamed synovial membrane. This new fibrinogen-induced arthritis mouse model may provide additional insight into understanding the disease mechanisms and developing novel therapeutic interventions for rheumatoid arthritis.

### OR-85. Murine Model of Mixed Connective Tissue Disease in HLA-DR4 Transgenic Mice Induced with U1-70kD Small Nuclear Ribonucleoprotein Autoantigen.

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Objective of study: We sought to develop a murine model of Mixed Connective Tissue Disease (MCTD) that replicated the immunologic and clinical features of this human autoimmune disease.

Materials and methods: Groups of 8–12 week old C57BL/6 mice transgenic for the extracellular domains of HLA-DR4 were immunized subcutaneously with 50 ug of a maltose binding fusion protein for U1-70kD ribonucleoprotein (70K-FP) in 50 ul of either U1-RNA in PBS (at 1 ug/ul) or CFA. Control groups also included mice injected with 50 ug of maltose binding protein lacking the 70K-FP with U1-RNA or CFA. Sera from mice were examined for autoantibodies using immunoblot and ELISA. Tissues were obtained at necropsy, stained with hematoxylin and eosin, and examined in a blinded fashion.

Results: Anti-U1-70kD and other anti-ribonucleoprotein (RNP) antibodies developed both in mice immunized with 70K-FP + CFA and 70K-FP + U1-RNA. MCTD-like interstitial and perivascular lung disease developed in groups of mice immunized with either 70K-FP + CFA (60%) or 70K-FP + U1 RNA (75%). Anti-RNP antibodies and lung disease was not observed in control mice. Injection of a single dose of U1-70kD RNP with its physiologically associated U1-RNA was adequate to induce autoimmunity in mice transgenic for the HLA-DR4 allele associated with susceptibility to MCTD.

Conclusions: A single injection of HLA-DR4 transgenic mice with 70K-FP+U1-RNA or 70K-FP+CFA induced anti-RNP autoimmunity and interstitial lung disease. Thus, this model replicates both the central immunologic and clinical features of MCTD.

### OR-86. Proteoglycan-Induced Arthritis: A New and Unique TCR Transgenic Arthritis Model.

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To characterize pathogenic effector T cells in arthritic mice, and to map T cell recognition sites in human and mouse cartilage proteoglycan (PG), we have generated PG-specific T cell hybridomas, which recognize dominant/arthritogenic T cell epitopes. Among these immortalized T cells, hybridoma 5/4E8 (specific for the consensus sequence of <sup>73</sup>GRVVRNSAY in human cartilage PG) induced arthritis upon adoptive transfer, which showed high similarities to the histopathology of the primary form of PG-induced arthritis (PGIA) and those described in peripheral joints of patients with rheumatoid arthritis (RA).

To better understand the role of antigen-specific T cells in the development of this autoimmune model of arthritis, we have inserted the V $\alpha$ 1.1 and V $\beta$ 4 chains of the T cell receptor (TCR) of hybridoma 5/4E8 into an *in vivo* expression vector. We generated transgenic (Tg) mice constitutively (over)-expressing both TCR chains. TCR-5/4E8-Tg mice were then backcrossed to the arthritis susceptible BALB/c strain and immunized with cartilage PG. Interestingly, a rapid onset of arthritis with severe clinical symptoms was detected in TCR-5/4E8-Tg mice after immunization with PG, which has never occurred in wild-type BALB/c mice. The arthritis was characterized by a chronic progressive disease course with intermittent spontaneous exacerbations and remissions reminiscent of the clinical appearance of RA. Histological analysis of inflamed joints showed extensive cartilage and bone erosions similar to that seen in arthritic joints of human patients. Both IL-4 and IFN- $\gamma$  cytokine-producing cells, with the predominance of IL-4-secreting cells, were detected during the prearthritic stage (initiation phase) of arthritis, which then shifted significantly toward a Th1 bias at the time of onset of arthritis. We also demonstrated that adoptive transfer of splenocytes from arthritic or non-arthritic TCR-5/4E8-Tg donor mice to syngeneic BALB/c-SCID or -RAG-2 knockout recipient mice could induce arthritis.

In conclusion, the presence of the large number of arthritogenic epitope-specific T cells with high expression level of epitope-specific TCR is sufficient to induce arthritis. These arthritogenic epitope-specific TCR-5/4E8-Tg mice are valuable and powerful tools, and are now used for further development of T cell directed immune modulating strategies.

**OR-87. BDC 12-4.1 (Anti-Insulin B:9-23) T Cell Receptor Transgenic Mice Are Lymphopenic but Entrain Early Insulinitis and Can Cause Early Diabetes Modified by Insulin 2 Gene Expression.**

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A series of studies suggests that insulin is a key target in the development of anti-islet autoimmunity in type 1 diabetes of mouse and man. The majority of the Wegmann CD4 T cell clones from islets of NOD mice react with insulin and more than 90% of these react specifically with insulin peptide B:9-23. Using the prototypic I-A<sup>g7</sup>-restricted T cell receptor (TCR) of the BDC 12-4.1 anti-B:9-23 clone, we have produced separately and combined  $\alpha$  (AV13S3 AJ53) and  $\beta$  (BV2S1 J $\beta$ 2.1) TCR transgenics. Expression of the V $\alpha$  chain was verified by RT-PCR of splenic mRNA and sequencing of the entire  $\alpha$  chain.

Flow cytometry of peripheral blood mononuclear cells demonstrates good expression of the  $\beta$  chain transgene in T cells. Approximately 98% of the peripheral CD4 T cells in tg FVB mice express the transgenic  $\beta$ -chain compared to 4% in non-transgenic mice. The transgenic FVB mice were crossed and back-crossed with NOD RAG1<sup>-/-</sup> mice. Homozygous RAG<sup>-/-</sup> TCR<sup>+</sup> mice are lymphopenic compared to heterozygous RAG  $\pm$  TCR<sup>+</sup> mice (mean lymphocyte count 500 lymphocytes/ul versus 4800 lymphocytes/ul;  $P = 0.02$ ). Heterozygous RAG  $\pm$  TCR<sup>+</sup> transgenic mice show insulinitis at every age tested (7–62 weeks) but do not progress to diabetes nor do they develop insulin autoantibodies. TCR<sup>+</sup> RAG<sup>-/-</sup> tg mice can develop diabetes at older ages (e.g., 32 weeks). Since the insulin2 gene (Ins2) is expressed in the thymus (the insulin1 gene is expressed minimally if at all in the thymus), the TCR transgenic mice were bred with NOD Ins2 knockout mice to create a TCR<sup>+</sup> RAG<sup>-/-</sup>Ins2<sup>-/-</sup> mouse. Diabetes developed much earlier in these mice (10 weeks for first diabetic observed) with partial restoration of peripheral lymphocytes. We believe the accelerated diabetes and higher peripheral lymphocyte counts of the Ins2 knock-out transgenic mice are due to lymphocytes escaping negative thymic selection due to the lack of insulin expressed as an autoantigen in the thymus. We are currently producing BDC 12-4.1 TCR congenics on the NOD and B6.NOD-H2<sup>g7</sup> backgrounds and will analyze the clonality of the T cell receptors of the infiltrating cells in insulinitic lesions. These experiments indicate that as a transgenic, the 12-4.1 T cell receptor confers diabetes susceptibility in immune-compromised mice and confers insulinitis susceptibility in immunocompetent mice.

**OR-88. Implication for the Pathogenesis and Immunoregulation in a Murine Model of Sarcoidosis.**

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Sarcoidosis is a systemic granulomatous disease with unclear etiology and limited treatment. Th1 cell activity plays prominent role in the multi-organ inflammation of sarcoidosis, but mechanistic study and therapeutic progress is hampered by the lack of an experimental animal model. We recently reported that G $\alpha$ i2<sup>-/-</sup> T cells produced chronic intestinal inflammation when transferred into RAG2<sup>-/-</sup> recipients. In this study, we demonstrate that re-transfer of splenic T cells from these recipients induce progressive systematic granulomatous disease. The inflammation involved skin, lungs, pancreas, and intestines. Environmental microbes are required for disease development. By flow cytometry, lymphocytes recovered from the mice with disease were increased in CD4<sup>+</sup> T cells with Th1 features. Surprisingly, co-transfer of wildtype mesenteric node (MLN) B cells prevented CD4 T cell expansion, inflammation, and disease activity induced by the immunopathogenic lymphocytes. The protective function of MLN B cells required genetic sufficiency for CD1d and IL-10. These results establish a mouse model for sarcoidosis, and reveal a new setting for protective B cell immunoregulation via cognate CD1d interaction and IL-10 production. This model provides an experimental system to delineate immune targeting and immunoregulatory deficits that may underlay pathogenesis in sarcoidosis. Supported by NIH DK46763, DK069434, and the Crohn's and Colitis Foundation of America.



### OR-89. A New Model of Endogeneous AML Following Irradiation and Allogeneic Bone Marrow Transplantation.

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**Background:** It has been shown that SJL/J mice given ionizing irradiation develop acute myelomonocytic leukemia. The one year-incidence (10–30%) of this radiation-induced AML (RI-AML) markedly increased (50 % and 75%), when irradiation was followed by treatment with corticosteroids or CSF-1 [1, 2]. Leukemogenesis was shown to be due to a deletion in one copy of chromosome 2, inducing a pre-leukemic state. A secondary proliferative stimulus then results in leukemic transformation [3, 4].

**Aim:** This study was initiated to investigate whether allogeneic bone marrow transplantation (allo BMT) can be used as a proliferative stimulus after irradiation of SJL/J mice, thereby creating a model of endogenous post transplant leukemia relapse.

**Materials and Methods:** SJL/J mice (H-2K<sup>s</sup>) were sublethally irradiated (8,5 Gy) and transplanted with 10<sup>7</sup> T-cell depleted Balb/c BM cells (H-2K<sup>d</sup>). Animals were monitored for weight loss, signs of leukemic disease and survival. At regular time intervals, peripheral blood was collected for evaluation of donor chimerism in different cell lineages (flowcytometry). Moribund animals were sacrificed and lymphoid and other tissues were prelevated for flowcytometric (CD3, CD4, CD8, CD11b, Gr1, c-Kit, Vb 8.3, H-2K<sup>d</sup>, IA<sup>d</sup>), histopathological and immunohistochemical studies (HE stains; MPO, B220 and CD3).

**Results:** BMT led to the development of mixed chimerism of 10 ± 4.5, 80 ± 17.3 and 97 ± 1.5 % in the T-cell, B-cell and myeloid cell lineage, respectively. From 3 weeks after BMT onwards, all mice developed weight loss and overt malaise (*n* = 65), resulting in 60% mortality between day 30 and 60 post BMT. Autopsy of these mice showed an enlarged spleen with nodules and a brownish discoloration of the liver with necrosis. Microscopic studies showed destruction of the splenic architecture by a uniform cell population, which was also found in the liver. The host-type origin and the immature myeloid nature of this population was demonstrated using immunohistochemistry (MPO+, B220-, CD3-) and flowcytometry (CD11b lo, Gr1 lo, B220 -, CD3 -, CD4 -, CD8- and H-2K<sup>d</sup> -) on spleen, liver and bone marrow. Ex vivo MLR failed to reveal increased alloreactivity and in vivo expansion of alloreactive T cell frequency (Vb8.3) was absent, arguing against graft-versus-host disease.

**Conclusion:** We describe a new model of endogeneous leukemia, in which irradiation and allo BMT in SJL/J mice gives rise to fatal AML. The model most likely involves a radiation-induced defect, while endogenous growth factors, produced after allo BMT, play a facilitating role in leukemogenesis. This model can be of value for the study of leukemogenesis and of immunotherapy in AML.

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### Costimulation

3:30 PM–5:30 PM, 5/15/2005

### OR-90. Monoclonal Antibody Targeting of TIRC7 Results in Significant Therapeutic Effects on T and B Cell Response in Collagen-Induced Arthritis in Mice.

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**Aim:** TIRC7 is an activation induced cell surface molecule on T and B cells that negatively regulates their function. TIRC7 is upregulated in vivo in patients with Rheumatoid Arthritis (RA) and mice with collagen induced arthritis (CIA) suggesting TIRC7 targeting might be a new therapeutic option of RA. **Methods:** We analysed the in vitro, ex vivo, and in vivo effects of anti-TIRC7 mAb particularly on memory T cell function under physiological and pathophysiological (CIA model) conditions. **Results:** Antibody targeting of TIRC7 *in vitro* significantly inhibited the memory T cell response to recall antigens in vitro and inhibited a delayed type hypersensitivity response in vivo (mouse). Most importantly, DAB mice with established collagen-induced arthritis (CIA) were treated with TIRC7 mAb alone or in combination with a TNF-alpha receptor-Ig-fusion protein. Anti-TIRC7 antibody administration demonstrated significant therapeutic efficacy in established CIA as monotherapy. Moreover, the combination of anti-TIRC7 antibody with a TNF alpha receptor-fusion protein revealed additional therapeutic effects in established arthritis in mice. Mice treated with anti-TIRC7 mAb also showed a significant reduction of IgG1 anti-collagen antibody responses together with reduced B cell numbers. **Conclusion:** The treatment of autoimmune diseases such as RA associated with exaggerated T and B cell response with an anti-TIRC7 mAb might be unique as TIRC7 targeting results in modulation of both T and B cell response. Moreover, unlike to other therapeutic pathways, anti-TIRC7 antibody therapy exhibits a significant inhibitory effect on memory T cell activation. TIRC7 targeting could offer a novel therapeutic strategy for RA patients that synergizes with TNF alpha receptor therapy and the combination of TIRC7 signaling pathway with TNF alpha blockade might be important for the clinical use as a large group of non- responders to anti-TNF alpha targeting therapy is existing.

### OR-91. Transgenic Expression of Program Death Ligand 1 Protects Islets from Autoimmune Diabetes in Nonobese Diabetic Mice.

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PD-L1, also known as B7-H1, is one of the ligands of programmed death 1 (PD-1) which can negatively regulate lymphocyte activation. PD-L1 is broadly expressed in mice and may contribute to the peripheral tolerance by interacting with PD-1. Based on this PD-1-mediated immune-inhibitory function, we investigate the preventive and/or therapeutic potential of PD-L1 in

autoimmune diabetes. In anti-CD3 stimulation experiment, proliferative response of splenocytes from non-obese diabetic (NOD) mice is down-regulated in a PD-L1.Ig dose-dependent manner. We further generated the transgenic NOD mice overexpressing PD-L1 in pancreatic cells and characterized the protective potential in these mice. In these transgenic mice, we observed an islet-specific transgene expression of PD-L1 both in transcriptional and translational levels. Strikingly, the severity of insulinitis in these transgenic mice is significantly decreased. Moreover, the disease onset is delayed as well as the diabetic incidence is decreased in these mice. To assay whether the protection of diabetes in these mice is differentially regulated by the Th1 and Th2 development, we crossed PD-L1 transgenic mice with T1 and T2 doubly transgenic NOD mice and directly investigated their Th1 and Th2 expression profiles. In the PD-L1/T1/T2 triply transgenic mice, the higher Th2 marker expression suggests that overexpressed PD-L1 may indirectly trigger the Th2 development. By enhancing the Th2 function, the original Th1-dominant autoimmune response can be suppressed in these PD-L1 transgenic mice. Furthermore, the transgenic islets had a higher transplantation success rate and survived for longer than wild-type islets. Our results support the theoretical basis for genetic manipulation in an organ-specific manner and provide a potential therapeutic approach mediated by PD-L1 in islet transplantation.

#### **OR-92. PD-L1/PD-L2 Expression Protects Against the Development of Autoimmune Diabetes.**

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PD-1, a member of the B7/CD28 family of costimulatory molecules, is expressed on activated T and B cells and plays a role in regulating tolerance and autoimmunity. PD-1 has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). PD-L1 is widely expressed on both immune and non-immune cells. PD-L2 has more restricted expression and is primarily found on macrophages and dendritic cells. PD-L1 is upregulated on islet cells in diabetic NOD mice. To investigate the role of PD-L1 and PD-L2 in progression to autoimmune diabetes, we crossed PD-L1/PD-L2 deficient mice onto the NOD background. The loss of PD-L1/PD-L2 precipitates early onset of diabetes in both male and female NOD mice. PD-L1/PD-L2 deficient mice become diabetic by 4–6 weeks of age, in comparison to >13 weeks in wild type controls. Both wild type and PD-L1/PD-L2 deficient T cells are diabetogenic when adoptively transferred into PD-L1/PD-L2 deficient NOD SCID hosts, while wild type and PD-L1/PD-L2 deficient T cells do not induce diabetes when adoptively transferred into wild type NOD SCID hosts. These data indicates that PD-L1/PD-L2 expression is required on host tissues (islet cells, endothelium, dendritic cells) in order to dampen autoreactive T cell responses.

#### **OR-93. ICOS Engagement of CD4 T-Cells Following Pulmonary Influenza Infection: Evidence for Negative Regulation of T-Cell Effector Function.**

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Respiratory viruses such as influenza A elicit an immune response involving the activation, proliferation and recruitment of

CD4 and CD8 T cells to the lung, the major site of viral infection. A major feature of the anti-viral response is the secretion of IFN- $\gamma$ , the signature Th1 cytokine, by activated effector CD4 and CD8 T cells. We have previously observed that these T cells can persist and spontaneously secrete IFN- $\gamma$  in the lungs for up to 30 days following viral infection, and have reported that IFN- $\gamma$  induced by viral infection contributes to major quantitative and qualitative alterations of pulmonary dendritic cells (*Nat. Immunol.* 5:337-43, 2004). In the current study, we have determined how the inducible costimulatory molecule, ICOS regulates CD4 and CD8 T cell responses following influenza A infection. Substantial levels of ICOS were observed on CD4 and CD8 T cells during the acute influenza viral infection, which persisted for at least 30 days post-infection, subsiding gradually in a manner that paralleled the expression of IFN- $\gamma$ . We neutralized ICOS-ICOS ligand interactions during acute influenza viral infection of BALB/c mice using either recombinant ICOS-Ig fusion protein or neutralizing anti-ICOS mAbs. Although there was initially a delayed recruitment of CD4 T cells, this was followed by substantially increased number of CD4 T cells in the lungs. Moreover, intracellular staining of these T cells demonstrated an elevated level of spontaneous production for both IFN- $\gamma$  and the immunoregulatory cytokine IL-10 for up to 30 days post-influenza infection. Thus, transient ICOS blockade dramatically alters the normal temporal expression of IFN- $\gamma$  by CD4 and CD8 T cells. To further investigate the effects of ICOS-ICOS ligand interactions *in vitro*, we activated ovalbumin (OVA)-specific DO11.10 CD4 T cells by co-culturing them with antigen and dendritic cells in the presence of blockade of ICOS/ICOS-ligand interactions. This blockade enhanced antigen-specific proliferation of the CD4 T cells and secretion of IFN- $\gamma$ . The *in vitro* primed CD4 T cells that received ICOS blockade were adoptively transferred *in vivo* and recipient mice were then sensitized and challenged with intranasal injections of OVA. CD4 T cells were purified from the pulmonary lavage of the mice and the adoptively transferred cells were found to express higher levels of IFN- $\gamma$  and IL-10 compared to adoptive transferred CD4 T cells that were not subjected to ICOS/ICOS-ligand blockade. Together, these results suggest a novel and previously unappreciated role for ICOS in negatively regulating both Th1 and regulatory cytokine production by T cells.

#### **OR-94. Tim-4 Is the Ligand for Tim-1, and the Tim-1/Tim-4 Interaction Regulates T Cell Expansion.**

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The newly identified Tim (T cell, immunoglobulin and mucin domain-containing molecule) gene family has been associated with the regulation of TH1 and TH2 immune responses. Tim-1 has been genetically linked to asthma, a TH2-mediated disease; however, its endogenous ligand has not been identified. We have found that Tim-4, which is expressed by antigen-presenting cells, is the ligand for Tim-1. An interaction between Tim-1 and Tim-4 can be observed between *in vivo*-derived cells and can be specifically blocked by anti-Tim-1 antibody. *In vivo* admin-

istration of soluble Tim-1 fusion protein (Tim-1Ig) during either a TH1- or TH2-biased immune response results in hyperproliferation and the preferential expansion of TH2 cells. Furthermore, soluble Tim-4Ig can costimulate T cell expansion both *in vitro* and *in vivo*. Our data suggest that the Tim-4/Tim-1 interaction delivers a signal necessary for the expansion of T cells.

**OR-95. Evidence for Early Anergy Followed by Rapid Peripheral Deletion of Donor-Specific CD8 T Cells after Non-Myeloablative BMT with Anti-CD154: In Search of a Tolerogenic Donor Cell.**

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**Aim:** Mixed chimerism and donor-specific tolerance can be achieved by bone marrow transplantation (BMT) with non-myeloablative conditioning using 3 Gy total body irradiation (TBI) and anti-CD154 mAb. CD4 T cells are needed during the first 2 weeks for CD8 T cell tolerance induction. We now investigated early CTL activity and the role of various donor cell populations in this tolerance model.

**Methods:** Recipient B6 mice were treated with TBI (3 Gy, day -1), one dose of anti-CD154 mAb (2mg MR1, day 0) and BMT from a fully allogeneic (B10.A) donor to induce mixed lymphohematopoietic chimerism. CTL function was assessed by <sup>51</sup>Cr release assay on days 4, 7 and 14 after BMT. To assess the role of specific donor cell populations in tolerance induction, recipient B10.S mice received the same conditioning followed by BMT from fully allogeneic B cell- or T cell-deficient mice ( $\mu^{MT}$  or TCR $\beta^{-/-}$ , B6 background). In a further experiment, transgenic mice expressing the diphtheria toxin receptor under a CD11c promoter were used as donors. In these mice diphtheria toxin injection leads to rapid depletion of CD11c<sup>+</sup> dendritic cells.

**Results:** In earlier studies we showed that donor-specific CD8 T cells are deleted from the peripheral repertoire within 10–14 days after BMT with this regimen. We now demonstrate donor-specific loss of CTL function in <sup>51</sup>Cr release assay already by days 4 and 7 after BMT in mice that received the tolerance protocol, but not in those receiving conditioning without BMT. This finding suggests an early phase of donor-reactive CD8 T cell anergy before deletion. In search of a tolerogenic donor cell population, we used either B cell- or T cell-deficient donor mice and also tested dendritic cell depletion in a transgenic model system. The absence of any of these cell populations from the donor marrow did not interfere with the establishment of lymphohematopoietic chimerism. Tolerance was further shown by durable mixed chimerism and acceptance of donor skin, but prompt rejection of third party skin.

**Conclusion:** Donor-specific alloreactive CD8 T cells are unresponsive within 4 days and deleted from the periphery within 10 days after BMT with TBI day -1 and anti-CD154 mAb treatment. Neither B cells, T cells nor dendritic cells of donor origin are critically required for tolerance induction in this model.

**OR-96. TIM-3 Mediated Enhancement of Anti-Tumor Immune Responses.**

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Effective immunity against tumors often results from the development of a vigorous cell-mediated immune response. Effective anti-tumor immunity is largely predicated on the contribution of Th1 cytokines (i.e., IFN- $\gamma$ , TNF- $\alpha$ ). While differentiation of T cells toward a Th1 or Th2 phenotype involves a complex chain of events, members of a novel class of molecules called TIMs (T cell Immunoglobulin and Mucin domain-containing proteins) have recently been shown to exert great influence over Th1/Th2 immune phenotype balance *in vivo*. Manipulating the action of TIM-3, one of the members of this novel protein family, was previously found to profoundly affect disease severity and outcome in animal models of autoimmunity and allograft transplantation, and promote hyperproliferation of antigen-activated T cells and the spontaneous production of Th1 cytokines. These results strongly suggested that agents targeting TIM-3 pathways could also enhance effective anti-tumor immune responses *in vivo*, presumably through one or more mechanisms favoring Th1 cells and the promotion of cell-mediated immunity. In the study described here, an antibody specific to TIM-3 was investigated for its ability to promote anti-tumor effects in mice. Using the EL4 thymoma tumor model, anti-TIM-3 was delivered as either a stand-alone therapeutic agent following establishment of tumors under the skin, or as an adjuvant to irradiated tumor cell vaccination prior to live tumor challenge. Anti-TIM-3 antibody promoted significant reductions in challenge tumor growth over time. Furthermore, including anti-TIM-3 as an adjuvant to tumor vaccination also allowed treated mice to fully reject subsequent live tumor challenge. Neither tumor rejection nor limited tumor growth was seen in mice receiving isotype-matched control antibody under either experimental protocol. By demonstrating a powerful capacity for TIM-3-specific antibodies to change the course of tumor progression in treated mice, these experiments further support the prospective role of TIM-3 to act as a critical regulator of cell-mediated immune function and Th1 responsiveness.

**Immunotherapy**

3:30 PM–5:30 PM, 5/15/2005

**OR-97. Apoptosis Induction as a Therapeutic Intervention to Eliminate Encephalitogenic T Cells; Antisense XIAP (AEG35169) in Murine Models of EAE.**

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MS is an inflammatory disease of the CNS white matter, with presumed autoimmune etiology. Therapies in current use show benefit for MS in targeting the T-cell autoimmune response. Apoptosis of auto-reactive T-cells is a fundamental immunoregulatory mechanism. If autoimmune T-cells were predisposed to death, MS could be alleviated. Increased expression of Inhibitor of Apoptosis (IAP) proteins protects cells from apoptosis. In particular, the X-linked IAP (XIAP) interrupts the apoptotic cascade in T-cells by directly inhibiting effector caspases. Inhibition of XIAP primes cells for apoptosis induced by multiple stimuli. We used repeated interperitoneal (IP) injection of XIAP antisense oligonucleotide (AEG35169) in mice to reduce XIAP

protein levels in peripheral blood leukocytes. AEG35169 was similarly administered to treat MOG p35-55 induced EAE in C57BL/6 mice. When given daily via IP injection, from time of symptomatic onset, AEG35169 reduced clinical scores within 5 days and prevented further disease progression in 84% of animals, compared to control groups receiving random or scrambled oligonucleotides or saline, 90% of which showed continued disease or increased severity ( $n = 16$ ). Anti-XIAP treated animals showed evidence of considerably increased leukocyte apoptosis, with high numbers of TUNEL positive cells in the spinal cord. A 5-day prophylactic treatment with AEG35169, prior to induction of EAE, followed by daily treatment, reduced the incidence of mild disease from 85% of animals to 9% ( $n = 57$ ), and of severe disease from 84% to 38% ( $n = 48$ ). Analysis of tissues at 40 days after immunization indicated no or very limited inflammatory infiltrates in anti-XIAP protected animals, and correlates of disease such as chemokine expression in CNS were reduced in treated animals. Amelioration of disease was not due to immune suppression, and there was no evidence for a Th1/Th2 shift in treated animals. Our data establish XIAP as a critical controller of the susceptibility of CNS-infiltrating T cells to apoptosis, such that experimental modulation of XIAP prevents or cures EAE. These studies increase our understanding of regulation of inflammatory pathology in the CNS and support XIAP as a novel target for therapeutic intervention in MS.

#### **OR-98. Blockade of TNF $\alpha$ Preferentially Inhibits Proliferation of Anti-CD3, Recall-Antigen Responsive and Autoreactive Human VLA-1+CD45RO+CD4+ T Cells.**

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The very late antigen (VLA)-1  $\alpha 1\beta 1$  integrin, a receptor for collagen, is induced on the surface membrane of activated T-cells (TC) and remains preferentially expressed by effector-memory Th1 cells. We recently showed that VLA-1+ T cells at sites of chronic autoimmune inflammatory arthritis express a restricted and unique T cell receptor (TCR) V $\beta$  repertoire, suggesting they are responding to a unique set of auto-antigens in tissues. Since VLA-1+ immunocytes are critical in immune mediated Th1 diseases that are ameliorated by monoclonal antibodies (mAb) to TNF $\alpha$ , we explored how an anti TNF $\alpha$  mAb affects the VLA-1+CD4+ TC subset. Anti TNF $\alpha$  mAb (Infliximab, 5–50  $\mu\text{g}/\text{ml}$ ) but not control immunoglobulins, neutralized TNF $\alpha$  during *ex vivo* mitogen (PHA or anti-CD3)-triggered activation of VLA-1- peripheral blood (PB) mononuclear cells (MC) (PBMC) and significantly reduced the percentage of VLA-1+ TC in 8–12 day cultures ( $36.9 \pm -20.3\%$  to  $26.9 \pm 15.7\%$  of the TC,  $n = 9$ ,  $p < 0.011$ ), but did not affect the VLA-4+ subset. Furthermore, CFSE-dye intracellular labeling revealed that the reduction was due to a preferential inhibition of VLA-1 expression among CD4+ TC that were induced to divide in the presence of anti CD3. Thus, dividing VLA-1+CD4+ T cells in the culture, were reduced  $66 \pm 22\%$  while non-dividing VLA-1+CD4+ TC were slightly increased. In contrast, the anti CD3 VLA-1-CD4+ responsive subset was inhibited to a lesser extent by TNF $\alpha$  blockade (40–50% inhibition,  $n = 5$ ). The addition, at a 1:2 cell:cell ratio, of washed MC from 8 day cultures of PBMC activated by anti CD3 mAb plus anti TNF $\alpha$ , but not, as a control, of VLA-1- anti CD3 triggered T cells in the absence of anti TNF $\alpha$ ,

likewise decreased the VLA-1+ subset emerging in autologous *de novo* anti-CD3-activated 8 day cultures, suggesting that the preferential inhibition of VLA-1+ CD4+ TC division by anti TNF $\alpha$ , may involve MC activated in the presence of anti TNF $\alpha$  ( $n = 3$  experiments). Importantly, anti TNF $\alpha$  mAb, also preferentially inhibited PB derived VLA-1+CD4+ TC dividing *ex vivo* in response to the recall antigen tetanus toxoid, while less potently inhibiting the VLA-1-CD4+ subset. Finally, non-antigenically or mitogenically stimulated, spontaneously dividing, (thus presumably autoreactive), synovial fluid (SF) VLA-1+CD4+, but neither VLA-4+CD4+ or CD25+CD4+ TC, from patients with autoimmune arthritis, were also dramatically and preferentially reduced by anti TNF $\alpha$  (85% inhibition,  $n = 6$ ) in *ex vivo* cultures. These data suggest that a critical immuno-modulatory effect of anti TNF $\alpha$  is mediated by its ability to preferentially target and inhibit mitogen, antigen or auto-antigen induced expansion of the VLA-1+ Th1 effector memory subset.

#### **OR-99. An Immunization Strategy for Generation of T Cell Receptor Mimics.**

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Antibodies endowed with specific recognition properties for HLA-displayed tumor associated antigens have been recently produced and shown to directly detect expression of HLA-tumor associated antigens on the surface of cancer cells. The application of these reagents for validation of T cell epitopes would greatly facilitate vaccine development. Yet many technical obstacles stand in the way of developing a consistent approach for making antibodies with T cell receptor (TCR)-like specificity. Particularly important would be to develop immunogenic forms of immunogen that could then be used for rapid and reproducible immunization of mice for the generation of polyclonal antibody responses reactive against peptide-A2 epitopes. Development of this technology might lead to more efficient creation of monoclonal antibodies (mAbs) specific for HLA-peptide. We hypothesized that HLA-A2-peptide immunogen presented to the immune system in a tetravalent form rather than a monovalent form would display enhanced immunogenicity and promote consistent generation of high titer IgG polyclonal antibody responses specific for the A2-peptide immunogen. To test our hypothesis we refolded *E. coli* produced insoluble protein and prepared purified forms of monomer and tetramer HLA-A2 peptide complexes displaying either human eukaryotic transcription initiation factor 4-gamma (eIF4G; VLMTEDIKL), a self-protein found to be upregulated in HIV infected T cells or human tumor suppressor protein p53 (264; LLGRNSFEV), a self-protein that is widely expressed in many cancers. We then immunized groups of Balb/c mice (5/group) 3 times with 2-week intervals with either monomer or tetramer forms of immunogen and assayed mouse sera for polyclonal IgG antibody response reactive for HLA-peptide by competitive ELISA. All mice (5/5 from both eIF4G- and 264-peptide-A2 groups) immunized with tetramers of immunogen showed specific anti-A2-peptide IgG antibody responses. In contrast, no specific polyclonal antibody response was detected from any of the mice (0/5) immunized with monomers of eIF4G- and 264-peptide-A2 immunogen. To confirm the specificity of the polyclonal anti-peptide-A2 response, we evaluated antibody from mice immunized with tetramer immunogen to stain T2 cells (HLA-A0201 positive)

pulsed with either eIF4G- or 264- peptide in a competitive binding assay. Our T2 cell assay results support the ELISA data and indicate that immunogen formulated as a tetramer is immunogenic and able to generate anti-peptide-A2 specific antibody responses in mice. Furthermore, we demonstrated by ELISA and T2 cell staining that tetramer forms of immunogen efficiently elicit IgG polyclonal antibody responses reactive against peptide-A2 within 4 weeks after initial immunization. Collectively, our findings support the hypothesis that tetravalent forms of peptide-A2 immunogen consistently lead to specific antibody responses against peptide-A2 epitopes. In addition, the ability to generate these probes in a rapid and reproducible manner will be invaluable for HLA class I epitope validation.

**OR-100. Differential Expression of Phosphorylated NF- $\kappa$ B/RelA in Normal and Psoriatic Epidermis and Downregulation of NF- $\kappa$ B in Response to Treatment with Etanercept.**

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Etanercept, a recombinant human TNF receptor fusion protein, is FDA approved for psoriasis and psoriatic arthritis. TNF $\alpha$  increases the synthesis of proinflammatory cytokines and leads to the activation of multiple signaling pathways, including NF- $\kappa$ B. The Rel/NF- $\kappa$ B transcription factors play a central role in numerous cellular processes, including the stress response and keratinocyte proliferation and differentiation. Utilizing a phosphorylation specific antibody we examined the expression of active nuclear NF- $\kappa$ B/RelA via immunohistochemistry in normal skin, non-lesional psoriatic skin, lesional psoriatic skin and lesional skin from patients treated with etanercept. There was no expression of active nuclear NF- $\kappa$ B in normal epidermis, whereas a basal level of constitutive active phosphorylated NF- $\kappa$ B/RelA was present in uninvolved epidermis from psoriasis patients. There was also significant upregulation of active phosphorylated NF- $\kappa$ B/RelA in epidermis from psoriatic plaques. Serial biopsies from psoriasis patients treated with etanercept at 1 month, 3 months, and 6 months demonstrated a significant down regulation of phosphorylated NF- $\kappa$ B/RelA which correlated with decreases in epidermal thickness, restoration of normal markers of keratinocyte differentiation, and clinical outcomes. These data suggest that activation of NF- $\kappa$ B plays a significant role in the pathogenesis of psoriasis and that a potential mechanism of action for TNF-targeting agents is down-regulation of NF- $\kappa$ B transcriptional activity.

**OR-101. Anti-IL-2R Therapy: An Alternative Strategy for Regulating CD40L Expression.**

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Monoclonal antibodies directed against the alpha chain (Tac/CD25) of the IL-2 receptor (IL-2R) are an emerging therapy in both transplantation and autoimmune disease. In a cohort of patients with autoimmune uveitis being treated with multiple immunosuppressive medications, monotherapy with daclizumab, a humanized anti-Tac antibody, was sufficient to control their disease without serious side effects. The basis of this antibody's therapeutic efficacy has not been established. Meanwhile, antibodies against CD40L that were shown to be efficacious in primate

transplant models were withdrawn from clinical use due to serious side effects associated with their administration. We have reported that CD40L expression on activated human CD4+ T cells is biphasic, consisting of an early CD28-independent peak, a subsequent nadir, and a second, CD28-dependent peak at 48 hr. The transient expression of CD40L is critical to the physiologic function of this costimulatory pathway, yet the mechanisms underlying the biphasic pattern of CD40L expression are largely unknown. We have also reported that the CD28-dependent second phase of CD40L expression is severely inhibited in vitro by daclizumab. We now show in primary PBMC cultures using blocking antibodies and flow cytometry that IL-2 does not impact late phase CD40L by acting indirectly through IL-2 regulated Th1 and/or Th2 cytokines as neither IL-12 nor IL-4 had any appreciable effect on either early or late expression of CD40L. In addition, CD28 signaling is not necessary for late phase CD40L expression as recombinant IL-2 or an agonistic anti-CD40 mAb could substitute for CD28 costimulation. Finally, in contrast to earlier reports, we observe that down regulation of early CD40L expression is not dependent upon interactions with CD40. These findings are in marked contradistinction to what has been reported in the mouse. Collectively, the data indicate that regulation of late CD40L expression is likely a direct consequence of IL-2R signaling. These results suggest that daclizumab, in combination with agents that can block early CD40L expression, may be a viable alternative to the use of anti-CD40L antibodies clinically.

**OR-102. Prophylactic Administration of Abatacept (CTLA4-Ig; BMS-188667) Prevents Disease Induction and Bone Destruction in a Rat Model of Collagen-Induced Arthritis.**

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**Background and Objectives:** Rheumatoid arthritis (RA) is an autoreactive disease in which activated T cells play an important role orchestrating the autoimmune responses giving rise to the inflammatory cascade responsible for joint inflammation and bone destruction. The CD28/B7 costimulatory pathway is critical for full T cell activation and modulating this pathway has been shown to inhibit T-cell activation leading to inhibition of these immune responses. Abatacept modulates T cell activation by interfering with the engagement of CD80/86 with CD28. Abatacept has been shown to provide significant improvement in the signs and symptoms of rheumatoid arthritis in a phase II trial. Here, we examine the effect abatacept administration has on disease induction, anti-collagen antibody production and bone destruction in a rat model of collagen induced arthritis.

**Methods:** Female DA rats were immunized s.c. on day 0 with 300  $\mu$ g of bovine type II collagen in incomplete Freund's adjuvant at the base of the tail. Immunized rats were administered either 1 mg/kg abatacept or a control human IgG IP on days-1, 0, 2, 4, 6, 8 and 10. Disease progression was monitored by measuring paw volume in mls. with a plethysmometer. Both hind paws were measured and the change in volume from base-line measurements (Day 0) were recorded. At the conclusion of the study (day 27) serum samples were collected from each animal for measurement of collagen specific antibodies by ELISA as well as serum cytokine measurements. Legs from the rats were removed and placed in formalin and prepared for histological analysis as well as analysis of bone morphology by micro CT.

**Summary:** By day 16 of the study, significant paw swelling was observed in the IgG treated control animals and continued to increase throughout the study until reaching a plateau (~ 3–3.5 mls.) on day 21. Administration of abatacept abrogated paw swelling throughout the course of the study. The IgG treated rats reached 100% incidence while no incidence was observed in the abatacept treated group. Serum anti-collagen antibody levels correlated well with the paw swelling data where abatacept administration resulted in 90% inhibition of collagen specific antibodies. We also found that abatacept decreased the expression of many of the circulating cytokines and chemokines which were upregulated in diseased animals. The micro-CT data revealed that abatacept treatment protects the bone from destruction as the knees and ankles of these rats appear to be normal.

**Conclusion:** Abatacept, a selective co-stimulation modulator significantly inhibited the onset and progression of disease in a rat CIA model. In these studies, paw swelling, collagen specific antibodies and bone destruction were all inhibited by the treatment.

### OR-103. Abatacept (CTLA4Ig) Modulates Human T-Cell Proliferation and Cytokine Production but Does Not Affect TNF $\alpha$ Production by Monocytes.

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**Background and Objectives:** Activated T cells play a central role in the inflammatory cascade leading to joint inflammation and destruction characteristic of rheumatoid arthritis (RA). The cytokines secreted by activated T cells can both initiate and propagate the immunologically driven inflammation associated with RA.

Abatacept, the first of a new class of agents that selectively modulate the co-stimulatory signal required for full T-cell activation, was evaluated *in vitro* for its ability to regulate human T-cell proliferation and cytokine production. The effect of abatacept on immune complex (IC)- or LPS-induced TNF $\alpha$  release from monocytes was also evaluated to distinguish the impact of abatacept on innate versus adaptive, antigen-specific responses.

**Methods:** T cells were isolated from normal healthy volunteers. The effect of abatacept on antigen-dependent T-cell activation was evaluated using either an irradiated human B-cell line (PM-LCL) as the antigen-presenting cells (APCs) for a primary mixed lymphocyte reaction (MLR), or autologous E-PBMCs as APCs, for a recall response to tetanus toxin (TT). Cytokines were measured at various times post activation, with proliferation determined on day 5. Monocytes were isolated by elutriation, challenged with LPS or ICs, and TNF $\alpha$  levels measured at 6 h. Chi L6 was included as a nonspecific Ig fusion protein control.

**Summary:** Abatacept significantly down modulated T-cell proliferation, in both primary and recall responses, at concentrations between 0.3 and 100  $\mu$ g/ml, with maximal inhibition (~60–80%) observed at ~3–10  $\mu$ g/ml. These concentrations are below the abatacept trough plasma levels observed in patients receiving a clinically effective dose.<sup>1</sup> Under conditions of maximal inhibition of proliferation, and similar to trough plasma levels in patients (30  $\mu$ g/ml), abatacept inhibited cytokine production in both primary and TT-dependent recall responses. However, the extent and rank order of cytokine inhibition by abatacept was markedly different between these two responses. Specifically, inhibition of IL-2 > TNF $\alpha$  > IFN $\gamma$  in a primary response whereas inhibition of IFN $\gamma$   $\geq$  IL-2, with a minimal effect on TNF $\alpha$  production in a TT recall

response. In contrast, abatacept did not inhibit IC- or LPS-induced TNF $\alpha$  production in human monocytes.

**Conclusion:** Abatacept, a selective co-stimulation modulator significantly inhibited the activation (as measured by cytokine production) and proliferation of human T cells in the context of a primary MLR or TT-dependent memory response. This inhibition occurred at concentrations below the serum C<sub>min</sub> levels observed in patients receiving a clinically effective dose of abatacept<sup>1</sup> (10 mg/kg monthly), consistent with suppression of T-cell activation *in vivo*. There was no effect of abatacept on TNF $\alpha$  production in monocytes challenged with LPS or ICs indicating that this agent may largely preserve innate immune responses.

#### Reference:

1. Kremer JM, et al. *NEJM* 2003; **349**:1907-1915.

### OR-104. Essential Role of IL-10 in Restricting Immunity during a Chronic Viral Infection.

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Viruses use a variety of strategies to suppress the anti-viral immune response leading to persistence in the host. Induction of immune suppression is one of the mechanisms by which viruses escape clearance and establish a persistent infection. The cytokine IL-10 has immunomodulatory properties and can down-regulate cellular immune responses by acting on APCs and T cells. To gain further insight into the role of IL-10 during viral persistence we studied lymphocytic choriomeningitis virus (LCMV) infection in its natural host. The LCMV isolate Clone 13 establishes a prolonged infection in Balb/c mice, which is associated with a less effective antiviral immune response and, in some studies, conditioning of dendritic cells. **Results:** Here, we report that a significant amount of IL-10 is being generated by CD4+ lymphocytes and some classes of APCs during persistent LCMV infection. Treatment with neutralizing IL-10R antibody on days 0, 7, and 14 post Clone 13 infection resulted in accelerated viral clearance. This was associated with a numeric increase of total spleen cells in comparison to non-treated mice and decreased levels of IL-10 were generated by such splenocytes. Lastly, overall clinical appearance was improved through this intervention as reflected in an increase in bodyweight, healthy shiny coat, and increase in physical activity. **Conclusion:** Our studies indicate that in persistent viral infections IL-10 plays an essential role in suppressing the anti-viral response and that systemic blockade can improve the clinical outcome. A similar strategy might be beneficial in other chronic infections associated with increased IL-10 levels, for example hepatitis C virus infection.

*This work was supported by an ADA mentor grant and a program project grant from NIAID for Matthias von Herrath.*

### Trafficking and Adhesion

3:30 PM–5:30 PM, 5/15/2005

### OR-105. Effects of Natalizumab (anti-VLA-4 Antibody) on Immune Cell Adhesion and Migration in Patients with MS.

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**Objective:** (1) To establish biological ‘proof of concept’ that *in vivo* anti-VLA-4 treatment of patients with multiple sclerosis (MS) results in decreased functional VLA-4 expression and migratory capacity of immune cells, and (2) Develop a simple *in vitro* assay that could be applied to monitor therapeutic response.

**Background:** Natalizumab (Tysabri®), a humanized monoclonal antibody directed against the adhesion molecule VLA-4, has recently been approved for the treatment of patients with relapsing remitting MS. It is presumed that beneficial effects in MS would be based on binding of Natalizumab to VLA-4 on the surface of circulating immune cells, thereby inhibiting their capacity to migrate into the CNS. To date, the impact of *in vivo* therapy with Natalizumab on the functional expression of VLA-4 on immune cells of MS patients has not been reported. The development of a simple assay to measure this effect could prove very useful in immune monitoring of patients on this emerging therapy.

**Design/Methods:** Consenting patients participating in the open-label phase of a clinical trial of Natalizumab in relapsing remitting MS, provided venous blood immediately prior to (Pre-infusion), and one hour following (Post-infusion), monthly Natalizumab infusions (300mg IV). Levels of VLA-4 surface expression on circulating immune cell subsets were assessed by flow cytometry. The migratory capacity of immune cells was evaluated in an established two-compartment Boyden chamber, known to capture VLA-4 mediated migration of human immune cells.

**Results:** We observed that expression of VLA-4 on circulating immune cells was significantly reduced after *in vivo* Natalizumab infusions ( $P = 0.004$ ;  $n = 12$ ). The effect was observed on all immune cell subsets but was greatest on T cells compared to B cells ( $P = 0.026$ ) or monocytes ( $P = 0.032$ ). In the functional assay, migration of post-infusion immune cells was significantly decreased compared to the migration of corresponding pre-infusion cells ( $P = 0.026$ ). The decrease in observed VLA-4 surface expression correlated well with the decrease in migratory function of the corresponding immune cells, following infusions ( $r = 0.71$ ;  $p < 0.05$ ). We confirmed that the migration assay can be carried out on frozen mononuclear cells (PBMC), providing a means for monitoring patients’ responses over time. We plan to present a batched analysis of prospectively collected samples from these patients, which should provide insights into the kinetics and stability of these *in vivo* effects.

**Conclusions:** Our study provides the first biological ‘proof of concept’ that *in vivo* Natalizumab therapy results in diminished VLA-4 functional expression and migratory capacity of circulating immune cells. The ability to reproducibly capture this effect in a relatively simple bioassay, and the validation that the assay can be applied to frozen PBMC, could provide a useful means to monitor patients on this promising therapy.

#### OR-106. Molecular Imaging of Adhesion Molecules in Experimental Autoimmune Encephalomyelitis (EAE).

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The infiltration of autoreactive T-cells into the central nervous system (CNS) requires a complex molecular interplay between immune cells and the blood brain barrier (BBB), especially involving vascular cell adhesion molecule (VCAM) 1 and intercellular adhesion molecule (ICAM) 1. We developed a new ultrasound based approach for the quantification of ultrasound contrast media in high concentrations, sensitive particle acoustic quantification (SPAQ). By combination of SPAQ with specific gas-filled microparticles (MP) targeted against VCAM and ICAM (VCAM-MP, ICAM-MP), we aimed to monitor the molecular changes at the blood-brain-barrier during the course of actively induced or adoptively transferred (AT) myelin basic protein (MBP)-EAE.

*Ex vivo* imaging of ICAM-1 expression in AT-EAE at the disease maximum proved the high sensitivity, specificity and spatial resolution of the method with the possibility of video-densitometric quantification. These results could be reproduced *in vivo* with a clear periventricular and cerebellar upregulation of ICAM1 and VCAM1 expression at the maximum of AT-EAE which could be suppressed by pretreatment of rats with corticosteroids ( $P < 0.008$ ). The imaging results were confirmed by parallel immunohistochemistry. Subsequent application of ICAM-MP after ICAM-MP or VCAM-MP injection did not influence follow-up measurements. Sequential imaging of ICAM-MP *in vivo* over the course of active and AT MBP-EAE revealed a significant upregulation of ICAM before the respective onset of disease (day 2 for AT-EAE, day 10 for active EAE). At that point of time no signal changes were observed on T2-weighted magnetic resonance images (MRI). Albumin staining for detection of BBB integrity and gadolinium enhanced MRI after sonification did not reveal a disturbance of the BBB thereby proving the safety of the method *in vivo*.

**Conclusion:** Based on these data, molecular imaging of adhesion molecules with SPAQ is a platform technology for quantification of changes at the BBB *in vivo* with a sensitivity superior to conventional MRI.

#### OR-107. Immunotherapy of Relapsing Experimental Autoimmune Encephalomyelitis (EAE) by Neutralization of CD4+ T Cell Chemoattractant Cytokine IL-16.

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Experimental autoimmune encephalomyelitis (EAE) is a CD4+ T cell mediated autoimmune disease. IL-16 is a CD4+ cell chemoattractant cytokine. Infiltration of the CNS by CD4+ Th1 cells precedes onset and relapses of experimental autoimmune encephalomyelitis (EAE). We reported that (B6 × SJL) F1 (H-2<sup>b/s</sup>) mice, with severe relapsing-remitting disease, had extensive infiltration by CD4+ T cells compared to C57BL/6 (B6) (H-2<sup>b</sup>) mice, which developed mild low-relapsing disease in response to myelin oligodendrocyte peptide 35–55 (MOG<sub>35–55</sub>). This observation led us to search for mechanisms that specifically regulate trafficking of CD4+ T cells in relapsing H-2<sup>b/s</sup> mice. In this report we show that the CD4+ cell chemoattractant cytokine IL-16, has an important role in regulation of relapsing EAE induced by MOG<sub>35–55</sub> in the (B6 × SJL) F1, (H-2<sup>b/s</sup>) mice. We found production of IL-16 within the CNS of mice with EAE. Levels of IL-16 in the CNS correlated well with prominent infiltration by CD4+ T cells and

B cells during acute and relapsing disease. Infiltrating CD4<sup>+</sup> T cells, and occasionally CD8<sup>+</sup> T cells and B cells contained IL-16 immunoreactivity. Pro-IL-16 (80 kD) and cleaved IL-16 (55 and 17 kD) were found in spinal cord of mice with active disease. During remission IL-16 levels were significantly decreased. In relapsing mice, CNS levels of IL-16 peaked and paralleled with activation of caspase-3 and CD4<sup>+</sup> T cell infiltration. We identified CD4<sup>+</sup>IL-16<sup>+</sup>active-caspase-3<sup>+</sup> T cells within CNS infiltrates. IL-16 (55kD), which co-immunoprecipitated with CD4 coreceptor (CD4R), was the most abundant form of IL-16 found during relapse. Our data suggested that IL-16 was produced by infiltrating CD4<sup>+</sup>T cells through caspase-3 dependent mechanism. It also indicated functional relationship between IL-16 and CD4R, consistent with CD4R specific chemoattractant properties of this cytokine. Based on these observations, we treated EAE mice with IL-16 neutralizing antibody. Treatment with neutralizing anti-IL-16 antibody successfully reversed paralysis and ameliorated relapsing disease. In treated mice, diminished infiltration by CD4<sup>+</sup> T cells, lesser demyelination and more sparing of axons were observed. Taken together, we show an important role for IL-16 in regulation of relapsing EAE. We describe a novel therapeutic approach to specifically impede CD4<sup>+</sup> T cell chemoattraction in EAE, based on IL-16 neutralization. Our findings have high relevance for the development of new therapies for relapsing EAE and potentially MS.

#### **OR-108. High Resolution Dynamic Visualization of Immune Cell Function In Vivo in Health and Disease.**

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Immune responses involve multiple cell-cell interactions. We have used explant and intravital confocal or multiphoton microscopy to collect 4D (XYZ and time) data on the interactions of antigen (Ag)-specific T cells with each other and with Ag-bearing dendritic cells (DC) in an intact lymph node (LN). Some naïve T cells move rapidly in the absence of Ag but show prolonged adherence to Ag-bearing DC, accompanied by immunological synapse formation. Activation and detachment from the antigen-bearing DC follows, along with cell division. These data suggest that T cell activation follows from prolonged lymphocyte association with individual antigen-bearing DC rather than summation of signals from brief encounters with such presenting cells. CD4 and CD8 T cells associate with a single DC when both antigens are present and direct CD4-CD8 T cell contact is also seen. Formation of these clusters appears to be non-random in nature. Rapid movement of DC dendrites is readily visualized, as is T-DC contact through these processes, followed by movement of the T cell towards the DC body. Quantitative analysis suggests an impact of self-MHC recognition on the time of naïve T cell-DC interaction. Intravital methods have permitted visualization of DC migration into LN and the egress of lymphocytes from HEV for initial contact with DC. Fluorescent reporter constructs (e.g., EGFP under the control of the IL-2 promoter) are revealing the consequences of T-DC interactions in real time within LN and fluorescent chimeric proteins are being used to track redistribution of key proteins during cell movement and interaction. Differential migratory behavior of lymphocytes and DC in distinct regions of the lymph node has been observed, as has the failure of rapidly moving T and B lymphocytes to cross rather strict borders between the T cell zone and B cell follicle. DC distribution and function are

being examined in non-lymphoid tissues such as liver (under steady-state conditions and during inflammation) and kidney. The behavior of subepithelial DC in the small bowel has been visualized under steady-state conditions and following infection with Salmonella, which elicits an active protrusion response from the DC. These studies are contributing to a more accurate picture of the molecular, cellular, spatial, and temporal aspects of cell interaction and signaling events in host immune responses.

#### **OR-109. Migration Matters: Regulatory T Cell Compartmentalization Determines Suppressive Activity In Vivo.**

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Regulatory T cells (Tregs) have fundamental functions for suppression of immune responses, however, the compartments at which they exert their suppressive functions in vivo are not known. The integrin  $\alpha_E\beta_7$  unravels a fundamental dichotomy between naïve-like and effector/memory-like CD4<sup>+</sup> Tregs, where only the latter are capable of suppressing Th1-mediated inflammatory immune responses. Strikingly, suppressive action of  $\alpha_E^+$  Tregs is completely dependent on their inflammation-seeking capacity: Tregs from fucosyltransferase-deficient animals, which lack selectin-ligands and fail to migrate into inflamed sites are unable to mediate suppression. In contrast, naïve-like  $\alpha_E$ -CD25<sup>+</sup> Tregs, which show an enhanced recirculation through lymph nodes, are more efficient in preventing priming of naïve CD4<sup>+</sup> T cells. These findings provide first conclusive evidence that appropriate localization is crucial for in vivo activity of Tregs.

#### **OR-110. The Anti-Inflammatory Action of Methotrexate Is Not Mediated by Lymphocyte Apoptosis, but by the Suppression of Activation and Adhesion Molecules.**

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Low-dose methotrexate (MTX) is an established and highly effective treatment for severe psoriasis and rheumatoid arthritis; however, its mechanism of action remains unclear. When used for the treatment of psoriasis, MTX was thought to act directly against the epidermal hyperproliferation, however, the poor efficacy of locally administered MTX and the effectiveness of agents that target T cells strongly suggest that the anti-proliferative effect of MTX is not responsible for its efficacy in psoriasis. Therefore, we investigated the effects of low-dose MTX on T cells and explored through which cellular pathways these effects are mediated.

Peripheral blood mononuclear cells were isolated and stimulated with streptococcal antigens, superantigens or the mitogen PHA in the presence of 0–10 mM MTX. T cell expression of adhesion molecules and activation markers, and the amount of T cell apoptosis in cultures, were determined flow cytometrically. The folate- and adenosine-dependent pathways of MTX action were manipulated using specific agonists and antagonists.

We show that MTX caused a dose-dependent suppression of T cell activation and adhesion molecule expression, and this was not due to T cell apoptosis. The suppression of intercellular adhesion molecule (ICAM)-1 was adenosine and folate-dependent, while MTX suppression of the skin-homing cutaneous lymphocyte-associated antigen (CLA) was adenosine-independent. The effect



of MTX on CLA, but not ICAM-1, required the constant presence of MTX in cultures.

The suppression of T cell activation and T cell adhesion molecule expression, rather than apoptosis, mediated in part by adenosine or polyglutamated MTX, or both, are important mechanisms in the anti-inflammatory action of MTX.

#### **OR-111. Conditional Deletion of Alpha (v) Integrins Causes Inflammatory Bowel Disease.**

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Integrins are heterodimeric transmembrane proteins that regulate cell-cell and cell-matrix interactions. The alpha (v) containing integrins represent a major family of RGD-binding integrins, and have been shown to have important roles in angiogenesis, tumorigenesis, neural development and wound healing. Alpha (v) beta (3) is expressed by many immune cells and surface expression is highest in tissue resident cells such as  $\gamma\delta$  T cells and B1 B cells. Various alpha (v) integrins are expressed by monocytes, macrophages and DCs, and have been shown by antibody blockade to regulate monocyte transmigration and phagocytosis of apoptotic cells by macrophages and DCs. However definitive *in vivo* studies of the role of alpha (v) in the immune system have been limited by the lethality of alpha (v) knockout mice. Here we describe the generation of conditional knockout mice to study the role of these adhesion molecules as regulators of leukocyte function. Conditional deletion of alpha (v) using mice expressing CRE from the Tie2 promoter generated mice lacking alpha (v) in endothelial cells and all hematopoietic cells. Although these mice appeared normal at birth, they developed signs of chronic disease and weight loss beginning at 8 weeks, which progressed such that 75% of experimental animals had died by 40 weeks. Pathological examinations revealed that the mice had developed spontaneous transmural gastro-intestinal (GI) and respiratory tract inflammation, ulceration and epithelial cell hyperplasia. These histological findings, in combination with the clinical observations of wasting and GI obstruction are consistent with chronic progressive inflammatory bowel disease (IBD). To further define this phenotype we have selectively deleted alpha (v) in specific leukocyte compartments and our results suggest that alpha (v) integrins regulate both cell migration and cell responses to pathogen derived ligands. In conclusion these data demonstrate that alpha (v) plays an essential role in regulating immune homeostasis in the GI and respiratory tracts, and that deletion of alpha(v) generates a new model of spontaneous, chronic IBD. We therefore propose a novel function for alpha (v) integrins in the normal regulation of inflammatory responses and immune homeostasis.

### **Cytokine Mediated Immunoregulation**

3:30 PM–5:30 PM, 5/15/2005

#### **OR-112. Phenotype of Cytokine Expressing Cells in Peanut Allergen-Primed Mice.**

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Food allergy is a significant health problem, particularly in Western countries. In the UK and the USA, peanuts are a common cause of food allergy, associated usually with high titer IgE antibody and consistent with the preferential activation of T helper (Th) 2 type cells. Using high IgE responder BALB/c strain mice, we have previously shown that sensitization with peanut lectin (a minor peanut allergen) is associated with an increase in allergen-specific IgE and changes in cytokine protein and mRNA expression indicative of a selective Th2 type response, with elevated levels of IL-4, but not IFN- $\gamma$ , cytokine expression. Here we have used flow cytometric analyses of intracellular cytokine expression patterns to determine the relative contributions of CD4 and CD8 T lymphocytes to the immune phenotype that develops following exposure to peanut lectin.

**Methods:** Mice were immunized by intradermal injection of 1mg/ml peanut lectin. Fourteen days following the initiation of exposure, draining auricular lymph nodes were excised and a single cell suspension prepared. Draining lymph node cells from peanut primed or naive mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) to identify proliferating cells, and restimulated *in vitro* with peanut lectin or with the T cell mitogen concanavalin A (con A) for various periods of time. Cells were stained with fluorescently-labeled anti-CD8 or -CD4 antibodies and following saponin permeabilization with fluorescently-labeled anti-cytokine antibodies.

**Results:** *In vitro* stimulation of peanut-primed cells with peanut lectin or con A induced proliferation of CD4 and CD8 cells from 48–120 hrs. In contrast, cells from naive mice responded only to con A. Moreover, allergen-specific CD4 cells expressed a Th2 profile with increased frequencies of IL-4 (6.3%) and IL-10 (5.9%) and relatively low levels of IFN- $\gamma$  (0.9%) positive cells compare with unstimulated controls or with cells cultured with con A, after 96 hrs in culture. CD8 cells displayed a Tc1 phenotype with high levels of IFN- $\gamma$  (6.5%) positive cells but few IL-4 (0.7%) and IL-10 (1.3%) positive cells regardless of whether restimulation was with con A or allergen.

**Conclusion:** These data suggest that CD4, rather than CD8, T lymphocytes are skewed towards a selective type 2 cytokine phenotype in a mouse model of peanut allergy.

#### **OR-113. Foxp3+ Regulatory T Cells can be Induced from CD4+CD25- TGF $\beta$ Transgenic Cells and are able to Condition Dendritic Cells to Suppress Naïve T Cells.**

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TGF $\beta$  is a highly conserved multifunctional cytokine that has diverse regulatory roles in the immune system. Regulatory T cells that secrete TGF $\beta$  and variable amounts of IL-4 and IL-10, termed Th3, can be induced by oral administration of myelin proteins and mediate recovery from EAE. Little is known about the differentiation, phenotype and function of these cells. We created an inducible TGF $\beta$ -1 transgenic (Tg) mouse model in which TGF $\beta$  is linked to the IL-2 promoter and thus allows tissue specific expression of TGF $\beta$  upon TCR stimulation. We found that antigen specific stimulation of naive peripheral CD4+CD25- T cells from TGF $\beta$  Tg mice induces Foxp3-expressing Th3 cells that are hyporesponsive and that have potent suppressive activities *in vitro* and *in vivo*. TGF $\beta$  Tg cells do not secrete IL-2, IFN- $\gamma$ , IL-13 or IL-10. Early expression of TGF $\beta$ , not IL-4 or IL-10, is critical for the differentiation of Th3 cells. In a MOG peptide TCR Tg adoptive transfer model, Th3 cells from TGF $\beta$  Tg mice not only

prevented the generation of pathogenic Th1 cells in wild type animals before the induction of EAE, but also greatly inhibited the effector functions if transferred at the time of disease onset. Using a two-step in vitro culture system, we found that antigen-presenting dendritic cells can act as 'temporal bridges' to relay the inhibitory signal from Th3 cells to naive CD4 T cells. Furthermore, Th3 cells inhibit the T cell induced up-regulation of CD80/CD86 costimulatory signals during activation but not the maturation. Thus, the suppression by Th3 cells is mediated by mature dendritic cells with altered antigen-presenting function.

**OR-114. Atorvastatin Prevents the Th1 Differentiation of Myelin-Reactive T Cells during EAE by Interfering with the Prenylation of Ras and the Activation of ERK.**

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Previously, we showed that treatment with the HMG-CoA reductase inhibitor Atorvastatin (AT) prevented the Th1 differentiation of myelin-reactive T cells and ameliorated clinical symptoms in mice with experimental autoimmune encephalomyelitis (EAE) (Youssef *et al.*, *Nature* 420: 78, 2002). HMG-CoA reductase is a critical enzyme in the mevalonate biosynthetic pathway that generates not only cholesterol, but also isoprenoid derivatives that function to attach certain signaling proteins and ubiquinone to cell membranes. The aim of the present study was to distinguish which of these pathway intermediates have a regulatory function in Th1 differentiation during the development of EAE. Here, we provide first-time evidence that oral administration of AT induces a Th2 bias CD4<sup>+</sup> cells by compromising the production of the isoprenoid lipids farnesyl-pyrophosphate (-PP) and geranylgeranyl-PP. In vivo depletion of these metabolic precursors by AT caused a transient (i.e., 4–12 h/d) redistribution of farnesylated Ras and geranylgeranylated RhoA GTPases from the membrane to the cytosol of T cells. This was accompanied by a reduction in the phosphorylation of ERK and the DNA binding of c-fos in response to T cell receptor activation. We also show that selective inhibition of the ERK pathway with the MEK inhibitor PD98059 shifted the balance in T cell cytokine production towards Th2. Since ERK activation has been shown to be required for transactivation of IFN- $\gamma$  and for repression of the IL-4 promoter (Jorritsma *et al.*, *J. Immunol.* 170: 2427, 2003), these results thus explain why CD4<sup>+</sup> cells undergo Th2 differentiation when activated by antigen in the presence of AT.

**OR-115. Regulation of T Cell Differentiation by IL-4R $\alpha$ -Chain Single Nucleotide Polymorphisms.**

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Chronic inflammation in rheumatoid arthritis (RA) is mediated by repeatedly activated pro-inflammatory Th1 cells. In contrast, Th2 cells that might down-modulate the chronic autoimmune response are rarely found in RA. It has been previously documented that RA T cells are severely impaired in their ability to differentiate into Th2 effectors while exerting enhanced Th1 differentiation. The mechanisms underlying this functional abnormality, however, have not been delineated. As interleukin-4 (IL-4) is a most critical determinant in regulating immune responses by

promoting Th2 cell development and inhibiting Th1 cell differentiation, we analyzed the role of single nucleotide polymorphisms (SNP) in the IL-4 receptor  $\alpha$ -chain, which is critical for binding of IL-4 and for IL-4 signal transduction, in the differentiation of human T cells. 361 healthy individuals were genotyped by allele specific PCR for the two IL-4R  $\alpha$ -chain SNPs that are located in functionally important regions of the IL-4R  $\alpha$ -chain—the I50V SNP50 and the Q551R SNP551 in the IL-4-binding and STAT6-binding domains, respectively. Naive and memory CD4 positive T cells were isolated from the peripheral blood of individuals who were homozygous for either allele at SNP50 and SNP551, and primed for five days with mAbs to CD28 and/or CD3 in the presence or absence of exogenous IL-4. The phenotype of the resulting differentiated effector cells was then analyzed by flow cytometric analysis of cytoplasmic cytokines. The SNP551 alleles did not affect T cell differentiation. In contrast, the inhibitory effect of IL-4 on Th1 cell differentiation was significantly diminished in CD4 T cells that were homozygous for the mutant allele at SNP50 (50V) as compared to those with the wild type allele (I50). Likewise, the augmenting effect of IL-4 on Th2 cell differentiation was enhanced on T cells that were homozygous for the wild type allele as compared to T cells expressing the mutant allele. These data indicate that the mutant allele of the IL-4R  $\alpha$ -chain at SNP50 is associated with a decreased T cell response to IL-4. To delineate a potential mechanism of different responses to IL-4 in the cells expressing different alleles of the IL-4R, T cells from individuals who were homozygous for either the wildtype or the mutant allele at SNP50 were primed with different concentrations of IL-4 and analyzed by flow cytometry for STAT6 and phosphorylated STAT6. Whereas STAT6 concentrations were not different between T cell expressing I50 or V50, STAT6 phosphorylation in response to IL-4 stimulation was significantly reduced in T cells expressing the V50 allele compared to T cells expressing I50. Thus, the V50 SNP50 allele of the IL-4R  $\alpha$ -chain might regulate T cell differentiation by diminishing T cell responses to IL-4, resulting in reduced STAT-6 phosphorylation and subsequently in diminished Th2 cell differentiation. The V50 SNP50 allele might thereby contribute to the development of unbalanced Th subset activation, as characteristic for autoimmune diseases, such as RA.

**OR-116-Diverging Roles for the IL-12 Family in T Cell Immunity: IL-12 and IL-27-EBI3 Have Similar Contributions to Pathogen Responses While IL-23 Is Unique to Certain Autoimmune Pathways.**

Jacqueline M. Benson,<sup>1</sup> Yevgeniya I. Orlovsky,<sup>1</sup> Tayseer Ghazzouli,<sup>1</sup> Mark R. Cunningham,<sup>1</sup> Kimberly A. Shamberger,<sup>1</sup> Mark A. Schneider,<sup>1</sup> Patricia A. Rafferty,<sup>1</sup> Peter J. Bugelski,<sup>1</sup> Don E. Griswold,<sup>1</sup> M. L. Mbow.<sup>1</sup> <sup>1</sup>Discovery Research, Centocor, Inc, Radnor, PA, USA.

The IL-12 cytokine family (consisting of IL-12, IL-23, and IL-27) is proposed to mediate Th1 immune responses. Therefore, we utilized subunit-specific neutralizing monoclonal antibodies or genetic knockout mice to distinguish the individual contributions of IL-12, IL-23, and IL-27 in established murine models of Th1 autoimmunity and pathogen responses, namely experimental autoimmune encephalomyelitis (EAE) and *Leishmania major* infection. Specific neutralization of IL-12p35, or mice genetically deficient in IL-27-EBI3 demonstrated no protection from the incidence or severity of EAE. However, they each exhibited transient susceptibility to *L. major* infection as demonstrated by

increased lesion size. These data suggest that IL-12 and IL-27-EBI3 each contribute to *L. major* immunity, although they are not required for EAE. In contrast, specific *in vivo* neutralization of IL-23 provided significant and long-lasting therapy of EAE when antibodies were administered prior to disease induction or onset, or during established EAE. Anti-IL-23 suppressed central nervous system inflammation and pathology even though antigen-specific proliferation and cytokine re-stimulation responses were not influenced by *in vivo* antibody treatment. FACS analysis demonstrated that *in vivo* IL-23 neutralization preserved a more naive (CD62L<sup>hi</sup>, CD45RB<sup>hi</sup>, CD69<sup>lo</sup>) CD4+ T cell phenotype. Thus, IL-23 appears to participate in the *in vivo* activation and/or trafficking of encephalitogenic T cells. Mice treated with IL-23 specific neutralizing antibodies maintained their protective T cell immunity to *L. major* infection. Thus, despite its critical role in EAE, IL-23 does not appear to contribute to effective *L. major* resistance. Overall, IL-12 and IL-27-EBI3 seem to be more closely related in function than IL-23. Further investigation will likely continue to delineate the roles of IL-12, IL-23, and IL-27 in autoimmune disease and pathogen immunity.

#### **OR-117. IL-4 Can Be a Key Positive Regulator of Inflammatory Arthritis.**

Koichiro Ohmura,<sup>1,2</sup> Linh T. Nguyen,<sup>1</sup> Richard M. Locksley,<sup>3</sup> Christophe Benoist,<sup>1</sup> Diane Mathis.<sup>1</sup> <sup>1</sup>*Immunology and Immunogenetics, Joslin Diabetes Center, Harvard Medical School, Boston, MA, USA;* <sup>2</sup>*Internal Medicine, Dohgo Spa Hospital, Center for Rheumatic Diseases, Matsuyama, Ehime, Japan;* <sup>3</sup>*Medicine, UCSF, San Francisco, CA, USA.*

**Objective:** Development of arthritis in the K/BxN mouse model is dependent on the induction of very high titers of antibodies (Abs) against the glycolytic enzyme glucose-6-phosphate isomerase, or GPI, promoted by CD4+ T cells expressing a transgene-encoded T cell receptor (TCR) specific for GPI. Our goal was to determine whether this unusually strong autoAb response, presumably reflecting unusually potent help, depends on T cell differentiation to the T helper (Th)1 or Th2 phenotype. The answer to this question might generate important insights into human arthritides, such as rheumatoid arthritis (RA), associated with the production of autoAbs.

**Methods:** The roles of cytokines known to control Th phenotype were investigated by introducing the interleukin (IL)-4 and IL-12p35 knockout mutations into the K/BxN model, and evaluating the impact of these deficiencies on clinical arthritis, autoAb production and T cell activation. The IL-4-expressing cell-types in K/BxN mice were revealed by crossing in a knock-in alteration resulting in green fluorescence protein (GFP) expression controlled by endogenous IL-4 gene regulatory elements. Transfer experiments permitted the identification of the IL-4-producing cell-type required for arthritis development. Finally, quantitative RT-PCR allowed determination of the cytokine profile of K/BxN T cells.

**Results:** While IL-12p35 appeared dispensable for the development of arthritis, IL-4 was crucial for full disease development. IL-4-deficient K/BxN mice had greatly reduced titers of anti-GPI Ab. The GPI-reactive TCR of standard K/BxN mice induced the transcriptional activation of the IL-4 locus in CD4+ T cells and in CD11b+ eosinophils. Yet, K/BxN arthritis is not a pure Th2 disease, as both Th1- and Th2-type cytokines were upregulated in K/BxN T cells, and the expression pattern of several cytokines in K/BxN T cells did not match that of conventional Th2 cells.

**Conclusion:** IL-4 is crucial for the development of anti-GPI-Ab-mediated arthritis in the K/BxN mouse model. However, the cytokine profile of initiating anti-GPI T cells does not fit that of a classical Th2 disease. The potential for IL-4 to promote the development of inflammatory arthritis should raise caution over proposed therapies for RA aimed at biasing T cells towards IL-4 production.

#### **OR-118. IL-12/23-Deficient Genotype Reveals Two Distinct Pathways Leading to Arthritis in Mice.**

H. Hess.<sup>1</sup> <sup>1</sup>*Molecular Pharmacology and Physiology, Biogen Inc., Cambridge, MA, USA.*

Collagen-induced arthritis (CIA) is an inflammatory joint disease in rodents. Its etiology involves pathogenic autoimmune responses, which are provoked by immunization with collagen type II (CII) together with adjuvant. Mycobacteria, as part of complete Freund's adjuvant (CFA), are potent inducers for IL-12 production by macrophages and DC. IL-12 is a key cytokine that instructs naive T cells, upon activation, to differentiate along the T helper type 1 (Th1)-pathway. CIA, like RA, claims to be regarded as a predominantly Th1-type autoimmune disease. However, as disease progresses, certain Th2-type features become detectable. It appears that CIA and perhaps RA are mixed type immune responses.

A recently described heterodimeric cytokine, IL-23, shares the p40 subunit with IL-12. Both cytokines are implicated in either initiating or sustaining Th1-type responses. Initial studies have shown that CIA development in IL-12/23p40-ko mice is markedly reduced. We have extended these studies and found that, provided that IL-12/23p40-deficient mice had been sensitized with pristane prior to priming with CII, severe CIA develops at 95% incidence.

When pristane sensitization precedes CII/adjuvant immunization, mycobacteria become dispensable for CIA induction. CIA incidence and severity in wild-type mice immunized with CII is comparable to the disease observed in pristane-sensitized IL-12/23p40-K/O mice. Notably, in wild-type mice, repetitive immunization with CFA can substitute for the requirement of pristane-sensitization to maximize CIA severity. IL-12/23-deficient mice do not respond to this provocation with significant CIA development. Taken together, the data suggest that pristane triggers an IL-12/23-independent pathway capable of enhancing the auto-immunogenic stimulus set by CII. Finally, it appears that both, IL-12 and IL-23 are sufficient, but not necessary to trigger severe arthritis.

#### **OR-119. Systemic Onset Juvenile Idiopathic Arthritis Is an IL-1 Mediated Disease.**

F. Allantaz, E. Arce, M. Punaro, J. Banchereau, V. Pascual.<sup>1</sup> <sup>1</sup>*Baylor Institute for Immunology Research, Baylor Institute for Immunology Research, Dallas, TX, USA.*

**BACKGROUND.** Systemic Onset Juvenile Idiopathic Arthritis (SOJIA) remains an enigmatic pediatric rheumatic disease. Most patients require systemic corticosteroids for prolonged periods to control the systemic manifestations, and half the patients develop chronic arthritis that is difficult to control even with methotrexate and anti-TNF agents. The IL-1 antagonist Anakinra is partially effective in the treatment of inflammatory chronic arthritis, but it has not been evaluated in SOJIA patients with systemic symptoms.

**METHODS.** Healthy PBMCs were incubated with the serum of SOJIA patients. Changes in gene transcription were assessed using oligonucleotide microarrays and real-time PCR. Genes whose expression was most significantly altered were identified and analyzed in the PBMCs of 16 SOJIA patients and 12 healthy

controls. Additionally, we have performed global gene expression analysis using blood PBMC RNA from 31 SOJIA patients to hybridize Affymetrix U133 gene arrays. One third of the patients were in remission, one third had systemic symptoms and the remaining one third had polyarticular arthritis with no systemic involvement at the time of analysis. We also compared the gene expression of these patients to children with systemic infections. PBMCs from healthy controls and SOJIA patients were exposed in vitro to PMA/Ionomycin to assess their cytokine secretion capacity. Twelve SOJIA patients were treated with Anakinra for 2–16 months.

**RESULTS.** SOJIA serum increased the transcription of IL-1a, IL-1b and other innate immunity genes. Several of these genes were upregulated in vivo in the PBMCs of SOJIA patients. PMA induced the release of IL-1b from the PBMCs of SOJIA patients but not from healthy PBMCs. Treatment of 12 SOJIA patients during the systemic and/or arthritic phase of the disease with Anakinra for a period of 2–16 months induced complete disease remission in 9/12 patients and a partial response in the remaining 3 patients.

**CONCLUSION.** SOJIA patients show a dysregulated IL-1 production and IL-1Ra is an effective treatment for this disease.

### Development of Immune Based Therapeutics

10:30 AM–12:30 PM, 5/16/2005

#### OR-120. A High Affinity, Soluble T Cell Receptor for Targeting Tumours Expressing NY-ESO and HLA-A2.

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Human tumours over-express a variety of TAAs (Tumour Associated Antigens), which are either absent or expressed at low levels in normal tissues. Peptides derived from TAAs are presented on the surface of tumour cells by Class I HLA molecules, and represent targets for cytotoxic or immunotherapeutic anti-cancer agents. NY-ESO is a TAA of unknown function, over-expressed in a number of tumour types, including melanoma and bladder. We have generated a soluble TCR (T Cell Receptor) specific for an NY-ESO peptide presented by HLA-A2. The TCR lacks transmembrane domains and is stabilised by a novel disulphide bond; it is expressed in *E. coli* as separate  $\alpha$  and  $\beta$  chains, and refolded from inclusion bodies. The natural affinity of the TCR is 24 $\mu$ M; in order to generate a molecule suitable for targeting tumours, the TCR was affinity matured using phage display technology. The TCR variant used in subsequent studies is estimated to have an affinity of 20pM by BIAcore analysis, and a half life on the HLA complex of 19 hours; it is highly specific for HLA A2-NY-ESO. We show data to demonstrate that the TCR binds specifically to NY-ESO peptide pulsed cells (by FACS analysis), and also that it targets tumour cells expressing NY-ESO, by fluorescence microscopy. Furthermore, the TCR specifically inhibits activation of a T cell clone by NY-ESO +ve tumour cells, measured by INF $\gamma$  ELISPOT. Immune activators, including cytokines, have been fused to the TCR  $\beta$  chain C terminus; these fusion proteins are suitable for development as immunotherapeutic agents, to treat NY-ESO +ve tumours.

### Immundiagnostic Disease Predictors

10:30 AM–12:30 PM, 5/16/2005

#### OR-121. GAD65- and Proinsulin-Specific CD4+ T-Cells Detected by MHC Class II Tetramers in the Peripheral

### Blood of Type 1 Diabetic Patients and Prediabetic Subjects.

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In type 1 diabetes the major loss of insulin producing beta cells is caused by autoreactive T-cells specific for antigens expressed by the pancreatic islets. Autoantibodies to insulin and glutamate decarboxylase 65 (GAD65) are strongly associated with type 1 diabetes. In this study we have analyzed the prevalence of GAD65- and proinsulin-specific CD4+ T-cells in type 1 diabetic patients, prediabetic subjects (positive for two or more autoantibodies) and in HLA-genotype matched islet-cell autoantibody (ICA) negative healthy children. Peripheral blood mononuclear cells, from DRB1\*0401, 0404 or 0301 positive children in these three study groups, were cultured in the presence of two different GAD65 peptides (557I; aa 555–567 and aa 274–286) or with a proinsulin (aa 24–36) peptide for 10–11 days. Thereafter the cells were restimulated with MHC class II monomers for 3 days. The monomers contained the same peptides as used in the primary stimulation. Binding of CD4+ T-cells to GAD65 or proinsulin-containing MHC class II tetramers was analyzed by flow cytometry. Our results show that 11 of 18 (61%) type 1 diabetic patients and 7 of the 20 (35%) prediabetic subjects were positive for one of the GAD65 or proinsulin-containing tetramers, whereas only 3 of 23 (13%) ICA negative healthy controls had tetramer binding cells. The difference between type 1 diabetic patients and healthy controls was statistically significant ( $P = 0.004$ , Chi-square test). The frequency of tetramer positive cells in the GAD65 or proinsulin activated CD4<sup>high</sup>/CD25<sup>+</sup> cells was higher in type 1 diabetic patients (0.00–9.19%) and in prediabetic subjects (0.00–53.60%) than in control subjects (0.00–2.84%) ( $P = 0.01$  and  $P = 0.03$  for respectively study group, Mann-Whitney U-test). In conclusion, type 1 diabetic patients and prediabetic subjects have a higher prevalence of GAD65- and proinsulin-specific CD4+ T-cells than HLA-genotype matched healthy controls.

### Poster Session 1

7:30 AM–1:30 PM, 5/13/2005

### Allergy/Asthma

#### F1.01. Prevalence of Aeroallergens in Allergic Rhinitis.

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**Background:** Allergic rhinitis is an extremely common disease world wide. Aeroallergens are very often involved in allergic rhinitis and their prevalence may vary in different regions. The causative allergens of allergic rhinitis in our area are uncertain. **Objectives:** The purpose of this study was to determine the aeroallergens which are prevalent in patients with allergic rhinitis in Shiraz. **Methods:** A total of 212 patients who were referred to Motahhari Allergy Clinic with chronic rhinitis were submitted to

skin prick test (SPT) with a series of common allergenic extracts including grasses, weeds, trees, house dust mites and moulds. **Results:** 132 subjects (62.2%) had positive SPT to at least one aeroallergen. Male to female ratio was 1.2 and mean age was 18.2 years. The prevalence rates for allergen groups were: pollens (92.4%), mites (22.7%) and moulds (8.3%). Among 122 patients reactive to pollens, 92 (75.4%) showed skin reactivity to weeds, 78 (63.9%) to grasses and 68 (55.7%) to trees. Polysensitization was common, with 75.7% of all sensitized patients positive to more than one aeroallergen.

### F1.02. Modulation of Immunological Functions of Mast Cells by Heat Shock.

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Mast cells play pivotal roles in immediate-type and inflammatory allergic reactions that can result in asthma. Cross-linking of the high-affinity receptor for IgE (FcRI) on mast cells activates a signaling pathway leading to Ca<sup>2+</sup> mobilization and is followed by degranulation and the release of histamine and other preformed mediators, as well as de novo synthesis of the arachidonic acid metabolites i.e leukotrienes, and prostaglandins. To investigate possible effects of heat shock on immunologic functions of bone marrow derived mast cells (BMMC), we studied degranulation and leukotrien production.

We found that heat shock inhibits degranulation of BMMC without effects on leukotriene production. To further elucidate the mechanism of suppression of degranulation, we studied the effects of heat shock on calcium influx and tyrosine phosphorylation. We found that heat shock inhibits calcium influx and tyrosine phosphorylation of Syk and SHIP. Since degranulation of mast cells play a role in allergic and non-allergic reactions our finding may have a relevance with respect to protective effects of heat shock response.

### F1.03. Naive CD4+ T Cell Activation by Antigen-Presenting Airway Eosinophils.

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In addition to the conventional "effector" functions of eosinophils, evidence that eosinophils function as antigen-presenting cells (APCs) has been increasing. A major distinction amongst potential APC types is between amateur and professional APCs. Amateur APCs stimulate only previously activated T cells and T cell hybridomas, whereas professional APCs are capable of initiating T cell responses. To investigate whether eosinophils are capable of initiating T cell responses in vivo, eosinophils were isolated from the spleens of IL-5 transgenic BALB/c mice by Percoll following MACS, and contamination with other APCs including dendritic cells was excluded. Co-culture of eosinophils with GM-CSF

increased their expression of costimulatory molecules including MHC-II. The GM-CSF stimulated eosinophils were allowed to take up OVA in vitro and then intratracheally injected into wild-type BALB/c mice that received intravenous injection of Ag-specific CD4+ T cells from DO11.10 OVA TCR transgenic BALB/c mice 24 h earlier. By alternatively using GFP-labeled eosinophils from IL-5 & GFP double transgenic mice and fluorescently conjugated OVA-beads, we demonstrated by fluorescence microscopy that the Ag-loaded eosinophil APCs were physically interacting with naive OVA TCR CD4+ T cells in the draining paratracheal lymph nodes (PLNs) 24 h after eosinophil transfer, while Ag-free eosinophils were randomly distributed across the PLNs with the donor CD4+ T cells. The physical interaction between Ag-loaded eosinophils and Ag-specific CD4+ T cells resulted in the activation of the naive CD4+ T cells, as measured by an early T cell activation marker CD69 by flow cytometry. However, this eosinophil APC function was completely impaired if eosinophils were pre-treated with RBC lysis buffer containing ammonium chloride, which inhibits antigen processing by eosinophils. Our data suggest that eosinophils may function as professional APCs to initiate T cell responses to a given antigen.

### F1.04. Transforming Growth Factor Beta-1 May up Regulate Interferon Gamma Production by Peripheral Blood Mononuclear Cells from Asthmatics and Normal Controls.

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#### Background:

TGF- $\beta$ -1, a multifunctional cytokine, has been shown to suppress immunoglobulin production in animal experiments. Plasma TGF- $\beta$  1 has been observed to be higher in some asthmatics compared to normal controls (Joseph *et al*, Ann of asthma Allergy). The effect of TGF  $\beta$ -1 on cytokine production by human peripheral blood mononuclear cells (PBMC) has not been investigated.

#### Methods:

PBMC from asthmatics and normal controls were isolated from heparinized blood by density-gradient centrifugation on Ficoll-Paque, washed three times in phosphate buffered saline and re-suspended at  $1 \times 10^6$  cells/ml in serum free medium (AIM-V). For cytokine measurements,  $1 \times 10^6$  PBMC/ml culture medium were incubated with phorbol-12-myristate 13-acetate (5ng/ml) and Ca<sup>2+</sup> ionophore (0.4mg/ml) in the presence of TGF  $\beta$ -1 (100pg/ml) alone and with antibody to TGF  $\beta$ -1. Following 72 hours of cell culture, supernatants were collected and stored at -800C until assayed for cytokines. ELISA kits from Pharmingen, USA were used to determine IFN- $\gamma$  in the supernatants from PBMC cultures.

#### Results:

So far results from 4 controls and 6 patients have been analyzed. The median IFN- $\gamma$  production in unstimulated cells (5.5 pg/ml) increased significantly following exposure to TGF  $\beta$ -1 (52 pg/ml) ( $p < 0.01$ ). Upon adding antibody to TGF  $\beta$ -1, the median IFN- $\gamma$  reduced to 10pg/ml ( $p < 0.01$ ). Following stimulation with PMA+ ionomycin, the basal median IFN-g production by PBMC (6144 pg/ml) significantly increased to 9599pg/ml upon exposure to TGF  $\beta$ -1 ( $p < 0.01$ ). Upon adding

specific antibody to TGF  $\beta$ -1, the median IFN- $\gamma$  level decreased to 5763 pg/ml ( $p < 0.01$ ). In the resting state, there was trend for PBMC from asthmatics to produce higher amount of IFN- $\gamma$  compared to PBMC from controls.

#### Conclusion:

TGF  $\beta$ -1 may up regulate the production of IFN- $\gamma$  by resting and stimulated PBMC in normal controls and asthmatics and this response was abrogated by specific antibody to TGF  $\beta$ -1.

#### F1.05. Increased Expression in CD30+ and CD57+ Molecules on CD4+ T Cells in Atopic Asthmatic Children: A Preliminary Report.

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Background: The phenotype of CD4+ T cells accumulated in chronically inflamed tissue, in allergic process, have been found to be mainly CD4+ memory T cells that express surface markers associated with IL-4 production. However the process of these cells and surface markers in peripheral blood have been not clearly determined. Objective: In this study we performed the frequency of surface markers on CD4+ T cells with IL-4 production in peripheral blood of atopic asthmatic children. Methods: Cross sectional study trial was carried out in 17 atopic asthmatic children and 12 healthy children as controls. The proportion of the peripheral mononuclear cells and surface molecules was studied by flow cytometry to identify surface molecules in CD4+ T cells (CD30, CD57, CD154, CD62L, and CD28), and IL-4. The analysis was performed on PBMC after PMA-Ionomycin stimulation, to examine IL-4 and INF-g production. Results: CD4+ CD30+ (median; 1.7, percentiles 25–75; 1.3–2.2), and CD4+ CD57+ (median; 3.3, percentiles 25–75; 2.2–4.4) T cells showed an increased production and relationship with IL-4 production in atopic asthmatic children. Conclusion: Although CD4+CD30+ T cells in peripheral blood have been observed in atopic dermatitis patients, in this work we identified similar cellular population in respiratory atopic diseases, and also CD57+ T cells, these cells seems to corresponds of CD4 T cells which expressing IL-4 under stimuli. That expressing markers could correspond early activation in atopic asthma.

Key words: Atopic asthma, children, CD4+ T cells, CD 30, CD57 and IL-4.

#### F1.06. CCR3 Expressed on CD4+ T Cells Correlations with IL-4 Production in Allergic Rhinitis Patients. Preliminary Report.

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In allergic process a number of studies have analysed the phenotype of CD4+ T cells that express surface markers preferentially associated with IL-4 production. However the repercussions of nasal challenge over these cells in peripheral blood have been not clearly determined. We found that CCR3 on CD4+ T cells correlated positively with IL-4 production. In conclusion the CD4 T house dust mite primed cells in allergic rhinitis patients expressing CCR3 that correlates with IL-4 production, these local challenge repercussion in peripheral CD4+ T cells could be observed only when those cells are stimulated with PMA-I.

#### F1.07. Mugwort-Pollen Allergy Represents an Ideal Candidate for Peptide Immunotherapy.

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Mugwort pollen allergens represent the main cause of pollinosis in late summer in Europe. Ninety-five percent of mugwort-allergic patients are sensitized to the major allergen Art v 1. In contrast to other common pollen allergens which contain multiple T cell epitopes, Art v 1 contains only one single immunodominant T cell epitope (Art v 1<sub>25–36</sub>). We characterized the minimal epitope of Art v 1<sub>25–36</sub> and investigated a possible association of Art v 1-reactivity with HLA class II-phenotypes.

Art v 1-specific T cell lines and clones were established from 51 patients with clinically defined mugwort pollen allergy and IgE specific for Art v 1. In 96% of the patients a cellular response to Art v 1<sub>25–36</sub> was obtained and a core region of 5–10 amino acids containing 3–5 amino acids essential for T cell reactivity was defined by using truncated and single-substitution analog peptides for T cell stimulation. The frequency of HLA-DRB1\*01 in patients recognizing Art v 1<sub>25–36</sub> was significantly increased as compared to healthy controls (69% vs. 21%; odds ratio: 8.45;  $p < 10^{-6}$ ). HLA-DRB1\*01 was identified as the main restriction element for the presentation of the immunodominant epitope using monoclonal anti-HLA antibodies and APC with defined HLA- DRB and DQB1-alleles.

In conclusion, allergy to Art v 1 is characterized by a uniform T cell reponse and the disease is associated with the HLA-DRB1\*01-phenotype. Therefore, mugwort pollinosis represents an ideal candidate for a peptide-based immunotherapy including the possibility of monitoring antigen-specific T cell responses during therapy by using HLA-DR-tetramers.

#### F1.08. Characterization of Human Cord Blood-Derived In Vitro Generated Mast Cells: Hemopoietic Antigens, Chemokine Receptors, Activation Markers, Tetraspanins.

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Mast cells (MC) play pathogenic role in allergic inflammation via releasing a broad spectrum of inflammatory mediators. We generated MC from human cord blood CD133+ hemopoietic precursors by culturing with rhSCF, rhIL-6 and rhIL-3 in Stem Span medium. To better characterize differentiation process, we mapped the expression of hemopoietic markers and chemokine receptors. Adhesion molecule ICAM-1 (CD54), IL-3 receptor (CD123), aminopeptidase N (CD13) and CD38 were present on MC and their precursors. Early hemopoietic markers CD133 and CD34, bright on freshly isolated precursors, disappeared within 2 weeks of differentiation. Development into mature MC was enhanced when cells underwent freezing/thawing cycle followed by culturing in the presence of 5% human serum. After 5 to 7 weeks they displayed typical features of mature MC: methachromatic staining with Gimsa-May Gruenwald, abundant expression of granular mast cell tryptase; surface expression of MC antigens c-kit (CD117) and FcεR1a; degranulation after cross-linking FcεR1a by IgE (+Ag). Both MC and precursors markedly expressed surface CXCR2 and CXCR4 and were negative for

CCR3. CCR5 was low to undetectable in precursors and absent in mature MC. Not reported before, MC and precursors substantially expressed surface CCR6 and some CCR7. Interestingly, we detected chemoattractant receptor homologous molecule expressed on Th2 cells, CRTH2, on the surface of MC and their precursors. As CRTH2 is a second receptor for prostaglandin D2 (PGD2), and PGD2 is a major prostanoid released from Ag-activated MC, our data suggest possible autocrine function of PGD2 for MC.

To find sensitive marker(s) of MC reactivity to Ag, we explored the correlation between MC degranulation and expression of activation markers CD63 (tetraspanin) and CD203c, both used for testing reactivity of basophils to allergens. We found both markers to be hardly detectable on the surface of MC precursors but high on mature MC both at the surface and intracellularly. This is the first evidence of CD203c presence on cord blood-derived MC. Expression of both CD63 and CD203c was further increased after IgE-dependent and independent stimulation, and this increase mirrored degranulation process.

We determined other members of tetraspanin family, CD9 and CD81, to be high on the surface of MC precursors. Expression of CD9 and CD81 was further augmented up to 10 fold with differentiation to mature MC. In contrast to CD63, surface expression of CD9 and CD81 diminished after stimulation with PMA/ionomycin but not after triggering with IgE (+Ag). Therefore, members of "tetraspanin web" could be differentially involved in MC activation.

These studies define potential targets for anti-allergic intervention and sensitive tools to monitor MC activation.

#### **F1.09. IL-4Ra Single Nucleotide Polymorphisms in Allergic Bronchopulmonary Aspergillosis.**

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**Rationale:** ABPA is a Th2 hypersensitivity lung disease resulting from bronchial colonization by *Aspergillus fumigatus* in asthmatic and cystic fibrosis patients. Previously, we reported HLA-DR2/DR5 restriction in ABPA patients. We propose that single nucleotide polymorphisms (SNPs) of IL-4Ra also play a role in the development of ABPA in asthmatic and CF patients.

**Methods:** DNA was extracted from cultures of B-cell lines of 26 ABPA and 29 non-ABPA patients and sequenced for IL-4Ra polymorphisms, including 1 extracellular (ile75val) and 4 cytoplasmic (glu400ala, cys431arg, ser503pro, and gln576arg) SNPs. IL-4 stimulated PBL from ABPA and control subjects were examined for the expression of CD23 on B cells by flow cytometry. HLA-DR genotyping was performed using standards techniques.

**Results:** The frequency of IL-4Ra SNPs was significantly increased in ABPA patients compared to non-ABPA subjects, 92% vs 55%. The ile75val SNP was identified in 77% of ABPA patients and was homozygous in 42%. Cytoplasmic SNPs were identified in 39% of the ABPA patients, and co-existence of extracellular plus cytoplasmic SNPs were observed in 27% of ABPA patients. ABPA subjects also had significantly increased expression of CD23 molecules per B cell of IL-4 stimulated PBL cultures compared to controls. In two ABPA patients we identified a previously unreported SNP in the IL-4 binding region at 468 A → C, asn98thr. In one ABPA patient, the

asn98thr SNP was associated with ile75val and ser503pro SNPs, and in the other patient the asn98thr SNP was isolated. This was also associated with up-regulation of CD23 expression on B cells by IL-4 stimulation.

**Conclusions:** The presence of IL-4Ra SNPs, particularly ile75val allele located within the IL-4 binding region may confer susceptibility to developing ABPA. In addition, a new SNP in the IL-4 binding region was identified in ABPA.

#### **F1.10. House Dust Mite Allergen (Der p1 and Blo t5) Levels in Asthmatics' Home in Hong Kong.**

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**Background:** House dust mite(HDM) allergen are involved in sensitization and development of allergic airway disease, particularly bronchial asthma and allergic rhinitis. Dermatophagoides pteronyssinus(Dp) and Blomia tropicalis(Bt) are the predominant inhalant allergens in most parts of the world. **Aim:** to measure Derp1 and Blot5 allergen levels in asthmatics' homes in HongKong.

**Methods:** Seventy houses were enrolled for a mite indoor environment study. Dust samples were obtained from two sites of each subjects' house: bed and floor. Derp1 and Blot5 levels were quantified by a two-site monoclonal antibody-based ELISA techniques. **Results:** The levels of Derp1 allergens were found in bed (GM: 3.43 ug/g of dust; 95%CI: 1.89–4.96 ug/g) and on floor (GM: 1.12 ug/g of dust; 95%CI: 0.71–1.53 ug/g) with significant differences( $P = 0.005$ ). However, the levels of Blot5 allergens were also found in bed (GM: 19 ug/g of dust; 95%CI: 0.89–38.9 ug/g) and on floor (GM: 6.14 ug/g of dust; 95%CI: 0.4–11.9 ug/g), with no statistically significant difference Blot5 allergens found in the different sites. In addition, Concerning the exposure index for Derp1 and Blot5 allergens found in bed and on floor, 17.6% in bed and 8.6% on floor had levels of Blot5 $\geq$ 10 ug/g of dust, higher than those obtained for Derp1 (7.2% and 0% in bed and on floor respectively,  $p < 0.05$ ); On the other hand, higher percentages in bed and on floor (25% and 35.7%) were observed for the levels of Blot5=0ug/g of dust as compared with Derp1 in bed and on floor (4.3% and 14.5% respectively,  $p < 0.05$ ). **Conclusions:** Der p1 and Blot 5 are the major sensitizing allergens in this region, Blot 5 is a more potent one in HongKong, probably reflecting the high level of exposure to Bt. The unique major Bt and Dp allergens should be included for precise diagnosis and effective immuno-therapeutic treatment of mite allergy in HongKong.

#### **F1.11. House Dust Mite Allergen Der f Increase Bronchial Epithelial Cell Cytokine Expression.**

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**Background:** The house dust mites (*Dermatophagoides farinae* (Derf)) are a major source of aeroallergens implicated in the expression of atopic disorders, including asthma, allergic rhinitis, and atopic dermatitis. In particular, strong circumstantial evidence suggests that house dust mites antigens are important precipitating factors of Asthma. Many house dust mite allergens are proteases that can elicit airway inflammation by stimulating the release of

cytokines in bronchial epithelial cells. **Objectives:** we have investigated whether Der f allergen proteases induced cytokine production from the epithelial cell line BEAS-2B. **Methods:** Cells were exposed to four different concentrations with serial additions of Der f (0.02, 0.2, 2, 20 ug/ml) were incubated for 24 h to 96 h. and compare with those without incubation of allergen. Cytokine in the supernatants were assayed by ELISA, Reverse transcription-PCR was also performed. **Results:** Cells treated with Der f allergen showed serial changes in the cohesiveness of the monolayer. There was a significant increase in the level of cytokine production compared with the untreated sample. Statistically Significantly increased with addition of Der f caused the release of IL-6 and IL-8 in time and concentration-dependent manner ( $p < 0.05$ , respectively). Levels of IL-6 and IL-8 were elevated 24 h and 48 h after allergen exposure, increasing with time, continued increased levels to be present of IL-6 and IL-8 in the supernatants at 72 h and 96 h. At the same time show the concentration dependence of induction of IL-6 and IL-8 expression as well as an increase in the expression of IL-6 and IL-8 mRNA. **Conclusion:** HDM-induced airway inflammation may include Der f-mediated release of inflammatory mediators, and the proteolytic activity of an allergen may stimulate the release of proinflammatory cytokines from human bronchial epithelium. Suggesting that IL-6 and IL-8 production by bronchial epithelial cells may play a role in the pathogenesis of allergic asthma.

#### **F1.12. *D. pteronyssinus* Extract-Treated Confluent A549 Cells (cA549) Secrete Mediators That Stimulate Human Pulmonary Fibroblasts To Increase Secretion of Vascular Endothelial Growth Factor (VEGF).**

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**Introduction:** The pathogenesis of airway remodeling involves altered interactions between epithelial and mesenchymal cells that lead to air wall thickening and edema (Davies D. *et al.*, J. Allergy Clin. Immunol., 2002; Frieri M. Allergy Asthma Proc. 2004). Airway remodeling is associated with increased VEGF and increased vascular permeability in the pulmonary submucosa (Lee KS *et al.*, J. Allergy Clin. Immunol 2004). 300, 600 and 1000 AU/ml dialyzed *D. pteronyssinus* extract (DpE) stimulated cA549 to secrete VEGF into serum-free conditioned media (CM) relative to DpE without cA549 (control media; CTLM) in 24 hours (Capetandes A. *et al.*, Am. J. Clin. Path., 2004). **Rationale:** Determine if mediators secreted into the CM by DpE-treated cA549 can stimulate NHLF to secrete VEGF relative to CTLM. **Methods:** Subconfluent NHLF were cultured for 24 hours with CM and CTLM generated with and without cA549, respectively, plus 0, 300, 600 and 1000 AU/ml DpE. The CM and CTLM were assayed for VEGF by ELISA. Cell number was measured by MTT incorporation at A550. Data is expressed as mean  $\pm$  2SD and analyzed by the two-tailed t test (two groups) or ANOVA ( $\geq$  three groups). **Results:** NHLF in serum-free media secreted VEGF relative to control ( $181 \pm 23$  pg/ml ( $n = 6$ ) versus  $3.3 \pm 0.5$  pg/ml ( $n = 4$ );  $P < 0.0001$ , t test); CTLM did not stimulate NHLF secretion of VEGF over control ( $n = 6$ ). Absolute VEGF levels were increased by the following conditions: CM + NHLF ( $1017 \pm 97$  pg/ml ( $n = 12$ ))  $>$  CM w/o NHLF ( $611 \pm 44$  pg/ml ( $n = 4$ ))  $>$  CTLM +NHLF ( $198 \pm 38$  ( $n = 12$ );  $P < 0.0001$  ANOVA). 1000 CTLM caused a decrease in NHLF cell number relative to CM 1000 ( $0.05 \pm 0.06$  versus  $0.15 \pm 0.014$  OD A550;  $n = 10$ ,  $P < 0.0001$ , t test. Relative

VEGF pg/ml: MTT with 1000 CM + NHLF was increased relative to 1000 CTLM + NHLF. **Conclusions:** cA549 secrete mediators that stimulate NHLF to increase secretion of VEGF. This suggests that the inhalation of aerosolized dust mite may stimulate alveolar epithelial cells to secrete mediators that stimulate fibroblasts to increase the secretion of VEGF. This may potentially contribute to the edema found in airway remodeling.

#### **F1.13. Skin Reactivity for Aeroallergens and Peripheral Eosinophilia in Children with Otitis Media with Effusion.**

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This study was performed to evaluate the probable effect of allergy in children with and without otitis media with effusion (OME). Is allergy more common in OME patients? Allergic patients might be helped with allergy treatment. Otitis media with effusion, a common childhood ear disease, has various predisposing factors that one of them may be allergy. Skin prick test (SPT) is able to determine allergic patients to common aeroallergens. The study was performed on 30 children with OME in khalili Hospital, a teaching hospital affiliated to Shiraz University of Medical Sciences in Iran. Myringotomy with or without ventilation tube insertion plus adenoidectomy were done for them. A group of 30 patients in the same age range (2–9 years) with normal middle ear whom underwent adenoidectomy was selected as control. Skin prick test for common aeroallergen (molds, grasses, weeds, trees and mites) was done. The presence of peripheral eosinophilia was also investigated in both groups. Peripheral eosinophil counts were significantly higher in the case group ( $p < 0.01$ ). 3 patients (10%) in OME group had positive SPT to weeds. SPT was negative in all children in the control group, however skin reactivity between two groups was not significantly different. We were not able to demonstrate a strong correlation between OME and positive skin test to common aeroallergens. We do not suggest SPT for evaluation of children with OME.

#### **F1.14. Recurrent Angioedema by Blastocystis Hominis Successfully Treated with Paromomycin.**

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**Background:** Published reports of urticaria associated parasitosis by Blastocystis hominis are uncommon. There have been no reported cases of angioedema by Blastocystis hominis. **Materials:** Clinical and immunological data of a patient with such association. **Case report:** The subject was a 21-year-old female with a 5-years history of episodic attacks of swelling of mouth, face and upper extremities accompanied by recurrent urticaria. She had been treated with different antihistamines and oral corticosteroids with only a partial response. Immunological tests performed in peripheral blood disclosed normal immunoglobulin levels (IgG 1160 mg/dl, IgA 182 mg/dl, IgM 120 mg/dl), normal percentages of lymphocyte subsets (CD3 77%, CD4 55%, CD8 18%, CD19 12%, CD56 7%); normal level of the complement factors C4 (19 mg/dl), C3 (100 mg/dl), FB (28 mg/dl) and of C1-inhibitor (15 mg/dl) as well as negative circulating immune complexes. Functional C1-inhibitor activity was also normal. IgE specific to ascaris, echinococcus and anisakis were negative.



Serologies for hepatitis virus B, hepatitis virus C, and HIV were negative. Complete blood and differential analysis as biochemical serum parameters were around normal ranges. Urinalysis was normal. Stool examination revealed *Blastocystis hominis* at 3 consecutive determinations. Both intestinal parasitosis as well as urticaria-angioedema responded successfully to paromomycin sulfate. Remission of urticaria-angioedema have been maintained after a 24-month of clinical follow-up. **Conclusion:** Diagnosis of *Blastocystis hominis* infection must be suspected in patients with otherwise frustrating chronic allergic skin disorder. Paromomycin might be of benefit in chronic persistent urticaria-angioedema associated with this parasitic infection.

### **F1.15. Exposure to Monomeric Human Myeloma IgE in the Absence of Known Specific Antigen Can Enhance Chemokine Production in *In Vitro*-Derived Human Mast Cells.**

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Two groups recently have reported that various mouse monoclonal IgE antibodies can induce mouse bone marrow derived mast cells to secrete mediators in the absence of known specific antigen. In this study, we investigated whether exposure to purified human myeloma IgE (catalog number A12162H, Biotest International, Kennebunk, ME) in the absence of known specific antigen had detectable effects on the mediator secretion of human mast cells that were generated *in vitro* from umbilical cord blood cells. Exposure to IgE at 2.5 micrograms/ml, but not IgG, significantly enhanced the release of chemokines, but not histamine or cysteinyl leukotrienes, from human mast cells. These results were obtained both with microcentrifuged preparations of IgE (which lacked large aggregates of IgE according to HPLC and mass spectrometry) and with HPLC-purified preparations of IgE monomers that were devoid of IgE dimers according to mass spectrometry. However, under all conditions of challenge tested, chemokine production in response to IgE alone was significantly less than that induced when aliquots of the same IgE-sensitized populations of human mast cells were stimulated by anti-IgE. The production of chemokines in response to exposure to IgE in the presence or absence of anti-IgE was inhibited by preincubation of the cells with dexamethasone. Overall, these results indicate that exposure to human myeloma IgE *in vitro* in the absence of known specific antigen can induce chemokine production by human mast cells at the concentrations tested. While the clinical relevance of these findings remain to be determined, one might speculate that effects of IgE on mast cells that are independent of known specific antigen can contribute to the pathogenesis of mast cell-associated disorders, particularly in subjects with high levels of IgE.

### **F1.16. Comparison of the Efficacy of Intranasal Phototherapy and Fexofenadine Hydrochloride for the Treatment of Seasonal Allergic Rhinitis.**

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We have recently showed that intranasal phototherapy using mixed low dose UVB, UVA and visible light (mUV/VIS) is effective in treating seasonal allergic rhinitis.

The aim of the present study was to compare the clinical efficacy of rhinophototherapy with fexofenadine hydrochloride. We performed an open study on 18 ragweed-allergic patients, during the ragweed season in Szeged. 11 patients received intranasal irradiation with increasing doses of mUV/VIS light for 2 weeks and 7 patients received 120 mg fexofenadine HCl once daily for the same period of time. Individual symptom scores and total nasal score (TNS) were recorded.

Rhinophototherapy resulted in a significantly better reduction of individual symptom scores for rhinorrhea ( $P = 0.0007$ ) and nasal obstruction ( $P = 0.014$ ) and of TNS ( $P = 0.004$ ) compared with fexofenadine HCl. No significant differences between the two treatments were observed in reducing symptom scores for sneezing, nasal itching, palate itching and eye symptoms. In addition, we have measured the wheal formation in skin prick test (SPT) by digital planimetry before starting the study and 10 days after ending the therapy. Interestingly, ten days after the end of the treatment, in the rhinophototherapy group the allergen-induced wheal formation was significantly reduced compared to baseline ( $P = 0.03$ ), in contrast in the fexofenadine treated group no differences were observed. No changes in histamine-induced wheal formation were observed. In our study, rhinophototherapy was significantly more effective than fexofenadine in treating allergic rhinitis. The prolonged inhibitory effect of rhinophototherapy on SPT suggests a long lasting effect that was not seen after fexofenadine treatment.

### **F1.17. Inhibition of the Peptidyl-prolyl Isomerase (PPIase) Pin1 Induces Caspase-3-Mediated Apoptosis of Human Peripheral Blood Eosinophils.**

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Eosinophils (Eos) are prominent cells in asthmatic inflammation. Once in the lung or airways, Eos show significantly prolonged survival. Anti-apoptotic activity is mediated by cytokines such as GM-CSF and IL-5, which are markedly increased in the asthmatic lung. Selective induction of Eos apoptosis has been proposed as a therapeutic approach for asthma. Previous studies have shown PPIase (cyclophilin A and FKBP) inhibitors suppress GM-CSF, IL-3 and TNF- $\alpha$  expression and function. These data implicate PPIase mediated *cis/trans* isomerization of pSer/pThr-Pro bonds in target proteins as a potential key regulator of cytokine expression. Recently, we have shown that inhibition of Pin-1, another PPIase, blocked the pro-survival effect of either GM-CSF or hyaluronic acid (HA). To identify the mechanisms underlying Eos apoptosis induced by Pin1 inhibition, we examined caspase-3 (Casp-3) activation. Eos were treated with the Pin-1 inhibitor juglone at 1.0  $\mu$ M and cell lysates examined for full-length Casp-3 proenzyme (p32) and active Casp-3 (p17) subunits by western blot analysis. As shown in previously published data, resting Eos underwent spontaneous (baseline) Casp-3 activation after 24 h in culture that was completely blocked by rhGM-CSF (100 pg/ml). Treatment of Eos with HA (100  $\mu$ g/ml), which is markedly increased in the airways of asthmatic lung, prevented spontaneous Casp-3 activation as well. However, treatment of Eos with juglone (1.0  $\mu$ M) induced Casp-3 activation, even in the

presence of rhGM-CSF or HA. Furthermore, apoptotic initiation by Pin1 inhibition was more apparent on Eos pre-activated with rhGM-CSF. Pre-incubation with high concentrations of rhGM-CSF or HA also failed to block juglone induced Casp-3 activation and cell death. Kinetic analysis showed that within 10 minutes juglone triggered extremely intense Casp-3 activation. Casp-3 activation was a very sensitive and early marker for the ultimate apoptosis of Eos. Trypan blue exclusion indicated that Eos viability remained high (between 86–97% at 4, 10 and 24 h) despite juglone treatment. These data indicate that Pin1 enzymatic activity is required for preventing Casp-3 activation and the initiation of apoptosis and does so downstream of the GM-CSF receptor.

#### **F1.18. Natural CD25<sup>+</sup>CD4<sup>+</sup> Regulatory Cells Inhibit Proliferation of CD25-CD4<sup>+</sup> Cells from Naive or Immunized BALB/c Mice When Stimulated by Dendritic Cells Pulsed with Fel d 1 or Major Fel d 1 T Cell Epitopes.**

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**Objectives:** Define in a BALB/c mouse *in vitro* model the dominant T cell epitopes of the major cat allergen Fel d 1. Test the effect of natural CD25<sup>+</sup>CD4<sup>+</sup> regulatory cells (T regs) on the response of CD25-CD4<sup>+</sup> cells from naive mice and mice immunized with Fel d 1. **Materials and Methods:** For the analysis of the proliferative immune response of naive mice and immunized mice, we used an *in vitro* system where myeloid-derived antigen-pulsed dendritic cells (DC) induced T cell proliferation. Immature DCs were harvested from mouse bone-marrow and matured *in vitro* for 7 days before being pulsed with antigen for 2 days. Lymphocytes were obtained from mouse spleen cells and added to the DC cells for 4 days, before <sup>3</sup>H thymidine addition and harvesting. Cell proliferation was measured for un-separated spleen lymphocytes and separated T cell subpopulations. CD25<sup>+</sup>CD4<sup>+</sup> and CD25-CD4<sup>+</sup> T cells were separated by magnetic beads and checked for purity by FACS. T cell stimulation was measured with whole Fel d 1 and 17 overlapping peptides. Immune BALB/c mice had been injected 3 times with Fel d 1 in Al(OH)<sub>3</sub>. **Results:** Un-separated splenic lymphocytes from naive mice did not give a significant proliferation when stimulated by Fel d 1 allergen or the 17 synthetic peptides. Un-separated lymphocytes from immunized mice gave significant stimulation indexes with Fel d 1 and peptide F 1.4 (aa 20–40 on chain 1). Purified CD25-CD4<sup>+</sup> lymphocytes from Fel d 1- immunized mice gave a significant stimulation with Fel d 1 and peptide F 1.4. When CD25<sup>+</sup>CD4<sup>+</sup> T regs were added to the CD25-CD4<sup>+</sup> cells, proliferation was inhibited. Purified CD25-CD4<sup>+</sup> cells from naive mice gave also a positive stimulation index when exposed to Fel d 1 or F 1.4 peptide. This proliferation was abolished by the addition of T regs from naive mice at a ratio of 1 T reg cell to 2 CD25-CD4<sup>+</sup> cells. **Conclusions:** The major cat allergen Fel d 1 seems to harbour one major T cell epitope containing region when tested in immunized BALB/c mice. Natural T regs from immunized mice inhibit CD25-CD4<sup>+</sup> lymphocyte proliferation, when stimulated by Fel d 1- allergen or F 1.4- pulsed DC. Natural T regs from naive mice inhibit CD25-CD4<sup>+</sup> proliferation from naive and immunized BALB/c mice when tested with Fel d 1- and F 1.4 peptide-pulsed dendritic cells.

#### **F1.19. Omalizumab Improves Asthma Outcomes Irrespective of Leukotriene Receptor Antagonist (LTRA) Use.**

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**Background:** Omalizumab (OMA) is a novel humanized monoclonal anti-IgE antibody for allergic asthma. OMA binds circulating IgE, leading to a reduction in high affinity IgE receptors on mast cells (MC), thereby reducing MC degranulation upon specific allergen exposure. This results in a decrease in MC release of allergic mediators such as histamine and leukotrienes (LTs). LTRAs block the effects of cysteinyl LTs responsible for some features of allergic asthma, however, they do not block other mediators or categories of LTs released by MC. To examine the effect of OMA in moderate-severe allergic asthma in patients using LTRAs, we evaluated asthma exacerbations and need for bursts of systemic steroids in a pooled analysis of two recently completed clinical trials.

**Methods:** INNOVATE (a 28 week randomized double-blind placebo-controlled study) and ETOPA (a 52 week open label trial) allowed concurrent LTRA use and were used for this analysis. Entry criteria and clinical outcomes were similar allowing for a pooled analysis. A total of 731 patients were in the intent-to-treat (ITT) population in the two studies. All patients received inhaled steroids (median dose 2000µg BDPE equivalent). Long acting betaagonists were used by all patients in INNOVATE and 87% of patients in ETOPA receiving concurrent LTRAs. Overall, LTRAs were used at baseline in 32.3% of patients (INNOVATE 34.8%, ETOPA 28.9%). Groups were compared using Poisson regression based on the ITT population, adjusting for baseline sex, age, use of oral steroids, FEV1 (>=80%, 60- <80%, <60%), study treatment and treatment-by-LTRA interaction.

**Results:** Patients on LTRAs tended to exhibit a greater level of asthma severity as evidenced by baseline history and trial incidence of clinical exacerbations, irrespective of treatment. The relative risk (RR) of asthma exacerbations (primary outcome; OMA vs. control) of the LTRA subgroup was 0.62 (95% CI: 0.42–0.91), which was somewhat lower than that observed for the overall study population. Similarly, the RR for use of systemic steroids (secondary outcome; OMA vs. control) for the LTRA subgroup was 0.5 (95% CI: 0.35, 0.72), similar to the effect size observed for the overall population.

**Conclusions:** In patients with moderate to severe asthma, OMA demonstrated efficacy in the LTRA subgroup that was similar to improvements shown in the overall population. Asthma morbidity, as assessed by clinical exacerbations, was improved in conjunction with significant reductions of systemic steroid bursts.

#### **F1.20. Administration of Chitin Down-Regulate Serum IgE Levels and Lung Eosinophilia in Ova-Albumin Allergic Mouse.**

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Inflammation in allergic asthma is generated and activated by endogenous proinflammatory cytokines including IL-4 and IL-5 produced by Th2-type lymphocytes. These allergen-induced Th2 responses enhance airway hyperreactivity in mouse models. In this study, we have shown that development of Th1/cell-mediated immune response significantly down-regulated Th2 responses by eliciting IFN-g production in experimental induced ova albumin (OVA) allergic BALB/c mice. Inhalation of chitin was made or mice were given chitin intravenously during the OVA-sensitization. Allergen-induced immunopathological responses, such as BALF cytology, anti-OVA humoral responses, and OVA-driven cytokines production were assessed. The administration of chitin significantly suppressed the immunopathological symptoms in OVA-sensitized mice. To dissect the inhibitory mechanisms of Th2 responses, spleen cells isolated from the chitin-treated or non-treated OVA-sensitized mice were cultured in the presence of OVA and/or chitin for 5 days. OVA alone stimulated the production of Th2 associated cytokines in both groups; in contrast, OVA/chitin stimulation resulted in the significantly increased production of IFN-g. Moreover, spleen cells isolated from the chitin-treated mice showed abundant amounts of IFN-g production with the stimulation of chitin, and less amounts of Th2 cytokine with or without OVA-stimulation, suggesting that the inhibited Th2 responses might explain the potential mechanisms, due to the changes in antibody isotypes and cytokines produced from splenocytes of mice receiving chitin. In summary, these results indicate that chitin-induced IFN-g responses successfully down-regulate Th2-facilitated IgE production and lung eosinophilia in the OVA allergic animals.

#### **F1.21. Allergic/Toxic Manifestations Associated with Ingestion of Stir-Fried/Cooked Mushrooms.**

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**Purpose:** The idea is to enlighten the series of allergic/ toxic manifestation with an un-expected onset on ingestion of cooked mushroom (*Cortinarius orellanus*), *Gyromitra*. *Gyromitra*, poisonings have also occurred after ingestion of commercially available morels contaminated with *G. esculenta*.

**Methods:** 10 cases (ages 16–50-years both sex) had been notified for treatment (1990–2000) to the emergency department of Al-Junaid hospital, with ingestion of cooked mushroom as vegetable (toxic species are confused with edible species), followed by adverse reactions (allergic/toxic) with a variable severity.

**Allergic manifestations** i.e., Itching burning flushing, tingling sensations, all over the body, Urticaria with variable severity.

**Toxic Manifestations:** Were Acute in onset (resulting from neurotoxins release) i.e., Nausea (15–30 min), vomiting (20–60 min) abdominal cramping, bloated feeling, watery diarrhea (20 min–13 h), prostration, dehydration, profuse sweating, coma convulsions hallucinations, excitement, depression, spastic colon, seizures (20 min–13 h), extreme thirst, and lack of urine production. Other symptoms included feeling of warmth, clamminess, numbness of the tongue and extreme thirst.

One case in series had concomitant intake of alcoholic beverage with toxic syndrome. clinical testing procedure i.e, using a 3H-radioimmunoassay (RIA) test kit had evidenced sub-nanogram levels of toxin in urine and plasma. 4/10 Patients survived this early phase & recovered without any complications with meticulous follow up, 6/10 with much severe manifestations/delayed in notification for treatment appeared to have recovered for a short

time, but this period was generally followed by a rapid and severe loss of strength, prostration, and pain-causing restlessness, sudden onset of abdominal discomfort (a feeling of fullness). Aggressive therapy resulted in survival 4/10 (out of 6/10) with variable degree of liver enlargement. The rest of 2/10 of (6/10) succumbed to death from irreversible damage to vital organs (hepato-renal insufficiency, cardiac, and skeletal muscle). The toxin affected primarily the liver, but there are additional disturbances to blood cells and the central nervous system.

**Results:** The degree of reversal of adverse effects depends upon the urgency of therapeutic notification. In the absence of dietary history, Allergic/toxic manifestations could be mistaken for symptoms of hepatic renal impairment as a consequence of other causes (e.g., viral hepatitis), therefore an urgent distinction be made, as the delayed onset of symptoms will be mistaken behind the idea that the organs have previously been damaged. The importance of rapid diagnosis is evident, *victims who are hospitalized and given aggressive supportive therapy immediately after ingestion have a mortality rate of only 5–10%, whereas those admitted 50 hours or beyond after ingestion have a 55–85% mortality rate.*

**Conclusions:** Mushrooms as per its names are alike, but dissimilar in their nutritional & toxicity nature, its haphazard selection/consumption could cost life of the consumer.

#### **F1.22. Effects of Tacrolimus upon T-Lymphocyte, NK Cell, and Eosinophil Activation.**

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Tacrolimus is a macrolide immunosuppressant used to prevent graft rejection in transplant patients and in the treatment of atopic dermatitis. The drug inhibits T-lymphocyte activation by preventing the transcription of IL-2. We wish to investigate whether tacrolimus also indirectly suppresses natural killer (NK) cell and eosinophil activation by inhibiting T-cells. **Lymphocyte Assay:** Heparinized blood was collected from healthy adult donors and was layered over a ficol-hypaque density gradient to isolate lymphocytes. Cells were cultured in-vitro at a concentration of  $1.0 \times 10^6$  cells/ml in IL-2 and increasing amounts of tacrolimus. The drug and cytokines were added bi-weekly to maintain the appropriate cell concentration. Weekly flow cytometric analyses were performed to detect tacrolimus' effects on T- helper cell (CD4+), T- cytotoxic cell (CD8+), and NK cell (CD16+ and CD 56+) populations. CD 69+ was also measured to assess lymphocyte activation by double staining for CD 4+/69+ and CD 8+/16+. Weekly assessments of <sup>51</sup>Cr discharges from K562 cells were performed to detect NK cell activity. **Eosinophil Assay:** Tacrolimus effect on eosinophil viability was investigated by isolating white blood cells from eosinophilia patients. Whole blood collected from patients was layered over a 75% percoll gradient. The isolated white blood cells were kept at a concentration of  $1.0 \times 10^6$  cells/ml and were treated with IL-5. Varying amounts of drug concentration were added to specific cultures in order to study its differing effects on cell activation. Cytokine and drug were added bi-weekly and flow cytometry was performed at 4, 7, and 14 day increments to monitor eosinophil activation through CD69+ expression and fluorescence. **Lymphocyte Activation:** Inhibition was observed in T-lymphocyte and eosinophil populations as well as NK cell activity. Flow cytometry analysis staining for

CD4+/69+ expressions indicate that with increasing time and concentration of tacrolimus, T-lymphocyte activation decreases. When compared to control (cultures treated with IL-2 alone), cultures from week 1 treated with tacrolimus at 50ng/ml and 500ng/ml displayed a decrease of 6.1 % and 8.3% respectively. At week 2, there was an 8.3% and 14.2 % decrease in CD4+/69+ activation levels with tacrolimus 50 ng/ml and 500ng/ml respectively. CD8+ overall activation level was unaffected with increasing treatment of drug and time, while <sup>51</sup>Cr assay suggested an overall decrease in NK cell activity. **Eosinophil Activation:** Compared to control, flow cytometry results staining for CD 18+/69+ indicate a 9% and 6% decrease in eosinophil activation at 4 days of incubation. At 14 days, there was a 19% and 23% decrease in cell activation with tacrolimus 50 ng/ml and 500 ng/ml. With increasing time and concentration, mean fluorescence for cells expressing the CD 69+ activation marker decreased. **Conclusion:** Although the primary effect of tacrolimus is on T- cells, it also may affect NK cell and eosinophil activation. The effect on eosinophils may explain the drug's beneficial effect in atopic dermatitis patients.

### F1.23. Hemopoietic Cells Accumulate in Murine Lungs in Response to Ectopic Grafts of Lung Tissue from Allergic and from IL-5 Transgenic Donors.

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**Background.** Hemopoiesis is an important factor in the pathogenesis of allergic asthma. Several studies suggest that extramedullary hemopoietic cells present inside the asthmatic lungs contribute to chronic airway inflammation. To define the factors responsible for the emergence of these cells, and their relationship to allergic inflammation, we isolated intrapulmonary hemopoietic cells from the lungs of allergic BALB/c mice, and showed that: a) their presence is strictly dependent on airway challenge of ovalbumin-sensitized mice (*Chest*, 2003, 123, 345S); b) they differ from hemopoietic cells in bone-marrow in their growth properties and sensitivity to steroids (*Intl. Immunopharmacol.*, 2005, *in press*). Here we evaluated the possible contribution to intrapulmonary hemopoietic cell accumulation made by systemically active signals originating in challenged lungs, and by the local allergic reaction. **Objectives.** To define: a) whether allergen-challenged lungs release factors responsible for intrapulmonary accumulation of hemopoietic cells; b) whether the production and activity of these factors can be dissociated from allergen-induced lung injury. **Methods.** We developed a transplantation model in which fragments of allergen-challenged, sensitized lung donors were ectopically implanted in syngeneic recipients, and hemopoietic cells inside the recipients' lungs were quantitated without allergen exposure of the recipients. The contribution of IL-5 released by the graft was assessed by ELISA, by neutralization and by IL-5-transgenic grafts. **Results.** In BALB/c mice, accumulation of hemopoietic cells occurred only when: a) donors were sensitized and challenged in the airways; b) recipients were sensitized through 2 sc allergen injections, but not airway-challenged.

Media conditioned by lung fragments from the appropriate donors contained biologically active IL-5, as well as immunoreactive IL-5 and eotaxin, and induced intrapulmonary accumulation of hemopoietic cells in sensitized recipients. The effect of the appropriate donor-recipient combination was prevented by the TRFK-5 anti-IL-5 antibody. Unlike BALB/c, lungs from IL-5 transgenic CBA/Ca mice contained a large number of hemopoietic cells, independently of sensitization and challenge. Lung fragments from naive, IL-5 transgenic donors (or their conditioned media), induced intrapulmonary accumulation of hemopoietic cells in nontransgenic, ovalbumin-sensitized recipients. **Conclusions.** a) intrapulmonary accumulation of hemopoietic cells is independent of local immunological injury induced by the allergen challenge; b) lung grafts systemically release IL-5, which is required for accumulation of hemopoietic cells in the recipients' lungs; c) IL-5 is fully effective only on sensitized animals.

### F1.24. Intracellular Stores of Interleukin-4 (IL-4) Receptor $\alpha$ Participate in the Secretion of IL-4 from Human Eosinophils.

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Traditionally considered terminal effector cells, eosinophils are currently emerging in more subtle roles relating to their influence on tissue milieu and immunomodulation. This relatively new appreciation stemmed in part from the realizations that eosinophils store arsenals of preformed cytokines and chemokines with well-defined immunomodulatory properties, and are capable of rapid release of these potent mediators in response to specific stimuli. Although multiple mediators are stored in close proximity within eosinophil specific granules, their release appears to be independently regulated (i.e. eotaxin induces release of IL-4 but not IL-12, while IFN- $\gamma$  induces secretion of IL-6 and RANTES without detectable release of IL-4). To date, mechanisms governing this selectivity have been elusive. Interestingly, eosinophils are responsive to many of the factors for which they are reservoirs, indicating that cognate receptors for these ligands are also expressed. Despite the dual expression of receptor/ligand pairs by eosinophils, few studies have addressed receptor expression in relation to the storage and release of cognate ligands. In this study we utilize flow cytometric analysis to monitor intra- and extracellular expression of IL-4 and the IL-4 receptor. In an approach combining electron microscopy, light microscopy and subcellular fractionation, we further visualize localization of the IL-4 receptor throughout eotaxin-induced secretion of its ligand, IL-4. Surprisingly, we discover that in addition to nominal surface expression, all components of functional types I and II receptor complexes are pre-formed and stored within freshly isolated eosinophils. Further, we demonstrate the IL-4 binding component (IL-4 receptor  $\alpha$  chain) is selectively mobilized in concert with IL-4 and likely participates in the trafficking of IL-4 out of the granule and through the vesicular compartment. This work represents the first indication of preformed internal stores of cytokine receptors within human eosinophils, and proposes a novel mechanism for the selectivity of mediator release.

### **F1.25. Anaphylaxis to Intravenous and Oral Cyclosporin in a Child and Successful Desensitization.**

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Hypersensitivity reactions to cyclosporin are rare. Cyclosporin formulations for parental and oral use are vital drugs after bone marrow transplantation (BMT), thus recognition of hypersensitivity reactions and guidelines for subsequent use are important in transplant surveillance. The purpose of this paper is to report a case of anaphylaxis to intravenous and oral cyclosporin successfully managed by oral desensitization also to present a review of different formulations of cyclosporin with the least drug reaction. Case report: This 9-year-old girl with thalassemia major was admitted post BMT, when developed an anaphylactic reaction (respiratory distress, hypotension and generalized urticaria) after second exposure with intravenous then oral cyclosporin. Fortunately she had a good response to immediate rescue treatment. There is not any immunosuppressive drug as effective as cyclosporin for the engraftment. Two available formulations of cyclosporin in Iran do contain Cremophor-EL (polyoxyethylated castor oil) in IV or poly-5-oleate (a chemically similar compound to cremophor-EL) in oral compounds. The previous reported cases of cyclosporin hypersensitivity were confirmed to be due to this solubilizing agent rather than the cyclosporin itself. The safest suggested formulation, corn-oil-based soft gelatin capsule, was not available for us, thus oral desensitization was started according to the classic penicillin desensitization protocol and tolerated appropriately by the patient. There are a few reports of cyclosporin desensitization in the literature. Cyclosporine anaphylaxis is rare but possible, and in the face with unavailability of the suitable formulation, desensitization should be considered.

### **F1.26. Skin Reactivity to Aeroallergens Is Not Related to the Nasal Polyp Tissue Eosinophil Inflammation.**

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The role of allergy in the pathogenesis of nasal polyposis is not clear. In this study we investigated the possible correlation of skin reactivity to aeroallergens, with the polyp tissue eosinophil inflammation. Twenty-five patients with nasal polyposis who were candidate for polypectomy under general anesthesia were enrolled. Polyp tissues were stained with hematoxylin-eosin for eosinophil count. Skin prick test (SPT) with at least 11 common aeroallergens (Allergopharma, Germany) including pollens, mites and molds were done for all patients. The positive SPT was defined as a reaction at least 3mm larger than the negative control (Glycerol).

12 of 25 patients had at least one positive SPT. In 18 patients eosinophil count in the polyp tissue was more than 50 percent of cells counted in the field. We did not find a significant correlation between the polyp eosinophil count comparing to the skin reactivity. It seems that polyp eosinophil inflammation is not a consequence of allergy to the aeroallergens in the nasal polyposis.

### **F1.27. Superior Safety of Polymerized Allergen Vaccines: Zero Systemic Reactions during 55,000 Injections of Polymerized Ragweed and Grass Vaccines in 500 Allergy Patients.**

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Allergen immunotherapy is the only treatment for allergic rhinitis and asthma that can reverse the immune imbalance in patients with these IgE-mediated disorders. This form of therapy involves gradual administration of increasing doses of allergens to patients who have been found to possess allergen-specific IgE reactivity. The treatment is 90% effective in reducing both allergy symptoms and medication use, while improving the quality of life for the allergy sufferer. Allergen immunotherapy with aqueous allergens, however, carries the risk of systemic reactions. Using a questionnaire of its membership, the Immunotherapy Committee of the American Academy of Allergy, Asthma and Immunology verified the potential risks of both death and near death reactions immediately following administration of aqueous allergens. Their findings confirmed that there had been 273 near death reactions and 20 deaths associated with aqueous immunotherapy from 1990 to 2001.

To reduce life-threatening reactions to allergen immunotherapy, Patterson developed the technique of glutaraldehyde polymerization of ragweed and grass allergens. In multiple studies, polymerized vaccines were found to be as effective as aqueous allergen extracts, and devoid of systemic responses. Here we extend Patterson's findings and demonstrate that polymerized ragweed and grass vaccines are superior in safety to aqueous materials. 500 allergy patients were given over 55,000 injections of polymerized ragweed and grass allergens, with zero systemic responses.

To eliminate the risk of death associated with aqueous allergen immunotherapy, while retaining its effectiveness, we strongly recommend that allergy patients receive polymerized ragweed and grass vaccines. Given that the use of aqueous allergen immunotherapy by untrained physicians and nurse practitioners is increasing nationwide, it may be safer for patients to have the FDA either eliminate the availability of aqueous ragweed and grass allergens altogether, or restrict their use to physicians board certified in Allergy-Immunology.

### **F1.28. Trans Fatty Acid and Atopic Eczema/Dermatitis Syndrome: The Relationship with a Free Radical cis-trans Isomerization of Membrane Lipids Trans LIPIDS in Atopic Dermatitis.**

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The formation of *trans* fatty acid residues in membrane lipids can be due to the radical-catalysed isomerization process of naturally occurring *cis* fatty acid moieties. Radical stress is well documented in atopic diseases but no data are still available on a

possible association with high levels of *trans* fatty acids in these patients. We investigated the presence of *trans* lipid isomers in erythrocyte and T-lymphocyte membranes of 24 children affected by atopic eczema/dermatitis syndrome (AEDS) taking advantage of the *trans* lipid library available from radical processes modelled *in vitro*. We found *trans* fatty acids both in erythrocyte and lymphocyte membranes and their total content reached the highest value of 3.0% of the main fatty acid residues. The high *trans* fatty acid levels correlated significantly with the increasing amount of palmitic acid and with the decrease of stearic acid. This fatty acid also correlated with the decrease of arachidonic level, and this scenario can fit with an inhibition of elongase enzymatic activity. Moreover, the highest *trans* fatty acid levels were detected in 12 out of 24 children which have atopic dermatitis not mediated by IgE (prick/RAST negative). A new significance of lipid impairment in AEDS can be proposed, which generally involves the role of *trans* isomers in human pathologies. This study aims to contributing to lipidomic researches regarding the double bond structure and the influence of a geometrical change of membrane lipids in physiology and diseases.

#### **F1.29. Proinflammatory Cytokines and Nitric Oxide in Exhaled Breath Condensate in Monitoring of Exacerbation Asthma in Children.**

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Proinflammatory cytokines and nitric oxide play important role in exacerbation of asthma. The aim of the study was to determine NO, IL-4, IL-6 level in exhaled breath condensate of asthmatic children.

**Material and methods.** The samples of exhaled breath condensate were collected in 31 children with asthma (16 females and 15 males, aged 8–18y, mean 13,3y) during 15min. breathing and then were frozen to (-) 70 °C. The examination of the exhaled breath condensate were done by EcoScreen equipment (Jaeger Comp.). Results were compared in 3 group of asthmatic children (I group- 7 children with asthma exacerbation, II group- 9 children without exacerbation of asthma, well controlled by steroids and  $\beta$ 2mimetic drugs, III group- 15 children with asthma improvement without drugs longer than 3 months) and control (IV-th group- 15 healthy children aged 11–17y, mean 14,5y).

**Results.** The highest value of NO, IL-6, IL-4 were found in I group of children. All parameters have shown significant differences between examination groups.

**Conclusions.** 1. Mean concentrations of NO and cytokines IL-6, IL-4 had strong correlation with exacerbation asthma in children.

2. The examinations of NO in exhaled breath condensate especially but also IL-6, IL-4 cytokines are useful, non-invasive method in monitoring exacerbation asthma in children.

#### **F1.30. Combined Skin Prick, Immediate Patch and Specific-IgE Testing in the Diagnosis of Peanut Allergy in Children.**

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**Introduction:** In children with probable peanut allergy, an 8-mm skin prick test (SPT) has been reported to be 100% specific. We aimed to determine the sensitivity and specificity, for peanut allergy,

of skin tests, peanut specific-IgE, and combinations of these, in children with a lower pre-existing probability of peanut allergy. **Methods:** Children attending the allergy clinic with a positive peanut SPT ( $n = 84$ ; age range 0.9–17.3 years; mean 4.5 years) were included in the study. Immediate skin application food tests (I-SAFT) using 1 gram of peanut butter (positive if any wheals were detected at 15 minutes), peanut specific-IgE levels and open-label peanut food challenges were performed. **Results:** Fifty-two of 85 peanut challenges were positive. SPT specificity was 67% at 8 mm and 100% at 15-mm. I-SAFT alone was 82% specific. A peanut specific-IgE level of 0.35 kU/L alone was 98% sensitive but 33% specific. A level of 10 kU/L was 100% specific. Combinations of an 8-mm SPT with a positive I-SAFT and a peanut specific-IgE > 0.35 kU/L were 88% specific. **Conclusion:** An 8-mm SPT cannot predict peanut allergy in children without a high pre-existing probability of peanut allergy. If a child without a recent history of a peanut reaction has a SPT of < 15-mm diameter, peanut specific-IgE should be measured. Challenge is not necessary if the level is < 0.35 or > 10 kU/L. Allergy test results should be interpreted in the context of a history or suspicion of food allergy.

#### **F1.31. Cow's Milk Allergy in Infantile Colic.**

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Cow's milk allergy has been considered as a cause of infantile colic. Clinicians frequently change the diet of these infants to a cow's milk free diet. In this study, we evaluated the role of cow's milk allergy in infantile colic in a group of exclusively breast fed infants. 114 exclusively breast fed infants between three weeks and three months of age, who were referred with infantile colic, enrolled in this study. Skin prick test with cow's milk extract (Allergopharma) and a stool exam for occult blood were done for all babies. Then, they were randomly selected as two groups of case and control. In case group (including two babies with positive skin prick test), we advised mothers not to consume cow's milk and other dairy products for two weeks. In control group, we did not change the diet of mothers. 77 babies came back for follow up, 35 babies in the case and 42 babies in the control group. Infants with colic whose mothers did not take dairy products, did not improve significantly in comparison with control group. Prevalence of positive skin prick test in colicky infants was 2.6 %, which is nearly similar to prevalence of cow's milk allergy in the population of infants below one year of age (2.2–2.6% on the basis of previous studies). Occult bleeding in stool was significantly higher in colicky infants in comparison with non-colicky infants. Cow's milk allergy does not seem to be a common cause of infantile colic. It is not advised to eliminate the dairy products from the diet of nursing mother.

#### **F1.32. 5-Lipoxygenase Localization to Cytosolic Lipid Bodies in Rat Basophil Leukemia Cells.**

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The metabolism of arachidonic acid via the 5-lipoxygenase (5-LO) pathway generates leukotrienes, eicosanoids with varied

paracrine and intracrine roles in inflammation. 5-LO in different cells may variably be present in the cytosol and/or the nucleus and may undergo activation-dependent translocation to sites, including the nuclear envelope. Lipid bodies are organelles that in leukocytes and other cells have roles in the local formation of both 5-LO- and cyclooxygenase pathway-derived eicosanoids. We have evaluated the expression of 5-LO in rat basophil leukemia cells (RBL-2H3). Resting RBL cells contained numerous lipid bodies, as identified by staining with Oil Red O and the incorporation of a fluorescent fatty acid analog. By immunocytochemistry, 5-LO was present in the cytosol and nucleus of resting RBL cells, as well as at punctate cytosolic sites, that co-stained as lipid bodies. Resting RBL cells were disrupted by nitrogen cavitation and subjected to subcellular fractionation with a protocol designed to isolate buoyant lipid bodies. By Western blotting of subcellular fractions, 5-LO was present in lipid body as well as cytosolic and nuclear fractions. To investigate the localization of 5-LO within RBL cells, cells were transfected a plasmid encoding an EGFP-5-LO fusion protein. Examination of cells as soon as 1 hr after transfection with EGFP-5-LO demonstrated very prominent focal green fluorescence at punctate cytosolic sites that stained as lipid bodies with Oil Red O. EGFP-5-LO fluorescence remained largely lipid body associated at 4 hrs post-transfection, when a lesser number of cells also began to exhibit diffuse cytosolic fluorescence. To ascertain whether cell activation altered the EGFP-5-LO distribution, cells were sensitized with anti-DNP IgE and activated with DNP. At both 1 and 4 hrs after IgE-mediated activation, lipid body numbers per cell increased ~50%. At 1 and 4 hrs after activation, EGFP-5-LO fluorescence exhibited almost exclusively punctate cytosolic localization with lipid bodies. Thus, lipid bodies in RBL cells constitute a discrete pool of 5-LO that is especially enriched in newly synthesized 5-LO. These findings provide additional evidence for the functions of lipid body organelles in the formation of eicosanoids pertinent to inflammation.

Category: allergy/asthma

Theme: immunoregulation

### F1.33. Approaches to Immunogenicity of Human Protein Products.

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Potentially serious immune reactions or loss of treatment effect may result from repeated exposure of patients to therapeutic proteins or peptides even when they are from human sources or based on human sequences. *Objective:* The objective of this investigation is to study the approaches taken in the development of human protein/peptide products available in the United States with respect to their immunogenicity potential. *Materials and Methods:* The package inserts of twenty therapeutic human protein/peptide products were examined for the following information: alterations to peptide sequence or polysaccharide attachment, manufacturing process, choice of excipients, viral validation, assay of antibody development, in vitro or in vivo correlates of cell mediated immunity, precautions and contraindications in specific patient populations, therapeutic effect, and adverse reactions. The products included hormones, cytokines, coagulation factors, immune globulins, as well as other blood components. *Results:* (1) Information on changes potentially affecting immunogenicity, such as differences in conforma-

tion of the therapeutic agent molecule and the possible adjuvant effect of certain excipients, is rarely presented except for some recombinant products. (2) Immunogenicity is most often studied for the development of serum antibodies to the therapeutic agent, but rarely in terms of cell-mediated immune responses or skin-sensitizing antibodies, unless the route of administration is dermal application. (3) Instructions on testing for antibodies to the therapeutic agent are often dictated by changes in treatment effect, and only occasionally by manifestations of adverse reactions, such as anaphylaxis. (4) Special populations, which are susceptible to the development of immune responses to the therapeutic agent, are generally addressed in the precautions, warnings and contraindications sections of labeling. However, systematic studies to explore immunogenicity in specific populations are often lacking. *Conclusions:* Information regarding immune reactions to human proteins/peptides remains inadequately pursued or presented for many available therapeutic products. Greater attention needs to be focused on this important issue, which has bearing on both safety and effectiveness of the products.

### F1.34. Plasma Concentration of Soluble IL-4 Receptor in Asthma Patients during Specific Bronchial Challenge with Dermatophagoides Pteronyssinus(Dp) Allergen.

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Soluble IL-4 receptor (sIL-4r), functioning as a decoy receptor, inhibits action of IL-4. The aim of this study was to evaluate serum concentration of sIL-4r in asthma patients during bronchial challenge with Dp allergen.

The study was performed on 51 asthma patients with a positive history of dust allergy symptoms, positive skin prick test results with Dp extract and with a significant bronchoconstrictive response to bronchial Dp challenge. Ten healthy persons with negative skin prick tests to common aeroallergens were used as controls. Bronchial provocation challenge with Dp extract was performed only in asthma patients. Blood samples were collected before, 1 hour (T<sub>EAR</sub>) after, 8 hours (T<sub>LAR</sub>) after and 24 hours (T<sub>24</sub>) after allergen challenge. Plasma concentration of sIL-4r was evaluated by ELISA (R&D Systems).

The mean plasma concentration of sIL-4r was greater in asthma patients (46.6 ± 18.6 pg/ml) than in healthy controls (29.1 ± 14.5 pg/ml). There was no difference in the mean plasma concentration of sIL-4r between patients who responded to allergen challenge with isolated early asthmatic response (single responders-SR) and those who responded with both early and late asthmatic responses (dual responders-DR). During the T<sub>EAR</sub>, a significant fall in plasma sIL-4r concentration was found in DR (to 33.7 ± 12.6 pg/ml; *P* < 0.001), but not in SR (45.8 ± 23.6 pg/ml). At T<sub>24</sub> the mean plasma concentration of sIL-4r was significantly greater in SR (57.9 ± 27.9 pg/ml) than in DR (41.1 ± 13.4 pg/ml), but was not significantly different from the baseline levels.

The fall in sIL-4r plasma concentration in DR seen at T<sub>EAR</sub> may result in increased activity of IL-4 which in turn may participate in the development of sustained allergic inflammation in these patients.

### F1.35. Interleukin-4 and Its Alternatively Spliced Variant (IL-4 $\delta$ 2) in Healthy Donors and Patients with Atopic Asthma.

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**Background.** The interleukin-4 (IL-4) splice variant (IL-4 $\delta$ 2) is known to antagonize many biological activities of IL-4. The aim of the study was to compare the IL-4 and IL-4 $\delta$ 2 expression ratio in patients with asthma versus healthy subjects.

**Materials and methods.** Eight healthy subjects and eight patients with atopic asthma confirmed by case history, skin tests and RAST were involved in the study. Informed consent from patients and healthy subjects to participate in the study was obtained. RNA was extracted from PBMC obtained by Ficoll-Urographin gradient centrifugation. cDNA was used for quantitative PCR with specific form-discriminating primers and SYBR Green I performed using iCycler iQ (BioRad, Hercules, USA).

**Results.** The IL-4:IL-4 $\delta$ 2 expression ratio was  $6.8 \pm 2.5$  and  $7.1 \pm 3.3$  for patients with atopic asthma and healthy subjects respectively. The IL-4:IL-4 $\delta$ 2 ratio did not correlate with age, sex, total serum IgE levels, or the presence of eczema, rhinitis or anaphylaxis in the patients with asthma.

**Conclusion.** There was no difference in relative expression of splice variant of IL-4 in healthy subjects versus atopic asthma patients.

### F1.36. B Epitope Assay of hIL-4 $\delta$ 2, an Alternative Splicing Variant of hIL-4.

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hIL-4 $\delta$ 2 is a natural alternative splicing form of human IL-4, derived from hIL-4 mRNA by the elimination of second exon encoding for the amino acid residues 22–37. The structural and biological properties of IL-4 $\delta$ 2 are poorly understood, and its direct (non-mRNA) quantitative measurement is currently not possible. The major purpose of this study was: a) to compare the 3D structures of hIL-4 $\delta$ 2 to the parent cytokine hIL-4; b) to attempt to reveal the dominant B epitope sequences in loop regions of hIL-4 $\delta$ 2 using synthetic peptides and computer-assistant 3D modeling; c) to prepare monoclonal antibodies (MAbs) specific for each cytokine form; and d) to evaluate the binding affinities of the antibodies. The hIL-4/hIL-4 $\delta$ 2 peptides were synthesized by solid-phase methods, characterized by analytical HPLC and mass-spectrometry. To increase immunogenicity, the peptides were conjugated to keyhole limpet hemocyanine (KLH) and used as immunogens. Polyclonal and monoclonal antibodies to both hIL-4 and hIL-4 $\delta$ 2 peptides in addition to whole recombinant proteins were produced in BALB/c mice. Analysis of reactivity of mouse antisera produced against hIL-4 and hIL-4 $\delta$ 2 showed very low reactivity to all synthetic peptides, while anti-peptide antisera obtained demonstrated noticeable reactivity to IL-4, especially the anti-

serum to the peptide mimicked splicing fragment consisting of sequence 22–37 of IL-4. ImmunoDot and ELISA demonstrated that anti-IL-4 MAbs produced were able to recognize only peptide 22–37. Reactivity of anti-IL-4 peptide antisera to hIL-4 $\delta$ 2 was completely absent. A 3D model of IL-4 $\delta$ 2 which was used as a template to design the peptide mimicked the unique B epitope which emerged in the splicing site. Assay of antibody binding of MAbs to I<sup>125</sup> labeled cytokines showed that K50 values varied from  $10^{-10}$  to  $10^{-6}$  M. These techniques may allow for identification and further characterization of differences between hIL-4 $\delta$ 2 and IL-4, including both quantitative and qualitative functional aspects.

### F1.37. LPS Differentially Modulates Th2 Cell Responses to Antigen during Acute and Relapse Allergic Asthma in Mice.

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Endotoxin has been shown to have a powerful effect on adaptive immunity. Although endotoxin, in particular, LPS is an important adjuvant during the priming of allergic immune responses, it has been shown to suppress recall Th2 immune responses. We tested the effect of LPS on in vivo priming and memory responses in a model of allergic asthma induced without adjuvants. Mice were immunized twice on days 0 and 21 with 10 mcg of ovalbumin (OVA) intraperitoneally. One week later, mice were challenged with a series of 4 aerosolizations with 1% OVA on 2 consecutive days. Groups of mice were either evaluated for acute disease or recuperated for at least 2 months before being rechallenged for relapse disease. We found that mice sensitized intraperitoneally with OVA with very low LPS content (removed with detoxigel; contained 50 pg/ml endotoxin) and rechallenged with OVA (Sigma, grade V; contained 190 ng/ml endotoxin), developed minimal allergic lung inflammation, mucus secretion, and OVA-specific IgE and IgG1, compared with animals immunized with high LPS containing OVA. In support of previous data, these results demonstrate that LPS has an influence during OVA priming. However, when mice were immunized using the same protocol with low-fat milk powder (containing endotoxin 1.3 ng/ml) and aerosolized with a 1% milk solution, responses were significantly higher than low endotoxin containing OVA primed mice. These data indicate that LPS may be critical for inducing Th2 responses to OVA but is not necessary for priming to milk proteins. To test the effect of LPS on in vivo memory responses that lead to disease relapse, we challenged recuperated mice with OVA and titrated doses of LPS (1.0, 100.0, 1000.0 ng/ml) in the aerosol OVA solution. We found that there was inhibition of allergic inflammation, mucus production, and immunoglobulin at 1.0 and 1000.0 ng/ml added LPS but not at 100.0 ng/ml. To determine whether the LPS suppression on memory responses with low and high LPS doses involved B cells, we did the same experiment in B cell deficient mice. Interestingly, in the absence of B cells, we found suppression with intermediate LPS doses but no effect with high and low doses suggesting that B cells play a role in the LPS effect on recall memory responses. In summary, our results show that LPS is necessary during priming with certain proteins, such as OVA, but not with others, such as with milk. Additionally, LPS has



an inhibitory effect on memory responses that appears to involve B cells.

### F1.38. Differential Th2 Immune Responses in C57BL/6 and BALB/c Mice in a Novel Model of Milk-Induced Allergic Asthma.

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Cow's milk allergy is a significant health problem during infancy and childhood. Patients are usually allergic to all the major milk proteins in cow's milk or formulas, including caseins, beta-lactoglobulin, and alpha-lactalbumin and as a result develop dermatitis, asthma, or anaphylaxis. Currently, there are no animal models of milk-induced allergic asthma. The advantages of milk as an allergen in experimental models is that it is natural and clinically relevant, broadens the scope and general applicability of mouse models of allergic asthma, induces an allergic response to a combination of proteins, and is cost effective (3 kg of milk powder in Vienna is 15.00 EUR; 50 g ovalbumin 900.00 EUR). To establish models of acute and relapse milk-induced allergic asthma in mice, we injected either BALB/c or C57BL/6 mice with 10 mcg of low-fat milk powder dissolved in PBS intraperitoneally three weeks apart. One week later we exposed mice to a series of 4 milk-aerosol challenges with a 2% solution of milk powder in PBS, on two consecutive days. Mice were either evaluated for disease during acute onset of disease or recuperated in the following 2 months and were then re-exposed to milk with a similar series of aerosol challenges to generate disease relapse. We observed increased lung inflammation, mucus secretion, and milk-specific IgE and IgG1 at acute onset disease (day 31) and during relapses (day 90) in both C57BL/6 and BALB/c mice. However, milk sensitization and aerosolization induced 10-fold higher infiltrating inflammatory cells in bronchoalveolar lavage fluid in C57BL/6 compared with BALB/c mice. The percent eosinophils in the airways were  $70 \pm 0.7\%$  for C57BL/6 and  $31 \pm 3.5\%$  for BALB/c mice, compared to no eosinophils in naive and recovered mice. Furthermore, the number of perivascular and peribronchial infiltrates and eosinophils within the lungs in tissue sections reflected these differences. Similarly, mucus hypersecretion and milk-specific IgE and IgG1 levels were higher in C57BL/6 compared to BALB/c mice and all observed differences between strains were apparent in acute and relapse disease. Recuperated C57BL/6 and BALB/c mice had lymphocytic infiltrates in the lungs, as previously demonstrated in ovalbumin-induced models. In contrast to ovalbumin-induced allergic asthma, milk sensitization and aerosolization resulted in a large difference between mouse strains, which is not yet fully understood. Here, we present a useful, inexpensive, and clinically relevant mouse model of milk allergy in C57BL/6 mice and demonstrate significant genetic differences in immune responses of C57BL/6 and BALB/c to cow's milk.

### F1.39. Response of Older Patients with Asthma to Omalizumab: A Pooled Analysis across 5 Clinical Trials.

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**Background:** Asthma in older adults is under-recognized and is often associated with allergic triggers. Anti-immunoglobulin E

(IgE) therapy with omalizumab (OMA) is indicated in patients ( $\geq 12$  years) with moderate to severe allergic asthma who continue to be inadequately controlled despite treatment with inhaled corticosteroids. Previous analyses have not focused specifically on efficacy in older adults. We examined treatment response to OMA on asthma exacerbations as well as patient-reported and investigator-reported global treatment effectiveness in patients 50 years and older.

**Methods:** Data were combined from 5 randomized double blind placebo-controlled (PBO) trials of patients with moderate to severe allergic asthma (confirmed by skin test or RAST testing); 4 were of 28 weeks and 1 was 32 weeks in duration. The pooled study population involved a total of 2236 patients (1136 treated with OMA, 1100 treated with PBO) who met entry criteria that included, at baseline, need for treatment with moderate to high dose inhaled corticosteroids. 601 subjects were  $\geq 50$  years of age. The relative risk (RR) of clinically significant asthma exacerbations (OMA vs. PBO; primary endpoint) was determined using Poisson regression, controlling for age category, study, sex, baseline IgE, and prior history of asthma exacerbations for the overall population and for patients  $\geq 50$  years. A similar approach was taken for evaluation of patient- and investigator-reported global treatment effectiveness (excellent, good, moderate, poor, worsening) using cumulative logistic regression for the two groups comparing OMA to PBO.

**Results:** The mean age of the older subgroup was 58 years; 61% were female; the mean IgE level was 184 IU/dl (range 19–743). For the overall study population the mean age was 40 years (range 12–79); 58% female; the mean IgE level was 211 IU/dl (range 19–1055). OMA was associated with a reduced risk of clinically significant asthma exacerbations in all 5 trials reviewed. Pooled analysis in the overall study population revealed a RR (OMA vs. PBO) of 0.79 (95% CI 0.62–0.97). In the subgroup of patients 50 or older, the RR was 0.72 (95% CI 0.48–1.09). The improvement shown with OMA was in agreement with patient- and investigator-reported global effectiveness which demonstrated significantly greater response ( $P < 0.0001$ ) on both measures in patients assigned to OMA relative to PBO irrespective of age.

**Conclusions:** In patients  $\geq 50$  years of age, omalizumab was associated with a RR reduction in clinically significant asthma exacerbations and significantly better patient- and investigator-reported global effectiveness ratings compared to placebo, suggesting that omalizumab is effective in older patients with moderate to severe allergic asthma. Treatments were generally well tolerated.

### F1.40. Development of a CD154-Dependent Model of IL-13 Producing B Cells.

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Our laboratory has demonstrated that human B lymphocytes synthesize IL-13, and that autocrine production of this cytokine appears to be essential for maintaining IgE production by these cells. To initiate IgE synthesis, contact between CD40 on B cells and CD40 ligand (CD40L or CD154) on Th2 cells is necessary. A culture system, using murine CD154-transfected fibroblasts (LTK) has been established for the propagation of human B cells *in vitro*. Our objective is to develop a model of IL-13-producing B cell using this co-culture system.

**Methods:** Human B lymphocytes were isolated from tonsils and purified by sheep red blood cell rosetting. LTK cells were stably transfected with a cDNA encoding for the wild-type form of the human CD154 protein. In the co-culture system,  $5 \times 10^5$  B cells were co-incubated with  $1.3 \times 10^5$  untransfected LTK (CD154-) cells or transfected LTK-4A1 (CD154+) cells and seeded in 24-well plates. Isolated B cells were also stimulated with soluble anti-CD40 antibody (1 $\mu$ g/ml). Recombinant IL-4 (200U/ml) was added to cultures and incubated up to 14 days to induce IgE production. After 5 days of co-culture, detection of intracellular IL-13 in B cells was assessed by flow cytometry. We also measured levels of phosphorylated STAT6 by flow cytometry.

**Results:** Using BrdU staining, we demonstrated that LTK-4A1 most efficiently supported survival of B lymphocytes with an increase in proliferation (41.36% versus to 9.59%) compared to soluble anti-CD40 antibodies. LTK-4A1 blocked apoptosis of B lymphocytes more efficiently than soluble anti-CD40 (39.7% vs. 13.77%). Most importantly, after 5 days in culture with LTK-4A1, the number of CD19+/IL-13+ B cells was significantly higher (47.9% versus 17.1%) compared to the soluble anti-CD40 Ab. The production of IgE by human B lymphocytes cultured with LTK-4A1, as assessed by ELISA, also increased significantly (13.4 ng/ml versus to 1.8 ng/ml) when compared to B cells stimulated with soluble anti-CD40 antibodies. In contrast, IL-13 receptor signaling was equally influenced by both culture systems. STAT6 phosphorylation in response to exogenous IL-13 was nearly equal 24 hours after co-culture with LTK-4A1 or with soluble anti-CD40 compared to the untransfected LTK control (75% of phospho-STAT6 positive cells in co-culture with LTK-4A1 or with soluble anti-CD40 versus 10% with LTK).

**Conclusion:** LTK-4A1 induces more efficient cross linking of CD40 than soluble CD40 antibodies. This leads, in turn to high levels of IL-13 positive B cells, not previously demonstrated. This likely suggests that IL-13 production by B cells is important *in vivo*. Th2 cytokine production by human B lymphocytes may be underreported due to incomplete stimulation via CD40.

#### **F1.41. Daclizumab Does Not Alter the Function of Healthy Human CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cell In Vitro.**

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##### **Rationale**

Daclizumab (Zenapax<sup>®</sup>), a humanized monoclonal antibody directed against the IL-2 receptor  $\alpha$  chain (CD25), is approved for the prevention of renal allograft rejection and is under evaluation for treatment of asthma, multiple sclerosis and other autoimmune diseases. Daclizumab inhibits activation of human T lymphocytes by blocking IL2-induced T cell proliferation, and by reducing production of Th2- and Th1-associated cytokines.

Naturally occurring regulatory T cells (T Regs) are thought to play an important role in the prevention of autoimmune diseases in man and mouse (Sakaguchi, *S. Annu Rev Immunol* **22**, 531-562 (2004)). Since T Regs are characterized by the constitutive expression of high levels of CD25, we evaluated the *in vitro* effect of daclizumab on the function of these cells.

##### **Methods**

CD4<sup>+</sup> T cells were enriched from whole blood obtained from healthy human donors using StemCell Technologies RosetteSep<sup>™</sup> system. T Regs were flow sorted as CD4<sup>+</sup>CD25<sup>bright</sup> (top 1.5% of CD25 staining intensity) and effector T cells (T Eff) were sorted as

CD4<sup>+</sup>CD25<sup>-</sup> (bottom 5% of CD25 staining intensity). The inhibitory activity of T Regs was assessed in a standard co-culture system. Briefly,  $2 \times 10^4$  T Eff alone, T Reg alone, and T Eff + T Reg were stimulated for 3 days in the presence of immobilized anti-CD3 in the presence of irradiated autologous APCs. Proliferation was measured by <sup>3</sup>H-thymidine incorporation during the last 16 hours of culture.

##### **Results**

T Regs stimulated for 3 days in the presence of daclizumab (10  $\mu$ g/mL) showed no or little proliferation, similar to T Regs stimulated in the absence of DAC. T Effs stimulated alone demonstrated substantial proliferation that was inhibited by daclizumab, on average by 40%. As expected, T Regs suppressed the proliferation of T Effs in the co-culture system. In this system, preincubation of T Regs with daclizumab did not affect the suppressive activity of these cells.

##### **Conclusions**

In these co-culture experiments, daclizumab had no effect on the function of T Reg cells but did inhibit T Eff cells from healthy human volunteers.

#### **F1.42. Respiratory Exposure to OVA Induce Functional Effector CD4 T Cells.**

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Respiratory exposure to environmental antigens such as OVA induces rapid expansion of antigen specific T helper cell population in mice. Primary intranasal challenge with OVA also induces differentiation of activated OVA specific effector Th cells from naive precursors. Effector function has been demonstrated in short-term culture with antigen re-stimulation. The effector Th cells are found both in draining lymph node and in circulation. However, secondary intranasal challenge with OVA fails to induce inflammation in the lung, even when the size of the Th cell population is at its peak. Based on these observations, Th cells primed in the respiratory system by environmental antigens in the absence of adjuvant are viewed as defective or anergic. In this study, we demonstrated the expression of effector function of intranasal primed Th cells in the lung following a challenge with antigen-bead emboli in C57BL6 mice. Importantly, secondary intranasal challenge with soluble antigen did not induce Th cell mediated inflammation in the lung. These results suggest that Th cells primed via respiratory route by environmental antigens are not anergic. More importantly, it is clear that the expression of Th cell effector function is tightly controlled by innate response in the lung. Bead emboli, but not soluble OVA, induced rapid increase of chemokine expression and rapid increase of the number of activated dendritic cells in the lung. However, it is not clear which innate events are critical for the expression of Th cell effector function in the lung. We propose that loss of innate regulation of Th cell effector function in a peripheral organ is necessary for T cell mediated organ specific disorders.

#### **F1.43. A-317491 Inhibits the Activation of Guinea-Pig Pulmonary Vagal Sensory Nerve Terminals by $\alpha$ , $\beta$ -methylene-ATP.**

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Extracellular adenosine 5'-triphosphate (ATP) is a local physiologic regulator. We have previously shown that ATP stimulates bronchopulmonary vagal sensory terminals of nociceptive C and stretch-sensitive A fibers, and that this action is mediated by P2X receptors (R) (J Physiol (Lond) 490:265-75, 1996, ditto 551:869-79, 2003). The stimulatory action of ATP on C and A fibers could be involved in ATP-induced bronchoconstriction and cough. Vagal sensory neurons in the nodose ganglion express homomeric P2X2R and P2X3R as well as heteromeric P2X2/3R. To further explore the P2XR subtype that mediates ATP-induced action potentials (AP) in nodose C and A fiber terminals in the lungs, the effects of A-317491, a potent and selective antagonist at P2X3R and P2X2/3R sites (Proc Natl Acad Sci USA 99:17179-84; 2002), on the activation of guinea-pig intrapulmonary vagal sensory nerve terminals by  $\alpha,\beta$ -methylene-ATP ( $\alpha,\beta$  mATP), a potent selective agonist at P2X3R and P2X2/3R sites, were studied in a perfused isolated lung preparation. The AP in C ( $n = 4$ ) and A fibers ( $n = 7$ ) induced by  $\alpha,\beta$  mATP (10  $\mu$ M, 1ml, bolus) in the absence and presence of A-317491 (1 and 10  $\mu$ M, 30 min) were quantified as discharge/sec; data are mean  $\pm$  SD.  $\alpha,\beta$  mATP induced AP in a non-desensitizing manner in both C and A fibers, the frequency was  $146 \pm 29$  and  $1543 \pm 285$ , respectively. A-317491 (10  $\mu$ M) reduced this response by  $62 \pm 5\%$  and  $88 \pm 5\%$ , respectively ( $P < .05$ ). At 1  $\mu$ M, A-317491 significantly inhibited the action of  $\alpha,\beta$  mATP in A fibers by  $59 \pm 12\%$ , but had no inhibitory effect on C fibers. Conclusion:  $\alpha,\beta$  mATP stimulates both nociceptive C and stretch-sensitive A fibers by acting on P2X2/3R. The present data could also indicate some difference in the nature of the P2XR subtype expressed on the two fiber phenotypes. In addition, since aerosolized ATP induces bronchoconstriction and cough in human subjects and more so in patients with asthma and COPD, A-31749 could constitute a novel therapeutic modality in the management of patients with chronic obstructive airway diseases.

Support: Duska Therapeutics, Inc., Bala Cynwyd, Pennsylvania.

#### F1.44. Abstract Withdrawn.

#### F1.45. Sodium Sulfite Activates Mast Cells and Basophils through Induction of Oxidative Stress.

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Sulfur dioxide is known to induce bronchoconstriction, and asthmatics are particularly sensitive. The cell physiological basis for this is unknown. We have investigated the biological basis for sulfite sensitivity in a mast cell line (RBL-2H3), human peripheral blood basophils, and airway epithelial cells. RBL-2H3 cells were exposed to varying concentrations of sodium sulfite in the presence and absence of anti-oxidants and inhibitors of redox pathways. Sodium sulfite induced mast cell and basophil degranulation to a level equivalent to that induced by IgE cross-linking and ionomycin. The response was independent of extracellular calcium influx. Using a redox sensitive fluorescent dye, 2'/7'-dichlorofluorescein diacetate, sulfite was shown to increase the generation of intracellular reactive oxygen species (ROS). Upregulation of sulfite-induced ROS generation was also demonstrated in the airway epithelial cell line, A549. Both ROS and degranulation induced by sulfite was inhibited by the free radical scavenger tetramethylthiourea and the flavoenzyme inhibitor diphenyleneiodinium. Overall, the data suggest that one potential mechanism of

sulfite-induced asthmatic symptoms may be due to activation of airway mast cells and epithelial cells through the generation of ROS via activation of the NADPH oxidase complex with increased generation of superoxide anion.

#### F1.46. Structural Basis for Epitopes Sharing between Group 1 Allergens of Cedar Pollen.

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Cedar pollen hypersensitivity is a major cause of seasonal airway symptoms in several regions of the Northern Hemisphere. Group 1 allergens have been isolated, cloned and sequenced from the pollens of at least six cedar species. We recently resolved the crystal structure and mapped the known IgE epitopes of Jun a 1 from mountain cedar (MC). Given the high degree of amino acid sequence identity and evidence for immunologic cross-reactivity between cedar allergens, we investigated the structural basis for sharing of IgE epitopes between two group 1 allergens. Cross-reactivity between Jun a 1 and Cry j 1 from Japanese cedar (JC) was probed with sera from JC-allergic patients by ImmunoCAP inhibition. Linear IgE epitopes were identified for Cry j 1 with an array of overlapping Jun a 1 peptides, using sera from patients allergic to JC. The binding of mouse monoclonal anti-Cry j 1 antibodies to these peptides were also tested. A 3-D model of the Cry j 1 protein was prepared with the MPACK suite, using the crystal structure of Jun a 1 as the template. ImmunoCAP inhibition indicated that about 1/3 of the IgE anti-JC pollen antibodies in the sera of JC-allergic patients reacted with Jun a 1. Peptides representing 3 of the 4 Jun a 1 epitopes bound human IgE anti-Cry j 1 antibodies. These epitopes mapped to regions of the Cry j 1 model that had similar surface exposure to homologous regions of the Jun a 1 crystal structure. One epitope, which maps to the beta-helical core of Jun a 1 was not recognized by the sera of JC patients, despite complete sequence identity and apparent similarity of surface exposure. Monoclonal antibodies to Cry j 1 identified another shared epitope that is probably conformational and one unique Cry j 1 epitope, which may be a glycopeptide structure. These findings indicate that the IgE antibodies to several linear and conformational IgE epitopes of group 1 cedar allergens cross-react with homologous allergens. The shared responses may recognize structural elements common to many plant allergens. These findings suggest that patients from genetically diverse population respond to similar linear and conformational epitopes of homologous allergens. Understanding the similarity and difference in the immune responses to groups of allergens will aid in the development of more effective allergy vaccines.

#### F1.47. Food Allergy Related Hospitalizations New York State Hospitals from 1994-2003.

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**Objective:** To profile hospital admissions for food allergy in New York state with respect to demographic characteristics including age, gender, race, and year. Also to compare food

allergy admissions with admissions related to other acute allergic diseases.

**Methods:** A database of all acute hospitalizations in New York state was examined from 1994-2003. The Statewide Planning and Cooperative Research System (SPARCS) database compiles mandatory reporting from acute care hospitals with an information input regarding diagnoses, disposition, procedures, insurance, demographics, and charges. Patient admissions with diagnosis (principal or otherwise) for food allergy (using ICD-9 codes V15.0, 995.6) or other allergic diseases including anaphylaxis, urticaria and allergy unspecified (ICD-9 codes of 995.0, 995.1, 999.4, and 708) were extracted. Demographic characteristics were tabulated for the hospitalizations and patterns were examined. Admissions for food allergy were compared to admissions for other acute allergic diseases.

**Results:** Over the decade examined, admissions for New York state hospitals which coded for allergic disease exclusive of food related codes increased only by less than 5%. However, the number of admissions for allergic conditions involving food allergy tripled. The median age for allergic disease related admissions increased over the years studied; however, the median age for food allergy admissions actually decreased. The increase in age for non-food related allergic disease admissions appeared to be primarily due to an increase in angioedema admissions where the age was greater. Food allergy related admissions increased more in non-African American patients than in African American patients over the decade study. In contrast, allergy related admissions exclusive of food related codes increased in African Americans, especially angioedema. Age related differences were observed with respect to specific foods causing anaphylaxis.

**Conclusions:** Food allergy related hospitalizations are being increasingly reported in New York State. Whether an increase in food allergy related hospitalizations relate to and increase in food allergy or a greater awareness of these conditions cannot be determined, but further research on patterns of food allergy related hospitalization is clearly warranted.

#### **F1.48. Vitamin E Supplementation Augments the Levels of Endogenous Antioxidants and Improves Lung Function in Asthmatic Patients.**

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Asthma is a chronic inflammatory disease with established oxidant-antioxidant imbalance. Antioxidant therapy might be a better strategy to augment endogenous antioxidants for better management of asthma. Vitamin E is a strong lipophilic antioxidant with multiple actions both at biochemical and cellular level. Effects of its supplementation with standard therapy have not been explored in asthmatics. We conducted a double blind standard therapy-controlled study to assess the role of exogenous supplementation of vitamin E on endogenous oxidant-antioxidant balance in asthmatics. Fifty six patients were divided into two groups: 1) placebo group, patients on standard therapy and 2) vitamin E-supplemented group, patients on standard therapy plus 400 I.U. of vitamin E capsules twice daily. Venous blood was collected on day 1 as baseline, then again after 8 weeks of respective treatments. The present study showed that standard

therapy as well vitamin E-supplemented group had lower levels of superoxide anion generation as compared to the baseline. Plasma glutathione peroxidase (GSH-Px) was increased in standard therapy group whereas no difference was found in plasma GSH-Px from baseline in vitamin E-supplemented group. Plasma lipid peroxides were increased and total antioxidant capacity was decreased in standard therapy group whereas vitamin E-supplemented group had significantly lower levels of lipid peroxides and higher total antioxidant capacity. Total blood glutathione was also decreased in standard therapy group whereas no significant difference was found in vitamin E-supplemented group. Plasma nitrates and nitrites (NOx) were decreased in standard therapy group whereas they were increased in vitamin E-supplemented group. Plasma protein sulfhydryls and red cell superoxide dismutase (SOD) levels were increased in standard therapy group, while there was no change from baseline in red cell SOD activity in vitamin E-supplemented group, the levels of the former remained increased in this group also. No significant difference was found in plasma protein carbonyls and red cell catalase in either standard therapy group or vitamin E-supplemented group. Plasma vitamin E levels increased more than two fold after vitamin E supplementation but no change was observed in standard therapy group. There was significant improvement in FEV1% predicted in both the groups after 8 weeks of respective treatments but vitamin E-supplemented group had greater degree of improvement in terms of % increase from baseline. Our study provides biochemical and clinical evidence for the first time that vitamin E augments endogenous antioxidant screen and improves lung function. So, it may be used as an adjunct therapy in the treatment of asthmatics.

#### **Bone Marrow or Stem Cell Transplantation**

##### **F1.49. Recovery of CD4 and CD8 Lymphocyte Subsets and Their Impact on Acute Graft-vs. Host Disease (GVHD) after Reduced Intensity Conditioning (RIC) Allogeneic Stem Cell Transplantation (allo-SCT).**

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RIC regimens (including highly immunosuppressive non-myeloablative therapy in replacement of high dose chemotherapy or radiotherapy) for allo-SCT, are being explored with good results concerning feasibility and engraftment. However, little is known about the immune recovery pattern in these patients, especially the different CD4 and CD8 lymphoid T cell subsets. Here, we assessed at different time points after allo-SCT, the kinetic of recovery of naïve (CD45RA+/CD27+), central memory (CD45RA-/CD27+), and terminally differentiated (CD45RA+/CD27-) CD4+ and CD8+ T lymphocytes in 64 patients from a single center, receiving HLA-identical RIC allo-SCT. Patients and graft characteristics are: age 48 y (27-63), diagnoses: 22 myeloid malignancies (34%), 22 lymphoid malignancies (34%) and 20 metastatic solid tumors (31%). 51 pts (80%) were considered as high risk. 49 pts (77%) received a fludarabine, busulfan and antithymocyte globulin-based RIC, while 15 pts (23%) received a low dose irradiation-based RIC. 91% of patients received a peripheral blood stem cell graft, with 42 (66%) receiving cyclosporine (CSA) alone for GVHD prophylaxis and 22 (34%) receiving CSA and MMF. 24 pts (38%) developed grade 2-4 acute

GVHD at a median of 49 d (26–85). In this series, in contrast to CD4+ T cell subsets, CD8+ T cell subsets had a progressive and sustained recovery in the first 3 months after allo-SCT, with acquisition of functional markers such as 2B4 and perforin. Among the different subsets analyzed, the recovery of naïve CD4+ T cells, and central memory CD4+ and CD8+ T cells, measured at day 28 after allo-SCT and before onset of grade 2–4 acute GVHD, showed a significant correlation with the risk of grade 2–4 acute GVHD ( $P = 0.001$ ;  $P = 0.002$  and  $P = 0.05$  respectively). Patients developing grade 2–4 acute GVHD recovered a median of 47 naïve CD4+ T cells/ $\mu\text{L}$  prior to onset of GVHD as compared to 11 cells/ $\mu\text{L}$  in patients with grade 0–1 acute GVHD. Naïve CD4+ T cell levels significantly decreased after appropriate acute GVHD treatment. In a Cox multivariate analysis taking into account all relevant risk factors for acute GVHD, early recovery of naïve CD4+/CD45RA+/CD27+ T cells in the first month after allo-SCT was the strongest parameter significantly predictive of grade 2–4 acute GVHD development ( $P = 0.006$ ;  $R_r = 4.0$ ; 95%CI, 1.5–11.0). Interestingly, there was a significant correlation between the total number of CD4+ T cells infused with the allogeneic graft and the early recovery of naïve CD4+ T cells ( $P = 0.001$ ), suggesting that graft manipulation might represent an attractive tool towards harnessing alloreactivity after RIC-allo-SCT.

#### **F1.50. Cross-Presentation of Minor Histocompatibility Antigens Requires Dendritic Cells and Immunoproteasomes.**

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CD8 T lymphocytes (CTL) play a major role in mediating allograft rejection in MHC-identical solid and bone marrow transplant settings. In such instances, alloreactivity is directed towards either donor- (solid organ) or recipient- (bone marrow) derived antigens that often represent only minor variations of self. Such alloantigens are called minor histocompatibility (minor H) antigens. These variant minor H antigens elicit robust CTL responses that in the most severe cases lead to graft versus host disease (GVHD) or graft rejection, and may result in death. The molecular mechanisms by which minor H antigens are processed and presented to donor or recipient CTL remain poorly understood. In this study, we have exploited mice deficient in various aspects of antigen presentation to demonstrate the roles of both donor- and recipient-derived dendritic cells and proteasomes in the acquisition, processing, and cross-presentation of minor H antigens. Our findings reveal an important role for recipient dendritic cells and donor proteasomes in the generation of an effective minor H antigen response. These findings have important implications not only for the understanding of GVHD, but for viral and tumor vaccine design as well.

#### **F1.51. Permanent Mixed Chimerism in Mice by Short, Low Toxicity, Radiation Free Treatment Allows Myoblast Allograft Tolerance across Fully MHC-Mismatched Barriers.**

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Duchenne muscular dystrophy (DMD) a fatal neuromuscular recessive disease is characterized by widespread muscle damage

throughout the body. No cure is currently available for DMD. Our research group is pursuing a research program to develop a treatment based on healthy donor myoblast transplantation (MT). A sustained FK506 immunosuppression is currently required to prevent rejection of allogeneic human MT. However, the major drawback of MT is that long-term use of immunosuppressive treatments is associated with adverse effects: nephrotoxicity, increased cancer risk etc. . .

During last years, transplantation tolerance has been obtained by development of stable donor-specific chimerism resulted by bone marrow transplantation (BMT). Induction of stable multilineage chimerism (SMLC) across fully MHC-mismatched barriers have been established by a busulfan myelosuppressive treatment in mice primed with an allogeneic spleen cells transfusion followed by a single cyclophosphamide (Cyp) dose, an immunosuppressive drug. We have developed a protocol to obtain same SMLC with donor BMT following by a conditioning regimen involving a treatment with single Cyp(200mg/kg) dose and low treosulfan (Treo) dose (3\*500mg/kg), a less toxic busulfan analog. We tried to obtain similar chimerism level in mdx mice, a dystrophic mouse model, with Cyp/Treo based protocol in order to prevent rejection of allogeneic healthy Balb/c myoblasts and of the muscle fibers that they formed in grafted *tibialis anterior* (TA).

All mice (9) treated, have variable mixed chimerism levels (0.5–55%) for leukocyte cells population (CD90) 230 days after the BMT. Most (5/9) treated mice have CD90 chimerism between 10 and 20%. All treated mice have variable chimerism level in (CD4+ or CD8+) T-cell population in same proportion as CD90 population. Consequently, mice have developed SMLC. Dystrophin positive fibers were present in chimeric TA mice 100 and 200 days after MT. Hybrid fibers number was equivalent to the one observed in TA sections of mdx mice treated with FK506 immunosuppression. No infiltration with CD4, CD8 T-cells was observed around dystrophin positive fibers at 100 or 200 days after MT. To test tolerance “resistance” capacity, we have done a challenge by initially grafting donor myoblasts in mice left (6) TA using Cyp/Treo protocol developed above. One hundred days later, a second MT from the same donor strain was performed in the right TA without any additional therapy. Mice were sacrificed 100 days after the second MT. In both TA grafted, we observed dystrophin positive fibers and no CD4 or CD8 T-cells infiltration. Thus, we conclude that first grafts can survive after challenge with donor antigen/MT without additional treatment.

Taken together, we show that Treo low toxicity associated with a protocol which does not require any irradiation and using only clinical use approved drugs, would permit to obtain safe sustained immunological tolerance of the DMD patients for donor MT. Accordingly, it could be applied as a conditioning regimen for several other organ or tissue transplantations.

#### **F1.52. Neonatal CD4+ CD25+ T Cells—Age Restricted Development of Immune Tolerance.**

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The regulation of immune responses is an intrinsic capacity of the developed immune system and is believed to be mediated by

specialised T cells. The CD4<sup>+</sup> CD25<sup>+</sup> T cell is the best defined subset with regulatory function. Being essential in maintaining immune balance in mice and having shown regulatory capacity in man, the prerequisites for their development are still controversial. However, understanding the developmentally needs for immune tolerance might facilitate its manipulation.

Having shown that the neonatal phase is pivotal to induce dominant tolerance to peripheral antigens, we built up a syngeneic bone marrow transplantation model to determine whether development of regulatory T cells is restricted to a certain developmentally window.

For that reason, rag<sup>-/-</sup> mice were transplanted with T cell depleted rag<sup>±</sup> bone marrow. After reconstitution, non-irradiated recipients showed immune dysregulation and died of wasting disease. Disease was driven by host derived T cells. Bone marrow derived T cells abolished disease, whereas TCR transgenic or neonatal recipients were not protected. Depletion of CD4<sup>+</sup>/CD25<sup>+</sup> cells alone resembled whole T cell depletion, whereas addition of bone marrow derived CD4 cells prevented, or even cured, disease up to 30days after BMT. Strikingly, T cells developed in bone marrow chimeras were ineffective. Taken together, these results demonstrate, that regulatory T cells might be helpful tools treating immune dysregulation. However, their development is restricted to a certain developmentally window in early live while its largely diminished in adults or after BMT.

Understanding development and role of regulatory T cells in immune reconstitution will allow us to improve GvT reactions while avoiding GvHD.

### **F1.53. In Vivo Neutralization of Both Interferon Gamma (IFN-g) and Interleukin-2 (IL-2) Accelerates Anti-Host Cytotoxic T Lymphocyte (CTL) Development and Acute Graft-Versus-Host Disease (GVHD) in the Parent-Into-F1 Model.**

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The parent-into-F1 model of acute GVHD is a useful model of in vivo CTL development. We have previously demonstrated that anti-host CTLs characteristic of acute GVHD in the B6-into-F1 model are impaired with selective blockade of either IL-2 or IFN-g. Surprisingly, preliminary experiments indicated that at 14 days after donor transfer, mice receiving combined IL-2 and IFN-g blockade exhibited more severe lymphopenia than untreated acute GVHD suggesting a paradoxical worsening of disease when compared to selective blockade of either IL-2 or IFN-g alone. To address the mechanism involved, acute GVHD was induced in B6D2F1 mice by the injection of 50 million B6 wild type (WT) parental spleen cells or 50 million B6 IFN-g<sup>-/-</sup> donors plus neutralizing anti-IFN-g mAb (XMG-6, 1 mg i.v. weekly). Neutralization of IL-2 was achieved with 2 mg anti-IL-2 mAb (S4B6) weekly i.v. Controls consisted of normal F1 mice or GVHD mice receiving IFN-g blockade alone, or control mAb alone (GL117). At 7, 10 and 14 days after parental cell transfer, mice were assessed for splenic lymphocyte subpopulations by flow cytometry and for ex vivo anti-host CTL activity. Our results indicate that peak donor CD8<sup>+</sup> T cell expansion (day 10) is 2-fold greater for GVHD mice receiving combined IFN-g and IL-2 blockade compared to untreated or control mAb treated WT

GVHD mice. Importantly, combined IFN-g and IL-2 blockade accelerated GVHD phenotype with peak anti-host CTL activity seen at day 7 (vs. day 10 for WT GVHD) and complete host B cell elimination and CTL downregulation seen at day 10 (vs. day 14 for WT GVHD). Of note, at day 7, GVHD mice receiving combined IFN-g and IL-2 blockade did not differ from IFN-g only blockade GVHD mice with respect to donor T cell engraftment or host B cell elimination. These results support the hypothesis that the early absence of IFN-g accelerates CTL development regardless of the presence of IL-2. In contrast, the absence of IL-2 after day 7 permits greater CD8<sup>+</sup> T cell expansion, consistent with the loss of an IL-2 dependent regulatory mechanism.

### **F1.54. Role of Apoptotic Cells in the Induction of Regulatory T Cells.**

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Extracorporeal photopheresis (ECP) is an immunomodulatory cell therapy being investigated as a treatment for various immune-mediated inflammatory disorders. Clinically, ECP involves the intravenous reinfusion of autologous, apoptotic peripheral blood leukocytes. In animal models, delivery of apoptotic cells has been shown to regulate immune responses through the modulation of cytokines, generation of regulatory T cells, and downregulation of antigen-presenting cell (APC) function. We and others have shown that activation of naïve T cells in the presence of APCs that have engulfed ECP-treated apoptotic cells leads to the generation of a T cell population with suppressive activity. In the present study, we demonstrate that the *direct* interaction of ECP-treated peripheral blood mononuclear cells (PBMCs) with naïve human CD4<sup>+</sup> T cells *in vitro* promotes a T cell phenotype with regulatory activity. The generation of human regulatory T cells by ECP is dependent on APCs and can be reversed by the addition of IL-2. CD25 expression is upregulated on these regulatory T cells and they proliferate in response to concanavalin-A. However, these regulatory T cells do not express increased levels of Foxp3 or IL-10, nor do they secrete significant levels of the pro-inflammatory cytokines IL-2, IFN $\gamma$ , IL-4, and TNF $\alpha$ . Transfer of ECP-derived regulatory T cells to a secondary mixed lymphocyte reaction results in a greater than 50% inhibition of syngeneic responder T cell proliferation and IFN $\gamma$  production at a Treg:responder ratio of 1:20. In addition, transwell assays demonstrate that inhibition of responder T cells by ECP-derived regulatory T cells is contact-dependent. Additional studies are underway to further characterize the phenotype of these regulatory cells and to determine their suppressive capacity *in vivo*.

### **F1.55. Distribution of CD4+25+ Cells in Fetal Sheep Early in Gestation.**

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Our laboratory has shown that transplantation of hematopoietic stem cells (HSCs) into fetal sheep results in long-term multi-lineage hematopoietic chimerism. This observation demonstrates that tolerance can be induced during prenatal period by early injection of HSCs (optimal age for transplantation = 55–65

days of gestation, term = 145 days). However, the relationship between hematopoietic chimerism and tolerance has remained obscure. In order to understand the ontogeny of immune system in fetal sheep, we analyzed various cell populations in the thymus, spleen, bone marrow (BM), small intestine (site of Peyer's patch formation), liver, and peripheral blood (PB) of fetal sheep, between days 39 to 82 gestation, by flowcytometry. These observations demonstrated that at day 39 the thymus contains up to 9% CD4+25+ cells. This population is minimally detectable at days 45 and 52 (0–1%); however increases to 11% at day 58 and then diminishes to 1–2% by day 65 and 2.5% at day 82. At day 58 of gestation, there is a peak CD4+25+ cells population in PB, BM, and liver as well. CD4+25+ cells are seen in BM, small intestine, and spleen at day 39 of gestation but not liver and PB. CD4+25+ expression in the small intestine increased from 8% at day 39 to 40% at day 82. However, in BM and spleen, CD4+25+ expression diminishes to 2% at day 82 of gestation.

The CD4+25+ cell population in peripheral lymphoid organs, at early gestational ages, express CD45R, likely a memory phenotype of T cells (as described before by Cooper *et al.* Journal of immunology, 1994, 152: 3098). Immunohistochemistry studies at day 58 show CD45R cells abruptly increase in the medulla of the thymus, but decrease at day 65 and gradually increase at day 82.

These data suggest that there is a population of CD4+25+45R+ cells (phenotypically consistence with T regulatory cells) in the primary and secondary lymphoid organs early in gestation. These data support the establishment of peripheral tolerance early in gestation that may have a role in tolerance induction following in utero HSCs transplantation.

### F1.56. Clinical Relevance of Recipient Leukocyte Infusion (RLI) Therapy.

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#### BACKGROUND

Surprisingly, anti-tumor responses can occur in patients who reject donor grafts following nonmyeloablative hematopoietic cell transplantation (Dey *et al.*, Biol Blood Marrow Transplant 7:604). In murine mixed chimeras prepared with nonmyeloablative conditioning, we previously showed that recipient leukocyte infusions (RLI) induced anti-tumor responses against host-type tumors (Rubio *et al.* Blood 102:2300).

To further investigate the clinical relevance of this RLI model, we:

1. Evaluated the effect of RLI from tumor-bearing mice
2. Compared RLI with allogeneic lymphocyte infusion in untreated mice.

#### METHODS

Mixed chimerism was achieved in BALB/c (H-2<sup>d</sup>) mice conditioned with depleting anti-CD4 and CD8 mAbs on Day-5, cyclophosphamide 200 mg/kg on Day -1 and 7 Gy thymic irradiation on Day 0 prior to transplantation of  $25 \times 10^6$  B10.BR (H-2<sup>k</sup>) bone marrow cells. Some groups received RLI ( $3 \times 10^7$  BALB/c spleen cells) seven weeks post-BMT. Some RLI donor mice received BALB/c A20 B cell lymphoma cells ( $1 \times 10^5$ ) two weeks before RLI. Some groups received RLI depleted of B cells by MACS column for purging tumor cells. A20 cells ( $5 \times 10^5$ ) were given i.v. one week after RLI in chimeras or after allogeneic lymphocyte infusion ( $3 \times 10^7$  B10.BR spleen cells) to untreated BALB/c mice.

### RESULTS

In the clinical setting, RLI would be obtained from tumor-bearing hosts. We therefore examined whether RLI is still effective when the lymphocytes are obtained from tumor-bearing mice. Recipients of RLI from tumor-bearing mice showed similar tumor survival compared to recipients of RLI from naive donors. Thus, with a purging procedure, the same anti-tumor effect was achieved with RLI from tumor-bearing hosts as from non-tumor-bearing hosts.

Allogeneic lymphocyte injection is a potentially feasible anti-tumor therapy. We therefore compared anti-tumor effects of allogeneic lymphocyte infusion into naive mice with that of RLI given to mixed chimeras. RLI recipients had longer survival than naive mice receiving allogeneic lymphocytes. This result suggests not only that the anti-tumor effect of RLI therapy is stronger than allogeneic lymphocyte infusion therapy but also suggests that rejection of allogeneic cells is insufficient and mixed chimerism is required prior to the induced rejection to achieve maximum anti-tumor effects.

### CONCLUSION

Together, these data reinforce the potential of RLI therapy to be a new HSCT strategy which does not have the risk of graft-versus-host disease.

### F1.57. Anti-Mouse Thymocyte Globulin Administration Prevents Acute Graft-versus-Host Disease in a Murine Model.

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Thymoglobulin<sup>®</sup>, a rabbit anti-human thymocyte globulin, is FDA approved for use in acute transplant rejection. Positive results in this setting suggest that Thymoglobulin<sup>®</sup> would likely be effective against graft-versus-host disease. To test this hypothesis preclinically, we generated an anti-mouse thymocyte antiserum (mATG) by immunizing rabbits with mouse thymocytes and purifying IgG from the resulting immunoserum. These methods were performed identically to those used to generate Thymoglobulin<sup>®</sup>, the human counterpart ATG. In addition, a model of acute graft-versus-host disease (GVHD) was developed. GVHD was induced by transfer of spleen cells from C57BL/6 mice into non-irradiated BALB/c RAG-2 deficient mice that lack mature T and B cells. Following transfer of the allogeneic cells, the C57BL/6 hosts displayed progressive weight loss which was accompanied by increased production of a variety of proinflammatory cytokines detected in the serum, hepatic necrosis and inflammation in several organs. In the absence of therapeutic intervention, control mice display significant disease within 3 weeks. We then applied mATG at various time points following cell transfer in the GVHD model to evaluate its effectiveness in disease prevention/reversal. Administration of mATG at days 0–3 post allogeneic cell transfer depletes most T cells from the circulation (FACS analysis) and completely prevents all evidence of acute GVHD. GVH mice treated with mATG showed no evidence of weight loss, elevated cytokine levels or target organ pathology. This protective effect of mATG on acute GVHD persisted for at least 12 weeks. We also tested the effectiveness of mouse ATG

treatment given after acute GVHD was well established. In contrast to mATG administration at the initiation of disease, treatment 10–13 days following allogeneic cell transfer showed no evidence of GVHD amelioration. Thus, we show that dependent upon timing of dosing, mATG treatment can be quite effective at preventing acute GVH in a murine model of disease. These results suggest that exploration of Thymoglobulin® dosing in relation to bone marrow transplant in human patients as prophylaxis for GVHD is warranted.

*This work is supported by Genzyme Corporation.*

#### **F1.58. IFN-Gamma Promotes Lymphohematopoietic GVH Reactions while Attenuating GVHD in Murine Allogeneic Hematopoietic Cell Transplantation Models.**

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The role of IFN-g in the induction of graft-versus-host disease (GVHD) after allogeneic hematopoietic cell transplantation (allo-HCT) has been elusive. IFN-g is protective in lethally irradiated mice receiving allogeneic T cells plus marrow cells, but deleterious in non-conditioned or sublethally irradiated recipients of allogeneic T cells alone (i.e., without marrow cells). We have recently observed that T cells from IFN-g KO donors induce more severe damage in parenchymal GVHD target tissues, but have reduced ability to eliminate recipient hematopoietic cells in lethally irradiated murine allo-HCT models. These results suggest that the deleterious effect of IFN-g in allo-HCT recipients of donor T cells alone might be due to facilitation of anti-host lymphohematopoietic GVH reactions, which result in destruction of host type hematopoietic cells and hematopoietic failure of the recipients. To test this hypothesis, we compared the development of GVHD in sublethally irradiated (6 Gy) B6D2F1 mice that received splenocytes alone or along with bone marrow cells (BMC) from IFN-g KO or wild-type (WT) B6 donors. B6D2F1 mice that received sublethal irradiation alone were used as non-GVHD controls and all survived long-term. Consistent with published studies, the recipients of WT B6 splenocytes alone (without BMC, so that the inoculum contained minimal numbers of hematopoietic stem cells) developed more severe GVHD compared to mice receiving a similar number of splenocytes from KO B6 donors ( $P < 0.05$ ). In contrast, when mice received donor spleen cells along with BMC, the recipients of KO allo-HCT developed severe acute GVHD and succumbed to death by 5 weeks, while most of the recipients of WT allo-HCT survived long-term ( $P < 0.01$ ). Unlike the recipients of WT allo-HCT, the addition of BMC to splenocytes did not significantly delay GVHD death in the recipients of GKO allo-HCT. In order to quantitatively evaluate the ability of WT vs. IFN-g KO donor T cells to induce lethal GVHD, we compared the survival of lethally irradiated B6D2F1 mice that received a fixed number of BMC along with titrated numbers of splenocytes from WT or IFN-g KO B6 mice. The minimal number of splenocytes needed to induce lethal GVHD was approximately 10 fold less for KO than WT donor cells. Taken together, our results indicate that WT allo-HCT mediates stronger lymphohematopoietic GVH reactions than GKO allo-HCT, but the latter cause more severe damage in parenchymal tissues. Since lymphohematopoietic GVH reactions may selectively eliminate host lymphohematopoietic cells, including lymphoma cells, a better understanding of the mechanisms by which IFN-g separates the two types of GVH reactions could lead

to novel approaches to separating GVHD and graft-vs.-leukemic effects.

#### **F1.59. Distinct Effects of Early and Delayed CTLA4-Blockade after Murine miHC-Disparate Allogeneic Bone Marrow Transplantation: Graft-Versus-Host Disease Versus Auto-Immunity.**

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**Background:** It has been shown that CTLA-4 blockade early after murine MHC-disparate allogeneic bone marrow transplantation (BMT) results in augmentation of donor or host alloreactivity (graft-vs-host disease (GVHD), resp. rejection). Later after BMT, CTLA-4 blockade in combination with DLI induced a graft-vs-leukemia (GVL) effect with a slightly increased risk for GVHD. **Aim:** To explore, in a model of MHC-compatible, miHC-disparate allo BMT, how CTLA-4 blockade can be used to modulate GVHD and to induce GVL effects. **Materials & Methods:** C3H × AKR 7,5 Gy radiation BM chimeras were treated with blocking anti-CTLA-4 MoAb (UC10-4F10) or irrelevant Hamster IgG, early (d-1 to 12) or late (d21 to 34). Mice were monitored for weight changes, clinical signs of GVHD and survival. Moribund animals were sacrificed for histopathology. FACS was used to study T cell chimerism and host/donor-reactive T cell-frequency, MLR to study ex vivo alloreactivity, ELISA to determine circulating antiDNA Ab. **Results:** CTLA-4-blockade early after non-T cell depleted (TCD) allo BMT induced acute and (>90%) lethal GVHD (increased donor T cell chimerism and alloreactive TCR-Vb6<sup>+</sup> cells), histopathologically confirmed. Late after non-TCD allo BMT, however, CTLA-4-blockade induced weight loss, alopecia, splenomegaly, lymphadenopathy and cachexia with 60% mortality. Treated and non-treated animals did not show any difference in chimerism or alloreactive T cell-frequency. Histopathology showed lymphoproliferation in spleen, lymph nodes but also in stomach, intestine, salivary glands and liver. In the liver, periportal infiltrates showed numerous plasmacells. AntiDNA Abs and liver-enzyme abnormalities were detected in the treated group. Ex vivo, splenocytes of diseased animals showed strikingly strong spontaneous proliferation but MLR reactivity towards host- or donor-type antigens was equal to that of tolerant non-treated chimeras. **Conclusions:** In a miHC-disparate context, CTLA-4-blockade, early after non-TCD BMT induces vigorous acute GVHD, consistent with activation of alloreactive T cells from the BM inoculum. In contrast, CTLA-4 blockade from day 21 onwards does not give rise to the breaking of transplantation tolerance, however, induces a lymphoproliferative syndrome with autoimmune manifestations. Our data suggest that after non-TCD allo miHC-disparate BMT, donor-host tolerance relies on clonal deletion which is insensitive to aCTLA-4 Ab. In contrast, tolerance to self is maintained by peripheral tolerance mechanisms, in which CTLA-4 signaling appears to play a major role. We are currently investigating if CTLA-4 blockade can elicit antileukemic reactivity and how the balance between autoimmunity and antitumor immunity can be modulated.



### F1.60. Successful Bone Marrow Transplantation in a Patient with WHIM Syndrome.

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WHIM syndrome is a rare primary immunodeficiency disease characterized by Warts, Hypogammaglobulinemia, Infections and Myelokathexis. Until now, there is no report on a patient with WHIM syndrome treated by allogeneic bone marrow transplantation (BMT). We performed nonmyeloablative BMT for an 18-year-old female patient who has a nonsense mutation (S338X) in the chemokine receptor CXCR4 which was identified as causative gene of WHIM syndrome, from her healthy HLA-matched sister who has no mutation of the gene. The patient had experienced recurrent middle ear, sinopulmonary, and urinary tract infections since 2 years of age. She was recognized as leukopenic (white blood cell counts of  $1.4 \times 10^9/L$ ), neutropenic ( $0.7 \times 10^9/L$ ) and hypogammaglobulinemic (IgG428mg/dl, IgA4mg/dl, IgM21mg/dl), when she was admitted to a local hospital at 7 years of age with the aim of operation for chronic otitis media. She was referred to our hospital for investigation of leukopenia. Examination of bone marrow aspirate revealed a marked granulocytic hyperplasia and bone marrow neutrophils contained extremely pyknotic nuclei and vacuolated cytoplasm. Her disease was diagnosed as WHIM syndrome. Treatment with daily subcutaneous injection of recombinant human G-CSF increased mildly her blood neutrophils and decreased frequency of her febrile episodes. Four months later, therapy was discontinued because of the development of splenomegaly and myelofibrosis. She has worn a hearing aid due to intractable otitis media since 11 years of age. Because her deafness was gradually aggravated, she was considered for allogeneic BMT at 18 years of age. This treatment plan was approved by the Institutional Review Board at Nagoya University Graduate School of Medicine and written informed consent was obtained from the patient, her parents and her sister. The conditioning regimen consisted of fludarabine ( $30\text{mg}/\text{m}^2/\text{d}$  on days -5 to -2) and TBI ( $2\text{Gy} \times 1$  on day -1). Because of the incompatibility between red-cell groups, a total of  $30 \times 10^7/\text{kg}$  erythrocytes-depleted bone marrow mononuclear cells were infused to the patient from her sister on January 16, 2002. GVHD prophylaxis consisted of tacrolimus and short-term methotrexate. The absolute granulocyte count in the peripheral blood was more than  $500/\mu\text{l}$  on day 27. Although there was no evidence of infection or no sign of acute GVHD, mild eczematous lesions were observed on the body on day 70. The diagnosis of chronic GVHD was confirmed by a skin biopsy. These skin lesions improved for 70 to 80 days by continuing only oral administration of tacrolimus. She had >99% donor cells in peripheral blood as determined by microsatellite typing on day 30. Thirty six months after BMT, she is doing well, with normal blood cell counts and no need for therapy. Because allogeneic BMT has the potential to cure primary immunodeficiency diseases affecting marrow-derived cells, we consider that nonmyeloablative BMT is the treatment of choice for patients with WHIM syndrome who have an HLA-matched sibling donor.

### F1.61. Characterization and Depletion of Human PBMNC Reactive with Murine Stimulator Cells.

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Human leukocytes respond vigorously when they encounter murine cells. One outcome of this response is the development of xenogeneic GVHD when human PBMNC are used to reconstitute immunodeficient recipient mice. It was hypothesized that preventing xenogeneic GVHD would permit the long term reconstitution of immunodeficient mice with functioning human cells. Two proposed approaches to prevent xenogeneic GVHD were depleting the xenoreactive cells prior to injection of PBMNC into the recipient mice or by generating recipient mice that lack the antigens that stimulate the xenogeneic GVHD responses by the human leukocytes. To characterize the human anti-murine response, the ability of unfractionated human PBMNC to proliferate in response to murine C57BL/6 stimulator cells lacking expression of murine H-2 class I, II or both was tested. The absence of H-2 antigen expression on the murine stimulator cells did not interfere with the proliferative responses of human PBMNC to these stimulator cells. These findings suggested that a number of human leukocyte subpopulations were able to proliferate in response to antigens other than H-2 antigens on xenogeneic murine cells. To characterize this response further, the cell surface markers expressed by activated CD69+ human leukocytes after incubation with murine stimulator cells were analyzed. These results indicated that the activated population of cells included T, B, NK, and NKT cells. Depletion of these activated CD69+ cells resulted in the inhibition of proliferative responses to the original C57BL/6 stimulating cells upon restimulation of the cells remaining after depletion. However the depleted cells were still able to proliferate to xenogeneic stimulator cells from other mouse strains and to allogeneic PBMNC. Additional studies will characterize the antigens responsible for inducing each of the responding lymphoid populations and test if administration of depleted populations prevents the development of xenogeneic GVHD.

## Poster Session 2

3:30 PM–5:30 PM, 5/13/2005

### Reproductive Immunology

#### F2.01. Cytokines in the Placenta of Pakistani Newborns with and without Intrauterine Growth Retardation.

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Objective: Intrauterine growth retardation (IUGR) is a major risk factor for increased neonatal mortality and morbidity. The prevalence of IUGR in newborns of Lahore, Pakistan was reported to be up to 24%, while in a Swedish population in about 1–4% of pregnancies. Despite the recognized problem with IUGR pregnancies, the mechanisms behind this condition remain unclear.

It has been shown that cytokines play a major role in normal pregnancy, but are also involved in IUGR and other pregnancy

dysfunctions. During normal pregnancy, a predominance of Th2 type cytokines prevails and is considered to protect the foetus, while an increase of Th1 type cytokines may have deleterious effects. In this study we wanted to evaluate the possible role of certain Th1 and Th2 cytokines in the development of IUGR. We analyzed the gene expression, gene polymorphisms and protein levels of these cytokines from IUGR and non-IUGR pregnancies in a Pakistani population, where IUGR is very common.

**Material and methods:** 45 IUGR and 55 control mother/infant pairs were studied. mRNA expression levels for IL-10, IL-8, TNF- $\alpha$ , TGF- $\beta$ , IL-6, IL-4, IL-1 $\beta$ , IL-12 and INF- $\gamma$  from the maternal (decidua) and the foetal (trophoblast) side of the placenta were quantified with RT-PCR. Cytokine and cytokine receptor gene polymorphisms for -1087IL10, -308TNFA, -174IL6, TGFB1, IL1RA, TNFR1, IL4RA 150V and -159CD14 were determined from genomic DNA by the combination of PCR and restriction enzyme cleavage. Serum levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, IL-12, TNF- $\alpha$  and TGF- $\beta$  were quantified in maternal and umbilical cord blood by ELISA and Luminex.

**Results:** There was a significant decrease of IL-10 ( $P < 0.0001$ ) and IL-12 ( $P < 0.008$ ), but an increase of TGF- $\beta$  ( $P < 0.009$ ) in the decidua and similarly a decrease of IL-10 ( $P < 0.03$ ), but an increase of TGF- $\beta$  ( $P < 0.009$ ) in the trophoblasts of the IUGR placentas compared to the non-IUGR placentas.

We found significantly lower levels of IL-1 $\beta$  in serum from the mothers of the IUGR infants ( $P < 0.008$ ) and of TGF- $\beta$  in serum of the infants with IUGR ( $P < 0.05$ ) compared to the non-IUGR infants.

**Conclusion:** We note that the IL-10 mRNA expression in the decidua was down-regulated, but the TGF- $\beta$  mRNA up-regulated in IUGR placentas of mothers from a population with multiple risk factors for IUGR. We propose that the low IL-10 in the placenta may be involved in the pathogenesis of IUGR and might possibly be treatable.

## F2.02. Setup of IgG MAR Methods and Determination of the Prevalence of Antisperm Antibodies in Semen of Immunoinfertile.

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**Background:** Infertility affects 10% to 15% of couples desiring children. Immunologic factors have been proposed to be involved in as many as 20% of otherwise unexplained cases of infertility. Due to importance of antisperm antibodies diagnosis in immunoinfertility, our main aim in this study is setup of IgG MAR and determination of the prevalence of ASA in Zanjan province-Iran.

**Methods:** out of 312 Semen Samples collected for this study. Setup IgG MAR with use of IgG-sensitized Erythrocytes were prepared. (From RBC O RH+ and incomplete IgG anti Rh D) and antihuman IgG serum. Coordinate time all samples were tested for ASA by means of the direct MAR test. Greater than 10% of motile sperm-bound to RBC was considered a positive.

**Results:** our finding showed that specificity and sensitivity of IgG MAR Versus commercial sperm MAR kits (Gold standard) are 100% and 94.7% respectively. In our study 18 cases from 312 samples (5.7%) were positive for ASA.

**Conclusion:** Our findings showed that ASA as an important causative factor in immunoinfertility field and Direct Mixed

antiglobulin reaction is a cheap, quick and sensitive screening test for detection of sperm-bound antibodies.

**Keywords:** Prevalence-Mixed antiglobulin reaction -Anti sperm antibody

## F2.03. Sildenafil Does Not Influence Natural Killer Cell Activity in Women with a History of Recurrent Spontaneous Abortion.

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**Introduction:** Endometrial growth seems to be dependent on uterine artery blood flow and the importance of endometrial development on pregnancy outcome has been previously reported. NK cytotoxicity has been reported to be predictive of subsequent pregnancy loss in women who had recurrent spontaneous abortions (RSA). Sildenafil citrate (VIAGRA), a type 5-specific phosphodiesterase inhibitor, augments the vasodilatory effects of NO by preventing the degradation of cGMP. Vaginal sildenafil improves uterine artery blood flow and sonographic endometrial thickness in patients with prior failed assisted reproductive cycles due to poor endometrial response. While improving uterine blood flow in the proliferative phase, NO may have detrimental effects at the level of the endometrium during the implantation window. The NO-mediated release of cytokines such as tumour necrosis factor from activated natural killer cells has been implicated as a cause of implantation failure. Therefore, the purpose of the study was to establish the effect of sildenafil on NK cell activity in women with a history of RSA, including women with multiple in-vitro fertilization failures. **Materials and Methods:** Fifteen nonpregnant women with the history of RSA and ten normal healthy women with the previous successful pregnancy outcome were studied. Measurement of uterine artery blood flow (pulsatility index, PI) was recorded using Doppler ultrasound by intravaginal probe in the study women. Natural killer cell activity was measured using flow cytometry. The following peripheral blood NK cells' surface antigens: CD16, CD56 were also studied using flow cytometry. NK cell activity before and after sildenafil therapy in RSA women were studied. In addition, influence of 10mg or 400 ng sildenafil on NK cell activity after in vitro culture were performed. **Results:** We determined that there is a positive correlation between increased natural killer cell activity and PI in women with the history RSA compared to normal healthy women ( $r > 0.5$ ,  $P < 0.05$ ). Our preliminary data suggest that sildenafil has no significant influence on NK cell activity. **Conclusions:** Our data suggest that increased natural killer cell activity can be associated with diminished uterine artery blood flow. Sildenafil might be interesting therapeutic option for women with reproductive failure. However, further studies are needed to determine the role of this therapy in human reproduction. This work was supported by grant nr 2 P05E 07926.

## F2.04. IgE Is Distributed on Macrophages Both in Human and Murine Term Placentae.

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**Objective:** High level serum IgE is the hallmark of immune response in allergic patients. Epidemiological studies indicate that the prenatal environment plays an important and decisive role in the development of allergy later in life. To test whether the impact of maternal atopy could be transmitted through the maternal-fetal interface, this experiment was conducted. **Methods:** Animal model was developed by administration of allergens before mating for the study of the impact of maternal allergy on the development of an allergic immune response in early life. Healthy pregnant women who have approved to have their placenta collected for medical investigation are invited to give birth at the university hospital. Twenty-five pregnant women who were demonstrated to be atopics, based on clinical symptoms of atopic disease together with a positive Phadiatop and/or skin prick test gave their birth at the same hospital. Placentas were collected both from mouse and human groups and kept at  $-70^{\circ}\text{C}$  until the preparation of slides was carried out. All slides were double-stained for the analysis with immunohistochemistry. To detect macrophages both in murine and human placentas, monoclonal antibodies, F4/80 and anti-CD14 Abs, were employed in immunohistochemical procedures. **Results:** CD14+ placental macrophages show an intensive positive reaction to anti-IgE monoclonal antibody. Surprisingly, there was no remarkable difference between healthy mouse and the atopic model mouse as well as the human counterparts on the distribution pattern of IgE in placentas. **Conclusion:** IgE is distributed on macrophages both in murine and human term placentas. The atopic mothers could not pathologically impose their babies by their high level serum IgE during the pregnancy.

#### F2.05. Complement Activation as a Mediator of Recurrent Immunologically-Triggered Miscarriages.

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Between 1% and 3% of women in the United States suffer recurrent miscarriages; 50% to 70% of conceptions fail. We have recently identified a novel role for complement activation in antiphospholipid antibody-induced pregnancy loss (J Clin Invest 112:1644, 2003). We now test the hypothesis that complement activation is a necessary intermediate in the pathogenesis of recurrent spontaneous miscarriage. DBA/2-mated female CBA/J mice are a well-studied model of immunologically-mediated peri-implantation pregnancy loss that shares many features with human recurrent miscarriage. Embryos from mating CBA/J females with DBA/2 males show an increased rate of resorption (30–40%), compared to rates of <10% observed in the control groups (CBA/J  $\times$  BALB/c) ( $P < 0.01$ ). Histological studies showed extensive C3 deposition, inflammatory infiltrates, and fetal debris in deciduas as early as day 6 of gestation. In contrast, there was no evidence of complement in decidua from CBA/J  $\times$  BALB/c. To provide direct evidence that complement is, in fact, a critical mediator in this model of spontaneous pregnancy loss, we attempted to rescue fetuses from DBA/2-mated CBA/J mice with complement inhibitors. To block C3 activation by alternative and classical pathways, we treated mice with a recombinant C3 convertase inhibitor Crry-Ig (3 mg ip day 2, 4 and 6). Crry-Ig completely rescued pregnancies in DBA/2-mated CBA/J mice (Crry-Ig vs

untreated  $8.5 \pm 6.3\%$  resorptions vs  $28.0 \pm 7.2\%$ ,  $P < 0.01$ ). Inadequate expression of complement regulatory proteins is a possible cause of increased complement deposition and embryonic death, but Western blotting of embryos and deciduas did not show such deficiency.

To examine the importance of complement activation at the level of C5, we treated DBA/2-mated CBA/J pregnant mice with anti-C5 mAb. Administration of anti-C5 mAb (1 mg ip on day 2 and 4) prevented pregnancy loss ( $8.6 \pm 4.4\%$  resorptions,  $P < 0.01$ ). To distinguish the role of C5a and C5a receptor (C5aR) from that of MAC seeded by C5b, we treated pregnant mice with a highly specific peptide antagonist of C5a receptor (C5aR-AP) AcPhe[L-ornithine-Pro-D-cyclohexylalanine-Trp-Arg]. C5aR-AP (100  $\mu\text{g}$  ip on day 2) significantly improved pregnancy outcomes ( $8.0 \pm 5.9\%$  fetal resorptions,  $P < 0.01$ ) suggesting that C5a-C5aR interactions play a critical role in fetal loss. Given the importance of alternative pathway in ischemic and antibody-triggered injury, we considered the role of factor B in our model. Treatment of pregnant DBA/2-mated CBA/J mice with a blocking anti-fB mAb improved outcomes to those of controls ( $8.4 \pm 6.6\%$  fetal resorptions,  $P < 0.01$ ). Our results show that complement activation plays an essential and causative role in damaging the fetal-placental unit: Factor B, C3, C5 and C5aR are required for miscarriage. These studies identify key innate immune effectors that mediate poor pregnancy outcomes and provide novel and important targets for prevention of recurrent pregnancy loss in patients.

#### F2.06. Prolonged Preterm Rupture of Fetal Membranes (PPROM), Is Associated with an Increased Maternal Anti-Fetal T-Cell Responsiveness.

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A fetus, although semi-allogeneic, is usually accepted by the maternal immune system. However, complications including allo-responsive mechanisms are thought to be potentially detrimental for a successful pregnancy. Therefore, we used the mixed lymphocyte culture (MLC) reaction to compare the allogeneic T-cell response of non-pregnant women ( $n = 96$ ) with the response of healthy pregnant ( $n = 68$ ) and pregnant women affected by different gestation-associated diseases such as prolonged preterm rupture of fetal membranes (PPROM) ( $n = 20$ ), uncontrollable preterm labor (PL) ( $n = 15$ ), preeclampsia ( $n = 22$ ) and intrauterine growth retardation (IUGR) ( $n = 12$ ). Peripheral blood mononuclear cells (PBMCs) of all three groups were stimulated with PBMCs from unrelated volunteers. Pregnant women had significantly reduced stimulation indices (SIs) (median value 21.8, range 1.3–167.0) compared to non-pregnant women (median value 62.5, range 8.4–379.5). Exposing PBMCs from pregnant women to PBMCs of their own fetus led to a further significant decrease of SIs (median value 2.2, range 0.6–51.5). Among the two groups of pregnant individuals, SIs of women with prolonged preterm rupture of fetal membranes (PPROM) were significantly higher (median value 6.3, range 1.3–56.8) in comparison to women with uncontrollable PL

(median value 1.4, range 0.9–8.5), women with preeclampsia (median value 1.8, range 1.1–14.9), women with IUGR (median value 1.9, range 0.7–38.1) and women with normal term delivery (median value 2.2, range 0.6–51.5) when the maternal PBMCs were stimulated with PBMCs of their own fetus. This phenomenon could not be observed after stimulation with PBMCs from unrelated volunteers. In addition, an increased humoral immune response was assessed for women with PPRM (40.5 % women anti-HLA-antibody positive) in comparison to women with uncontrollable PL (15.2 % women anti-HLA-antibody positive). Our results revealed a strongly reduced allogeneic T cell response of PBMCs from pregnant women that was further downregulated when PBMCs from their own fetus were used as stimulators. The same finding could be observed in pregnant women who suffer from different gestation-associated diseases. An exception were women with PPRM who exhibited a comparatively high alloresponse implying an increased anti-fetal reaction under these conditions.

### **F2.07. STAT3 Knock Down Reduces the Invasion of Choriocarcinoma Cells.**

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**Objectives:** The Signal Transducer and Activator of Transcription 3 (STAT3) is involved in the invasiveness of carcinoma cells. In an earlier study, we found that invasiveness correlates with STAT3-DNA binding activity in trophoblast and choriocarcinoma cells. Aim of this investigation was to verify the role of STAT3 in these observations by RNA interference induced STAT3 knock down. **Methods:** By using short interference RNA (siRNA), STAT3 was knocked down in the Jeg-3 choriocarcinoma cell line. Oligonucleotides were designed to interfere exclusively with STAT3 mRNA. For control scrambled oligonucleotides were designed to not interfere with any known human protein. The successful knock down was analyzed by polyacrylamid gel electrophoresis and western blot. Matrigel was used for an in vitro invasion assay. Invasion of cells was measured by counting cells on the filter beyond the matrigel as well as in the medium beyond the filter. **Results:** By applying a concentration of 66nM of siRNA oligonucleotides the invasion of Jeg3 choriocarcinoma cells was reduced by 57%. Invasion of non-transfected cells was increased by stimulation with Leukemia Inhibitory Factor (LIF; 64%) and Interleukin-6 (IL-6; 12%). In STAT3-knock down cells these effects were completely blocked for IL-6 and significantly reduced for LIF (16% invasion). **Conclusion:** STAT3 plays a key role in the invasion of choriocarcinoma cells. The slight remaining invasive capacity of Stat3 knocked down cells may be due to a not complete elimination of Stat3 or to further invasion triggering pathways.

### **F2.08. Evaluation of Four Different Methods of Sperm Surface Antigens Extraction Using Biotinilated Sperm Due to Finding a Better Method for ELISA Technique.**

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**Introduction:** One of the most important problems in detection of antisperm antibody (ASA) is to design an international

correct method with at least false answers. It seems that Enzyme-Linked Immunosorbent Assay (ELISA) will be more sensitive, specific and with more diagnostic value for detection of ASA, if it is used sperm surface antigens without contamination with sperm inside antigens and nonspermic antigens as coated antigens in ELISA technique. In this way, some techniques such as using different specific detergents are recommended. This study has focused on different methods of sperm surface antigens extraction to determine a better method of antigens extraction for ELISA technique. **Materials and Methods:** We extracted sperm surface antigens by four different method: Sonication method, using NaCl salt, SDS detergent and using LIS detergent on sperm prior treated by biotin and then we evaluated all four extraction method by blotting and ELISA technique to assess the better exposing surface antigens. **Results and Conclusion:** Our results have demonstrated that extraction method by LIS detergent expose sperm surface antigens better than other three methods and we concluded that this method is superior for using in ELISA technique.

### **Other**

### **F2.09. The Changes of T Lymphocyte CD Markers in Patients Exposed to Mustard Gas.**

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**INTRODUCTION:** The immunodepressive effects of mustard gas was detected in previous studies. The present study was performed on 31 Iranian chemical casualties, after 10 years of exposure to mustard gas, to determine the changes of absolute lymphocyte count and T cell CD markers compared to normal control group.

**METHODS:** CBC and absolute lymphocyte count was determined by H 1 automatic system. T cell CD markers (CD2, CD3, CD5, CD7) was analyzed by Flow cytometric method using conjugated monoclonal antibodies.

**RESULTS:** Absolute lymphocyte count were increased but lymphocyte CD2, CD3 and CD5 markers were decreased significantly ( $P < 0.05$ ) compared to normal control group (The decrease in CD4 and CD8 markers were reported by other investigators previously).

**CONCLUSION:** The increase susceptibility to respiratory and other infections in these patients, could be due to decrease in T lymphocyte subsets. Increase in absolute lymphocyte count could be due to lymphoproliferative changes in B or NK lymphocyte lineage, and requires another investigation.

### **F2.10. Binding of Phage Displaying Multimeric CD147 on U937 Cell Conducts Apoptotic Signal.**

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Molecular effect of CD147, leukocyte surface molecule, on the neighbor cells is largely unclear, currently. Herein, multivalent

CD147 displaying on VCSM13 phage was generated to enhance the functional affinity of CD147 for its partner (s) and to study the ligand-receptor signaling in U937 monocytic cell line. Cellular morphological change of U937 incubated with multivalent CD147 phages had been observed since 24 h of cultivation and cell propagation was ceased after 48 h. This phenomenon was presumably related to the anchoring of multivalent CD147 phage to U937 surface molecule (s) proven by either ELISA or immunocytochemistry. Interestingly, the apoptotic nucleus was found to coordinate with U937-bound phage. Cytotoxic activity of CD147 phage on U937 was further analyzed by EthD-1/calcein AM double stains. In contrast to wild-type phage, dual-colour fluorescent in U937 induced with recombinant phage, which associated with the apoptotic characteristic, was indicated. The level of cleaved caspase-3 in U937 incubated with multivalent CD147 phage was not as high as in U937 stimulated with cisplatin when determined by flow cytometry. Nevertheless, the signal was apparently stronger than uninduced U937 and VCSM13-incubated U937. Quantified by immunocytochemistry, only 12.8% of multivalent CD147-activated U937 with apoptotic nucleus harbored substantial amount of cleaved caspase-3, whereas 41% was resulted from cisplatin-induced U937. Accordingly, the caspase dependent pathway supposed to partially involve in CD147-provoked cell death program. A novel function of CD147 in triggering apoptosis implied the existence of CD147 counter-receptor (s) on U937 membrane.

#### **F2.11. Sensitivity of Soil Bacteria towards Cadmium Metal and Its Effect on Mung Bean Plant.**

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##### **Introduction**

Pollution is now a days a tremendous threat to the universe, progress in different scientific fields helps at one side to the world but on technologist hand it causes great dangerous effects on human and plants. Heavy metal pollution (hanaeklaus., 1999) in aquatic system has become a serious threat today. Heavy metals are wide spread pollutants of great environmental concern as they are non degradable and thus persistent (Trueby.P., 1990) Many bacteria are facultative anaerobes. Nitrogenfixing facultative bacteria are generally only capable of fixing nitrogen when they are growing in anaerobic environments. Examples of such bacteria include some of the Enterobacteriaceae, such as *Enterobacter* species (*Camb. Univ. press*). Chemical treatment of water resources are very expensive, in this endeavour, microbial biomass has emerged as an option for developing economic and ecofriendly waste water treatment process but presence of heavy metal can damage microbial life. The present study was carried out to check the toxicity of Cd (cadmium) metal on bean plant and their physiological process in relation with nitrifying bacteria in soil and roots and shoots of the plant.

##### **Methodology**

Plants were analysed after 15 days of germination, the contents of chlorophyll a, b carbohydrates, proteins and amino acids were determined by standard method (Chapman.S.B., 1976). Mineral ions were analysed by flame photometry and atomic absorption technique. The toxicity of different concentrations of Cd metal on nitrifying bacteria was determined by SPC (standard plate count) method.

##### **Results and discussion**

Cd was found to be highly toxic to the seedling as well as for the microbial life present in the rhizosphere of bean plant at all concentrations used. Morphology of plant was effected while colour of plant turns yellow. The growth of the plant was inhibited and the length of root and shoot was found to be decreased, compared with control plants. Reduction in the processes of the photosynthesis was observed as yellow coloured leaves appeared instead of green coloured because of the decline in the chlorophyll a and b contents.

Nutritive values of plants decreases with the increase in the concentration of Cd metal which may be due to the absence of nitrifying bacteria which are normally found in symbiotic association with these plants where they fix atmospheric nitrogen in the roots of leguminous plants (Dakora and Donald, 2002) but in the presence of Cd metal nodule formation was inhibited results in the decrease in the proteins contents and absence of amino acids at high concentrations of Cd. Potassium and sodium (Hsiao.T.C., 1986) were investigated and low percentages showed that accumulation of Cd was higher in the roots as compare to the other mineral ions due to which plants were not able to stand in the erect position. Iron Magnesium Magnese which were responsible for metabolic activity, transpiration and translocation were also effected and their percentages were also found to be decreased at higher concentration of Cd.

#### **F2.12. Surgical Trauma and the Mannan-Binding Lectin (MBL) Pathway of Innate Immunity.**

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**Introduction:** The mannan-binding lectin (MBL) pathway of innate immunity is important in host defense against pathogens. Major surgical trauma is known to influence various immune functions and in the present study we investigate the effect of major surgery on two central components of the MBL pathway; MBL and the associated protease MASP-2, compared with the effect on IL-6 and CRP levels.

**Methods:** Sixty patients were randomized to open or laparoscopic colectomy for benign or malignant disease. Serum levels of MBL, MASP-2, IL-6 and CRP were determined preoperatively, and 1, 2 and 6 hours following incision, and at postoperative day 1, 2, 8, and 30.

**Results:** All four parameters showed a slight decrease in serum levels within the first two hours after incision. For MBL and MASP-2 a minor, but significant ( $P = 0.01$  and  $P = 0.04$  respectively) peak was found on postoperative day 8. Compared to the preoperative level a significant, 10-fold increase of IL-6 was found 6 hours after incision ( $P = 0.0001$ ), and with levels significantly lower on day 30 ( $P = 0.0005$ ). For CRP a significant increase was seen on postoperative day 2 ( $P = 0.0001$ ), whereas the levels on day 30 were not statistically different from preoperative levels ( $P = 0.08$ ). The levels of IL-6 and CRP were significantly correlated ( $r = 0.71$ ,  $P < 0.0001$ ), whereas no other significant correlations were detected between the parameters. No significant differences between the responses to the two surgical techniques were revealed.

**Conclusion:** In contrast to the marked effects on the levels of IL-6 and CRP major surgery only marginally influenced the MBL pathway. There was no difference in the response to the two different surgical techniques.

### F2.13. The Theory of Hemaimmune Reaction Road Map.

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**Objective:** To study the theory of hemaimmune reaction.

**Methods:** Cancer cells or yeast cells (or NS) were added into human fresh anticoagulant whole blood (or blood cells and plasma) treated by citric acid, and incubated for 30 minutes at 37°. Main Outcome Indexes: adhering rate. IL-8, CD35, DARC (Fy6), CXCR4 et al.

**Results:** It was found that cancer cells (dead cells) and yeast cells can activate a war of hemaimmune reaction (HIR). In time of war against cancer cells and yeast cells, the level of indexes (adhering rate, IL-8, CXCR4) was significantly higher than that in time of peace (NS control group). In time of war against cancer cells and yeast cells, level (IL-8) of HIR in white blood cell group with plasma added was significantly higher than that (IL-8) in white blood cells group without plasma. Level (IL-8, CXCR4) of HIR in white cells group with red blood cells added was significantly higher than that (IL-8, CXCR4) in white blood cells group without red blood cells added.

**Conclusion:** human hemaimmune reaction looks like a modern war in the body. The results suggest that the complement and red blood cell play a vital role in blood immune reaction, and there is a road map of hemaimmune reaction: Antigens (cancer cells or yeast cells) can activate complement in plasma, then the antigens which were opsonized by complement C3b are mainly adhering to red blood cells, and then they are adhering to white blood cells to activate hemaimmune reaction system (see Figure 1). Furthermore, it can provide useful information for studying innate and adaptive immune and for establishing experimental (or war type) system of hemaimmune reaction road map.

### F2.14. A Novel CD70+ APC Imprints a Unique Pattern of NK Receptors on Gut Mucosal CD8 T Cells.

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We have identified a novel antigen presenting cell population in the intestinal lamina propria that constitutively expresses the costimulatory molecule CD70. Here we show that stimulation via this APC induces a unique pattern of NK receptors on the mucosal CD8 T cells. Resident CD8 T cells in the gut mucosa in naïve mice exhibited an activated phenotype and expressed the Ig family NK receptors 2B4 and gp49B1 but did not express the lectin-like CD94-NKG2 heterodimeric receptor. In contrast, activated CD8 T cells in the peritoneal exudate lymphocytes and spleen following Vaccinia or Listeria infection expressed gp49B1 and CD94-NKG2 and but did not express 2B4. Similar differences in NKR expression were also found on antigen-specific CD8 T cells generated at different sites during the same infection. The NKR expression profile in the gut mucosa was dependent on stimulation via CD70+ APC since 2B4 expression could be dramatically reduced by administration of blocking CD70 antibody. CD70 antibody treatment however, had no effect on NKR expression by activated CD8 T cells at peripheral sites, where CD70+ APC do not occur. However, when mice were

intraperitoneally immunized with CD70 expressing allogenic P815 cells, 2B4 was induced on CD8 T cells accumulating in the peritoneal cavity. Thus, CD70 costimulation via CD70+ APC imprints unique NK receptors on mucosal T cells.

### F2.15. CAUSAL Protein Signaling Networks Derived from Multiparameter Single-Cell Data.

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We demonstrate the application of Bayesian networks for computational elucidation of causal intermolecular influences in signaling networks, using simultaneous multivariate measurements of phosphorylated proteins and phospholipids in populations of single human primary CD4+ T cells. Selective perturbations, both activating and inhibiting, were important to inferring the direction of influence between signaling components. We identified most classically reported signaling relationships and predicted novel influence connections, including inter-pathway crosstalk from the kinase Erk1 to the kinase Akt (confirmed experimentally). These results manifest the feasibility of data-driven construction of causal signaling network models from primary cell data at the single cell level and may have utility in understanding patient specific signaling alterations in disease states.

### F2.16. Don't Judge a Cell by Its Surface Only: Combination with Functional Assays.

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Immunophenotyping of peripheral blood mononuclear cells (PBMCs) is of limited value for assessment of most clinical states. As a more informative alternative, immunophenotyping may be combined with functional assays as a correlate of clinical status. Most functional assays, however, are tedious or require prolonged culture periods. One simple, highly informative, and rapid approach is to examine cell signaling within individual cells following brief stimulation. Abnormalities within the signaling pathways of specific cell types could provide important insights concerning pathological conditions. This study focuses on the JAK/STAT pathway as it is central to host defense, cell growth, and apoptosis. Dysfunction of this pathway has been observed within cancer cells of various types. We have developed conditions allowing dual labeling of cell surface markers and intracellular phosphorylated STAT members within individual, normal PBMCs, which have undergone rapid cytokine stimulation *in vitro*.

The PBMCs are first incubated with a primary antibody against cell surface CD4, CD8, CD14, or CD56. Cytokine [IFN $\gamma$ , IL-2, IL-4, or IL-13] treatment is then applied to induce STAT phosphorylation. The cells are immediately fixed with 2% PFA and then permeabilized with a cocktail of saponin, methanol, ethanol, isopropanol, or acetone at varying concentrations. The cells are labeled with primary antibody against several pSTAT proteins. Analysis of the efficacy of extracellular and intracellular labeling is completed using a BD FACSAArray flow cytometer.

With all of the reagents tested, it appears that there is a trade-off between intracellular and extracellular labeling of cells. 90%

methanol, which was previously used in our lab and in the labs of others, gives a good signal for phospho-STATs, but does not allow identification of many cell surface molecules. Other permeabilization methods, such as saponin, and lower alcohol concentrations, allow for better cell surface labeling, but simultaneously cause a decrease in the pSTAT signal. Of the reagents tested, 70% methanol allows for the best dual labeling of both intracellular and extracellular proteins. These methods will permit rapid analysis of complex cell populations, including PBMCs. Interrogation of signaling pathways in individual cell types will allow more profound evaluation to gain additional insight into abnormalities causing or arising from the presence of immune dysfunction.

## F2.17. Cigarette Smoke Extract Impairs CD40

**Ligand-Induced Maturation of Human Dendritic Cells.**  
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Dendritic cells (DC) are key regulators of innate and acquired immunity. DC maturation is a critical event in the DC life cycle, conferring to it the capacity to both simulate T cell proliferation and polarization. DCs may be matured by Toll receptor ligands (like LPS), as well as by T cell dependent mechanisms via CD40 ligand-mediated (CD40L) activation. We have previously demonstrated that cigarette smoke extract (CSE) inhibits several maturation events induced by lipopolysaccharide (LPS), including the upregulation of co-stimulatory molecules, and production of the key Th-1 polarizing cytokine IL-12p70. In the current study, we hypothesized that CSE also impairs DC maturation mediated by T cell dependent pathways through CD40L stimulation. CSE was generated by bubbling the smoke from one cigarette (1R3F University of Kentucky) through 10 ml PBS. Immature DCs were generated from human monocytes cultured with IL-4 (5ng/ml) and GM-CSF (800U/ml). During the final 48 hours of culture, DCs were incubated with CSE at concentrations that do not diminish cellular viability (as measured by Annexin V and Propidium iodide staining). During the final 24 hours, DCs were matured with interferon-gamma (50ng/ml) and CD40L (500ng/ml). The expression of costimulatory molecules was determined by flow cytometry and IL-12p70 concentration was determined by ELISA. PGE<sub>2</sub> levels were also analyzed by ELISA. CSE-conditioned DCs matured with CD40L, expressed lower levels of CD-86 and CMRF-56 compared to control DCs. In addition, IL-12p70 production by CD40L matured DCs was inhibited in a dose-dependent fashion by CSE conditioning. CSE-conditioned DCs produced more PGE<sub>2</sub> than controls, suggesting a mechanism by which CSE alters DC maturation. Our data illustrate that CSE alters DC maturation induced by both LPS and CD40L. The inhibition of IL-12 production by CSE illustrates an important mechanism by which smoking inhibits essential adaptive immune responses relevant to the pathogenesis of cancer and certain infections.

## F2.18. Isolation of Lactobacilli, Bacillus and Pseudomonas from Yogurt and To Determine Their Bacteriocin Activity Against Various Clinical Isolates.

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Bacteriocin are low molecular weight single polypeptide or polypeptide complexes having an antibacterial activity, synthesized

in ribosomes and secreted by bacterial cells or "the group of heterogenous substances ranging from low molecular weight compounds to high molecular particles resembling bacteriophage protein components (Rasool & Khan, 1991)."

In 1967 (Bradly) classified bacteriocins into two broad types:

- A group of low molecular weight, trypsin sensitive, thermostable bacteriocin.
- A group of high molecular weight, trypsin resistant, thermostable bacteriocin.

Microorganisms in yogurt and the subsequent creation of organic acids and bio-active proteins inhibit the growth of many pathogenic microorganisms.

### Methodology

Different yogurt samples were used for isolation of bacterial strains. After making the dilution of yogurt samples, the diluted samples were spreaded on nutrient agar and incubated at 37 °C. Isolated colonies were identified by using different conventional methods. Ten reference strains were used to check bacteriocinogenic activity. The isolates includes *E. coli*, *Staph.aureus*, *Klebsiella*, *Salmonella*, *Enterococcus*, *Micrococcus*, *Bacillus*, *Staph. epidermidis*, *Pseudomonas*. After this bacteriocin production was detected against reference strains by using agar well and cross streak methods. The titres of bacteriocin produced were quantified by two fold serial dilutions of bacteriocin.

### Results and discussion

The average colony forming unit per ml calculated as  $8.4 \times 10^6$  Cfu/ml. The bacteria isolated were identified by using different conventional methods. Bacteriocin of Lactobacilli was active against Shigella, Bacillus and Micrococcus.

The bacteriocin of bacillus was active against *S. aureus*, *St. epidermidis*, *Enterococcus* and *E. coli*.

Bacteriocin of Pseudomonas was active against Shigella, *S. aureus*, *St. epidermidis*, *Enterococcus*, *Micrococcus*, *Bacillus* and *S. typhi*.

The arbitrary unit of bacteriocin of Lactobacilli against Shigella is 1:4, against *S. aureus* is 1:2, against *St. epidermidis* is 1:8, against *Enterococcus* is 1:8, against *Bacillus* is 1:2.

The arbitrary unit of bacteriocin of Pseudomonas against Shigella is 1:6, against *S. aureus* is 1:2, against *St. epidermidis* is 1:4.

The arbitrary unit of bacteriocin of Bacillus was not found.

It was found that yogurt contain high number of bacteria which include both Gram negative and positive species of bacteria. The bacteriocin of lactobacilli are broad spectrum as active against both Gram negative and positive bacteria and best inhibitory activity was observed against *St. epidermidis*.

*Pseudomonas* produce powerful bacteriocin which inhibit the growth of majority of clinical isolates where bacteriocin of *Bacillus* and *Lactobacilli* were not as effective.

We also observed those bacteria which produce bacteriocin line of diffusion after removal of growth and also bacteriocin activity was best observed when the plates were first refrigerated and then cross streaked which indicate that refrigeration temperature facilitated the diffusion of bacteriocin in medium.

## F2.19. Dendritic Cells Overexpressing FasL Induce Anti-Tumoral T-Cell Response In Vivo.

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We previously showed that FasL retrovirally transduced bone marrow-derived DC increase allospecific cytotoxic activities and Th1 cytokines production *in vivo* thanks to a FasL dependent neutrophils recruitment. The aim of our study is to test if the expression of FasL by DC can promote a stronger cytotoxic activity and a better anti-tumoral immunity compared with DC expressing a control Ag. We used the tumor cell EL4 isolated from C57BL/6 mice and transfected with the cDNA encoding ovalbumin (EG7-OVA), the predominant Ag expressed by the tumor. We first evaluated a therapeutic approach which consisted in subcutaneous injection of EG7-OVA tumor cells and FasL or control-DC in C57BL/6 mice. We studied the consequences of the treatment on the tumor growth. We realised histological analysis on tumor sites and we measured the anti-tumoral T cell response. We observed that co-injection of FasL-DC and EG7-OVA cells inhibit tumor growth. Histology of tumors sites harvested from mice co-injected with control-DC and EG7-OVA cells showed only a moderate inflammatory infiltrate composed essentially of mononuclear cells. In contrast, tumor sites coming from mice co-injected with FasL-DC and EG7-OVA cells reveal a massive destruction of the tumor associated with apoptosis and neutrophils recruitment. We demonstrated that *in vivo* EG7-OVA apoptosis could not be explained only by the tumoricid activity of FasL-DC since we observed that only 6 to 7% of tumor cells were lysed by FasL-DC *in vitro*. We demonstrated that neutrophils contribute to the tumor eradication in FasL-DC and EG7-OVA co-injected mice as anti-Gr1 monoclonal antibodies treatment restore tumor growth. Finally, mice co-inoculated with FasL-DC and EG7-OVA tumor cells displayed a higher Th1 cytokines production and were protected against tumor challenge. Indeed, we observed that the antitumoral protective response was mediated by OVA-specific CD8<sup>+</sup> T cells. We now evaluate the role of neutrophils recruited by FasL-DC in the induction of the OVA-specific T cell response.

## F2.20. CD8aa + T-Cells Represent a Long-Lived Memory T-Cell Subset in Patients with Melanoma Undergoing Epitope-Based Tumor Vaccination.

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Tumor vaccines are targeting CD8<sup>+</sup> T-cells capable of differentiating into activated effector cells which mediate tumor-destruction, and memory cells crucial for long-term tumor-immune surveillance. The CD8ab heterodimer represents the predominant CD8 form in the peripheral blood circulation, although some CD8<sup>+</sup> T-cells express the homodimeric CD8aa. Recent data suggest that CD8aa cells may represent a biologically important subset of memory T-cells. We tested longitudinally sampled peripheral blood mononuclear cells (PBMCs) from patients with melanoma, vaccinated with the differentiation antigen Melan/MART-1, for i) the presence of CD8aa expressing T-cells, ii) for T-cell differentiation and homing markers (CD45RA/CCR7) and iii) antigen-specific

T-cells using AAGIGILTV (naturally processed peptide) and ELAGIGILTV (superagonist) peptides loaded into HLA-A2 tetramers. MART-1/Melan-A reactive T-cells were present in CD8ab and CD8aa expressing T-cells. Although CD8ab and CD8aa cells show a different composition based on CD45RA and CCR7 expression, the examination of tetramer Melan/MART-1 specific T-cells in the two CD8 subsets shows a terminally differentiated phenotype (CD45RA+/CCR7-) which is maintained over time. These data suggest that CD8aa cells represents a memory T-cell pool, contributing to a long-lived immune protection. We could consolidate this hypothesis using transfected recipient surrogate (TCR-/CD8-) cells which express either the MART-1/Melan-A specific TCR and CD8aa or CD8ab as a transgene: TCR interaction with HLA-A2/peptide complexes shows different requirements in TCR+/CD8ab cells as compared to TCR+/CD8aa cells. *In vitro* culture of PBLs from vaccinated patients with melanoma, using different cytokines and peptide stimulation, showed that IL7 or IL2 lead to a differential expansion of the CD8aa and CD8ab Melan/MART-1 specific T-cell population, respectively. This represents the first *ex vivo* report of anti-tumor directed CD8aa T-cells in patients with melanoma undergoing peptide vaccination. The assessment of this memory T-cell population will be relevant for immunomonitoring of patients with tumors and aid to design improved tumor vaccines capable of stimulating long-lived cellular immune responses.

## F2.21. Human Adipocyte and Its Participation in Innate Immunity.

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**Introduction:** In addition to the well know role of adipocyte in energy homeostasis, some recent studies suggest that this cell is also involved in immunity. To further investigate the immune capacities of adipocyte, we studied the expression of Toll Like Receptors (TLR), which are important players in innate immunity. In addition, the functionality of TLR2 and TLR4 was tested, by using one of the TLR ligand in adipocytes culture.

**Methods:** Human subcutaneous primary preadipocytes and mature adipocyte were used as cellular model. The presence of different TLRs was determined at RNA level by reverse transcriptase polymerase chain reaction (RT-PCR) and at protein level by Western Blotting and immunocytochemistry. Furthermore, cells were stimulated with lipopolysaccharide (LPS), and cytokines gene expression were quantified at different times by real time PCR, in order to assess adipocytes response to one of the TLR ligand.

**Results:** We demonstrated that human subcutaneous adipose cells and tissue expressed TLR 2 and 4 mRNA. These results were confirmed by the presence of the corresponding proteins. The same results were observed in mature adipocytes and in the tissue. After stimulation with LPS, an increase in mRNA level for Cyclooxygenase-2 (Cox-2) and interleukine 6 (IL-6) was observed in culture.

**Conclusion:** Our results showed for the first time the presence in adipocytes of TLR 2 and TLR4, two important receptors involved in innate immunity. These receptors are present not only on preadipocytes but also in mature adipocyte and on the adipose tissue. Furthermore, we demonstrate that these receptors on



adipocytes are able to signal an immune response such as cytokine production. These results suggest that adipose cells could respond to bacterial infection via the interaction between bacterial component and TLR and participate in innate immunity. Finally, to better understand the mechanisms of adipocyte response to infection, analysis of TLR signalling pathways from subcutaneous adipocyte are in progress.

### F2.22. HDL-Reverse Cholesterol Transport and Signal Transduction in T-Cells.

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HDL-mediated Reverse Cholesterol Transport (RCT) serves to regulate plasma cholesterol levels as well as cholesterol exchange with circulating cells. We have previously shown that membrane cholesterol is increased by 2-folds in T-cells from elderly donors. This was accompanied by defects in cellular activation leading to immune senescence. RCT is well-known in the case of atherosclerosis but its role in T-cells cholesterol metabolism as well as T-cells signalling is still unknown. RCT is a lipid rafts-dependent mechanism that involves a fast and a slow pool of membrane cholesterol. In this study we sought to determine the role of HDL in cholesterol metabolism of T-cells. We separated T-cells from young (<25 years) and elderly (>65 years) healthy SENIEUR subjects. We studied cholesterol uptake by the tritiated cholesterol and for extrusion by HDL-driven reverse cholesterol transport. The cholesterol uptake was decreased in T-cells of elderly. The fast cholesterol pool is extracted from lipid rafts microdomains, but separated lipid rafts from elderly donors still contain a high amount of cholesterol. The incubation of T-cells with HDL induced a signalisation that involved Jak-2 after 90 min that corroborates with the fast pool cholesterol (lipid rafts) while a long lasting activation of ERK is observed after 24h that corroborates with the slow pool cholesterol (non-rafts). In the case of elderly donors, the activation of these pathways is differentially altered. These data are the first to show that HDL-mediated RCT is impaired in aging explaining the changes in lipid rafts properties. Moreover, it has been suggested recently that lipid rafts play an important role in the cholesterol exchange mediated by HDL. Thus, lipid rafts may auto-regulate their cholesterol content and changes in lipid rafts properties with aging may explain defects in T-cell activation.

### F2.23. The Effect of Treatment Recurrent Herpes Simplex with Larifan.

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**Introduction.** Viral infection of herpes has captured a growing attention during the last decade that shall be explained with the ever-increase of herpes viruses throughout the world, frequent relapses and unsatisfactory results of the usually prescribed therapy of Herpes simplex viruses.

Various researches deal with the immunology of herpes virus attempting to more accurately define the impact of immune regulation factors.

The therapy of Herpes simplex infection more excessively suggest immune modeling remedies that directly or indirectly affect the response of immune competent cells.

**Aims of the research.** To evaluate the efficiency and safety of Larifan rectal suppositories for the treatment of frequently relapsing infections of simple Herpes as well to detect the concentration of circulating interferon during therapy.

**Methods.** The research comprises the analysis of a variety of Larifan- suppositories, a general and local antiviral medicine synthesized in Latvia. Larifan is a medicine of natural origin of doubly spiraled ribonuclein acid that forms up in *Escherichia coli* cells that are infected with bacteriophages. Larifan is wide scope antiviral medicine. In vitro proves its capability to slow down the reproduction of encephalomyelitis in Venezuela horses and other viruses, reducing the output of viruses for 100 000 times. The animals involved in experiments proved to have both preventive and therapeutic affects in case of tick encephalitis, mice encephalomyocarditis, rabies, influenza and other viral infections.

Out of the total number of 36 patients involved in the research, all of them had frequently relapsing simple herpes (6 times and more per year), 19% ( $n = 7$ ) had genital herpes, 81% ( $n = 29$ ) had labial herpes. The involved patients ranged from 18- 70 years of age. All of them had 1 rectal suppository consisting of 4 mg active substance before sleep on the 1<sup>st</sup>, 2<sup>nd</sup>, 6<sup>th</sup>, 10<sup>th</sup> and 14<sup>th</sup> days. All of patients had 5 such courses, between each of them was 4 weeks without treatment. Circulating interferon was detected in sera of 23 patients before therapy and on the 7<sup>th</sup> day during the 1<sup>st</sup> course. Circulating interferon was detected by the biological standart micromethod.

**Results.** 30 of 36 patients ended the therapy completely. 6 discontinued therapy of various reasons. None of them discontinued therapy because of side effects of Larifan. 66% ( $n = 20$ ) patients had no relapses during therapy, 17% ( $n = 5$ ) patients had 1 relapse, 3% ( $n = 1$ ) had 2 relapses, 7% ( $n = 2$ ) had 3 relapses, 7% ( $n = 2$ ) had 5 relapses.

The increase of circulating interferon was detected in 87% ( $n = 16$ ) patients, only 13% ( $n = 7$ ) of patients it remained low.

**Conclusion.** The results prove that a suppository variety of Larifan combines both the local and systemic immune modeling properties. Larifan would be an efficient medicine to treat frequently recurring herpes infections during their aggravations. To evaluate the significance of Larifan suppositories in the treatment of frequently recurring herpes infections further long term researches are needed and are carried out.

### F2.24. Multidimensional Liquid Phase Separations of Intact Proteins as an Alternative to 2D Gel Electrophoresis for Proteomics.

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In the field of expression proteomics, the dominant separation technology has been Two Dimensional Gel Electrophoresis (2DGE) frequently coupled with Mass Spectrometric identification methods. Although an extremely powerful separation technique, 2DGE suffers from a number of drawbacks including lack of speed and automation, poor reproducibility and quantitation and fundamental limitations in linearity resulting in a bias towards highly expressed proteins. Alternative approaches based on Multidimensional HPLC separation of proteolytic digest of complex samples have emerged combining nanoscale reversed phase separations of strong cation exchange fractions. Although these approaches have demonstrated utility, the complexity of the resulting mixture represents a considerable chromatographic challenge. Furthermore,

pooling the proteolytic fragments from a large number of proteins eliminates any association of a given peptide with its parent proteins and thus configurations of multiple Post Translational Modifications.

In an effort to overcome the limitations of existing techniques, we have been evaluating the use of multidimensional liquid phase separation of intact proteins as an alternative. In this approach, 96 fractions from a first dimension are collected and re-fractionated by a second, orthogonal separation mechanism collecting 16 fractions resulting in a total of 1536 fraction per sample. As 1<sup>st</sup> dimension modes, we have coupled strong anion exchange chromatography with a 2<sup>nd</sup> dimension based on high speed separations using a 5mm 300Å Macroporous Reversed Phase material at high temperatures (60°C) for very high resolution and recovery. This material allows rapid diffusion of large molecular proteins resulting reversed phase cycle times of 5–10 minutes/fraction. The combinations of these chromatographic modes are largely orthogonal, utilize compatible mobile phases and yield resolution of several hundred proteins in a single analysis.

Following the 2D Fractionation of intact proteins, all of the collected fractions are reduced, alkylated and proteolytically digested for subsequent analysis by Nanoscale electrospray LC/MS based on a microfluidic ChipMS System for Ion Trap Mass Spectrometry. Although each fraction generally contains multiple proteins, it is feasible to identify the components using rapid Nanospray LC-MS/MS information. In this workflow, the LC-MS/MS is the most time consuming, so a key feature of this approach has been the development of tools to visualize and compare the 2D separations of intact proteins to identify and prioritize fractions for subsequent analysis by LC-MS/MS. Following collection of the MS/MS data, the pooled spectra are processed and compared to protein library data bases using SpectrumMill software to identify proteins present in the samples.

This method is demonstrated with the analysis of a protein extract of Murine Immunodepleted *Sera* from NOD and NOD.B10 Mice. Discussion is made of optimization and current limitations of the method as well as future potential.

### F2.25. Screening of Immunostimulatory Oligosaccharides by Using a New HPLC-Chip/MS Technology.

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Oligosaccharides are involved in a host of cell-cell processes and play a key role in the immune system. Changes in glycosylation or the formation of aberrant oligosaccharides accompany a host of diseases from infection to cancer (eg. ovarian cancer, diabetes, cystic fibrosis, arthritis, autoimmunity, respiratory capacity diseases). In this presentation, we introduce a new fully automated and integrated analytical platform for oligosaccharide profiling consisting of a chip-based chromatography system in conjunction with time-of-flight mass spectrometry. The microfluidic HPLC chips are made of laser ablated and laminated biocompatible polyimide films. Sample enrichment, separation and nanoelectrospray tips are fully integrated in the chip device. High resolution separations of oligosaccharides are achieved with porous graphitized carbon (PGC) column material packed in the chip device. Oligosaccharide isomers are readily separated and resolved. Specific oligosaccharide structures are identified by

accurate mass measurements and highly reproducible retention times. In this way, over 100 different oligosaccharides are simultaneously identified and monitored. Those known to have immunostimulatory effects were unambiguously identified. This new advanced chip-based analytical platform is also capable to analyze other aspects related to diseases like changes in the phosphorylation pattern or changes at the metabolomics level.

### F2.26. The Role of Mannose-Binding Lectin in Natural IgM Mediated Ischemia/Reperfusion Injury.

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Ischemia/reperfusion (I/R) injury consists of an acute inflammatory response involving complement which is activated upon the recognition of natural IgM to self-antigen exposed by ischemia. This study dissected the role of lectin versus classical complement pathways in an intestinal I/R model. When RAG-1<sup>-/-</sup> mice were reconstituted with I/R-specific clonal IgM, mannose-binding lectin (MBL) co-localized with IgM in the injured tissue. Further, MBL<sup>-/-</sup> mice were protected from I/R injury (40min ischemia, 3hour reperfusion) with the absence of IgM and C3 deposition. In contrast, C1q<sup>-/-</sup> mice were injured similarly as WT animals with deposition of MBL and IgM in the damaged tissue. Reperfusion in MBL<sup>-/-</sup> mice for 15min showed IgM deposition in intestinal villi, and subsequent proteomic analysis identified specific self-antigen bound to IgM. Preliminary results from in vitro study show that murine MBL binds to IgM. This study reveals that I/R pathogenesis is initiated by IgM response to ischemic antigen followed by direct activation of downstream lectin pathway of complement.

### F2.27. Effect of Plaferon LB on the Damaged Peripheral Nerve Regeneration.

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Axonal repair of mature neurons involves complex molecular changes. Critical role is played by Schwann cells as well as macrophages and inflammatory cells. One of the most important molecules, which determine degeneration/regeneration processes in damaged nerve, is nitric oxide (NO). NO influences on many aspects of nervous system physiology, being either detrimental or beneficial. As it is already revealed, the possible NO targets are neurofilaments and myelin sheaths of interrupted axon, newly formed neuromuscular endplates and Schwann cells in the distal nerve stump.

Manipulation of NO supply may offer interesting therapeutic options for peripheral nerve lesions recovery. The possibility of regeneration of transected sciatic nerve axon under the influence of Plaferon LB (PLB), inhibiting inducible nitric oxide synthase activity *in vitro* was investigated.

Experiment was conducted on twenty white rats. Animals were daily treated by PLB or saline. Treatment was begun three days

before transection. Ten animals were treated with PLB, others were undergone a course of saline treatment. Operation was carried out by microsurgery; sciatic nerve was transected and then sewed up.

Animals were sacrificed seven and thirty days after operation. The nerve was removed for nitric oxide (NO) measurement (after 7 days) and morphological examination (after month). NO was measured by electron spin resonance method using NO trap.

For morphological investigation, preparations were stained by hematoxylin-eosin methods, neurohistological method-Nils and immunocytochemical method by using monoclonal antibody S100.

Our findings suggest that PLB may play an important role in the regeneration of the injured peripheral nerve. In the injured nerve intense increase of NO production as well as the quantity of Schwann and mast cells was observed. According to our results PLB prevents the induction of NO production in axotomized sciatic nerve. Quantitative analysis showed that under the influence of PLB increase of Schwann and mast cells was statistically high reliable.

### F2.28. Activation of the Lectin Pathway of Complement in IgA Nephropathy.

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IgA nephropathy (IgAN) is characterized by glomerular co-deposition of IgA and complement components. The complement system can be activated via three activation pathways, the classical pathway, the alternative pathway, and the more recently identified lectin pathway. Complement activation leads to tissue deposition of activated complement components and release of pro-inflammatory factors. IgA can activate the complement system via the alternative pathway but not the classical pathway. Furthermore, recent data indicate involvement of the lectin pathway of complement in IgAN. The LP can be activated by binding of mannose-binding lectin (MBL), L-ficolin and H-ficolin to carbohydrate ligands, followed by activation of MBL-associated serine proteases (MASPs) and C4. We studied the potential role of the lectin pathway in IgAN and the interaction between MBL and human IgA as a possible explanation for lectin pathway activation in IgAN.

Renal biopsies of IgAN patients ( $n = 60$ ) were stained for IgA1, IgA2, MBL, L-ficolin and complement. Polymeric and monomeric serum IgA of IgAN patients ( $n = 14$ ) and healthy controls ( $n = 8$ ) was purified by affinity chromatography and gel filtration. Binding of MBL to IgA was studied by ELISA.

All IgAN cases showed mesangial deposition of IgA1 but not IgA2. Glomerular deposition of MBL was observed in 15 out of 41 cases with IgAN (25 %) and showed a mesangial pattern. All MBL-positive cases, but none of the MBL-negative cases, also showed glomerular deposition of L-ficolin, MASP-1/3, C4-binding protein and C4d. Glomerular deposition of MBL and L-ficolin was associated with significantly more mesangial proliferation, interstitial damage, and proteinuria.

In vitro studies demonstrated binding of MBL to IgA from healthy controls and from IgAN patients, with a strong inter-

individual variation. MBL binding was observed to polymeric but not to monomeric IgA. The interaction between MBL and IgA was dose-dependent and could be inhibited by EDTA and D-mannose but not L-mannose, indicating involvement of the lectin domain of MBL.

Together, these findings strongly point to a role of the lectin pathway of complement in glomerular activation of C4 in IgAN, and suggest a contribution for both MBL and L-ficolin in the progression of the disease. Furthermore, the findings support the hypothesis that glomerular MBL binding and complement activation in IgAN is based on an interaction of MBL with IgA.

### F2.29. Characterisation of Antibody Titres in an Intravenous Immunoglobulin Concentrate (Flebogamma®).

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To evaluate the IgG antibody levels in an intravenous immunoglobulin concentrate (Flebogamma®) against several pathogens.

From 3 to 217 lots of Flebogamma®, product at 5% IgG concentration containing 5% sorbitol as excipient, were studied. The titres against Cytomegalovirus (CMV), hepatitis A and B, measles, varicella (VZV), parvovirus B19, poliovirus type I, II and III, rubella, Epstein Barr (EBV), herpes simplex type 1 and 2, influenza A and B, parainfluenza type 1 and 2, adenovirus, mumps, coxsackie, echovirus, tetanus, diphtheria, *streptococcus pneumoniae*, *candida albicans*, *bordetella pertussis*, *helicobacter pylori*, *campylobacter jejuni*, *chlamydia*, *borrelia burgdorferi* and *toxoplasma gondii*, were studied. ELISA tests were used except for antibodies to poliovirus type I, II and III, diphtheria and measles, which were performed by neutralization tests. The results are shown as IU/ml or Elisa Units (U/ml) when no international reference is available. For determination against poliovirus, measles and diphtheria, the CBER lot 176 and US Diphtheria antitoxin standard F4505 reference preparations were used.

The most relevant results obtained are as follows: hepatitis A and B ( $35 \pm 4$  and  $1.1 \pm 0.5$  IU/ml respectively), VZV ( $12 \pm 1$  IU/ml), tetanus ( $22 \pm 4$  IU/ml), parvovirus B19 ( $141 \pm 21$  IU/ml), CMV ( $31 \pm 6$  IU/ml). For all these agents, the titres in the final product are 6- to 9-fold the value observed in the starting plasma. The results obtained for neutralizing antibodies against poliovirus type I, II and III and measles were  $0.34 \pm 0.11$ ,  $0.71 \pm 0.13$ ,  $0.59 \pm 0.10$  and  $0.30 \pm 0.08$  sample/reference ratio, respectively, and against diphtheria  $4.4 \pm 0.5$  U/ml. The presence of neutralizing antibodies is demonstrated and the results meet FDA's requirements for these preparations. With regard to the other antibodies studied, since no international reference is available, the quantitation is performed in relation to the cut-off or positivity limit of the ELISA method used. Thus, the activity against EBV ( $644 \pm 50$  U/ml), herpes ( $9290 \pm 735$  U/ml), mumps ( $1890 \pm 169$  U/ml), *helicobacter* ( $436 \pm 41$  U/ml), adenovirus ( $62 \pm 9$  U/ml), rubella ( $323 \pm 37$  U/ml), *St. pneumoniae* ( $449 \pm 57$  mg/L), *influenza*, etc., is 5-fold the cut-off limit. Finally, antibodies are also detected, even though to a lower extent, against *Candida* ( $50 \pm 5$  U/ml), *Bordetella* ( $28 \pm 2$  U/ml), *Campylobacter* (90 U/ml), *Coxiella* ( $42 \pm 21$  U/ml) and *Coxsackie* ( $220 \pm 11$  U/ml).

Flebogamma® contains IgG antibodies against a wide panel of pathogens, between 6- and 9-fold the normal plasma values for a high number of agents.

### F2.30. FLEBOGAMMA® Intravenous Immunoglobulin; Evaluation of Neutralizing Antibodies to Vaccinia Virus.

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Immunocompromised individuals may be at risk for Vaccinia infection if widespread smallpox-immunization programs are needed. The aim of this study was to determine the level of neutralizing antibodies to Vaccinia virus in Human Intravenous Immunoglobulin (IVIG) manufactured by Instituto Grifols. Thirteen batches of Flebogamma® were evaluated for the presence of anti-Vaccinia antibodies by a Vaccinia Plaque reduction neutralization assay. The Vaccinia virus working stock (Western Reserve-WR strain) was mixed with IVIG preparations, and after incubation at 37°C for 1h were inoculated onto monkey cells (BSC-40) for detection. An international standard from NIBSC (63/024) (potency of 1000 IU/ml) was used as the Antibody-positive Control preparation. Sera from non-smallpox vaccinated donors was the negative control preparation used. There is a linear correlation between the rate of virus neutralization and the antibody concentration. The neutralizing titer for a specific sample was expressed as the sample dilution that reduced the number of virus plaques to 50% (determined in triplicate) as compared with the number seen in the virus positive control (without IVIG added).

The results of the experiments provided a neutralizing potency of 35 (range 18 to 68) anti-Vaccinia IU/ml IVIG. Anti-Vaccinia antibodies in the product were also determined by ELISA and WB techniques revealing that the product was able to react against several Vaccinia virus proteins. There were no relevant differences among batches in the anti-Vaccinia titers.

The results of this study are consistent with the work of Goldsmith et al. (Vox Sang. 2004, 86:125-9). The titers of anti-Vaccinia antibodies found in this preparation have biological relevance since they provided protection against Vaccinia infection to immunocompromised mice.

### F2.31. Compatibility Study of Two Intravenous Immunoglobulin Preparations with Plastic Containers.

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The customization of intravenous immunoglobulins (IVIG) therapeutical doses to each patient's needs, by unifying the content of several bottles in a single container, is more and more frequent.

This study deals with the compatibility of two IVIG preparations sharing formulation (5% sorbitol) with two types of plastic container.

Nine lots of two preparations manufactured by Grifols, Flebogamma® (licensed) and 5% IGIV3I (under clinical trial), both containing 5% sorbitol as excipient, were filled in Griflex-polypropylene (PP) or Gribag-PVC sterile bags, at a rate of 40 ml solution/100 ml bag, what implies worst case conditions as refers to product-plastic contact ratio. The Grifill® system, which ensures sterile filling, was used. The product, filled in the plastic containers, was stored at 5 °C, 30 °C and 40 °C for a period between 10 and 15 days. For each temperature, tests before and after transfer from the original container (glass bottle) to the plastic container (PVC or PP), as well as at different storage time points, were scheduled. The following parameters were evaluated: appearance, pH, turbidity, osmolality, total protein (Bio-Rad), molecular distribution (HPLC),

anticomplementary activity (ACA), prekallikrein activator (PKA), antibodies titration against tetanus and hepatitis B (ELISA), antibodies against poliovirus type I (neutralization), DEHP (gas chromatography, mass spectrometry) and other polypropylene plastic additives: BHT, Irganox 1010 and 1073 and Ethanox 330 (HPLC), sorbitol (HPLC), pyrogens (injection into rabbits), toxicity (intraperitoneal injection into guinea pigs and mice), sterility (Steritest system, from Millipore) and bags weight control.

The results of the tests performed do not show significant variations in the products' characteristics after transferring them to the plastic containers. The results at temperatures of 5 °C and 30 °C do not show any sign of incompatibility with the plastic material, the antibody titres studied remaining perfectly stable. The markers used to assess the possible migration of plastic components to the product (DEHP for PVC) and PP additives are undetectable or very low (the DEHP content shows values below 5 µg/ml in all instances and the PP markers are undetectable). The mean weight loss is between 0.1% and 0.2% after 10–15 days of storage at 5 °C and between 0.6% and 1.3% at 30 °C. In the accelerated temperature studies (40 °C) a higher weight loss is noticed (between 1.2% and 2.9%), but no significant variations are detected in the other parameters studied.

5% sorbitol-formulated IVIG shows compatibility with PVC and PP during short time periods, between 2 °C and 30 °C.

### F2.32. Peripheral Blood Lymphocyte Immunological Profile of Patients with Autoimmune Hepatitis in Different Stages of Immunosuppressive Therapy.

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**Introduction:** Autoimmune hepatitis (AIH) is a rare disease characterized by progressive necro-inflammatory hepatocyte injury caused by breakdown of immune tolerance to self-antigens however pathogenic mechanisms underlying the development of AIH are still unclear. The aim of our study was a complex analysis of autoimmune phenomena involving activation and apoptosis as well as transfer of co-stimulatory signals of particular lymphocytic subpopulations in peripheral blood of patients with AIH in different stages of immunosuppressive therapy.

**Material and methods:** Examinations were carried out in 26 AIH patients divided into two groups. The first group consisted of 13 patients with *de-novo* diagnosed AIH in which immunophenotyping of peripheral blood lymphocytes was done twice i.e., before and after five months of immunosuppressive therapy. The second group consisted of 13 patients showing clinical and laboratory features of AIH remission (single immunophenotyping).

The functional state of lymphocytes was examined using three-color flow cytometry technique with 17 monoclonal antibodies repertoire. The reference ranges for activation and apoptosis markers were obtained from immunophenotyping of 30 healthy volunteers.

**Results:** The percentage of T cells, Tc with surface marker CD95 was significantly lower in AIH *de-novo* diagnosed patients than in healthy subjects. The percentage of activated Th, as well as B cells were significantly higher in AIH *de-novo* diagnosed than in healthy subjects.

After five months of immunosuppressive therapy following changes with comparison to initial data were found: a) increase of

percentages of T, Th and Tc cells, b) increase of percentage of Th cells with surface CD95, c) decrease of percentage of NK cells, d) decrease of percentage of activated B and B1a cells.

After 2 to 4 years of immunosuppressive therapy no significant differences in percentages of T, Th, Tc and NK cells were found between AIH patients and healthy subjects.

#### Conclusions:

1. The activated T lymphocytes appear during clinical exacerbation of AIH.
2. The percentage of peripheral blood NK cells decreases after 5 months of immunosuppressive treatment and normalizes after 2–4 years of treatment.
3. The percentage of activated B cells was increased in active phase of disease and significantly decreased following 5 months and 2–4 years of immunosuppressive therapy.
4. Exacerbation of AIH is associated with a decrease number of activated Tc lymphocytes which probably results from accumulation of these cells in the liver.
5. Activation of lymphocytes T via CD69 antigen seems to be a principal immune event in pathogenesis of AIH exacerbation.
6. Immunosuppressive therapy lasting 2–4 years leads to normalization of amount of basic T lymphocyte populations in peripheral blood. No such effect was observed after 5 months of immunosuppressive therapy.

### F2.33. Identifying Common “Innate Signature” from Gene Expression Profile in Innate vs. Adaptive Lymphocytes.

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Innate and adaptive immune responses are two radically different strategies the host immune system takes against pathogen assaults. This dichotomous view of the immune response has now been widely accepted. However, it is not clear what the definition, or hallmark, of innate immunity is, and what really distinguishes it from adaptive immunity. In order to address this question, we took a broad approach and compared gene-expression profiles in innate vs. adaptive immune cell populations. The players in innate immune responses include neutrophils, macrophages, natural killer (NK) cells, and dendritic cells (DCs), whereas those in adaptive responses include the classical T and B cells. However, comparing two extreme cell-types (e.g. neutrophils vs. T cells) could end up highlighting superficial differences such as TCR and downstream genes. Therefore, we have explored differences in gene expression by comparing close, but distinct, paired innate vs. adaptive populations; CD3<sup>+</sup>CD4<sup>+</sup>NK1.1<sup>+</sup> T cells (NKT) vs. CD3<sup>+</sup>CD4<sup>+</sup>NK1.1<sup>-</sup> T cells (CD4T); IgM<sup>hi</sup>IgD<sup>int</sup>CD11b<sup>+</sup>B cells (B1) vs. IgM<sup>int</sup>IgD<sup>hi</sup>CD11b<sup>-</sup> B cells (B2); TCRb<sup>+</sup>CD8a<sup>+</sup>CD8b<sup>-</sup> cells (CD8aa) vs. TCRb<sup>+</sup>CD8a<sup>+</sup>CD8b<sup>+</sup> cells (CD8ab). These six populations were sorted from wild-type C57BL/6 mice or TCR transgenic mice, and mRNA was hybridized to Affymetrix U74Av2 chips. The genomic analyses were done in two ways: a mathematical/geometrical analysis of the cell population coordinates based on their gene expressions (Reference Population Plot; RPP), and a bioinformatic analysis of the over-expressed genes based on their molecular functions. The RPPs indicate that innate lymphocytes have active-, memory-, NK-, and regulatory-like characters. We found 22 genes over-expressed >1.5 fold in the intersection of three innate vs. adaptive comparisons, a number which is significantly higher than the background level (1 gene) in randomized datasets. We also defined 33 genes that are under-expressed in innate cells. These two sets of

genes successfully sort multiple lymphocyte populations into innate and adaptive subgroups in the RPP, indicating that expression levels of the 22 genes can be designated as an “unbiased” innate signature. The molecular characterization of the genes induced in innate populations pointed to multiple molecular pathways being activated. We have found a limited set of functional entities to be enhanced across the populations of innate lymphocytes: essentially the intracellular vacuole trafficking and interferon-inducible GTPase systems. This finding prompts one to speculate that the innate immune system might deploy a unique intracellular recognition system to detect subtle changes in the tissue environment. Our analyses have revealed shared genes and pathways induced in innate lymphocytes, and thus a common “innate signature” that distinguishes innate lymphocytes from adaptive ones. These findings are not only instrumental for genomic definition and classification of “innateness” in various immune cell-types, but also have implications for our understanding of the origin and evolution of innate immunity.

### F2.34. Different O-glycosylated Structures Are Expressed in T Cell Activation.

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Previously, we reported that the saccharidic structure (Gal, GalNAc) recognized by *Amaranthus leucocarpus* lectin (ALL) is expressed on activated T cells. Gal, GalNAc structure is also recognized by Peneaut agglutinin (PNA). The objective of this work is to determine if the structure recognized by ALL is similar to the structure recognized by PNA.

PBMC were obtained from healthy donors and cultured in presence of 1 mg/ml of Concanavalin A (Con A). Then, the cells were recovered every 24 h during 4 days. After that, the cells were labeled with the FITC conjugated-lectins *Amaranthus leucocarpus* (ALL) or Peneaut agglutinin (PNA) and against different mAb such as CD3-Cychrome, CD69-PE or CD25-PE. In some cases the cells were treated by neuraminidase to eliminate the sialic acid camouflage. The results were analyzed by flow cytometry.

Results: After stimulation with Con A, the percentage of ALL+ T cells increases from 3% to 69% at 48 h, diminishing gradually, whereas the percentage of cells PNA+ T cells increases from 1% to 90% at 72 h and maintaining this percentage until 96 h. The expression of CD69 and CD25 and we observed that the CD69 and CD25 expression was 3 times more on ALL+ T cells. However on the neuraminidase treated cells the expression of CD69 and CD25 was diminished in both, ALL+ and PNA+ T cells.

Conclusions: Our results suggest that saccharidic structure recognized by ALL is different to the saccharidic structure recognized by PNA.

### F2.35. Short-Term Atorvastatin Treatment Enhances Specific Antibody Production Following Tetanus Toxoid Vaccination in Healthy Volunteers.

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Statins are a class of HMG CoA reductase inhibitors used by millions of Americans with high cholesterol. Several lines of evidence suggest that these drugs possess anti-inflammatory and immunomodulatory properties beyond their cholesterol-lowering effects. Recent reports have shown that statins affect immune responsiveness by skewing the  $T_H1/T_H2$  balance and interfering with MHC class II expression. While inhibition of inflammatory processes can be protective, suppression of immune responses such as antigen presentation may weaken host defense. To determine whether HMG CoA reductase inhibitor treatment has an effect on *in vivo* acute phase response and antibody production following tetanus vaccination in normal healthy volunteers in a double blind, parallel, placebo controlled study. Twenty healthy volunteers were randomized to receive atorvastatin 40 mg (AT) or calcium placebo (PL) for 10 days and all volunteers received tetanus toxoid (TT) vaccine on day 5 of the study. The acute phase response was evaluated by determining changes in ESR, CBC with differentials, and serum concentrations of acute phase proteins ( $\alpha_1$ -antitrypsin, C-reactive protein, and  $\alpha$ 1-acid glycoprotein) and cytokines (IFN  $\gamma$ , IL-4, IL-6, and IL-10). The humoral response to vaccination was assessed by measuring total immunoglobulins and specific anti-TT antibodies. Baseline measurements of all variables were similar in both groups. Following the ten-days of study drug or placebo, subjects in the atorvastatin group had a significant reduction in total cholesterol (AT:  $-44.4 \pm 9$  mg/dL; PL:  $22.7 \pm 8$  mg/dL;  $P < 0.0001$ ) and LDL cholesterol (AT:  $-46.2 \pm 9$  mg/dl; PL:  $13.2 \pm 7$  mg/dl;  $P = 0.0001$ ) compared to the control group, demonstrating the effectiveness of atorvastatin. The baseline anti-TT antibody concentrations and the number of years elapsed since last vaccination were similar in both groups. Unexpectedly, the production of specific antibody against TT (predominately IgG<sub>1</sub>) was more than 3 fold higher in volunteers treated with atorvastatin 15 days post-vaccination (AT:  $2306 \pm 468$  units; PL:  $713 \pm 21$  units,  $P = 0.0085$ ). Atorvastatin suppressed the post-vaccination rise in platelet counts (AT:  $-5.2 \pm 3.1\%$ ; PL:  $9.63 \pm 4.4\%$ ;  $P = 0.0132$ ) and lymphocyte counts (AT:  $-7.4 \pm 9.1\%$ ; PL:  $33.4 \pm 10.5\%$ ;  $P = 0.0089$ ) consistent with its anti-inflammatory properties. There were no significant changes in ESR, serum levels of acute phase reactants or cytokines in the treatment and control groups. This study demonstrates that statins augment specific immune responses following vaccination in healthy volunteers rather than suppress them. Beyond their applications in reducing cholesterol and suppressing inflammation, it is possible that statins may benefit groups who respond poorly to vaccinations, such as the elderly or immunocompromised patients. Further clinical studies are needed to determine if these groups demonstrate a similar response to atorvastatin as well as to determine the mechanism(s) that mediated this effect.

### **F2.36. An Immunology Syllabus to Student's Medicine.**

*Osmel Gaspar Guerra Segura.* <sup>1</sup>Immunology Lab, Teach. and Gen. Hosp. Dr. A. Neto, Guantanamo, Guantanamo, Cuba.

We did a pedagogical diagnostic of the knowledges of Immunology to the students of second year of Medicine before and after the teaching the topics in Microbiology and Pathology, then the diagnostic was done to the six year students and Internal Medicine residents. To accomplish the objectives were designed some pedagogical instruments (interview, questions, Test's knowledges) to students and professors which taught and received the subject. The 93 % of the knowledges were acquired by the second year students, and the Internal Medicine residents obtained the lowest marks. The highest integration of contents of Immunology

with other subjects is only about 40 %. That's right we propose an Immunology syllabus in agreement with social and professional needs of Cuban doctors and from other countries.

### **F2.37. Use of C1 Inhibitor Concentrate for Treatment of Angioedema Attacks in Patients with C1 Inhibitor Deficiency. Survey of 1102 Infusions in 503 Patients.**

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C1 inhibitor deficiency is a rare condition that can be either inherited (hereditary angioedema, HAE) or acquired (acquired angioedema, AAE) and is clinically characterized by recurrent, self-limiting skin and intestinal edema and life-threatening upper airway obstruction. Replacement therapy with C1-INH concentrate is the treatment of choice for severe acute attacks of angioedema in Europe.

We report the need and efficacy of C1-INH concentrate in 477 patients with HAE and 25 with AAE observed for: 30–25 years 50 patients, 24–20 in 44, 19–15 in 48, 14–10 in 96, 9–5 105, <5 in 159. Efficacy was evaluated as the time to beginning of resolution of symptoms. Side effects were recorded. C1-INH concentrate was used in 149 of 477 HAE patients (31%) for a total of 1013 infusions (mean infusion/patient 6.8, range 1–84) and by 8 of 25 AAE patients (32%) with a total of 89 infusions (mean infusion/patient 11.1, range 1–27). The dose ranged from 500U to 2000U for HAE, 1000U–12000U for AAE.

In 431 laryngeal attacks time to beginning of resolution was between 30 and 60 minutes in 424 episodes (98%) and between 1 and 2 hours in 6 episodes. One HAE patient underwent tracheostomy for laryngeal edema despite treatment with C1-INH concentrate. In 475 abdominal attacks time to beginning of resolution was within one hour in 469 episodes (99 %), between 1 hour and 12 hours in 6 episodes. Among 21 episodes of edema of the oral mucosa time to beginning of resolution was within one hour in 20 (90 %) and between 1 and 48 hours in 2. In 39 facial attacks the time to beginning of resolution was within 60 min in 26 (67%), between 1 and 2 hours in 7 and between 2 and 12 hours in 5. In 45 peripheral attacks the time to resolution was within one hour in 23 episodes (51 %), between 1 and 2 hours in 8 episodes and between 2 and 48 hours in 14 episodes. In 5 patients with AAE (54 infusions) beginning of resolution was always within one hour; in the remaining 3 patients (35 infusions) the response became progressively slow 3 (3 hours or more) requiring higher doses of C1-INH concentrate. Two anaphylactoid reactions were reported in 2 HAE patients. Prevalence of HCV in treated patients dropped from 71% to 3% with the introduction of virucidal methods. No HIV infection was ever detected. Our data indicate that treatment with C1-INH concentrate is highly effective in angioedema of the laryngeal or abdominal mucosa; its effectiveness is reduced when the skin is involved and particularly in peripheral attacks. Patients with AAE may become resistant to the treatment. Safety is generally good and transmission of blood borne infection has drastically reduced since viral inactivation procedures have been introduced.

### **F2.38. Immunonutritional Assessment of Special Plasma's Donors.**

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We did a prospective study of special plasma's donors with more than a year in the plasmapheresis program of hiperimmune anti TT (tetanic toxoid) plasma in the provincial Blood Bank of Guantanamo. We have evaluated the donors nutritionally with the CERES program from Hygiene of Foods Institute from Hanvawich have an special form for the collection of data in the relation to high, weight and race. From the immunohematologic and biochemist point of view, we value each fractions of Protein Electrophoresis, Eritro, Hemoglobin and in particular the specific antibodies (anti TT) and positiveness to antigens of Hepatitis B and C and HIV test. From the nutritional point of view the lipids supply is the only deficiency in those donators where antropometric and hematologic parameters are agree with literature. Specific antibodies titles (anti TT) vary in the 98 % of donors.

### **F2.39. Presentation of the Human Hepatitis B Surface Antigen- Loop by the Cpn10 Scaffold Induces a Specific Antibody Response in Mice by Genetic Immunization.**

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#### *Introduction:*

Cpn10 proteins belong to a widely distributed protein family found from mammals to bacteria. Because of their conservation between species they exhibit low immunogenicity. This property is advantageous when fusion proteins are used in genetic immunizations: antibodies against the fusion partner and not against Cpn10 are generated. Cpn10 spontaneously forms a heptafold scaffold and each subunit of this scaffold displays an exposed polypeptide loop. This native loop can be replaced by a foreign loop sequence.

An important application of this "loop replacement" strategy lies in presenting extracellular loops of transmembrane proteins like G-protein coupled receptors (GPCR) for which it has been proved difficult to generate antisera against their short extracellular loops. The broad interest on the GPCR family is based upon the fact that 50–60% of approved drugs elicit their therapeutic effect by selectively addressing GPCRs.

As a proof of principle for the applicability of the Cpn10 scaffold we have replaced the Cpn10 loop by the "a" determinant of the Hepatitis B surface antigen (HBsAg). The "a" determinant is a small loop of nine amino acids, which is highly immunogenic when presented in the context of the full length HBsAg. In this study, we wanted to analyze whether presentation of this HBsAg loop in the Cpn10 scaffold can elicit a specific immune response upon genetic immunization and whether the scaffold is able to mimic the native conformation. Therefore we immunized mice with a plasmid coding for the Cpn10-HBsAg scaffold and analyzed the antisera for specific anti-HBsAg antibodies.

#### *Methods:*

The murine Cpn10 cDNA and the nucleotides coding for the "a" determinant were cloned into an optimized immunization vector. BALB/c mice were immunized once in a two week interval over a period of 12 weeks using a Helios<sup>®</sup> gene gun. Blood samples were taken from each animal every two weeks and analysed for the presence of specific antibodies using a cell-based ELISA assay.

#### *Results:*

Screening the antisera in the cell-based ELISA showed that all mice elicited specific antibodies against the plasmid-encoded Cpn10-HBsAg antigen. It could also be demonstrated that this immune response was not directed against the scaffold itself

proving the low immunogenicity of the scaffold in the murine system. Therefore the detected antibody response seems to be specific for the "a" determinant of the HBsAg.

#### *Discussion/Conclusion:*

We could show for the first time that the murine Cpn10 scaffold can be used in genetic immunization experiments for presenting a foreign peptide loop and for eliciting an immune response against this encoded antigen-loop. Whether this scaffold allows formation of the native loop conformation needs to be further investigated in an ELISA, where the recombinant HBsAg protein should be detectable by the sera. Further experiments have to be performed to establish whether this approach can be extended and used e.g. for the directed generation of antibodies against GPCR loops.

### **F2.40. Angioedema without Urticaria in 929 Italian Patients: Proposal for a Diagnostic and Therapeutic Approach.**

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Angioedema without major urticaria flares is poorly characterized, it is caused by different conditions and few data exist on the underlying etiopathogenetic mechanisms. Here we report our experience on patients with this symptoms and propose a diagnostic-therapeutic approach.

#### **Methods**

929 consecutive patients were examined at our outpatient clinic for recurrent angioedema without urticaria between 1993 and 2002. Known causes of angioedema were identified by clinical history with detailed information about personal and familial allergies; relationship of angioedema to potential causative agents were search (food, drugs, insect stings); complete physical examination, routine laboratory tests (blood cell count, protein electrophoresis, erythro sedimentation rate, stool for ova and parasites, pharyngeal and urine cultures, sinus and dental film, anti-tissue autoantibodies, rheumatoid factor), complement parameters (C1 inhibitor, C4, C1q) were performed. Further testing was done when pertinent based on clinical finding. When all was negative, response to H1-antihistamine was considered.

#### **Results**

According to our testing angioedema were classified as follow: related to external agents (drugs, insect stings, food) 209 (22.5%) (in particular 85 of them were related to treatment with an ACE-inhibitor); associated to autoimmune diseases or infections 77 (8.2%); hereditary C1 inhibitor deficiency 183 (19.6%); acquired C1 inhibitor deficiency 14 (1.5%); idiopathic histaminergic 253 (27.2%), idiopathic non histaminergic 40 (4%). 153 patients (16.4) dropped out from the study and could not be classified.

#### **Conclusion**

Based on the frequency of the different diagnosis we propose the following progression of testing: 1. anamnestic identification of causative agents, 2. testing for complement abnormalities, 3. H1 antagonist treatment, 4. complete diagnostic workup.

### **F2.41. Dynamic Control of T Cell Homing by Gut and Peripheral Dendritic Cells.**

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T cell activation by intestinal dendritic cells (DC) induces gut-tropism. We show that, reciprocally, DC from peripheral lymph nodes (PLN-DC) induce homing receptors promoting CD8 T cell accumulation in inflamed skin, particularly ligands for P- and E-selectin. Differential imprinting of tissue-tropism was independent of Th1/Th2 cytokines and not restricted to particular DC subsets. Fixed PLN-DC retained the capacity to induce selectin ligands on T cells, which was suppressed by addition of live intestinal DC. By contrast, fixed intestinal DC failed to promote gut-tropism and instead induced skin-homing receptors. Moreover, the induction of selectin ligands driven by antigen-pulsed PLN-DC could be suppressed 'in trans' by adding live intestinal DC, but PLN-DC did not suppress gut homing receptors induced by intestinal DC. Reactivation of tissue-committed memory cells modified their tissue-tropism according to the last activating DC's origin. Thus, CD8 T cells activated by DC acquire selectin ligands by default unless they encounter fixation-sensitive signal(s) for gut-tropism from intestinal DC. Memory T cells remain responsive to these signals allowing for dynamic migratory reprogramming by skin- and gut-associated DC.

(Pew Fellowship to J.R.M. & NIH grants HL62524, HL54936, HL56949, AI-061663 to U.H.vA.).

#### **F2.42. Isoation and Characterization of Water Isolates for Bacteriocinogenic Activity.**

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A Prospective study has been carried out to study the bacteriocin producing ability of water isolates a total of 27 isolates were screened for their bacteriocinogenic potential against intragenic and intergenic microorganisms 20% of the isolates were found to exert broad range bacteriocinogenesis (bioactivity). However none of the producers was antagonistic against the related *streptococcal* strains.

Bacteriocinogenesis was demonstrated by 1) stab over lay 2) cross streak 3) patch test 4) Agar well. Titration of bacteriocine was done by arbitrary unit(AU) method. In first method test strains were stabbed into Luria agar plate and incubated at 37°C for 24 hrs, chloroform was used to kill the bacteria, plates were then overlaid by soft agar (0.6%) containing 5–10 micro liter of 2-3 hours grown indicator strains and incubated at 37°C for 24 hrs and examined for zone of inhibition In second Test strains were streaked across the surface of a Luria agar plate and incubated at 37°C over night the growth of test strain was removed and plate was exposed to chloroform as described earlier, indicator strain was then cross streaked. Plate was incubated at 37°C over night, and examined for inhibition of growth. In 3rd Luria agar plate was overlaid with 3 ml soft agar containing 5–10 micro liter of indicator strain. Plate was kept at 37°C for 2 hrs for drying. A fresh colony of strain was stabbed into indicator lawn. Plate was incubated at 37°C for 24 hrs in 4th Test strain was grown in Luria broth, culture was centrifuged at 3000 rpm for 20 min, supernatant was collected. Luria agar plate was overlaid with 3 ml soft agar containing 5–10 micro liter indicator strain. Wells were made and 100–200 micro liter supernate was added in the well. Plate was incubated at 37°C for 24 hrs, zone of inhibition of indicator strain around the well was

measured. For titration, Inoculate the nutrient broth by different producers strains and incubated at 37°C, next day centrifuge those tubes for 20 minutes then make 2 fold dilutions (1:2-1:4, so on) on nutrient agar make 5 wells and marked according to dilution tubes (1:2-1:32). Take 50 micro liter from 1:2 dilution tube and transfer in 1:2 marked well and this process was repeated with all dilutions. Next day check the zone of inhibition. Among many results most appropriate results obtained about *Listeria monocytogenes* and *Streptococcus Pyogenes* Bacteriocinogenic organisms gave this activity against some Gram negative stains such as *E.coli*, *S.typhi*, *Klebsiella*, *Pseudomonas* and some G+ve stains such as *S.aureus*, *S.fecalis*, *M.leutus*, *Bsubtilis*. Streptococin titer was estimated to be 1:64 A.U. The lacuna percentage (which determine the number of Streptococin producing clones in a population) was found to be 14.6 elevated temperature 40°C mediated curing of the producing isolates revealed the chromosomal location of bacteriocinogenic determinants as reported in the past studies.

#### **F2.43. Inhibition of Pericardic Adhesions by the Amoebian Anti-Inflammatory Pentapeptide (MLIF). Preliminary Results.**

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Cardiac surgery is usually followed by adhesions, which still represents an incompletely solved problem. Different treatments have been proposed, the fibrin seal probably being the best so far, reducing adhesions by about 50%. *Entamoeba histolytica* produces an anti-inflammatory peptide (Met-Gln-Cys-Asn-Ser) termed *Monocyte Locomotion Inhibitory Factor (MLIF)* for its first described effect. Monocytes are key cells of the late inflammation and also modulates the cicatrization stage of the wound-healing process. Thus, *MLIF* could regulate wound-healing. The purpose of this study is to prevent the formation of experimentally induced pericardic adhesions in rats (a model originally developed to induce cardiac colateral circulation). Epicardiectomy and 3x soft sandpaper scratches over 0.5 cm<sup>2</sup> apical area of the heart were performed in four groups of eight male Wistar rats each under anesthesia and sterile conditions. Groups received either; *MLIF* 100 µg in 0,05 ml of pyrogen free saline solution, fibrin seal solution (0.1 ml), both, or none, the final volume adjusted to 0.1 ml with saline solution. All the animals recovered and were humanly sacrificed at day ten with an anesthetic overdose. Systematized necropsies and photographic registers were done, and were blindly evaluated by three independent participants. Control animals (without *MLIF* and or fibrin seal) developed strong, nodular and extensive adhesions. The fibrin seal allowed only *focal, -yet strong-* adhesions (++++ to +, p<0.05). *MLIF* inhibits the strong adhesions (++++ to +, P < 0.05) but allowed the development of *soft, -and extensive-* adhesions (++++ to +++, P = n. sig.). *MLIF* together with fibrin seal completely inhibits the pericardic adhesions in this model (++++ to 0, p<0.05). That combined *MLIF* and fibrin seal inhibited adhesions but either substance alone induced only a partial- and qualitatively different- reduction of adhesions suggests that these factors have different target mechanisms and act synergically, namely *MLIF* reducing activation signals from monocytes to fibroblasts, while fibrin seal modulating the



ciatrization stage of wound-healing. (supported by CONACYT 28363-M and IMSS- FOFOI 2004-432 grants).

#### **F2.44. Attenuation of the GAA-Specific Immune Response in 6<sup>neo</sup>/6<sup>neo</sup> GAA Knockout Mice, a Model of Pompe Disease.**

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Pompe disease is an autosomal recessive lysosomal storage disorder characterized by the deficiency of acid alpha-glucosidase (GAA). The absence or decreased expression of GAA results in the accumulation of glycogen in muscle tissue. Infantile Pompe patients experience life-threatening manifestations such as cardiomegaly and late-onset patients experience progressive myopathy with or without respiratory insufficiency. The use of recombinant human alpha-glucosidase (rhGAA) is being investigated as a therapeutic agent for Pompe disease. In our clinical studies, it has been observed that patients treated with rhGAA can seroconvert in response to the therapeutic protein. The role of these rhGAA-specific antibodies is unclear, but they could potentially inhibit the activity of the therapeutic protein or alter the biodistribution, thereby decreasing enzyme efficacy. We have previously demonstrated that weekly intraperitoneal administration of rhGAA in 6<sup>neo</sup>/6<sup>neo</sup> GAA knockout (GAA KO) mice results in high rhGAA-specific IgG titers. We have shown that high doses of methotrexate (MTX) were able to suppress these titers ten-fold when administered 24 and 48 hours following the first eight weekly rhGAA treatments. Further studies were conducted in GAA KO mice to both model intravenous administration of rhGAA which is the clinical route of therapeutic administration and to identify a lower efficacious dose of methotrexate. We have observed that intravenous delivery of rhGAA in GAA KO mice significantly reduces rhGAA-specific IgG titers from those observed upon intraperitoneal administration of rhGAA. In addition, we have demonstrated that MTX is most effective at reducing this immune response when administered 0, 24 and 48 hours following the first eight weekly rhGAA treatments. Moreover, lower weekly doses of MTX are as effective as the higher dose at inhibiting the rhGAA-specific immune response when given 0, 24 and 48 following rhGAA treatment. These studies suggest that the timing of MTX treatment influences its ability to inhibit the rhGAA-specific immune response. This approach may be effective in minimizing drug-specific antibody responses in patients receiving protein therapeutics.

#### **F2.45. The Effect of Electro Active Dental Metal Fillings Replacement on Lymphocyte Activity in Patients with Oral Discomfort.**

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The release of metal ions from dental metal fillings is being supported by presence of galvanic cell in the oral cavity and such a way can cause local or general pathological problems in sensitive and genetically susceptible individuals.

**Aim of the study:** To investigate the *in vitro* lymphocyte responses to metals before and after replacement of electro active dental metal fillings in patients with oral discomfort.

**Methods:** Sixty-eight patients with oral discomfort and dental metal fillings were investigated. They were divided into two groups. Group A (38 persons)-patients with pathological levels of galvanic currents and group B (30 persons)-patients with physiological levels of galvanic currents and voltage. The galvanism measurement was performed by the equipment Odontologic 2000 (Embitron CZ). The lymphocyte activity was tested by the lymphocyte proliferation method modified for metals (MELISA) in 33 patients from Group A and 24 patients from group B. The electro active metal fillings replacement was performed in 18 patients from group A. Follow up MELISA at least half a year after fillings replacement was performed.

**Results:** The higher lymphocyte activity to metals was discovered in both examined groups. The highest levels of proliferation activity were found to Ni, Hg and Mo in Group A and to Hg, Ni and Au in Group B. In general, lymphocytes of patients in group B were sensitized more than the ones in group A. The follow up results after electro active fillings removal show that lymphocyte reactivity to at least all metals tested decreased. The highest decrease was found in reaction to inorganic mercury.

**Conclusion:** The long-time influence of galvanism can, only in sensitive and genetically susceptible individuals, influence the lymphocytes proliferation. The beneficial effect of dental metal fillings replacement was confirmed by decrease of lymphocyte reactivity to metals. Our findings support the hypothesis that suffering from the oral discomfort is not only a subjective feeling of the patient but that it can be based on a real cell discomfort due to release of metal ions from dental metal fillings and due to galvanism. But the activity of lymphocytes to metals can be influenced by a couple of other factors as well.

**Acknowledgment:** The study is supported by the grant of Czech Ministry of Health nr. NR 8324-3.

#### **F2.46. A Review of the Allergic /Toxic Adverse Reactions to Anti-Tuberculous Drugs(ATD), A 10 Years Therapeutic Survey.**

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**Introduction:** Various un-towards reactions (Allergic/Toxic) had been observed during the course of treatment for patients with pulmonary/extra pulmonary tuberculosis.

**Methods:** The old theme about tuberculosis as 'untreatable' has been replaced by the 'curable' with introduction of the effective anti-tuberculosis drugs, but still their effectiveness is with in the limit of careful assessment of patients for ATD. Allergic & toxic manifestations are as under.

*Rifampicin:*<sup>1</sup> (Urticaria, red skin with orange discoloration).

<sup>2</sup>(Febrile reactions), <sup>3</sup>(Hepato),<sup>1</sup>(Neuro&Nephrotoxicity, blood dyscrasia haemolysis and shock, blurring of the vision, confusion, ataxia/peripheral neuritis, Pseudomembranous colitis).

*Ethambutol:*

<sup>R</sup>(Skin rashes, itching/burning sensations, anaphylactoid reactions). <sup>1</sup>(Nausea vomiting, abdominal pain, anorexia), <sup>1</sup>(head ache peripheral neuropathy, hallucination dis-orientation, mental confusion) <sup>2</sup>(Visual disturbance),<sup>1</sup>(transient impairment of liver function) <sup>1</sup>(gouty arthritis).

*Isoniazide:*<sup>R</sup>(Itching/Skin rashes, Slurred speech, hallucination, coma, generalized convulsion, status epilepticus, peripheral neuropathy, hypotension respiratory failure, severe metabolic acidosis),<sup>1</sup>(fever, nausea, vomiting, Hepatitis).<sup>R</sup>(hemolytic anemia).

*Pyrazinamide*: <sup>R</sup>(skin rashes) <sup>2</sup>(Hepatotoxicity, gouty arthritis),  
*Streptomycin*: <sup>1</sup>(Wide spread Skin rashes, hives, nausea vomiting, headache), <sup>2</sup>(hypotension), <sup>1</sup>(ear & kidney damage). In some rare with paralysis by interfering with calcium transport in the central nervous system.

*Ethionamide*: <sup>2</sup>(Severe skin rashes, acne, alopecia, photosensitivity), <sup>1</sup>(nausea vomiting anorexia, diarrhea, excessive salivation, metallic taste), <sup>2</sup>(hepatotoxicity). <sup>1</sup>(Mental depression, anxiety/psychosis, **Systemic** encephalopathy with pellagra-like symptoms), <sup>1</sup>(dizziness, drowsiness, headache, convulsion, peripheral neuropathy, tremors, paresthesias). <sup>1</sup>(Optic neuritis, optic atrophy, diplopia, <sup>1</sup>Olfactory disturbances), <sup>1</sup>(Deafness), <sup>R</sup>(Hypothyroidism), <sup>1</sup>(impotence, menorrhagia, gynaecomastia), <sup>R</sup>(hypoglycemia, hypotension), <sup>R</sup>(Thrombocytopenia, Rheumatic pains).

*Thiacetazone*: <sup>1</sup>(Skin rashes, nausea, vomiting, Jaundice), <sup>1</sup>(giddiness, bone marrow suppression, agranulocytosis).

% incidence of adverse reactions as observed R=0-1%, 1=5-10%, 2=>10-30%, 3=>30%-80%.

**Results:** Careful selection with Appropriate diagnosis of the infectious patients are less likely associated with adverse effects.

**Conclusions:** Incidence of adverse effects had been relatively less frequent with first line than second line ATDs treatment.

#### **F2.47. Defective Intrathymic T Cell Development and Early Onset of IBD in Mice Devoid of WASP and N-WASP.**

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**BACKGROUND:** Wiskott-Aldrich syndrome protein (WASP) family members (WASP, N-WASP, WAVES) are key molecules that regulate cytoskeletal changes in response to cell surface signals. These events are critical for various biological processes including cell migration and immune synapse formation. We recently generated chimeric mice deficient for N-WASP (NWKO) and mice double-deficient for WASP and N-WASP (DKO) employing Rag-2-deficient blastocyst complementation. NWKO chimeric mice, like WASP KO (WKO) mice, did not demonstrate any change in T cell development. In contrast, WASP/N-WASP DKO mice have a marked defect in T cell maturation associated with a block in the CD4/CD8 double-negative stage, and a consequent decrease of T cell numbers in the peripheral lymphoid organs.

**AIM:** To elucidate the mechanism underlying the aberrant T cell development in WASP/N-WASP double-deficient mice.

**METHODS:** We deleted N-WASP selectively in T-cells from WKO mice by using the Cre-loxP system. In this approach we mated conditional N-WASP-KO with WKO and lck-Cre transgenic mice (creDKO). Lymphoid organs were processed for phenotypical analysis employing standard developmental and activation markers. We also assessed thymocyte migratory function driven by CXCL12 and CCL19 by transwell migration assays and thymocyte apoptosis by Annexin V staining. Colonic samples were obtained for histological analyses and cytokine measurements.

**RESULTS:** The lck-driven N-WASP deletion in WKO mice (creDKO) during T cell development resulted in a significant

decrease in thymic cellularity. We also found increased apoptotic cell death in the creDKO animals compared to WT and WKO mice. CD69+ cells were substantially decreased in both CD4 and CD8 single-positive thymocytes from creDKO mice- which may correlate directly with the increase in apoptosis. In addition, creDKO thymocytes have a significant migration defect when compared with WKO thymocytes, which are also defective in migration compared with WT thymocytes. Consequently, we found significantly decreased numbers of T cells in the spleen of creDKO mice. However, we observed that the majority of these T cells present an effector phenotype (CD44hiCD62Llo). Interestingly, creDKO mice present with colitis at an earlier onset which may be associated with increased severity.

**CONCLUSIONS:** These studies demonstrate redundant functions for WASP and N-WASP with a critical combined role for both proteins in T cell development and function. Together the functional analyses of creDKO thymocytes suggest that the decreased migratory activity of these cells might be related to decreased signaling resulting in increased cell death and consequent reduced thymocyte numbers. Furthermore, the finding of significant increased proportion of effector T cells in the periphery of cre-DKO may be associated to the early onset of inflammatory bowel disease.

#### **F2.48. FAS Induced Apoptosis- A Model System for BiFAR™ Implementation.**

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FAS induced apoptosis, a key signaling pathway in many diseases, was studied using a functional genomic approach in joint study by Quark Biotech and Agilent Technologies. Quark's proprietary BiFAR™ platform is based on the discovery of essential functional genes using Gene Inhibitory Elements (GIEs; antisense, shRNAs or dominant negative peptides). Abundance of GIEs is expected to change based on their ability to inhibit certain genes. GIEs that are protective to a certain treatment will be enriched and GIEs sensitizing to a certain treatment will be depleted.

In this study, PC3 cells were transduced with a GIE library, propagated and then exposed to high or sub-lethal doses of anti-Fas antibody. Using a custom oligonucleotide Agilent microarray, designed to match the specific GIE library, the relative abundance of GIEs before and after anti-FAS treatment was measured. Analysis of functional GIEs identified GIEs that were protective to FAS and others that were sensitized to the treatment. Protective GIEs inhibiting genes involved in the interferon response, phosphoinositol metabolism, vesicle transport and chaperons were found. Sensitizing GIEs included those inhibiting anti-apoptotic genes.

The BIFAR platform, implemented on Agilent's microarrays, allowed functional dissection of FAS induced apoptosis, a process known to participate in the killing of virally infected cells, transformed cells and peripheral auto-reactive T-cells, as well as other physiological processes. The discovery of the inhibition of genes leading to accelerated FAS induced apoptosis can be implemented in therapeutics that are aimed at the enhancement of tumor suppression or tolerance.

This basic approach can be applied to a variety of other functional systems using a variety of GIE types in proliferating cells as well as fully differentiated cells, to elucidate signal transduction pathways and identify novel drug targets.

### F2.49. Protective Effect of Thalidomide in Rats with Granuloma in the Cerebral Amygdala and Pentylentetrazole-Induced Epilepsy.

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Chronic brain inflammation by persistent infections, traumas or extensive surgeries (e.g. tumoral resection) is a main cause of secondary epilepsy. To evaluate the preservation of functional neural tissue by chronically administered antifibrotic and immunomodulating drugs in damaged brain, we induced a granuloma in the cerebral amygdala of 130 Wistar rats, by stereotaxic injection of aluminum silicate (interaural 6.2 mm, lateral 5.0 mm, height 8.5 mm, according to Paxino's Atlas), which were randomly distributed in 9 groups ( $n = 13$ ), treated with: vehicle (water, 5% alcoholic-water or maize oil); methylprednisolone acetate (4 mg/kg/week, IM); colchicine (300 µg/kg/day, orally); thalidomide (160 mg/kg/day, orally); cyclosporine (10 mg/kg/day, orally); mixture of colchicine-methylprednisolone (300 µg/kg/day-4 mg/kg/week); thalidomide-methylprednisolone (160 mg/kg/day-4 mg/kg/week); cyclosporine-methylprednisolone (10 mg/kg/day-4 mg/kg/week) during forty five days. Two weeks after the end of the treatment, experimental epilepsy was induced by PTZ subthreshold increasing doses (20–70 mg/kg), administered according 48 hours intervals, until the development of generalized seizures. Cortical electrodes were implanted in three animals from each group. Electrical activity was registered during 20 minutes. Time elapsed since the application of the drug until the first myoclonic and tonic-clonic seizure (latency), as well as the number and duration of afterdischarges were measured and compared between groups.

Histological and imaging analyses and collagen quantification of granulomatous lesion were also made. Evident contrast of all measures was found in rats that received thalidomide. Dose required to develop tonic-clonic seizures in this group was higher ( $P < 0.05$ ) than that required in the rest of groups. The latency for generalized tonic-clonic seizures was also longer in thalidomide treated rats than in controls. Afterdischarges in the control group were more frequent than those in the thalidomide group, in which we also found sleep-like high voltage slow waves and isolated spikes that didn't burst in a real afterdischarge. Thalidomide seems to preserve functional brain tissue surrounding a granulomatous lesion and would be a promissory alternative to prevent secondary epilepsy.

### F2.50. Self-Limitation of Th1-Mediated Inflammation by IFN- $\gamma$ .

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**Objective of the study:** IFN- $\gamma$  is a pro-inflammatory effector cytokine of cell-mediated immunity that plays an essential role in both innate and adaptive phases of an immune response. Interestingly, in several Th1 dependent autoimmune models lack of IFN- $\gamma$  is associated with an acceleration of disease. The aim of

the study was to investigate how IFN- $\gamma$  could be involved in the regulation of a Th1 dependent inflammation.

**Materials and methods:** To study the influence of IFN- $\gamma$  on effector T cells we used an adoptive transfer model of differentiated antigen-specific Th1 cells. To differentiate the impact of IFN- $\gamma$  we analyzed the antigen specific delayed type hypersensitivity (DTH) reaction in different KO strains.

**Results:** IFN- $\gamma$  displayed a dual function in a Th1-dependent immune reaction. In the early phase, IFN- $\gamma$  accelerated the inflammation, whereas in the late phase it mediated the process of self-limitation. IFN- $\gamma$  negatively regulates Th1 homeostasis after antigen challenge. Studies in IFN- $\gamma$  R KO and iNOS KO mice revealed that IFN- $\gamma$  could act via the receptor of host cells and that NO is involved downstream. Transfer experiments into IFN- $\gamma$  KO mice showed that Th1 cells control both themselves and the corresponding inflammation.

**Conclusion:** Our results show that IFN- $\gamma$  is an important player in the regulation of a Th1 dependent inflammation. The proinflammatory cytokine can act as a negative feedback regulator and control the self-limitation of a Th1 dependent inflammation.

### F2.51. Origin and Generation of Distinct CD4<sup>+</sup> Regulatory T Cell Subsets.

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**Objective of the study:** CD4<sup>+</sup> regulatory T cells (Tregs) are increasingly recognised to play an important role in the maintenance of T cell homeostasis and self-tolerance. We recently reported a phenotypic and functional dichotomy among naturally occurring murine Treg subsets characterised by the expression of the integrin  $\alpha_E$  and CD25.  $\alpha_E$ -expression on Tregs correlates with an effector/memory-like phenotype, in contrast to naive-like  $\alpha_E$ -CD25<sup>+</sup> Tregs. These phenotypic characteristics have a crucial impact on the migratory properties of Tregs *in vivo* and ultimately result in a differential potency to suppress immune responses within distinct anatomical compartments. In the current study we intend to elucidate the relationship between  $\alpha_E^+$  and  $\alpha_E^-$  Treg subsets. We want to clarify whether  $\alpha_E^+$  Tregs originate from the thymus as a distinct lineage or whether they are generated in the periphery under certain conditions.

**Materials and methods:** We performed BrdU-labelling experiments to follow *in vivo* proliferation of different Treg subsets under normal and athymic conditions. In transfer experiments we investigated the stability of their phenotype and conditions of their generation.

**Results:** The effector/memory-like phenotype of  $\alpha_E$ -expressing Tregs suggests antigen-specific differentiation or expansion in the periphery. Indeed, BrdU-labelling experiments in both normal and thymectomised mice revealed a high frequency of proliferating cells within this subset. Interestingly,  $\alpha_E$ -expressing Tregs were comparatively enriched in adult-thymectomised mice, as they sustained stable cell numbers even in the absence of thymic output, while mild lymphopenia was observed in the remaining T cell compartment.

We transferred distinct Treg subsets into non-lymphopenic recipients and observed that under such conditions  $\alpha_E$ -expressing Tregs represent a stable subset or differentiation stage of Tregs rather than a transient population. By transferring TCR transgenic cells and subsequently applying antigen, we want to identify the

cellular precursor of  $\alpha_E$ -expressing Tregs and the conditions of their generation *in vivo*. Initial experiments indicate that  $\alpha_E$ -expression is indeed induced on a fraction of transferred cells under tolerogenic conditions. Interestingly, this induction appears to be confined to certain compartments.

**Conclusion:**  $\alpha_E$ -expressing effector/memory-like Tregs represent a stable population in the normal immune system. They contain cells, which are constantly cycling, presumably in response to self-antigen and/or environmental antigen. These effector/memory-like Tregs have the potential to maintain a stable population size in the periphery even in the absence of thymic output. Preliminary results suggest, that this phenotype is induced in the periphery after antigen-specific differentiation under distinct conditions. A better understanding of the fundamental physiology of the generation of Treg subsets may aid in the design of future therapeutic strategies for the treatment of ongoing autoimmune diseases.

### F2.52. Correlation between Histamine and Mast Cells and Presence of IgE.

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**Aim:** According to presence of immune system factors in chronic periapical lesions, hypersensitivity reactions may have some role in pathogenesis of these lesions. Up to now, among the factors participating in type I hypersensitivity reaction, only one of them has been studied. So the aim of this study was to evaluate the relationship between presence of IgE & number of mast cell and presence of IgE and histamine in human dental chronic periapical lesions.

**Materials and Methods:** 40 specimens were collected from 39 patients. After extraction of the lesions, they were divided into two sections. Half of the sections were used for explant culture and were maintained for 3 days. Then sandwich enzyme linked immunosorbent assay (ELISA) was used to determine the presence and concentration of IgE and histamine. Based on the presence of IgE, the samples were divided into case (with IgE) and control (without IgE) groups. The other sections were used for estimating the number of mast cells by histopathological technique.

**Results:** The average percent of mast cells in case and control groups was respectively,  $10.25 \pm 7.02$  and  $6.9 \pm 4.09$ . Statistical analysis (Mann Whitney-U test and Spearman correlation coefficient) showed that there is no difference between the case and control groups regarding the number of mast cells and histamine concentration. Also there was not any correlation between IgE and mast cells or IgE and histamine.

**Conclusion:** It is concluded that hypersensitivity reaction type I possibly does not have any role in pathogenesis of chronic periapical lesions.

### F2.53. Neutrophil Chemotaxis and Dental Caries.

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**Aim:** In addition to microorganisms, several other factors such as host immune responses are also involved in pathogenesis of dental caries. Among the factors of immune system, the role of neutrophil in prevention or pathogenesis of dental caries is not well

studied. So the aim of this study was to investigate the neutrophil chemotaxis in patients with dental caries.

**Materials and Methods:** For this purpose, fifty of dental students with dental caries were selected. 10 ml of heparinized blood were collected from subjects for neutrophil chemotaxis test by modified Boyden chamber method.

**Results:** The amount of neutrophil chemotaxis in the above subjects was  $94.38 \pm 12.08$  micrometer. Statistical analysis did not show any significant difference regarding to neutrophil chemotaxis in different degrees of DMF. But by comparing of neutrophil chemotaxis with the degree of active caries, significant differences were shown between 0 and 5 degrees; and 1 and 5 degrees of active caries regarding to the mean of neutrophil chemotaxis.

**Conclusion:** It is suggested that not only deficiency in neutrophil chemotaxis is not assumed in pathogenesis of dental caries, but also there is a direct correlation between neutrophil chemotaxis and the degree of active caries. Of course more studies are needed in order to prove this hypothesis.

### F2.54. Correlation between Neuropeptides Concentration and Different Parts of Human Gingiva and the Effects of Neuropeptides on Neutrophil Death.

Mandana Sattari,<sup>1</sup> Saeed Khalili.<sup>1</sup>  
<sup>1</sup>Immunology Dept, Shaheed Beheshti University of Medical Science, Tehren, Islamic Republic of Iran.

**Objective:** It is not well defined up to now that why some parts of gingiva is more susceptible to periodontal diseases. Since these regions are adjacent to the foramen of some nerves and paths and regarding to the important role of neuropeptides in inflammation and specially gingival and periodontal inflammation. On the other hand it was shown that substance P (SP) and calcitonine gene related peptide (CGRP) have some role in periodontal diseases. Since it was established that neutrophil is one of the most important defense line against periodontal diseases. So the aim of this study was to determine the correlation between concentration of neuropeptides and different region of human healthy gingival and their effects on neutrophil death.

**Materials & Method:** In the first section twenty gingival samples from first maxillary incisor and molar regions and twenty gingival samples from other regions were collected during periodontal surgery. Tissue samples were immediately transported to sterile tubes filled with tissue culture media. After culturing for 72 hrs the supernatant fluid were extracted and after diving them in microtubes, were frozen at  $-70$  degree of centigrade. EIA was used for detection of neuropeptides and in the second section we collected heparinate blood from a healthy individual neutrophil were collected from it. For this purpose, 5ml heparinate blood mixed with five ml dextran was maintained at laboratory temperature for 45 minutes and the high concentration and low concentration extract of neuropeptides (regarding to the concentration of SP and CGRP in first section) were collected with neutrophils Annexin V flourescence kit was used for detecting the death of neutrophils under flourescence microscope.

**Finding:** we find significant difference between different gingival regions regarding to CGRP concentration and it was also a significant reverse correlation between CGRP and different of Gingiva We could not find any significant correlation between SP and different regions of gingiva. Both SP and CGRP were present

in the all of the samples. In the second part we find that SP and CGRP significantly induce apoptosis in neutrophils.

**Conclusion:** It is concluded these neuropeptide probably participate in the regulation and maintenance of gingival health and based on the low level of CGRP in the gingival of upper and lower 6 dental region prevalence of local aggressive periodontitis distributed to low level CGRP and SP in this region we could suggested a possible role for the susceptibility of these regions to localized periodontitis and in the second part of this study we can concluded that both of SP and CGRP induces apoptosis instead of necrosis in the neutrophil, this is also may be parallel with regulatory role of these neuropeptides.

### F2.55. Human MyD88s Is the Result of MyD88 Splicing like That Reported in the Mouse.

A. Dominguez,<sup>1</sup> J. L. Ventura,<sup>2</sup> A. Zentella,<sup>2</sup> R. Kretschmer,<sup>1</sup> J. R. Velazquez.<sup>1</sup> <sup>1</sup>Investigation in Medical Immunology, Instituto Mexicano del Seguro Social CMNSXXI, Mexico City, DF, Mexico; <sup>2</sup>Biochemistry, Instituto Nacional de Nutrición Salvador Zubiran, Mexico City, DF, Mexico.

MyD88, the first adaptor protein that was discovered, possess a TIR-domain that interacts with a Toll-like receptor and with IL-1R, leading to activation of the NF- $\kappa$ B and JNK signaling systems. MyD88 is highly conserved in nature. Mouse and human MyD88 genes reveal a 82% homology at protein level. Human and murine genes are organized into five exons and four introns. A splicing variant of MyD88 lacking exon 2 the so called MyD88s, has been reported. In the mouse MyD88s is a protein isoform that regulates IL-1 and LPS signaling pathways through its inability to bind or to activate downstream signaling molecules. The murine MyD88 splicing has been established as the generator of MyD88s, there is not information yet on such human MyD88s processing. By nucleotide sequencing analysis of U937 and human peripheral blood mononuclear cells we noticed that human MyD88 splicing is comparable to that reported in the mouse. Human MyD88s is the result of exon 2 excision. It therefore lacks the ID domain (aa 110-154). In the human mononuclear cells tested, MyD88s was constitutively expressed as a transcript and as a protein. Murine and human MyD88s are similar and are processed in comparable ways. However, the regulatory mechanisms in which they are involved could be exquisitely different.

### F2.56. Relationship of School Attendance with Quality of Life, Physical Function, Disease Activity and Damage in Pediatric Systemic Lupus Erythematosus.

L. N. Moorthy,<sup>1</sup> M. G. Peterson,<sup>2</sup> M. J. Harrison,<sup>2</sup> K. B. Onel,<sup>3</sup> M. J. Baratelli,<sup>1</sup> D. R. Mohan,<sup>1</sup> Thomas Lehman.<sup>2</sup> <sup>1</sup>Robert Wood Johnson University, New Brunswick, NJ; <sup>2</sup>Hospital for Special Surgery, New York, NY; <sup>3</sup>La Rabida Children's Hospital, Chicago, IL.

**Background:** Pediatric systemic lupus erythematosus (SLE) is a multisystem disease that significantly impacts quality of life (QOL) of children. SLE's impact on school attendance, an important outcome and potential predictor of other outcomes, has received little attention.

**Objective:** Examine the relationship of school attendance with QOL, physical function, SLE activity and damage in children with SLE.

**Design/Methods:** In this cross-sectional study, children with SLE (6–18 years) and parents completed child/parent versions of:

Childhood Health Assessment Questionnaire (CHAQ), Pediatric QOL Inventory (PedsQL Generic 4.0 and Rheumatology 3.0 modules). Physician completed: SLE Disease Activity Index (SLEDAI) and Systemic Lupus International Collaborating Clinics/ACR Damage Index (SLICC). Number of days over the prior 30 the child missed school due to physical/mental health reasons was recorded using PedsQL family information form. Spearman correlations were determined between number of missed school days and other variables.

**Results:** 24 children (23 girls) with SLE (mean age  $15 \pm 3$  years, mean education 9<sup>th</sup> grade, mean SLE duration  $46 \pm 30$  months, SLEDAI 0-24, SLICC 0-6, median CHAQ 0.3, mean PedsQL-Generic  $67 \pm 20$ ), and 19 parents (median CHAQ 0, mean PedsQL-Generic  $69 \pm 18$ ) participated. 4 children were excluded because school attendance was inapplicable. In 30 days prior to participation, 10 children (50%) missed school with a mean of  $3 \pm 7$  days. Mean number of days too ill to play =  $3 \pm 7$  and mean number of days needed someone =  $3 \pm 6$  days. The number of missed school days moderately correlated with decreased QOL as reported by parents and as measured by the PedsQL-Generic module ( $r$  0.56,  $p$  0.02), but did not correlate significantly with Rheumatology module, CHAQ, SLEDAI, SLICC, or any of the child reported scores.

**Conclusions:** Number of missed school days is correlated with decreased general QOL in children with SLE as perceived by their parents. However, parallel correlation between missed school days and overall QOL as perceived by children was not identified. Discrepant perception between parents and children warrant further investigation in a larger cohort. Lack of correlation of school attendance with other scales suggests that generic scale captures a less tangible element of SLE.

### F2.57. Improvement of Antimycobacterial Therapy Due to IL-10 Activity Blockage Is Strain Dependent.

S. Roque,<sup>1,2</sup> C. Nóbrega, R. Appelberg,<sup>1</sup> M. Correia-Neves.<sup>1,3</sup> <sup>1</sup>Laboratory of Microbiology and Immunology of Infection, Institute for Molecular and Cell Biology (IBMC), Porto; <sup>2</sup>Mestrado de Imunologia Clínica, Universidade da Beira Interior; <sup>3</sup>Instituto Superior de Saúde do Alto Ave (ISAVE), Fontarcada, Portugal.

*Mycobacterium avium* infection is a common opportunistic infection in immunocompromised patients. Treatment against these infections is complex and not always effective. Antimycobacterial treatment implies a combination of two or more drugs administered for long periods. Furthermore, this chemotherapy repeatedly fails to induce sterile cure, and the occurrence of antibiotic-resistant bacteria is not a rare event. IL-10 is a cytokine with pleiotropic activities. IL-10 major effects are associated with its anti-inflammatory and immunosuppressive properties. Particular interest on IL-10 results from its ability to increase susceptibility to infection in several mouse models. In fact, previous studies from our group have shown that abrogation of IL-10 activity improves the efficacy of antimycobacterial drugs in BALB/c mice.

Surprisingly, in the present study we show that this IL-10 effect seems to be strain specific. Combination of anti-IL-10 receptor mAb administration and antimycobacterial therapy (clarithromycin, rifampicin and ethambutol) in BALB/c and C57BL/6 chronically infected with *M. avium* (strain 2447) only increased response to treatment in the BALB/c strain. Whether this represents strain differences in the immune response to *M.*

*avium* during antimycobacterial treatment is currently being investigating.

## **F2.58. Quality Control of DNA with the Agilent 2100 Bioanalyzer for Oligonucleotide Array CGH (aCGH).**

Samar Lightfoot,<sup>1</sup> Hans Brunnert,<sup>2</sup> Carsten Buhlmann,<sup>2</sup> Paige Anderson.<sup>1</sup> <sup>1</sup>Agilent Technologies, Palo Alto, USA; <sup>2</sup>Agilent Technologies, Waldbronn, Germany.

Comparative genomic hybridization (CGH) measures copy number variations at multiple loci simultaneously, providing an important tool for studying cancer and developmental disorders, and for developing diagnostic and therapeutic targets. We have recently developed an oligonucleotide array platform for array based comparative genomic hybridization (aCGH) analyses that can detect and map copy number alterations in the human genome, including single copy losses and gene specific homozygous deletions, as well as amplicons of varying sizes.

The application of this technology to the study of human disease requires adequate quality control of DNA sample preparation prior to hybridization. The Agilent 2100 bioanalyzer and associated RNA assays are now established industry standards for checking the integrity of RNA samples. However, in addition to RNA sample analyses, the bioanalyzer has on-chip electrophoresis capabilities for DNA analysis. Here we investigated the use of the bioanalyzer for monitoring critical steps in the workflow of an aCGH experiment including DNA amplification, digestion of template and labeling. Our results demonstrates that the bioanalyzer can robustly monitor the quality and quantity of DNA templates used in aCGH experiments.

## **F2.59. Abstract withdrawn.**

### **Poster Session 1**

7:30 AM–1:30 PM, 5/14/2005

### **Autoimmune Neurologic Diseases**

#### **Sa1.01. Receptor-Modified T Cells as Novel Therapeutic Approach for Multiple Sclerosis.**

I. Moisini, T. L. Geiger. <sup>1</sup>Pathology, St. Jude Children's Research Hospital, Memphis, TN, USA; <sup>2</sup>Pathology, University of Tennessee Health Science Center, Memphis, TN, USA.

Multiple sclerosis is a chronic autoimmune disease mediated by T cells reactive to different antigenic peptides of the myelin sheath. Experimental autoimmune encephalomyelitis induced with peptides such as MBP, MOG, PLP emulsified in CFA recapitulates the human disease in mice. T cells re-directed against the antigen-specific T-lymphocytes that mediate immunopathology have been previously shown to be selective and effective as a targeted therapy of experimental autoimmune encephalomyelitis (Mekala et al. Nature Biotechnology 2002). To develop humanized receptors capable of re-directing therapeutic T lymphocytes against potentially disease-causing cells, we linked the immunodominant epitope, 84-102, of the human myelin basic protein MBP to the extracellular and transmembrane domains of the HLA-DR2 beta chain (DRB1\*1501) and the TCR-zeta cytoplasmic domain. We similarly linked the DR2 alpha chain (DRA\*0101) to the TCR-zeta. The alpha and beta chain constructs (denoted as HC2) were linked using the TMV 2A sequence and subcloned into the MSCV-I-GFP retroviral vector. GFP positive, retrovirally transduced 4G4 hybridomas and

C57Bl/6J CD8<sup>+</sup> T cells were isolated by flow cytometry. Western and flow cytometric analysis showed the expression of the construct. In order to assay the specificity of effector HC2 cells we used two different types of target cells: Ob hybridoma specific for human peptide MBP<sub>84-102</sub>/MHC II DR2 (courtesy of Dr. Lars Fugger, Skejby Hospital, Århus) and hMBP<sub>84-102</sub>-specific T cell line derived from humanized DR2xTCR double-transgenic mice. Recognition of the chimeric receptor by hMBP-specific cells was shown by the production of specific cytokines (interleukin-2 and interferon-gamma) by the HC2-transduced T cells. Recognition of target cells by HC2-transduced T cells also induced proliferation of the transduced therapeutic cells. We have also shown that cytotoxic HC2 cells could specifically recognize the cognate TCR of MBP<sub>84-102</sub>-reactive target cells and kill the target cells *in vitro*. *In vivo* studies in a humanized murine model system, validating the use of these modified T cells as a cellular immunotherapeutic agent capable of selecting targeting pathologic antigen-specific T cells, are ongoing.

#### **Sa1.02. The Role of Leukemia Inhibitory Factor (LIF) in Experimental Autoimmune Encephalomyelitis (EAE).**

R. A. Linker,<sup>1</sup> A. Wiczarkowicz,<sup>1</sup> S. Weikard,<sup>2</sup> N. Kruse,<sup>1</sup> C. Kleinschnitz,<sup>2</sup> B. Holtmann,<sup>3</sup> M. Sendtner,<sup>3</sup> R. Gold.<sup>1</sup> <sup>1</sup>Institute for Multiple Sclerosis Research, University of Goettingen and Gemeinnuetzige Hertie-Stiftung, Goettingen, Germany; <sup>2</sup>Dept. of Neurology, Clinical Research Group for Multiple Sclerosis, University of Wurzburg, Wurzburg, Germany; <sup>3</sup>Institute for Neurobiology, University of Wurzburg, Wurzburg, Germany.

LIF is a neurotrophic cytokine, belonging to the IL-6 family of cytokines. It plays a role in oligodendrocyte survival, but also in stem cell biology. Here we searched for possible immune functions of LIF.

RT-PCR analysis proved the presence of LIF receptor beta on murine macrophages, dendritic cells and T-cells and its upregulation by activation. We studied myelin-oligodendrocyte glycoprotein peptide aa 35-55 (MOG 35-55) induced EAE in LIF knockout mice (LIF <sup>-/-</sup> mice). LIF <sup>-/-</sup> mice suffered from a less severe course of EAE in comparison to wild-type mice until day 70 p.i. ( $n = 10/10$ ). In primary lymph node proliferation assays, lymphocytes from LIF <sup>-/-</sup> mice responded less to MOG 35-55 than those from wild-type mice, but exhibited a similar mitogen response. The defect in antigen-specific proliferation could not be restored by addition of LIF or IL-6 to cell culture. Supernatants from primary lymph node cultures were used to investigate cytokine production after immunization with MOG 35-55. LIF <sup>-/-</sup> mice displayed decreased levels of MCP-1 and IFN- $\gamma$  whereas no difference in the production of IL-12, IL-6 or IL-5 was observed. Finally, a histological analysis was performed to investigate the inflammatory reaction *in situ* after immunization with MOG 35-55. The blinded quantification of CD3 and Mac-3 positive cells revealed significantly lower numbers of T-cells and macrophages in the spinal cord of LIF <sup>-/-</sup> mice on day 60 p.i. These results were paralleled by a decrease of MCP-1 and GM-CSF mRNA levels in the spinal cord of LIF <sup>-/-</sup> mice on day 40 p.i.

In summary, our results are in line with a defective antigen-specific T-cell priming and a selective defect in cytokine/chemokine production in LIF <sup>-/-</sup> mice. This may result from impaired recruitment of antigen presenting cells (APC) and disturbed APC-T-cell interaction overall leading to a less severe course of MOG 35-55 EAE. The direct effects of LIF on T-cells are currently under investigation.

**Sa1.03. Novel Immunotoxin: A Fusion Protein Consisting of Gelonin and an Acetylcholine Receptor Fragment as a Potential Immunotherapeutic Agent for the Treatment of *Myasthenia gravis*.**

M. Hossann,<sup>1</sup> Z. Li,<sup>2</sup> Y. Shi,<sup>2</sup> U. Kreilinger,<sup>1</sup> J. Buettner,<sup>1</sup> P. D. Vogel,<sup>1</sup> Y. Jingming,<sup>2</sup> J. G. Wise,<sup>1</sup> W. E. Trommer.<sup>1</sup> <sup>1</sup>Chemistry, TU Kaiserslautern, Kaiserslautern, Germany; <sup>2</sup>Biotechnology, Shanxi University, Taiyuan, China.

In continuation of our attempts for antigen-specific suppression of the immune system (Urbatsch, I.L., Sterz, R.K.M., Peper, K., and Trommer, W.E. (1993) Eur. J. Immunol. 23, 774-779) a fusion protein composed of amino acids 4-181 of the extracellular domain of the  $\alpha$ -subunit of the human muscle acetylcholine receptor and the plant toxin gelonin was expressed in *E.coli*.

The fusion protein formed inclusion bodies but could be solubilized in the presence of guanidinium chloride. It exhibited a rather native structure as shown by antibodies recognizing a conformational epitope. Half maximal inhibition of translation was achieved at 46 ng/mL as compared to 4.6 ng/mL for native and 2.4 for recombinant gelonin.

Its use as therapeutic agent for the treatment of *Myasthenia gravis* was investigated in an animal model. Female Lewis rats were immunized with complete acetylcholine receptor from the electric ray *Torpedo californica* and developed thereafter experimental autoimmune *Myasthenia gravis*. Quantitative assessment of the disease was achieved by repetitive stimulation of the sciatic nerve. Rats showed no more symptoms of *Myasthenia gravis*, neither visually nor electrophysiologically after treatment with the fusion protein as determined one and seven weeks after the second application.

**Sa1.04. Fumarate Therapy Ameliorates Chronic Experimental Autoimmune Encephalomyelitis (EAE).**

S. Schilling,<sup>1</sup> S. Goelz,<sup>2</sup> R. A. Linker,<sup>1</sup> F. Luhder,<sup>1</sup> R. Gold.<sup>1</sup> <sup>1</sup>Institute for Multiple Sclerosis Research, University of Goettingen and Gemeinnuetzige Hertie-Stiftung, Goettingen, Germany; <sup>2</sup>Biogen Idec, Cambridge, USA.

**Background:** Treatment with fumaric acid derivatives is well established as an effective therapy in severe psoriasis, a Th1 mediated skin disease. It has been proposed that one of the underlying ways that fumaric acid works is by inducing a Th1 to Th2 shift. In this study we investigated the clinical and molecular effects of Dimethyl fumarate (DMF) and Monomethyl fumarate (MMF) in chronic EAE, a model for multiple sclerosis.

**Methods:** C57BL/6 mice were immunized with myelin-oligodendrocyte glycoprotein peptide aa 35-55 (MOG 33-35)/ CFA and received pertussis toxin. 3 groups of 8 mice/group were treated from day 3 to day 30 twice a day with 5 mg/kg body weight Dimethyl fumarate (DMF), Monomethyl fumarate (MMF) or the vehicle alone. Medication was administered by oral gavage. Animals were weighed and scored for clinical signs of disease on a daily basis over 30 days. Mice reaching paraplegia had to be sacrificed due to animal experimentation laws. Blood samples were taken from all mice before immunization, at the peak of the disease (day 11) and in partial remission (day 21), plasma protein concentration of 60 cytokines, chemokines and other markers was measured by Multi-Analyte Profile (MAP) testing.

**Results:** In the control group and MMF treated group, 5/8 mice suffered from paraplegia, whereas in the DMF group only 2/8 mice reached this clinical endpoint. Onset of disease was earlier in the

MMF treated group (mean: day 11,8) compared to DMF (mean: day 13,6) and control group (mean: day 12,3), this was not significant. DMF treated mice had a significantly less severe clinical course in this preventive treatment approach,  $p < 0,001$ . Blood samples are currently analyzed using a Multi-Analyte Profile including 60 cytokines and chemokines, and histological studies are ongoing.

**Conclusion:** Our data suggest that DMF has a beneficial effect in chronic EAE. We will present cytokine and histological results pending further analyses.

**Sa1.05. Beneficial Autoimmunity Restrains Destructive Immunity in a Regulatory Manner.**

Nathan Karin,<sup>1</sup> Yaniv Zohar,<sup>1</sup> Uri Weinberg,<sup>1</sup> Rachel Anunu,<sup>1</sup> Gizi Wildbaum.<sup>1</sup> <sup>1</sup>Immunology, Rappaport Faculty of Medicine, Technion, Haifa, Israel.

In previous studies we have shown that targeted DNA vaccines encoding selected proinflammatory mediators, particularly chemokines and cytokines, could effectively suppress experimentally induced autoimmune diseases in a selective manner. For example: targeted DNA vaccine encoding the CC chemokine RANTES (CCL5) could selectively suppress experimentally induced rheumatoid arthritis, but had no beneficial effect on experimental autoimmune encephalomyelitis (EAE), whereas DNA vaccines encoding MCP-1 (CCL2) effectively suppressed both diseases. The beneficial effect of each vaccine could be transferred by chemokine-specific autoantibodies developed in protected donors.

Throughout these studies we have repeatedly observed an unexpected phenomena. The elicitation of beneficial autoantibody production, during ongoing autoimmunity, is very rapid. In the current presentation we shall show that this rapid response results from an amplification of a regulatory response induced by the disease itself. We shall show the high relevance of this regulatory mechanism to human disease and its therapeutic implications for autoimmunity and cancer.

**Key reference:**

Wildbaum, G., Nahir, M. & Karin, N. Beneficial autoimmunity to proinflammatory mediators restrains the consequences of self-destructive immunity. *Immunity*, 19, 679-688 (2003).

**Sa1.06. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Regulatory T Cells Are a Highly Apoptosis Sensitive T Cell Population with an Impaired Suppressive Function in Multiple Sclerosis.**

B. Fritzsche,<sup>1,2</sup> N. Oberle,<sup>1</sup> N. Eberhardt,<sup>1</sup> J. Haas,<sup>2</sup> M. Korporal,<sup>2</sup> B. Wildemann,<sup>2</sup> P. H. Kramer,<sup>1</sup> E. Suri-Payer.<sup>1</sup> <sup>1</sup>Department of Immunogenetics, German Cancer Research Center, Heidelberg, Germany; <sup>2</sup>Section of Molecular Neuroimmunology, Department of Neurology, University of Heidelberg, Heidelberg, Germany.

CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells suppress human and murine T-cell function. The role of regulatory T cells in human disease and its modulation are of major interest. Enhanced survival and expansion of regulatory T cells would be beneficial in autoimmune disease. In contrast, increased depletion by apoptosis would be advantageous in cancer. In addition to their described sensitivity to IL-2 deprivation, we show that freshly isolated FACS-sorted human regulatory T cells are highly sensitive towards CD95-dependent apoptosis in contrast to their CD4<sup>+</sup>CD25<sup>-</sup> T cell counterparts. However, restimulation of expanded regulatory T cells revealed a reduced sensitivity towards activation induced cell death (AICD) in contrast to AICD-sensitive CD4<sup>+</sup>CD25<sup>-</sup> T cells. Simultaneously, expanded regulatory T cells remained highly

sensitive towards CD95L-triggered apoptosis. Murine CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells displayed a similar sensitivity. Our data suggest a model in which CD4<sup>+</sup>CD25<sup>-</sup> effector T cells could modulate the number of regulatory T cells via the CD95L/CD95 system in the contraction phase of an immune response. Furthermore, we found a defective suppressive function of regulatory T cells in Multiple Sclerosis (MS) patients. Given known alterations of the CD95 system in MS we are investigating whether an altered sensitivity of regulatory T cells towards CD95-dependent apoptosis could be critical for the modulation of defective regulatory response in Multiple Sclerosis.

#### **Sa1.07. Accumulation of CD4+CD25+ Regulatory T Cells in the CNS during Recovery from EAE.**

*M. J. McGeachy,<sup>1</sup> L. A. Stephens,<sup>1</sup> S. M. Anderton.<sup>1</sup>* <sup>1</sup>*Institute of Immunology and Infection Research, University of Edinburgh, Edinburgh, United Kingdom.*

Many studies focus on the role of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs) in preventing the onset of autoimmunity. Here we have investigated the regulation of activated pathogenic T cells in a spontaneously remitting model of autoimmune disease- experimental autoimmune encephalomyelitis (EAE). EAE was induced by immunization of C57BL/6 mice with the central nervous system (CNS) autoantigenic peptide MOG(35-55). In our hands this leads to the development of EAE from 7 days post-immunization, with peak of disease in the second week, followed by a recovery phase with most mice free of clinical signs by 30 days post-immunization. The mice are subsequently resistant to a second round of disease when re-immunized with MOG(35-55). Spinal cord and draining lymph node (LN) cells were purified from mice at different time-points during disease. Cells were phenotypically examined by FACS analysis, and functionally tested for their ability to proliferate or suppress proliferation and cytokine production of naïve or primed cells in vitro. We found that the proportion of CD4<sup>+</sup> cells expressing CD25 in the CNS increased during the course of disease, correlating with recovery. These CD4<sup>+</sup>CD25<sup>+</sup> cells preferentially produced IL-10, while IFN $\gamma$ -secreting CD4<sup>+</sup> cells were CD25<sup>-</sup>. In vitro, CD4<sup>+</sup>CD25<sup>+</sup> CNS cells were anergic, but proliferated in response to anti-CD3 in the presence of IL-2. These CD25<sup>+</sup> cells could also suppress proliferation of responder CD25<sup>-</sup> cells in vitro. Anti-CD25 (PC61) antibody was used to deplete CD25<sup>+</sup> cells in vivo 3 days prior to EAE induction. This depletion led to exacerbated disease with greatly delayed recovery, supporting a functional role for Tregs in remission. Interestingly, CD25-depletion after recovery rendered mice fully susceptible to reinduction. Moreover, transfer of CNS-derived CD25<sup>+</sup> cells led to accelerated recovery from subsequent EAE. In conclusion, accumulation of CD4<sup>+</sup>CD25<sup>+</sup> Tregs (producing IL-10) in the CNS during the recovery phase of EAE suggests a role in the control of myelin-reactive pathogenic T cells. Our hypothesis is that autoantigen-reactive regulatory T cells are expanded or induced in draining lymph nodes, then recruited to the effector site to aid in the resolution of this autoimmune disease.

#### **Sa1.08. The Pathology of Progressive Multiple Sclerosis.**

*A. E. Kutzelnigg,<sup>1</sup> C. Lucchinetti,<sup>2</sup> W. Brueck,<sup>3</sup> M. Schmidbauer,<sup>4</sup> H. Lassmann.<sup>1</sup>* <sup>1</sup>*Department of Neuroimmunology, Brain Research Center, Medical University Vienna, Vienna, Austria;* <sup>2</sup>*Department of Neurology, Mayo Clinic, Rochester, MN, USA;* <sup>3</sup>*Institute of Neuropathology, University of Goettingen, Goettingen, Germany;* <sup>4</sup>*Department of Neurology, Municipal Hospital Lainz, Vienna, Austria.*

Multiple sclerosis is defined as inflammatory demyelinating disease of the white matter of the central nervous system. While the functional neurological deficit of patients with acute or relapsing-remitting MS can be explained by the focal white matter lesions in the CNS, this is not the case for patients with primary or secondary progressive MS who experience a gradual accumulation of their clinical deficit. So far no pathological feature of the disease has been described which clearly distinguishes relapsing-remitting from progressive disease in MS patients.

In the present study, we systematically analyzed cortical and white matter pathology in a large sample of multiple sclerosis brains with different disease courses (11 cases with acute MS, 6 with RRMS, 20 with SPMS and 14 with PPMS). Hemispheric and double hemispheric tissue sections were examined for cortical demyelination and pathological changes in the white matter which offered the unique opportunity to evaluate disease involvement of large tissue areas.

Cortical demyelinated plaques were abundant in patients with primary or secondary progressive MS, but were virtually absent in patients with acute or RRMS (percentage of demyelinated cortical area: acute MS: 0,36; RRMS: 4,54; PPMS: 14,89; SPMS: 21,22;  $P = 0,0014$ ). Focal load of demyelinated lesions in the white matter was almost the same in the four groups.

Similar results were obtained from our analysis of the cerebellar cortex (percentage of demyelinated cortical area: acute MS: 0,85; RRMS: 1,89; PPMS: 33,28; SPMS: 38,2;  $P = 0,042$ ).

Especially in primary, but also in secondary progressive MS cases, myelin pallor was observed in the normal appearing white matter, which was associated with significant inflammation as well as microglia and macrophage activation. These pathological changes were sparse in acute and RRMS brains (inflammatory infiltrates per mm<sup>2</sup>: acMS: 0,05; RRMS: 0,07; PPMS: 0,20; SPMS: 0,30;  $P = 0,019$ ). No correlation between size and location of white matter plaques and cortical demyelination or diffuse white matter damage was observed.

In conclusion, we suggest that in MS brains three different pathological processes occur, which are stage-specific and occur at least in part independently from each other: focal white matter plaques, cortical demyelination and diffuse damage of the white matter.

All pathologies occur on the background of inflammation although the type of inflammation is different. Focal white matter lesions seem to be due to new waves of inflammation entering the brain with BBB damage. With chronicity of the disease, inflammatory cells accumulate gradually throughout the whole CNS, leading to diffuse damage in the "normal" white matter and the cortex.

#### **Sa1.09. Endoneural CD34+ Fibroblasts: Putative Regulators of Macrophage Activation in the Peripheral Nerve?**

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Using mouse models of distinct hereditary demyelinating neuropathies (heterocytous P0-deficiency, P0<sup>+</sup>- and homocytous CX32-deficiency, CX32<sup>-</sup>) we could recently demonstrate that T-cells and macrophages are involved in the pathogenesis of hereditary demyelinating disorders. Beyond these immune cells we have also found blood-derived CD34<sup>+</sup> fibroblast-like



cells in the endoneurium of P0+/- mice. We supposed that these fibroblast-like cells might be identical to the recently described population of CD34+ peripheral blood fibrocytes and might therefore comprise another cell type of potential importance for immune regulation in hereditary demyelinating neuropathies.

In this study we further characterized this novel cell population in the endoneurium of P0+/- mice. We observed that these fibroblast-like cells show close contacts with endoneurial macrophages. Contact sites between these two cell populations were found regularly under normal and demyelinating conditions. The interaction between these two cell populations seems to play a role for immune regulation within the peripheral nerves since immunodeficient P0+/-RAG- mice lack an age-related increase of CD34+ cells that parallels the occurrence of pathological changes within the peripheral nerves of P0+/- mice. We suppose that this lack of CD34+ fibroblast-like cells might result in altered macrophage activation.

In conclusion we could show that macrophages and fibroblast-like cells have close contacts within the endoneurium. According to our morphological observations we hypothesize that CD34+ fibroblast-like cells are involved in regulation of macrophage function under demyelinating conditions.

#### Sa1.10. Decreased T-Cell Immunity to Epstein-Barr Virus in Multiple Sclerosis.

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**Introduction:** Increasing evidence indicates that infection with the Epstein-Barr virus (EBV) has a role in the pathogenesis of many human chronic autoimmune diseases, including multiple sclerosis (MS). We have proposed a new hypothesis that chronic autoimmune diseases occur in individuals genetically susceptible to the effects of B-cell infection by EBV, and that the EBV-infected B cells not only produce autoantibodies but also inhibit activation-induced apoptosis of autoreactive T cells in the target organ (Trends Immunol 24 (2003) 584-588; <http://eprint.uq.edu.au/archive/00001146>).

**Aims:** This study aims to determine whether patients with MS have an increased frequency of EBV-infected immortalized B cells and defective immunity against latent EBV infection, which may lead to the development of MS.

**Methods:** Sera from patients with MS (not on immunomodulatory therapy) and healthy controls are tested for the presence of anti-EBV viral capsid antigen (VCA) IgG or anti-EBV nuclear antigen (EBNA) IgG using ELISA. We are using real-time PCR to quantify the EBV DNA load in the cerebrospinal fluid (CSF) and peripheral blood mononuclear cells (PBMC) of patients with MS. We are also performing IFN-gamma ELISPOT assays to measure peripheral blood T-cell reactivity against major histocompatibility complex (MHC) class-I-restricted latent and lytic EBV peptides, MHC class-II-restricted EBV peptides and human cytomegalovirus (HCMV) peptides.

**Results:** Our preliminary results show detectable levels of EBV DNA in the CSF in 3 of 8 patients with MS. Furthermore, our results from the IFN-gamma ELISPOT assays indicate a reduction in the T-cell response to MHC class-I-

restricted latent EBV peptides in MS patients compared to healthy controls.

**Conclusions:** Our findings of the presence of EBV in the CSF of MS patients and of reduced T-cell immunity against latent EBV antigens may shed light on the role of EBV infection in the pathogenesis of MS.

#### Sa1.11. HLA-DR2 Is Associated with Chronic Inflammatory Demyelinating Polyradiculoneuropathy (CIDP) in Females, but Not Males.

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CIDP is an inflammatory and demyelinating disease affecting the peripheral nervous system. It is related to the Guillain-Barré syndrome (GBS), but is a chronic condition and is distinguished from GBS by its temporal pattern and potential for clinical relapse. CIDP occurs more commonly in males than in females, and is thought to be autoimmune in nature, although this still remains unproven. Because it is thought that CIDP might have an autoimmune component, several studies have investigated HLA molecules carried by patients with CIDP. These studies have shown a trend towards increased carriage of HLA DR2 and DR3; however, since CIDP is relatively uncommon, the number of individuals studied has been small and the majority of patients have been male. Because studies in other autoimmune diseases have shown that sex-related factors appear to influence the risk associated with carriage of different HLA molecules, we set out to test a larger cohort of patients to determine if there are HLA associations with CIDP, and whether these differ depending on the gender of the patients.

We have investigated carriage of class II HLA molecules in a cohort of 100 CIDP patients (60 male), and compared this to carriage of these molecules in 90 healthy controls and 71 patients with GBS. DNA was extracted from 5 ml whole blood, which was collected after written informed consent had been obtained. Dynal low-resolution SSP kits were used to type for HLA-DR, DQA and DQB molecules, to a resolution equivalent to that of serotyped subgroups.

In comparison to female healthy controls, there was an increased carriage of DR2 by female patients with CIDP ( $P < 0.05$ ), but not by female patients with GBS. Upon further subtyping of DR2<sup>+</sup> individuals, the majority of individuals in all 3 groups were found to carry DRB1\*1501. There were no differences in the frequency of carriage of DR2 between males in any of the 3 groups. There was a trend in male CIDP patients towards increased carriage of DR6 compared to male controls, whereas there was a trend towards decreased carriage of DR6 in female CIDP patients compared to controls. Our results did not confirm any association between carriage of DR3 and development of CIDP.

There were no significant differences for frequency of carriage of DQA or DQB molecules between healthy controls, CIDP patients, or GBS patients when groups were considered as a whole, or subdivided based on gender.

These results show that gender-specific HLA-DR associations occur in CIDP. They reflect similar findings in studies of other autoimmune diseases and provide additional evidence for an autoimmune disease mechanism in CIDP. The HLA typing in this study was only done to a low resolution, and further associations between CIDP (particularly in males) and carriage of particular

HLA molecules may become apparent if the typing were done to the molecular level.

**Sa1.12. Antibodies Specific for Myelin Proteolipid Protein Are of Potential Pathogenic Relevance in Myelin Opsonization in Multiple Sclerosis.**

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Myelin proteolipid protein (PLP) is the most abundant protein of central nervous system myelin and is a putative target antigen in multiple sclerosis (MS). Increased levels of T cell reactivity directed against PLP have been well-documented in MS. We have found that there are also increased levels of antibodies specific for PLP, particularly for the extracellular loop consisting of amino acids 180-230, in patients with MS compared to healthy controls and patients with other neurological diseases (OND). The aim of the current study was to determine whether these antibodies could have any pathogenic relevance in MS.

Sera from 70 patients with MS (12 with primary progressive MS, 14 with secondary progressive MS and 44 with established relapsing-remitting MS or a first attack of MS), 25 patients with OND and 25 healthy controls were screened for their ability to opsonize DiI-labelled purified human myelin and thereby increase the uptake of the myelin into macrophages. The percentages of individuals in each group whose sera induced levels of myelin uptake greater than 2 standard deviations higher than the mean of the healthy control group were 69% for MS, 0% for healthy controls and 16% for OND patients. Over 70% of the sera from secondary or primary progressive MS patients induced increased levels of myelin uptake by macrophages. The percentage of patients with established relapsing-remitting MS or a first attack whose sera induced increased levels of myelin uptake by macrophages was slightly lower (59%).

Of those sera that induced increased myelin uptake, 60% contained antibodies specific for the PLP180-230 region, as assessed by ELISA. Preadsorption of sera with peptides from this region of the PLP molecule was found to be able to inhibit partly or wholly the opsonizing potential of those sera that showed PLP reactivity in ELISA. Thus, approximately 40% of patients with MS contain PLP-specific antibodies in their sera that can opsonize myelin for phagocytosis.

Antibodies could have functional pathogenic effects in MS via a variety of mechanisms such as causing demyelination by antibody-dependent cell-mediated cytotoxicity or complement-dependent lysis, or via opsonization of myelin resulting in phagocytosis. In addition, they may induce upregulation of MHC and costimulatory molecules in the CNS, inhibit remyelination, or cause axonal damage and modulation of synaptic transmission. An antibody that could bind to extracellular epitopes of intact myelin (such as PLP180-230) would be more likely to be directly pathogenic than one that recognizes only disrupted myelin, although the later could be involved in escalating inflammation by activating the complement cascade. Our results show that PLP-specific antibodies can exhibit at least one of these potential pathogenic effects.

Identification of MS patients exhibiting elevated pathogenic antibody responses may be an important step in selecting patients for treatment with intravenous immunoglobulin or plasmapheresis to decrease this activity.

**Sa1.13. Autoantigen Specific T Cells Inhibit Glutamate Uptake in Astrocytes by Decreasing Expression of Astrocytic Glutamate Transporter GLAST- A Mechanism Mediated by Tumor Necrosis Factor- $\alpha$ .**

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Glutamate excitotoxicity is increasingly being recognized as pathogenic mechanism in autoimmune inflammatory disorders of the central nervous system (CNS). Astrocytes are the predominant players in clearing the extracellular space from glutamate and normally have extensive spare capacities in terms of glutamate uptake. We asked what might be the basis of glutamate accumulation in T cell-triggered autoimmune inflammation. In vitro, exposure of primary rat astrocytes to antigen-activated myelin basic protein (MBP)-specific T cells resulted in the decrease of astrocytic glutamate uptake rates as far as  $v_{max}$  was concerned. In parallel, the amount of the astrocytic  $Na^+$ -dependent glutamate transporter GLAST was reduced in a time range of 48 to 60 h. Similar decreases of GLAST protein were observed in astrocytes re-isolated after co-culture with T cells activated by MBP and astrocytes as antigen presenting cells (APCs) or after co-culture with T cell blasts pre-activated in the presence of splenocytes beforehand. Since incubation of astrocytes with cell-free supernatant from MBP-activated T cells also resulted in a reduced expression of GLAST, a humoral factor appeared to be the driving agent. In blocking experiments using neutralizing antibodies and by exposure of astrocytes to recombinant cytokines, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) was identified to be responsible for the down-modulation of GLAST. GLAST was also down-regulated in the CNS of autoimmune encephalitic rats, but not in animals suffering from systemic inflammation. Since the loss of GLAST was not confined to inflammatory infiltrates, here too, a humoral factor seemed to be causative. In conclusion, T cell-derived TNF- $\alpha$  impaires glutamate clearance capacity of astrocytes in vitro and probably also in vivo in autoimmune inflammatory disorders providing a pathogenic link to glutamate excitotoxicity that may be responsible for early axonal dysfunction remote from active inflammatory demyelination.

**Sa1.14. The MHC Is the Major Determinant for the Requirement of B7.1/7.2-Costimulation in the Induction of Experimental Autoimmune Encephalomyelitis (EAE).**

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Full activation of T-cells requires costimulatory signals such as B7.1/B7.2-CD28/CTLA-4 interaction besides MHC-peptide-TCR signalling. We have previously shown in B7.1/B7.2-deficient (DKO) mice that the genetic background (SJL/J versus C57BL/6J) determines the requirement for B7 costimulation in induction of EAE. Whereas, DKO on the B6 background were highly resistant to the development of EAE following immunization with the encephalitogenic MOG 35-55 peptide, DKO mice

on the SJL mice remained susceptible to EAE when immunized with the PLP 139-151 peptide. Since the two strains differ in their MHC molecules, thus differ in peptide binding and the selection and expansion of the encephalitogenic T cell repertoire, we examined the role of the MHC as a determinant for the requirement of B7 costimulation in EAE. In 88 [SJLx(SJLxB6 F1)] DKO back-cross 1 (BC1) mice we found that homozygosity of the *s* haplotype is a strong susceptibility factor for clinical and histological PLP139-151-induced EAE in these mice. In order to detect further susceptibility regions we studied MOG 35-55-induced EAE in 204 (SJLxB6) F2 DKO mice which were selected for at least one *b* allele at the MHC locus. No additional susceptibility regions were identified. Our results indicate that the expression of class II molecules (homozygosity of the *s* allele at the H2-A locus) is the major susceptibility factor which determines the requirement and dependence for B7-costimulation for the induction of EAE.

#### **Sa1.15. Anti-CTLA4 scFv, a Single-Chain Antibody, Attenuated Host Humoral Immune Responses during Repeated DNA Vaccination in Immune Competent Animals.**

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**Objectives:** To induce tolerance in immune-competent animals with a plasmid (p4F10-eB7) expressing a membrane-bound single-chain antibody against CTLA4. **Materials and Methods:** Thirty 9-week old immune-competent C57B/L6 mice were divided into three groups. Ten mice received an intramuscular injection of endotoxin-free plasmid, pCBLacZ, at the left anterior tibialis. The plasmid encodes a potent immunogen, *Escherichia coli* beta-galactosidase. Ten mice received a co-injection of p4F10-eB7 and pCBLacZ. Another ten mice received five consecutive intramuscular injections of the plasmid. Five of them received injection of pCBLacZ alone. The other five received coinjection of two plasmids. **Results:** For single injection, we examined the expression of beta-galactosidase in muscle fibers on the 6<sup>th</sup> and 12<sup>th</sup> days after injection. Significant difference of transduced muscle fibers was only found 6 days after single injection. However, serum IgG against beta-galactosidase was much lower in the animals received consecutive injections of two plasmids as compared with those in the control group. **Conclusions:** Expression of anti-CTLA4 antibody in muscle cells protected the expression of beta-galactosidase in immune-competent mice. This membrane bound single-chain antibody attenuated host humoral immune responses during repeated immunization. The probable mechanism is peripheral tolerance induced by anergy of activated T cells after interaction with the single-chain antibody. This novel strategy targeting on activated T cells may be used for treatment of acquired autoimmune diseases.

#### **Sa1.16. Memory CD4+ T Cells and EAE.**

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The memory T cell pool functions as a dynamic repository of antigen-experienced T lymphocytes that accumulate over the

lifetime of the individual. Previous studies indicate that T cells which are reactive to myelin antigens are mainly memory cells in MS patients. We hypothesize that there are specific mechanisms of disease mediated by memory T cells. Here, we developed an animal model of memory CD4+ T cell-inducing EAE. Wild-type (WT) or MOG TCR transgenic memory CD4+ T cells were generated from WT B6 or 2D2 transgenic mice respectively, immunized with MOG/CFA for more than 100 days. CD4+CD44hi cells were sorted and immediately transferred into TCR ab<sup>-/-</sup> mice followed by immunization with MOG/CFA. A control group of mice immunized with MOG peptide were used to generate CD4+CD44hi effector cells. Our data indicate that disease induced by memory cells is more severe when compared to that induced by effector cells. Confocal double-labeling images showed CNS infiltration of CD4+ memory cells associated with astrocytic activation and dramatic demyelination as shown by GFAP-positive cells and SMI32 staining, respectively. Furthermore, sorting of memory CD4+CD44hi according to the expression of either CD62L or CCR7 providing central (TCM) or effector (TEM) memory T cells demonstrated more severe EAE induced by TEM than TCM associated with increase proliferation and high production of IFN $\gamma$  in response to MOG peptide in vitro. Costimulatory signal blockade of T cells in this model, demonstrated that while CTLA4Ig suppressed effector CD4+CD44hi-induced EAE, it did not significantly inhibit disease induced by memory CD4+CD44hi cells. By contrast, anti-ICOS-L treatment worsened effector cell-induced EAE, but inhibited memory cell-induced disease. Our data suggest the presence of specific mechanisms of disease mediated by memory CD4+ cells and has relevance to the treatment of human autoimmune disease by costimulatory signal blocking agents.

#### **Sa1.17. Therapeutic Effects of Glycosylated $\beta$ Interferon ( $\beta$ IFN) on Childhood Adrenoleukodystrophy (ALD CCER).**

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Childhood Cerebral Adrenoleukodystrophy (ALD CCER) is a genetic disease. Based on some bibliographic research and our own experience we conjecture that the lesions it causes in the central nervous system (CNS) are the result of an autoimmune demyelinating reaction resembling the Multiple Sclerosis (MS) inflammatory reaction.  $\beta$ IFN has shown a positive therapeutic action on MS; for that reason, we developed a clinical trial to test its effect on ALD CCER. Thirteen boys affected by the disease were divided into three groups: Group A, 5 patients with a Behavior Performance Index (BPI) of over 80 points, received  $\beta$ IFN; Group B, 5 patients with a BPI of under 80 points, received  $\beta$ IFN; and Group C, 3 patients with a BPI of under 80 points, did not receive medication. The  $\beta$ IFN was supplied by Ares-Serono International. The dosage schedule was 3 to 6.106 IU of  $\beta$ IFN by intramuscular injection, at 8 p.m. on three consecutive days every week. The patients were followed for 24 months via monthly neurological, psychological and immunological evaluation and by telephone follow-up thereafter to assess survival and general clinical and neuropsychological performance. The results of our small clinical trial strongly suggest that  $\beta$ IFN can change the natural history of ALD CCER.  $\beta$ IFN improved the clinical conditions of 4 out of 5 patients from the first group for almost three years, and has maintained these conditions for two of these patients up to the

present (six years after stopping the bIFN treatment). The treatment also slowed the evolution of the disease in the second group. MRI, immunological and psychometric data confirmed these findings, which support the theory that the interaction of immune cells with nervous tissue cells plays a crucial role in the CNS repair, as well as that bIFN may be a therapeutic agent for it.

### **Sa1.18. Anti-Myelin Antibodies as an Immune-Modulatory Marker for IVIG Treatment in Patients with Progressive Multiple Sclerosis.**

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Multiple Sclerosis (MS) is the most common Neurological Autoimmune Disorder diagnosed in young adults and is characterized by the repeated occurrence of demyelinating lesions within the central nervous system (Pohlau, et al., 1998).

Part of the mechanism of MS is an abnormal production of auto-antibodies, specifically producing cross-reactive epitopes, which leads to an auto-immune reaction against myelin.

It is our belief that IVIG can act as a neuro-immune modulator for patients diagnosed with MS. Current research has demonstrated that, in relapsing MS, an association exists between high dose IVIG and reductions in relapse rates, exacerbations and the number and size of brain lesions, as well as increases in functional improvements.

A recent study from researchers at the University of California found that the anti-myelin antibody is found in virtually all patients with progressive multiple sclerosis (Brown, 2002). We theorize that the anti-myelin antibody may be used as a marker for the treatment of patients diagnosed with progressive MS on high dose IVIG treatment.

In our study, we report on a 43-year-old patient with a diagnosis of progressive multiple sclerosis; the disease that has resulted in rapid regression over the past 5 years. The subject presented to our clinic with a history of countless treatment's and therapies that failed to improve his condition or stall his regression. An extensive immune work-up revealed extremely low CD3 and CD8 numbers along with low lymphocyte stimulation function, an IgG3 subclass deficiency and positive anti-myelin antibodies; past records indicated the presence of positive anti-myelin antibodies for over 5 years. Upon completion of a thorough physical examination and work-up, the patient was started on high dose IVIG: 2g/kg divided over three consecutive days. Subsequent testing, conducted after a short period of time, revealed negative anti-myelin. This patient has now been on IVIG for three months. As a result of this treatment, he is beginning to display increased energy, decreased muscle spasticity and improvement in his ability to walk.

Based upon the preliminary data, we suggest that: 1) IVIG may have an immuno-modulatory effect in MS and 2) anti-myelin AB may serve as a marker for the immuno-modulation effect.

### **Sa1.19. T Cell Tolerance Induced by Cross-Reactive TCR Ligands Can Be Broken by Superagonist Ligand Resulting in Anti-Inflammatory Cytokine Production.**

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Cross-reactive activation by high affinity non-self ligands, of T cells with the potential to recognize self, may be important in breaking self-tolerance. In a TCR transgenic mouse that shows a broad cross-reactivity to a number of ligands, we have previously shown that a hyperstimulating superagonist ligand, L144 induced unresponsiveness to a self-ligand, proteolipid protein (PLP) 139-151 peptide. In a further examination of the role of cross-reactive superagonists in an autoimmune response, we demonstrate here that a superagonist ligand can break tolerance induced by the lower affinity cognate antigen. In our proposed "altered" hierarchical anergy model, T cells tolerant to cognate ligand, Q144, responded to superagonist, L144 by proliferation and the production of mainly IL-4 and IL-10 in vitro. In contrast, T cells that were tolerized to the superagonist, were unable to respond to any peptide that cross-reacted with the transgenic TCR. In vivo, low-dose immunization with superagonist was able to break tolerance to the cognate ligand, and resulted in a blunted proliferative response and Th2 cytokine production following a re-challenge in vitro. A combination of different T cell responses may thus provide a multilevel defense against autoimmunity during a T cell response against cross-reactive high-affinity foreign antigens, and may serve as an important regulatory mechanism to prevent auto-immune disease.

### **Sa1.20. Agonistic Anti-CTLA4 Antibody Inhibits T Cells Expansion, Cytokine Production and Development of Autoimmunity.**

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We generated a novel agonistic anti-CTLA4 monoclonal antibody, by immunizing Lou/M rats with the CTLA4Ig fusion protein and screened the hybridoma supernatants for binding to CTLA4 in ELISA and CHO expressing CTLA4 on their surface. Of the three antibodies thus generated, one antibody 3C1.B5 was agonistic in that inhibited T cell expansion and IFN $\gamma$  secretion in vitro and in vivo. This contrasts with the previously described anti-CTLA4 antibody 4F10, which promotes T cell clonal expansion and IFN $\gamma$  secretion presumably by blocking the inhibitory signal delivered by CTLA4. We compared the effects of both anti-CTLA4 antibodies in murine relapsing remitting autoimmune disease experimental autoimmune encephalomyelitis (EAE), a demyelinating disease mediated by PLP139-151 specific CD4<sup>+</sup> T cells in SJL /J mice. Whereas, administration of the 4F10 antibody in vivo exacerbated EAE, the agonistic anti-CTLA4 antibody 3C1.B5 ameliorated disease. To understand the molecular basis for the difference in the functional effects of the two antibodies, we undertook epitope mapping and binding studies. We demonstrate that the Tyrosine residue in the MYPPPY domain of the CTLA4 utilized for binding by the B7 molecules, is also crucial for binding of the blocking anti-CTLA4 (4F10) antibody but not for the agonistic anti-CTLA4 antibody (3C1.B5). Cross-blocking studies show that the agonistic anti-CTLA4 antibody recognizes a closely related but distinct epitope on the CTLA4 molecule. Thus binding of the two anti-CTLA4 antibodies to a closely related but distinct epitopes gives distinct functional T cell responses.

### Sa1.21. Tumor Necrosis Factor- $\alpha$ Gene Polymorphism in Iranian Patients with Multiple Sclerosis.

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Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) as an important immune regulator and inflammatory cytokine is implicated in the pathogenesis of multiple sclerosis (MS). A single nucleotide polymorphism, G to A, at position -308 in the promoter has been previously shown. This polymorphism is associated with TNF- $\alpha$  production level. To study the effect of this polymorphism on susceptibility to multiple sclerosis, we screened genomic DNA samples from 98 definite MS patients and 97 unaffected ethnically matched individuals as healthy controls, using sequence-specific primers (PCR-SSP). The results indicated that the frequency of GG genotype (related to low production of TNF- $\alpha$ ) was significantly increased in MS patients compared to controls while the frequency of GA and AA genotypes (related to high production of TNF- $\alpha$ ) decreased (df = 2,  $P$  = 0.04). Our findings suggest that polymorphism at position -308 and production levels of TNF- $\alpha$  could influence susceptibility to MS.

### Sa1.22. Corticotropin-Releasing Hormone Contributes to the Peripheral Inflammatory Response in Experimental Autoimmune Encephalomyelitis.

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Corticotropin-releasing hormone (CRH), a hypothalamic neuro-peptide, is thought to have peripheral pro-inflammatory effects outside the central nervous system. Studies have shown that T lymphocyte can produce CRH, and CRH binding site presents on macrophages and lymphocytes. Upregulation of CRH peptide production has been shown in peripheral sites with acute or chronic inflammation. Our *objective* was to study the peripheral role of CRH on T lymphocyte and antigen-presenting cell (APC) activation in an immune mediated disease model of experimental autoimmune encephalomyelitis (EAE). *Material and Method*: *Crh*<sup>-/-</sup> mice on a C57BL/6  $\times$  129 genetic background were immunized with MOG 35-55 peptide together with complete Freund's adjuvant and pertussis toxin. The severity of EAE was scored on the scale of 0-5 as described previously. To eliminate the anti-inflammatory effect of corticosterone, astressin, a peripheral CRH antagonist with no glucocorticoid inhibition effect was given to wild-type mice on day 11-16 post immunization. In comparison between *crh*<sup>-/-</sup> and *crh*<sup>+/+</sup> T cells, T cell proliferation assay and the ELISA assay of cytokine production were conducted on either naive CD4<sup>+</sup> T cells with anti-CD3/anti-CD28 TCR stimulation or MOG-specific CD4<sup>+</sup> T cells from immunized mice in response to  $\gamma$ -irradiated MOG-loaded APC stimulation. The antigen presentation function was examined by MOG-specific wild-type T cell proliferation in response to MOG-loaded *crh*<sup>-/-</sup> or *crh*<sup>+/+</sup> APCs. Anti-CD3/anti-CD28 stimulated I $\kappa$ B $\alpha$  phosphorylation in *crh*<sup>-/-</sup> splenocytes was tested by western blot analysis. *Result*: We found that *crh*<sup>-/-</sup> mice as well as astressin treated wild-type mice are resistant to EAE with decrease in clinical score as well as decreased cellular infiltration in the central nervous system.

Furthermore, antigen-specific responses of primed T cells as well as anti-CD3/anti-CD28 TCR costimulation response of naive T cells were decreased in *crh*<sup>-/-</sup> mice with decreased production of Th1 cytokines, IFN- $\gamma$  and IL-2 and increased production of Th2 cytokine IL-5. Wild-type mice treated *in vivo* with a CRH antagonist astressin showed a decrease in IFN- $\gamma$  production by primed T cells *in vitro*. This effect of CRH is independent of its ability to increase corticosterone production, since adrenalectomized wild-type mice had similar disease course and severity as control mice. Furthermore, a decreased antigen-specific T cell response was observed in wild-type MOG-specific T cells when incubated with MOG-loaded *crh*<sup>-/-</sup> APCs compared with MOG-loaded *crh*<sup>+/+</sup> APCs. We found that I $\kappa$ B $\alpha$  phosphorylation induced by TCR cross-linking was delayed and compromised in *crh*<sup>-/-</sup> T cells. *Conclusions*: Peripheral CRH exerts a pro-inflammatory effect in EAE with selective increase in Th1-type responses. This effect CRH is likely the result of optimal activation of T cells through activation of NF- $\kappa$ B pathway.

### Sa1.23. Individual Statins Differ in Immunomodulatory Potential and Capability To Treat Th1-Mediated CNS Autoimmune Disease.

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*Rationale*: Oral HMG-CoA reductase inhibitors differ in their ability to treat hypercholesterolemia. Recent studies have shown that certain statins can promote a Th2 bias and prevent or reverse relapsing and chronic paralysis in experimental autoimmune encephalomyelitis (EAE). However, it is not clear whether some statins are more effective than others in treating this autoimmune disease. Furthermore, some studies have shown purified statin administered either orally or parenterally (i.p.) can also have immunomodulatory effects. In this study we examined whether statins differ in their ability to modulate autoimmunity in EAE. We further investigated whether differences in formulation (prescription vs. purified) and route of administration (oral vs. parenteral) effect treatment and the development of Th2 bias.

*Methods*: Relapsing-remitting EAE was induced in (PL/J  $\times$  SJL/J) F1 mice by immunization with MBP Ac1-11. Prescription formulation of atorvastatin, rosuvastatin, lovastatin, simvastatin, pravastatin, and placebo was administered orally in PBS vehicle one time daily. Prescription form was administered at one and ten times the equivalent of the highest-approved, human dose. Purified atorvastatin was administered orally or parenterally. Th1 and Th2 cytokine profiles of T cells isolated from treated mice were evaluated by ELISA. Phosphorylation of STAT signaling molecules was measured by Western blot.

*Results*: Control and placebo-treated mice developed EAE with similar incidence and severity. While all statins tested ameliorated EAE, atorvastatin and rosuvastatin were the most effective when administered orally in prescription formulation. All statins inhibited antigen-specific proliferation, secretion of Th1 cytokines and IL-12-dependent STAT4 phosphorylation. Atorvastatin and rosuvastatin induced Th2 cytokines, which was associated with increased phosphorylation of STAT6. Immunomodulatory effects were reversed by treatment with L-mevalonate. The route of administration was tested using prescription and purified atorvastatin. Orally administered prescription atorvastatin was superior to purified atorvastatin administered either parenterally or orally.

**Conclusions:** Our results show that all statins can ameliorate EAE progression, although individual statins appear to vary in the strength of their immunomodulatory effects and only some statins induce a Th2 bias. Atorvastatin and rosuvastatin, the most potent of the statins in lowering cholesterol, are the most robust in both amelioration of EAE as well as induction of a Th2 T cell phenotype, which likely reflects differences in enzymatic binding efficiency, half-life, and lipophobicity. Drug formulation and route of administration are important factors in determining in vivo immunomodulation by atorvastatin. This study highlights the importance of selecting the type of statin for testing in treatment of multiple sclerosis and other autoimmune diseases.

#### **Sa1.24. fMRI Correlates of Strategic Planning in Multiple Sclerosis.**

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Recent work using functional magnetic resonance imaging (fMRI) indicates that patients with multiple sclerosis (MS) show altered brain activation on motor and cognitive tasks relative to controls. However, few studies have examined MS patients' brain activation patterns on fMRI probes of executive cognitive functioning. The present study used a modified version of the Tower of London task to examine potential alterations in the neural circuitry of strategic planning in MS. Six MS patients and four healthy controls were administered an fMRI Tower of London task, which included easy (2–3 move solution), hard (4–5 move solution), and control conditions. The hard greater than easy contrast was of particular interest in our analyses. fMRI data were obtained on a 1.5T GE scanner. Data were analyzed using a random effects model in SPM99. While both MS participants and controls activated predicted regions during task performance, including right dorsolateral prefrontal cortex, MS patients showed increased anterior right hemispheric activity relative to controls ( $P < .01$ ,  $k=3$ ). In contrast, controls tended to show more posterior right hemispheric activation than patients ( $P < .01$ ,  $k=3$ ). Behaviorally, controls tended to perform better than patients across task conditions. These preliminary findings support previous reports of altered brain activation patterns associated with cognition in MS. Our group and others are engaged in longitudinal research to determine the relationship between changes in neural activity and cognitive task performance, and to examine the potential utility of fMRI for tracking progression of brain changes associated with cognitive decline in MS.

#### **Sa1.25. A New Clinically Relevant Approach To Expand Myelin Specific T Cells.**

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**Intro:** Human self-reactive T cells are postulated to play an important role in many autoimmune diseases. Although detection of self-reactive T cells directly ex-vivo has been achieved, in-depth functional characterization of these cells is impeded by their low precursor frequency. Hence, researchers have expanded self-

reactive T cells in vitro in order to characterize and compare these cells from autoimmune patients and controls. Antigen specific T cells need to be re-stimulated on a regular basis with new antigen presenting cells (APCs) and antigen (Ag). This has been usually achieved by using autologous fresh or frozen irradiated peripheral blood mononuclear cells (PBMCs) or EBV-immortalized B cells or dendritic cells in the presence of Ag. This approach requires either one large blood sample from which cells are frozen for later use or repetitive blood draws. Evaluation of self-reactive T cells from many donors is thus hindered by the large volume of blood necessary. We explored a method successfully applied for tumor antigens to present myelin antigen to T cells using in vitro expanded autologous B cells (Lapointe et al. 2003).

*Our goal is to assess the feasibility of expanding human myelin specific T cells from one small blood draw.*

**Methods:** Myelin specific T cells were expanded from 50–75 ml of blood. PBMCs were put in culture with myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) peptide. A week after the first round of stimulation T cells were separated into CD8<sup>+</sup> and CD4<sup>+</sup> using beads (Miltenyi) prior to be re-stimulated. In parallel, a fraction of donor's PBMCs was stimulated with irradiated CD40L-expressing fibroblasts in the presence of IL-4; B cells proliferated and after few rounds of stimulation were the only cell type in the culture and expressed high levels of MHC molecules and co-stimulatory molecules, essential hallmarks of efficient APCs. Expanded B cells were loaded with Ag, irradiated and then used as APC to stimulate T cells for multiple subsequent rounds of stimulation. CD4<sup>+</sup> T cell lines were re-stimulated every 10–14 days and CD8<sup>+</sup> T cell lines every 7–9 days. Proliferation was measured by thymidine incorporation and cytokine release by ELISA.

**Results:** At the second or third round of stimulation T cells were tested for their specificity by comparing their proliferation and IFN- $\gamma$  secretion in the supernatant when put in cultured with Ag-loaded B cells vs. unloaded B cells. T cell lines exhibiting Ag-specific proliferation and/or IFN $\gamma$  secretion at least two times higher than in the absence of Ag were considered Ag-specific and were expanded further. MBP and MOG specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines were obtained from multiple donors in comparable number than those obtained with a traditional approach (fresh PBMCs) and kept in culture for many weeks.

**Conclusion:** Human myelin specific T cell lines both CD4<sup>+</sup> and CD8<sup>+</sup> can be expanded in vitro from a relatively small blood sample facilitating immunological studies of such cells in multiple donors.

#### **Sa1.26. IL-15 and IL-2 Fusion Proteins Attenuate Experimental Autoimmune Encephalomyelitis.**

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**Objective:** To investigate the effect of therapy with IL-15Fc and IL-2Fc fusion proteins on experimental autoimmune encephalomyelitis (EAE). **Background:** Peripheral T cell activation and their migration into the central nervous system (CNS) are involved in the pathogenesis of EAE. Targeting the IL-2 receptor, which shares the  $\gamma$  and  $\beta$  portions with the IL-15R, suppresses immune responses. The IL-15R $\alpha$  chain is specific for IL-15, and is expressed on CD8<sup>+</sup>, natural killers, macrophages, endothelial cells and tissues

such as brain, spleen and thymus. We used two fusion proteins to induce protection in EAE: IL-2Fc that binds to activated T cells and induces cytolysis, and IL-15Fc that binds to the IL-15R $\alpha$ . **Methods:** C57BL/6 mice were immunized with MOGp35-55 in CFA to induce EAE. Daily i.p injections of either IL-2Fc (5ug/dose), IL-15Fc (5ug/dose), IL-2Fc+IL-15Fc or control IgG2 were given for 10 days, starting either on day 0 or on day 10 postimmunization, to target the priming or effector stages of disease, respectively. Outcomes measured included clinical disease, cell proliferation measured by thymidine incorporation, cytokine profiles measured by ELISPOT assays, and immunohistological staining of the CNS at day 15, 30 and 40 post immunization. **Results:** Early treatment with the combination fusion proteins decreased disease severity (MMG 0.6;  $p < 0.0001$ ) and incidence ( $P = 0.01$ ) significantly compared to the control Ig group (MMG=2.4). Injection of IL-15Fc alone also attenuated disease (MMG 1.0;  $P < 0.0001$ ) while IL-2Fc (MMG = 2.1) showed no disease attenuation. Treatment with any of the infusion proteins induced a high background proliferation of splenocytes; but MOG peptide-specific proliferation was suppressed in splenocytes from IL-15Fc ( $P = 0.002$ ) as well as combination therapy ( $P < 0.0001$ ) treated mice. Protected mice showed an increase in MOG-specific IL-4 production when compared to the control group ( $P < 0.03$ ). Staining for inflammatory cells in the CNS showed a decrease in CD4<sup>+</sup> and macrophage infiltrates in the protected groups, particularly in those receiving combination therapy. These scarce infiltrates were positive for IL-4 and IL-10. In contrast, IFN $\gamma$  was highly expressed in the CNS of control mice as well as those treated with IL-2Fc alone. Injection of any of these fusion proteins during the effector stage of the disease (d10-20) did not show any protection. **Conclusions:** These results suggest that blocking the specific IL-15R $\alpha$  is protective in EAE and is more effective when combined with the cytolytic effect of IL-2Fc fusion protein. Protection is associated with an increase in Th2 cytokines both in the periphery and the CNS. The additive effect of combination therapy may be due to the cytolytic effect of IL-2Fc on MOG-specific T cells, and decreased cell migration into the CNS mediated by IL-15 blockade. Further studies need to be done in order to fully understand the mechanisms by which these molecules protect in EAE.

#### **Sa1.27. Dendritic Cells from the Mesenteric Lymph Nodes of OVA Fed Animals Stimulate Naïve T Cells In Vitro To Express Latency-Associated Peptide.**

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Oral exposure of soluble antigens leads to the induction of systemic hyporesponses, a phenomenon known as oral tolerance. In this study, we isolated dendritic cells (DCs) from the Peyer's Patches (PPs), mesenteric lymph nodes (MLNs) and spleens of fed mice and measured their ability to support CD4<sup>+</sup> T cell proliferation, cytokine production and differentiation. We found that the ability of DCs to support T cell proliferation appeared in PPs first, followed by MLNs and spleen. Moreover, PP DCs promoted significant increases of Th1 cytokines (IL-2, IFN $\gamma$  and TNF $\alpha$ ) but no increases of Th2 cytokines (IL-4 and IL-5). However, MLN DCs promoted significant increases of both Th1 and Th2 cytokines. Although DCs from all these lymphoid tissues were able to induce naïve T cells to express surface TGF $\beta$ , as determined by the up-regulation of latency-associated peptide

(LAP+), MLN DCs were the most efficient. At their peak time (~12hrs after last feeding), MLN DCs induced a ~5-fold increase of CD4<sup>+</sup>LAP<sup>+</sup> cells. These studies demonstrate that MLN DCs acquire fed antigens and induce the expansion of CD4<sup>+</sup>LAP<sup>+</sup> T cells in situ which then may participate in specific tolerance to fed antigens and regulation of autoimmune processes associated with oral tolerance to autoantigens.

#### **Sa1.28. Immature Dendritic Cells Can Reduce Experimental Autoimmune Encephalomyelitis in the Mouse.**

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Multiple Sclerosis (MS) is a chronic autoimmune disorder that affects the central nervous system. Although the aetiology of the progressive neurological loss has not yet been fully elucidated, it is believed that genetically determined susceptibility and environmental triggers are both implicated in the detrimental immune response against components of the myelin sheath, where myelin-specific T cells are thought to play a central role. As an animal model for this disease, Experimental Autoimmune Encephalomyelitis (EAE) can be induced in C57BL/6 mice. EAE is an inflammatory demyelinating disease that is primarily mediated by CD4<sup>+</sup> T cells and shares most of the clinical and histopathological aspects of MS. Due that dendritic cells (DCs) are professional antigen presenting cells important for the activation of self-reactive T cells, we are interested in evaluating their therapeutic potential in the regulation of autoimmune responses. DCs have a central role in maintaining peripheral tolerance to self and alterations in their physiology are likely to be responsible for defective immune regulatory mechanisms. We first observed that immature DCs are able to promote tolerance in the EAE model. To further enhance the tolerogenic capacity of these cells, we used drugs that interfere with NF $\kappa$ B activity, such as andrographolide, a bicyclic diterpenoid lactone and Rosiglitazone, a PPAR $\gamma$  agonist. In vitro, these molecules were able to interfere with DCs maturation and with their ability to present antigens to T cells. T cell activation by DCs was completely abolished by exposing DCs to andrographolide and Rosiglitazone during antigen pulse. Injections of immature DCs treated with either andrographolide or Rosiglitazone showed an enhanced capacity to reduce EAE symptoms. Our results indicate that injection of immature DCs can prevent myelin-specific T cells activation in EAE. These data suggest that pharmacological approaches that promote a tolerogenic phenotype in DCs could be useful as a potential strategy in the design of new therapies to prevent or treat detrimental immune responses.

#### **Sa1.29. CD4+CD25+ Regulatory T Cells Mediate Recovery from Experimental Autoimmune Encephalomyelitis in Association with LAP+ TGF- $\beta$ Secreting Cells.**

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In SJL/J mice, PLP139-151 is the immunodominant encephalitogenic epitope and induces a relapsing-remitting form of EAE. It has been reported that CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells play a role in affecting the onset and progression of EAE, as transfer of CD4<sup>+</sup>CD25<sup>+</sup> cells at these stages ameliorate disease.

The role of CD4+CD25+ regulatory cells in the natural recovery from disease has not been well defined. Here we show that EAE-recovered SJL/J mice have an increase number of Forkhead box P3 (*Foxp3*)-expressing CD4+CD25+ T cells. These cells were anergic and inhibited the proliferative response of CD4+CD25- T cells against PLP139-151 or anti-CD3 stimulation. Depletion of CD4+CD25+ T cells prior to the recovery phase exacerbated disease, resulted in the expansion of IA<sup>s</sup>/PLP139-151-tetramer-positive cells and enhanced IFN- $\gamma$  production. In addition, transforming growth factor (TGF- $\beta$ ) was shown to be involved in the recovery from EAE as the percentage of CD4+CD25+ cells expressing TGF- $\beta$  latency associated peptide (LAP) on the cell surface increased significantly in blood and spleen of EAE-recovered mice as compared to the naïve mice ( $P < 0.001$  and  $P < 0.01$ , respectively) and in vivo neutralization of TGF- $\beta$  abolished recovery from disease. Our results demonstrate that CD4+CD25+ regulatory cells mediate recovery from PLP139-151-induced EAE in SJL/J mice in which TGF- $\beta$  plays an important role.

### Sa1.30. Genetic Markers of Disease Risk, Prognosis and Treatment Response in Multiple Sclerosis.

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Multiple Sclerosis (MS) is a multifactorial, polygenic disease that manifests itself as a chronic inflammation of the CNS. In addition to influencing the presence or absence of disease, genetics may also play a significant role in individual variations in prognosis and differential response to therapy among patients.

In order to better understand the genetic variation involved in these heterogeneous aspects of the disease we selected 43 SNPs from 22 genes that were associated with risk or severity of MS in at least one published study. These loci were genotyped using DNA from a large MS patient registry which was established as a longitudinal study aimed at finding biomarkers of disease risk, prognosis and response to therapy.

Our case-control association analysis utilized 190 patients enrolled to date and an ethnically-matched cohort of healthy controls ( $n = 363$ ). The DR15 allele of the HLA-DRB1 locus associated very strongly with presence of disease ( $P = 0.000004$ , OR=2.439, CI<sub>95</sub>=1.671-3.561), as did VDR ( $P = 0.001$ , OR=1.845, CI<sub>95</sub>=1.271-2.680), PTPRC ( $P = 0.009$ , OR=1.594, CI<sub>95</sub>=1.099-2.311), TNFRSF6 ( $P = 0.002$ , OR=1.778, CI<sub>95</sub>=1.236-2.557), SH2D2A ( $P = 0.00005$ , OR=15.937, CI<sub>95</sub>=2.719-93.099) and CNTF ( $P = 0.007$ , OR=1.728, CI<sub>95</sub>=1.141-2.616).

When we compared patients with benign vs. malignant disease course ( $n = 37$  vs. 27) we observed an association of MBP with disease prognosis ( $p=0.003$ , OR=5.319, CI<sub>95</sub>=1.727-16.393). Paradoxically, the DR15 allele also associated with the presence of benign disease ( $P = 0.004$ , OR=5.447, CI<sub>95</sub>=1.628-17.945); it is therefore a risk factor, while at the same time providing a better prognosis.

A comparison of responders vs. non-responders to beta-interferon or glatiramer acetate therapy ( $n = 38$  vs. 28) showed a significant association of IL1B with response ( $P<0.1$ , OR=5.182,

CI<sub>95</sub>=1.821-14.747). Linear regression analysis of genotypic association with age of onset revealed a potential involvement of TNFSF6 ( $P = 0.002$ ).

All analyses were controlled for gender. Among several markers that exhibited significant sex-specific associations, TNF was associated with malignant disease only in female patients ( $P = 0.01$ ), and TNFSF10 was significantly associated with risk of disease in males ( $P = 0.0016$ ).

These results illustrate the complex genetic etiology of MS, which involves a variety of sometimes gender-specific factors combining to influence not only the presence but the progression of the disease. These markers may eventually improve our understanding of the disease and provide better clinical and diagnostic indicators of disease risk and prognosis.

### Sa1.31. Investigating Anti Human MOG Antibodies in Multiple Sclerosis Using Stably MOG Transduced Cell Lines.

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Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). Although the cause of MS is still uncertain, an ongoing autoimmune response against myelin antigens seems most likely. Myelin Oligodendrocyte Glycoprotein (MOG) is considered the most promising candidate autoantigen in MS. MOG induces experimental autoimmune encephalomyelitis in several rat and mouse strains. Recent findings in MS suggest that the development of antibodies against MOG is associated with progression of disease during early MS. However, studies in humans and animal models are based on a fragment of the MOG protein expressed in *E. coli*. To achieve physiological MOG expression with proper glycosylation, we cloned the human MOG isoform 2 gene in a lentiviral expression vector. We transduced primary cells and several human and murine tumor cell lines. Transduced cell lines expressed high levels of MOG on the surface. Stable expression was achieved in murine lymphoma and several human glioma cell lines. These transgenic lines allowed fast and reliable detection of anti-MOG antibodies at lower nanogram concentration comparable with the sensitivity of ELISA assays. Further studies are ongoing to determine anti-MOG antibodies in MS patients and controls to obtain further insights into the role of anti-MOG antibodies in multiple sclerosis.

### Sa1.32. Role of CD46 in Multiple Sclerosis.

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CD46 acts as a costimulatory molecule for human T cells and coligation with CD3 promotes T cell proliferation. Furthermore, addition of IL-2 induces a Tr1 phenotype, characterized by a large production of the regulatory cytokine IL-10. However, in a murine transgenic model, the two intracytoplasmic isoforms of CD46 (Cyt1 and Cyt2) that are produced by alternative splicing, have antagonist roles in an in vivo T cell-mediated inflammation. While Cyt1 decreases the inflammation, Cyt2 increases it. This difference has been linked to their differential effects on CD4+ T cell proliferation, CD8+ CTL cytotoxicity, as well as IL-2 and IL-10 production. Hence, CD46 is a crucial regulator of T cell activation, and depending on the ratio of the intracytoplasmic isoforms present, a different outcome in T cell activation might result in



humans. As multiple sclerosis (MS) is an autoimmune disease with a direct involvement of T cells, we investigated the role of CD46 in MS. While as previously described, IL-10 could be induced upon CD46 stimulation in healthy donors (5/5), T cells from most patients with MS did not produce IL-10 or to a much lesser extent (8/11). We then investigated the ratio of the two intracytoplasmic isoforms of CD46 in T cells of patients with MS as compared to healthy donors. Interestingly, we found that upon CD46 costimulation the Cyt1/Cyt2 ratio is altered in MS patients when compared to healthy individuals, which might correlate with the lack of IL-10 secretion observed upon CD46 stimulation. These preliminary data suggest that a dysregulation of CD46 activation in human T cells may be involved in MS etiology.

### Sa1.33. Myelin-Specific Cytokine Responses Show a Dramatic Gender Bias in Multiple Sclerosis (MS).

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Most autoimmune diseases disproportionately affect women. Unfortunately, most animal models and *in vitro* studies show that steroid hormones (estrogens and androgens) are *suppressive* for autoreactive responses. This raises the question: what is the evidence for a heightened autoimmune response in women that would help explain their increased susceptibility to autoimmune disease? To address this question, we examined sex differences in antigen-induced cytokine-secreting cells between untreated relapsing-remitting multiple sclerosis (RRMS) patients and controls. We studied Th1 (IFN $\gamma$  and TNF $\alpha$ ) and Th2 (IL-5 and IL-10) cytokine responses to MS-relevant epitopes of myelin (MBP, PLP, MOG) as well as irrelevant epitopes. We observed significant *female* skewing ( $P \leq 0.005$ ) in the IFN $\gamma$  response to 3 PLP peptides: PLP 40-60, 103-120, 195-206. PLP 40-60 and 195-206 showed a simultaneous *male* skewing ( $P \leq 0.007$ ) in IL-5 responses. For PLP 40-60, this resulted in a IFN $\gamma$ /IL-5 ratio of 174.4 in MS females, whereas the MS male IFN $\gamma$ /IL-5 ratio was only 0.3. Similarly, for PLP 195-206, the MS female IFN $\gamma$ /IL-5 ratio was 26.8, and the MS male ratio was 1.7. MBP responses also showed strong gender interactions: MS females had very strong MBP-IFN $\gamma$  responses and virtually no MBP IL-5 response ( $P = 0.004$ ) while the reverse was true for MS males: high IL-5 and extremely low IFN $\gamma$  responses ( $P = 0.0023$ ). For MBP, this gave an IFN $\gamma$ /IL-5 ratio of 77.4 in MS females compared to a ratio of 0.8 in MS males. For MOG 64-96, MS females gave an IFN $\gamma$ /IL-5 ratio of 82.9, whereas MS males gave a ratio of 1.0. In contrast, mitogen-stimulation gave IFN $\gamma$ /IL-5 ratios with less than a 2 fold difference between MS females and MS males. Control females showed slightly elevated IFN $\gamma$  responses compared to control males, however, IFN $\gamma$ /IL-5 ratios were  $<9$ . These results show that *MS-relevant* myelin proteins can induce female MS patients' lymphocytes to secrete inflammatory cytokines, whereas the very same myelin epitopes induce males to secrete anti-inflammatory cytokines. These interactions suggest that disease and gender are not independent factors in the immune response, but rather they interact to promote gender bias in cytokine responses that may explain why women are more susceptible to MS. A gender bias in cytokine responses could have implications for designing clinical trials involving antigen-specific therapies in MS.

Supported by the National MS Society grants FA 1459-A-1 and RG 3263-A-3, and NIH grant ROI NS 41972.

### Sa1.34. Intrathymic Expression of Torpedo Acetylcholine Receptor Alpha Subunit.

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We have developed an approach to test a new hypothesis of the intrathymic pathogenesis of myasthenia gravis. The hypothesis posits that acetylcholine receptor alpha subunit (AChR $\alpha$ )-specific CD4<sup>+</sup> T cells, which escape central deletion, migrate to the thymus where, during the course of a nonspecific inflammatory reaction, they are activated by intrathymically expressed autoantigen. This approach entails expressing Torpedo AChR $\alpha$  (T-AChR $\alpha$ ) as a neo-self-antigen in the thymus of adult C57Bl/6 (B6) mice. B6 AChR $\alpha$  CD4<sup>+</sup> T cells see an immunodominant T-AChR $\alpha$  peptide that does not cross-react with mouse AChR $\alpha$ . Thus, this epitope can only be encountered in the B6 mouse thymus if we express T-AChR $\alpha$  there. However, it is widely regarded that 1) membrane expression of AChRs requires the proper assembly and folding of constituent subunits and 2) T-AChR subunits are assembled and expressed only at below 26°C. We reasoned that even though T-AChR $\alpha$  would not assemble with mouse subunits at 37 C, it was possible that a small amount of T-AChR $\alpha$  could be expressed on the surface of mammalian cells *in vivo* at murine body temperature. We transiently transfected the tsA201 cell line with T $\alpha$ /pRBG4, a mammalian vector that expresses T-AChR $\alpha$  cDNA. In additional experiments, tsA201 cells were transfected with either 1) mouse AChR $\alpha$  alone, 2) T-AChR $\alpha$  + mouse AChR $\beta$ , AChR $\delta$ , and AChR $\epsilon$ , or 3) mouse AChR  $\alpha$  + mouse AChR $\beta$ , AChR $\delta$ , and AChR $\epsilon$  (positive control). Expression of AChR $\alpha$  protein was investigated by flow cytometry using mAB 210, a rat IgG mAB that sees an epitope on the extracellular domain of Torpedo as well as mammalian AChR $\alpha$ , followed by FITC-goat anti-rat IgG antibody to detect expression of the of the transfected protein. We also used FITC- $\alpha$ -bungarotoxin as an independent marker for detecting the alpha subunit. Mock-transfected cells served as negative controls. We observed 1) a low, albeit statistically significant, number of viable cells that had surface staining of mAB 210 after transfection with only mouse AChR $\alpha$  relative to mock transfected cells, 2) a similar magnitude of T-AChR $\alpha$  expression on cells transfected only with T-AChR $\alpha$ , 3) no augmentation of T-AChR $\alpha$  expression when the mouse AChR $\beta$ , AChR $\delta$ , and AChR $\epsilon$  subunits were co-transfected and 4) many of the positive control cells (transfected with murine AChR  $\alpha$ ,  $\beta$ ,  $\delta$ , and  $\epsilon$  subunits) expressing mouse AChR $\alpha$ . Transfected cells that were permeabilized by treatment with saponin/paraformaldehyde expressed considerable amounts of T-AChR $\alpha$  intracellularly. Thus, despite the temperature requirements unique to the assembly of T-AChR subunits and their membrane expression, a small amount of T-AChR $\alpha$ , like mouse AChR $\alpha$ , can be transported to the cell membrane where it is expressed as a single entity. A greater amount of the unassembled T-AChR $\alpha$  or mouse AChR $\alpha$  subunits was detected intracytoplasmically. These results indicate that unassembled T-AChR $\alpha$  can indeed be expressed on/in mammalian cells *in vivo*. They lay the groundwork for determining whether T-AChR $\alpha$ -specific B6 CD4<sup>+</sup> T cells can be activated when they encounter their cognate autoantigen in the thymus in a context that promotes activation.

### Sa1.35. The Cytokine Pattern of Glatiramer Acetate Reactive CD8+ T Cell Lines Derived from MS Patients and Healthy Volunteers.

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**Background:** Multiple Sclerosis (MS) is the most frequent disabling neurological disease in young adults. Glatiramer Acetate (GA) is a synthetic amino acid copolymer that has been shown to reduce the relapse rate in patients with relapsing-remitting MS. The proposed mechanism of action of GA is functional suppression of autoreactive T cells specific for myelin antigens. GA reactive CD4+ T-cells have been generated from MS- patients and controls by us and others. As CD8+ T cells may be involved in the pathogenesis of MS, we established an experimental system to generate human, GA reactive CD8+ T- cell lines and clones, which demonstrated an antigen specific, dose dependent proliferation und IFN gamma production. The aim of our current study was to investigate the cytokine profile of GA-reactive CD8+ T cell lines derived from MS patients and controls. **Results:** So far 88 CD8+ T cell lines from healthy volunteers, 61 lines from GA treated MS patients and 47 lines from untreated MS patients were generated. GA reactive CD8+ T cells of untreated MS patients produced more IFN gamma ( $P < 0.05$ ) and IL- 4 ( $P < 0.001$ ), but less IL-5 ( $P < 0,01$ ) than CD8+ T cells derived from healthy volunteers. Cells derived from GA treated MS patients showed less TNF alpha ( $P < 0.001$ ), lower levels of IL- 10 ( $P < 0.001$ ), less IL- 4 ( $P < 0.001$ ), but more IL- 5 ( $P < 0.05$ ) production than T cell lines generated from untreated MS patients. **Conclusions:** The data presented here demonstrate that the cytokine spectrum secreted by GA reactive CD8+ T cells differs in untreated MS patients and healthy controls and that this cytokine shift may be partly corrected by initiation of GA treatment in patients suffering from RRMS.

**Acknowledgments:** This work was in part supported by TEVA Pharma Deutschland GmbH to FW and AD and from the Gesellschaft für Nervenheilkunde Mecklenburg- Vorpommern to AV and AD

### Sa1.36. An Exploration of the Role of TLR2 and TLR4 in CD14+ Cells in Multiple Sclerosis.

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Multiple sclerosis (MS) is a chronic disease of the central nervous system characterized by inflammation and areas of

demyelination. The role of the innate immune system in this disease is emerging. One critical family of molecules in innate immunity is the Toll-like receptor (TLR) family which plays a fundamental role in pathogen recognition and activation of the innate immune response. The TLRs have been implicated in several autoimmune diseases, but their role in MS remains unclear. We present an initial exploration of the role of two of these molecules, TLR2 and TLR4, using a retrospective study of immunophenotypes captured by the MS Natural History Database at the Partners MS Center in Boston. We analyzed data from forty-four (44) patients with MS by McDonald criteria; each subject had data from three different visits to the MS Center over the course of one year. At each visit, peripheral blood mononuclear cells (PBMCs) were collected and stained using monoclonal antibodies against CD14, TLR2 and TLR4. The expression of these molecules on PBMCs was measured both ex vivo and after stimulation with lipopolysaccharide (LPS) and ionomycin. These data were then correlated with the associated clinical phenotypes that are available in the database, including disease subtype, course, and activity as well as MRI volumetric data. Large interindividual and intraindividual variability in TLR2 and TLR4 expression on CD14+ cells was observed despite the precision of such measurements in our clinical laboratory. This variability could not be explained solely by disease activity in subjects as reported in the database. The role of TLR2 and TLR4 on CD14+ cells in MS remains unclear. However, these data demonstrate that TLR2 and TLR4 expression on CD14+ cells may undergo large intraindividual fluctuation over time, a fact that needs to be taken into account in any future association of these molecules with human disease.

### Sa1.37. Regulation of Expression of Matrix Metalloproteinases in CNS Inflammation Versus CNS Injury.

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Matrix metalloproteinases (MMPs) are a family of 22 proteases, including 6 membrane-bound MMPs (MT-MMPs). Their physiological inhibitors are tissue inhibitor of metalloproteinases (TIMPs). MMPs are thought to mediate cellular infiltration in CNS inflammation, which is an integral part of the pathogenesis of Multiple Sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). MMPs may also mediate infiltration, and possibly repair, after CNS injury. We have investigated the differential expression of selected MMPs in spinal cord from mice with adoptively transferred EAE, and in a model of CNS axonal injury in the hippocampus, using real-time RT-PCR to profile changes in expression. MMP-12 is a secreted MMP, which is virtually absent in the uninfamed CNS. It is highly up-regulated in EAE, where it is expressed almost exclusively by infiltrating macrophages. After axonal transection, MMP-12 expression correlated with kinetics of macrophage infiltration measured by flow cytometry in the lesion-reactive hippocampus. In CC chemokine receptor-2 (CCR-2) deficient mice, macrophage infiltration is absent. Consistently, there was no up-regulation

of MMP-12 in the lesion-reactive hippocampus of CCR-2 deficient mice.

After axonal transection, TIMP-1 was up-regulated prior to leukocyte infiltration of the lesion-reactive hippocampus, implicating an endogenous source. TIMP-1 may be expressed to counteract potential damaging effects of MMPs. Whereas the majority of secreted MMPs were up-regulated in EAE, 4 of 6 MT-MMPs were down-regulated. Conversely, none of the 3 MT-MMPs investigated so far were affected in the lesion-reactive hippocampus. We sorted CD45dim CD11b+ microglia from CNS of mice with EAE and found expression of 4 out of 6 MT-MMPs down-regulated. This suggests that activation of microglia in the course of neuroinflammation is correlated with reduced expression of some MT-MMPs. Activated microglia in the lesion-reactive hippocampus may differentially regulate MMP expression, possibly because axonal transection leads to activation of microglia in the absence of inflammation.

This work was funded by the MS Society of Canada and a CIHR-IHRT grant.

### Sa1.38. Searching for Biomarkers in Multiple Sclerosis: The Need for Natural History Studies.

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Multiple sclerosis (MS) is a chronic autoimmune disease whose main pathophysiological features are lymphocyte infiltration and inflammation, demyelination, and axonal damage of the central nervous system. This disease is heterogeneous in its clinical manifestation, prognosis, and response to different treatments. Although several therapies are available to treat patients with MS, tools to predict the course of the disease or the success of a particular treatment are still missing. The combination of different therapeutic strategies directed at the different pathophysiological processes of the disease might be necessary. In order to achieve this goal we need to develop reliable biomarkers of disease activity and response to treatment. In this study we examined immunological biomarkers by analyzing relevant molecules on peripheral blood mononuclear cells (PBMC) from patients with MS, both *ex vivo* and after *in vitro* stimulation using flow cytometry-based assays. We measured a battery of cytokines, chemokines and their receptors, activation markers, and costimulatory molecules longitudinally in more than one hundred patients with MS enrolled in the Multiple Sclerosis Natural History Study at the Partners MS Center in Boston. The patients in different categories of disease and in different treatment subgroups were recruited and followed prospectively for up to three years with consecutive measurements of immunological markers, clinical assessment and MRI measures. Changes in biomarkers were correlated with clinical and MRI measures and with response to treatment. Our preliminary analysis shows a differential effect of various therapies on different immunological targets, some increasing anti-inflammatory response while others dramatically reducing pro-inflammatory responses at different time points after initiating therapy.

This prospective multi-parameter analysis of immune markers with clinical responses and MRI support represents a systematic approach for the identification of surrogate markers of disease activity and treatment response in patients with MS.

### Sa1.39. Protection in a Rodent Model of Multiple Sclerosis by Carbon Monoxide Release.

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**PURPOSE:** Iodoform is metabolized by the cytochrome P-450 oxidative system to CO and CO<sub>2</sub>. *In vivo*, the anti-inflammatory effects of CO have been previously demonstrated in LPS-induced shock, graft rejection, and lung injury models. In EAE, increased CO has a variety of potentially beneficial effects including the protection from 1) nitric oxide synthase, 2) cellular infiltration, 3) macrophage activation, and 4) inflammatory processes including TNF- $\alpha$  production. Experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS), was chosen to study the effect of this compound in acute inflammatory autoimmune encephalomyelitis.

**METHODS:** EAE was induced in Lewis rats after immunization on day 0 with myelin basic protein (MBP) from guinea pig. On day 10, the rats developed an inflammatory encephalomyelitis with lymphocyte infiltration and subsequent progressive paralysis. Rats injected with MBP were randomized to receive by vehicle or iodoform (250 mg/kg/day) by oral gavage. Daily clinical scores were determined on a 0–5 scale. Brain and spinal cord samples were obtained at early and peak clinical signs from the vehicle control group and corresponding treated group for cytokine and histological analysis. In addition, pharmacokinetics and blood chemistries were also determined.

**RESULTS:** Iodoform reduced the severity of EAE in a dose dependant fashion. Significant reduction in severity was evident after 5 days of therapy ( $P = 0.001$ ). Iodoform ameliorated pathological damage as determined by image analysis. Carboxyhemoglobin levels peaked within a few hours of administration but normalized within 24 hours without cumulative pharmacokinetic consequences. Analysis of brain cytokine levels indicated that iodoform increased IL-10 in EAE animals with no effect on TNF- $\alpha$  or IFN- $\gamma$ .

**CONCLUSIONS:** These results demonstrate a powerful CO-mediated amelioration of EAE and suggest that further study of halothanes would be warranted to define their potential benefits in the treatment of human autoimmune diseases such as MS.

### Sa1.40. IFN $\gamma$ -Dependent Regulation of the Neutrophil-Activating Cytokines IL-17 and IL-18 in EAE.

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Inflammation is an important aspect of autoimmune disease that contributes to the pathology of conditions such as multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis (EAE). The processes that underlie inflammation in the CNS, including migration of cells and release of their effector molecules, are co-ordinated by a network of cytokines and chemokines released by resident brain cells as well as infiltrating immune cells. Following induction of EAE, mice that lack IFN $\gamma$  or its receptor develop a very severe disease that progresses rapidly to paralysis and death,

compared to a milder relapsing-remitting phenotype in wild-type littermates. The severe disease observed in IFN $\gamma$ -deficient mice is characterized by lesions in the spinal cord and brainstem that are larger and more diffuse than in wild-type mice. Furthermore, inflammatory CNS infiltrates consisted primarily of macrophages/microglia and T cells in wild-type mice, but included large numbers of polymorphonuclear cells (mainly neutrophils) in IFN $\gamma$ -deficient mice. It is not clear why IFN $\gamma$  deficiency results in more neutrophils infiltrating the CNS. We hypothesized that this is due to a change in the cytokine networks. Two possible candidate molecules are IL-17 and IL-18, both of which promote neutrophil migration and activation. We induced EAE in wild-type and IFN $\gamma$ -deficient mice by active immunization with PLP139-151 peptide in CFA, and investigated expression of these two cytokines by real-time PCR. IL-17 mRNA was not detectable in the spinal cord of unimmunized animals, but was induced with onset of EAE. IL-17 mRNA was expressed at significantly higher levels in the spinal cords of IFN $\gamma$ -deficient mice compared to wild-type mice. IL-18 mRNA and protein was expressed constitutively in the spinal cord of unimmunized mice and did not change with onset of EAE. We also investigated expression of IL-18 binding protein (IL-18BP), an endogenous, potent inhibitor of IL-18 that is regulated by IFN $\gamma$ . IL-18BP mRNA was dramatically up-regulated after onset of EAE in the spinal cord of wild-type but not IFN $\gamma$ -deficient mice. This study demonstrates that IFN $\gamma$  deficiency could result in aberrant IL-18 actions due to a failure to up-regulate IL-18BP. Thus, the enhanced neutrophil infiltration could be due to enhanced IL-17 expression or a lack of inhibition of IL-18. Elucidation of these pathways will help understand how perturbations of the cytokine network can impact on cellular infiltration and CNS pathology.

#### **Sa1.41. Differential Reactivity Against Solid-Phase and Liquid-Phase Myelin/Oligodendrocyte Glycoprotein in Multiple Sclerosis and Experimental Allergic Encephalomyelitis.**

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**Background:** Myelin/oligodendrocyte glycoprotein (MOG) is a potent encephalitogenic antigen in experimental allergic encephalomyelitis (EAE), the animal model of multiple sclerosis (MS); it is a known target for pathogenic demyelinating antibody responses in EAE. In humans, anti-MOG antibodies have been described as prognostic markers in early MS. However, anti-MOG antibodies are not specific and can be found in a high proportion of healthy individuals, when analyzed by different methods.

**Objective:** To validate a novel, easy to perform liquid-phase based immune assay for the detection of serum anti-MOG antibodies and to assess the different immunological properties of MOG in solution- and in solid-phase immune assays.

**Design/Methods:** Sera of 37 MS patients with different disease stages, and 13 healthy control subjects (HC), chosen according to their reactivity against recombinant extracellular human MOG (rhMOG<sub>125</sub>) determined by solid-phase ELISA, were tested for the presence of anti-rhMOG<sub>125</sub> IgG by incubation with the biotinylated rhMOG<sub>125</sub> in solution. Immune complexes were captured by plate-bound Protein G and detected by peroxidase-labeled streptavidin. Biotinylated tetanus toxoid (TT) was used as a positive control. A

panel of monoclonal fab fragments (Fabs) and sera of 15 non-human primates *C. jacchus* marmoset immunized with either human whole white matter (HWM), recombinant ratMOG or linear 20-mer MOG peptides ( $n = 5$  per group) were used to validate the assay.

**Results:** No reactivity against solution-phase rhMOG<sub>125</sub> was detected in humans, in contrast to high anti-TT reactivity. In ELISA sera reacted equally against rhMOG<sub>125</sub> and TT. For anti-TT reactivity the correlation between LP and ELISA was highly significant ( $P < 0.001$ , Pearson's correlation), validating the assay technically.

In the marmoset EAE model, sera of MOG peptide immune animals reacted significantly weaker to solution-phase rhMOG<sub>125</sub> as compared to sera of ratMOG- or HWM-immune marmosets (mean binding ratio 1.9 vs. 33.9 (ratMOG-immune) and 14.3 (HWM-immune), respectively;  $P < 0.001$ , SNK T-test). The binding ratios of ratMOG- and HWM-immune sera were indistinguishable from each other in solution- or solid-phase assays. By means of monoclonal antibodies and fab fragments, we were able to detect a 7-32 fold lower antibody affinity against soluble rhMOG<sub>125</sub> compared to solid-phase rhMOG<sub>125</sub>.

**Conclusions:** We conclude that (1) immunodominant epitopes are less well presented on soluble rhMOG<sub>125</sub>, (2) antibodies against linear epitopes of MOG do not bind to MOG in solution, and (3) antibody assays employing solution phase rhMOG<sub>125</sub> are hence not suitable for serum antibody studies in MS most likely because of the biological nature of this membrane-bound, water insoluble antigen.

#### **Sa1.42. Characterization of B Cell Immunoglobulin Variable Region Genes Derived from Muscle Tissue of Subjects with Inclusion Body Myositis.**

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The inflammatory myopathies are putative autoimmune disorders characterized by muscle weakness and the presence of inflammatory infiltrates in skeletal muscle. In inclusion body myositis (IBM), the inflammatory cells that are infiltrating muscle have been characterized as predominately CD8+ cytotoxic T lymphocytes. While there does not appear to be significant B cell infiltrates as indicated by CD20 immunohistochemistry, we recently demonstrated the presence of significant muscle infiltrates of CD138+ plasma cells. Here, we examined the immunoglobulin variable region sequences of these cells. B cell immunoglobulin variable region gene libraries of IBM muscle were created by reverse transcriptase-polymerase chain reaction followed by sequencing of the whole immunoglobulin cDNA variable region, allowing identification of somatic mutations. There was significant oligoclonal expansion found in IBM muscle, in contrast to libraries from peripheral blood mononuclear cells that showed no oligoclonal expansion. Moreover, a pattern of B cell affinity maturation was evident in that clones sharing the same CDR3 had accumulated varying numbers of common and unique somatic mutations within the CDR regions and this clonal variation was also observed within groups comprised of CD19+ and CD138+ B cells. These data suggest that a local inflammatory response occurs within the muscle tissue of patients with IBM that may indicate the presence of a humoral antigen-specific response present in muscle.

**Sa1.43. Analysis of Genomic DNA from Human Single Cells Using Multiple Displacement Amplification: Application to B- and T-Cell Receptor Variable Region Genes.**

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Interest in examining the genetic nature of single cells has been shared by investigators in different fields. The limited amount of genetic material from a single cell specimen presents a technical challenge even for the most efficient and specific PCR protocols. In the present report we show the application of a recently developed method for whole genome amplification (WGA), the multiple displacement amplification (MDA) (Dean, Gen Res 2001), to single cells FACS-sorted from human blood. This procedure allows the interrogation of a single cell's genetic information through the production of several copies of the single cells genome followed by specific PCR experiments on a small fraction of the MDA product. We also compare MDA to whole genome amplification of single cells using the primer extension pre-amplification (PEP) method.

This technique can be applied to the analysis of the clonality and antigen-specificity of T-cells and B-cells which infiltrate tissues in autoimmune diseases. In fact, it allows the sequencing of both chains of immunoglobulin and T-cell receptor V-(D)-J rearranged genes from single cells isolated from frozen human tissue. Single cells are dissected from the stained tissue with the use of a laser capture microdissection device, their DNA is amplified with MDA, then a small fraction of the amplification product is used as template for the specific PCRs. Furthermore, the combination of laser capture microdissection and MDA allows to study single cells/cell types from paraffin-embedded tissues, where the RNA is mostly degraded but the DNA remains intact.

**Sa1.44. Characterization of B Cell Immunoglobulin Variable Region Genes Derived from Lesions of Patients with MS.**

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The notion that the process of B cell affinity maturation with ensuing production of potentially pathogenic autoantibodies may occur inside the CNS of patients with multiple sclerosis (MS) is supported by the presence, within lesions, of oligoclonal B cells and cell surface markers capable of supporting such a local immune response. Although B cells carrying somatic mutations of Ig variable (V) region genes have been detected in the CSF and CNS tissue of patients with MS, more direct evidence for the process of B cell affinity maturation confined within this compartment is needed. Here, we have characterized the B cell Ig variable (V) region genes derived from multiple lesions within the white matter of patients with MS. Analysis of variable region gene libraries revealed evidence of significant oligoclonal expansion of local B and plasma cells. Control tissue and PBMCs revealed no such oligoclonal expansion. Moreover, a pattern of B cell affinity maturation was evident in that clones sharing the same CDR3 had accumulated varying numbers of common and unique somatic

mutations within the CDR regions and this clonal variation was also observed within groups comprised of CD19+ and CD138+ B cells. These data suggest that a local inflammatory response occurs within the CNS tissue of patients with MS which includes differentiation and affinity maturation. Auto- or exogenous antigens resident in the CNS need to be considered as driving such a response.

**Sa1.45. Genetic Resistance to Autoimmunity Is Mediated by Autoantigen-Specific CD4+CD25+ Regulatory Cells.**

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SJL mice are highly susceptible to the induction of experimental autoimmune encephalomyelitis (EAE) with myelin proteolipid protein (PLP) peptide 139-151, whereas H-2 congenic B10.S mice are resistant. Immunodominance and susceptibility to PLP 139-151-induced EAE was found to be associated with a high precursor frequency of PLP 139-151-specific T cells in the naive repertoire of SJL mice. To understand the mechanism for disease resistance in B10.S mice, we determined the precursor frequency of PLP 139-151-reactive T cells in both strains of mice using IAs / PLP 139-151 tetramers. Both SJL and B10.S mice had similar frequencies of tetramer-reactive T cells in the naive peripheral repertoire. However, in SJL mice the majority of PLP 139-151 tetramer-positive cells were in the CD4+CD25- population whereas there were more tetramer-positive cells in the CD4+CD25+ population of B10.S mice, suggesting that there were more PLP 139-151-specific T cells in regulatory population. Depletion of CD4+CD25+ cells in vivo facilitated expansion of PLP 139-151-reactive cells with production of TH1 cytokines in EAE-resistant B10.S mice. Furthermore, depletion of CD25+ T cells with anti-CD25 antibody treatment prior to immunization with the encephalitogenic peptide resulted in induction of EAE in these otherwise resistant mice. These data indicate a role for autoantigen-specific CD4+CD25+ cells in genetic resistance to autoimmunity.

**Autoimmune Rheumatologic Diseases**

**Sa1.46. A 20 Year Old with Knee Arthritis Develops Multiple Organ Dysfunction: Think Zebras, Not Horses.**

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A 20 year-old female with Systemic Onset Juvenile Idiopathic Arthritis (SoJIA) presents to University Hospital with right knee arthritis and pharyngitis previously treated with naproxen and penicillin with no response. She denied flares for the past 7 years. Vancomycin and Ceftriaxone were started after bilateral arthrocenteses (each appearing inflammatory) were performed. Following the second arthrocentesis she experienced right shoulder and pleuritic chest pain. Naproxen and prednisone were started. Labs showed: CRP 196 mg/dL; normal liver and renal functions. WBC was normal but bands increased from 15%-56% and hemoglobin fell 3 grams since admission. HIV, ANA, double stranded DNA, RPR and RF were negative. By hospital day (HD #) 8 she reported fatigue, malaise, nausea and vomiting. New cervical and

inguinal adenopathy, splenomegaly, abdominal distention and right upper quadrant pain were noted. A light erythematous, non-pruritic, macular rash covered her anterior neck and shoulders. Over three days acute renal failure and liver function abnormalities developed; non-steroidals were discontinued. Bilateral infiltrates evolved on her chest radiograph. On HD #11 she had a generalized tonic-clonic seizure, was intubated and transferred to the intensive care unit (ICU). The next few days were marked by hypotension, tachycardia and persistent fevers (>101°F). She developed clonus, asymmetric plantar reflexes and obtundation. Laboratory data revealed pancytopenia with bands of 88%, and marked coagulopathy. All cultures and viral studies returned negative. Despite her condition, ESR was only 5, but CRP was 24 and ferritin was 152,197. Macrophage Activation Syndrome (MAS), a complication of SoJIA was considered and high dose solumedrol and cyclosporin were started. Blood product support, hemodialysis and pressor management were instituted. By HD #14 she was extubated and by HD #20 she was downgraded from ICU. She was discharged home on HD #31 on a prednisone taper and cyclosporin. Bone marrow biopsy during her ICU course showed prominent hemophagocytosis. TNF $\alpha$ , IL2 and IL6 levels were markedly elevated during her critical illness, but normalized by the time of discharge. MAS is a secondary hemophagocytic syndrome most commonly associated with SoJIA. Its symptoms are attributed to activation and proliferation of well-differentiated macrophages precipitated by a change in medication or infectious cause. Presenting symptoms include pyrexia, changes in mentation, organomegaly, lymphadenopathy, bleeding, bruising and purpura; paradoxically arthritis and serositis can improve. Laboratory abnormalities can include pancytopenia, transaminase elevation, coagulopathy, decreased ESR (hypofibrinogenemia), hypertriglyceridemia, hyponatremia, hypoalbuminemia and hyperferritinemia. Histological evaluation of bone marrow shows macrophage hemophagocytosis. Mortality exceeds 20%, but with rapid diagnosis and the institution of high dose steroids, cyclosporin and other immunomodulators, good outcomes can be achieved.

#### **Sa1.47. Differential Expression of Co-Stimulatory Molecules on PBMCs from Patients with Active Systemic Lupus Erythematosus and Rheumatoid Arthritis.**

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**Background:** Following activation of antigen-presenting cells (APCs), co-stimulatory pathways including B7-1 and B7-2 recognizing CD28 and CTLA-4 play a key role in the activation of autoreactive lymphocytes, also CD40 ligand is expressed by activated T cells and is considered to be a critically T-cell marker, and their increased expression might further contribute to cognate T-B cell interactions and autoantibody production. To succeed an appropriate interaction between T and B cells is required an appropriate signaling of co-stimulatory molecules, being the broadly studied; B7-CD28 and B7-CTL4 who are crucial in both pathways IL-2 production and tolerance induction. **Objective:** The aim of this study was to explore, which preferably co-stimulatory via in APCs interacting with CD4+ T cells, and which are the correlation with activity disease in Systemic Lupus Erythematosus and Rheumatoid Arthritis patients. **Methods:** Cross sectional study

was carried out in SLE and RA patients with activity disease. The proportion of peripheral mononuclear cells was studied using flow cytometry in order to measure the percentage of surface molecules in CD4+ T cells, and antigen presenting cells such CD14+ and CD19+. **Results:** CD4+ CD152+ T cells showed a high expression only in Lupus patients, CD80+ and CD86+ in both type of cells CD14+ and CD19+ showed increased levels of those molecules predominantly in Lupus patients.

**Conclusion:** Costimulatory molecules play an essential role in the activation and regulation of T cell immune responses in lupus and arthritis patients through CD28, CD30, CD152, CD154, CD80 and CD86, they could be used to monitoring the activity disease and also could be a target for therapeutic manipulation of the costimulatory system in order to beneficial effects in clinical autoimmune disease

**Keywords:** PBMCs, CD4+, CD28+, CD14+, CD19+, CD152, CD154, co-stimulatory molecules, CD80, CD86, disease activity, Systemic Lupus Erythematosus and Rheumatoid Arthritis.

#### **Sa1.48. Effects of Taxus Yunnanensis for the Pain on Patients with Rheumatoid Arthritis.**

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The extract from *Taxus yunnanensis* (TY) had many beneficial effects in certain clinical settings, in which physicians can easily use on patients without interrupting therapy. We will show the long-term effect of TY on patients with severe pain due to rheumatoid arthritis (RA). After prescribing the TY extract, the extent of morning stiffness, joint swelling and pain were reduced, and the lowered patients' quality of life (due to severe pain caused by RA) was significantly improved with increasing daily life activities. However, the beneficial effects of TY did not appear shortly, although TY had an immediate relieving effect on allergic diseases. Plasma cytokine levels were not decreased promptly. Although walking ability of the patients got better after 3 days, it took around 8–20 days and 1 month to improve morning stiffness and joint pain, respectively. Therefore, a longer prescribing period seems to be necessary to demonstrate the beneficial effect of TY. The following two patients were good responders to TY; they were prescribed TY extract in addition to the usual therapeutics for RA. It is noteworthy that their plasma IL-6 levels reduced to the normal level after taking TY.

Case 1 (Class 4 disability): Plasma IL-6 level dramatically reduced from 165pg/ml to the normal level 32 weeks after prescribing TY. CRP became negative simultaneously. RF also became negative at 20 weeks.

Case 2 (Class 3 disability): Plasma IL-6 decreased from 6.8pg/ml to the normal level 16 weeks after prescribing TY. CRP level also decreased from 4.4 to 0.6mg/ml within 8 weeks. RF activity in the serum improved from 220 to 78U/ml.

#### **Sa1.49. Protection from Collagen-Induced Arthritis by Complement Receptor CR2/CR1 Deficiency.**

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In mice, complement receptors CR2 and CR1 are expressed on B cells, T cells, and follicular dendritic cells, linking innate

complement and adaptive T cell and B cell immune responses. Upon binding of its ligand C3d, CR2 lowers the threshold for B cell activation; however, the function of CR2 on T cells is unknown. Mice deficient in CR2 and CR1 (*Cr2*<sup>-/-</sup>) demonstrate altered humoral immunity in response to foreign and self antigens as well as an altered natural antibody repertoire. Collagen-induced arthritis (CIA), a model of autoimmune arthritis, depends upon complement activation, effective collagen presentation by antigen presenting cells, autoantibody production by B cells, and cytokine production by T cells. Therefore, CIA provides a model in which the roles of lineage-specific expression of CR2 and CR1 in autoimmune disease may be dissected. DBA/1j mice were immunized with bovine type II collagen (CII) emulsified in complete Freund's adjuvant (CFA) on days 0 and 21 to establish CIA. On day 35, draining lymph nodes from mice with CIA demonstrated a greater percentage of CD4<sup>+</sup> cells expressing CR2 compared to naïve mice (3.59% vs. 1.29%, *P* = 0.005, respectively), suggesting a role for CR2 expression on T cells during autoimmune disease. To determine the importance of CR2/CR1 for the development of CIA, *Cr2*<sup>-/-</sup> and *Cr2* ± mice backcrossed 5 generations onto the DBA/1j strain were generated. Mice were immunized with CII in CFA on days 0 and 21 and evaluated in a blinded fashion for the development of arthritis. *Cr2*<sup>-/-</sup> mice (*n* = 20) had significantly reduced severity (2.5 ± 0.9 vs. 5.5 ± 1.2, *P* = 0.05) and incidence (45% vs. 64%) of arthritis compared to *Cr2* ± mice (*n* = 25). However, anti-bovine and anti-murine CII antibodies did not differ significantly between the two groups of mice, nor did cellular proliferation and cytokine production in response to CII restimulation *ex vivo* differ between the two groups. C3 deposition within the joints of *Cr2*<sup>-/-</sup> mice was significantly reduced, suggesting that activation of complement in these mice was altered. These results demonstrate that CR2 and CR1 are required for robust development of CIA, likely because of altered T cell activation and trafficking to the joint or because of defects within the anti-CII antibody repertoire.

#### **Sa1.50. Leptin Modulates Autoimmune Reactivity in (NZB × NZW)F<sub>1</sub> Lupus Mice.**

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Leptin is an adipocytokine that links the metabolic status to several important immune functions. We and others have recently shown that leptin, similarly to other pro-inflammatory cytokines, can promote the differentiation of T helper (Th1) cells and can contribute to the onset and progression of organ-specific autoimmunity in several animal models of autoimmune disease. Nonetheless, the role of leptin in systemic autoimmunity, and in particular in systemic lupus erythematosus (SLE), remains elusive. We studied here whether leptin exerted some influence on the development and progression of systemic autoimmunity in lupus-prone (NZB × NZW)F<sub>1</sub> (BWF1) mice. We found by ELISA that that the circulating levels of serum leptin increased progressively with age in untreated female mice (*P* < 0.0002 at 13 and 20 weeks vs 1 week of age). This increase correlated with development of autoantibodies and kidney disease. Importantly, treatment of BWF1 mice with recombinant leptin promoted Th1 autoreactivity (as indicated by predominant IgG2a rather than IgG1 anti-double stranded (ds)DNA antibody responses). Histological studies indicated that leptin accelerated production of autoantibodies and favored deposition of immune complexes and kidney glomerular damage in the mice treated with leptin, as compared to saline-

treated control mice. Interestingly, intraperitoneal injection of a single dose of 100 µg of anti-leptin antibodies to severely nephritic mice (proteinuria ≥ 300 mg/dl) delayed progression of renal disease and significantly prolonged survival of the treated mice (*P* < 0.001 by the Mann-Whitney *U* test at six weeks post-treatment). Taken together, these studies point to an important role of leptin in the development and progression of lupus in BWF1 mice and raise the possibility to consider leptin antagonists as novel therapeutic tools for immune intervention in systemic autoimmunity.

#### **Sa1.51. Hyperhomocysteinemia in Hughes Syndrome.**

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**Background.** Hyperhomocysteinemia is a risk factor for thrombosis and recurrent pregnancy loss. Similar mechanisms appear to mediate thrombosis and pregnancy loss associated with hyperhomocysteinemia and with antiphospholipid antibodies.

**Objective.** To report on the case of a patient with the association of hyperhomocysteinemia and antiphospholipid or Hughes syndrome; and to assess the prevalence of increased levels of homocysteine in a group of patients with Hughes syndrome.

**Materials and Results.** A 41-year old woman with a previous history of migraine, placental abruption, arterial hypertension, unstable angina and multinodular goitre was admitted because of two consecutive transitory ischemic attacks. Laboratory investigations revealed the presence of elevated titres of anticardiolipin antibodies (66 GPL-units/ml) and significant hyperhomocysteinemia [homocysteine concentration, 36 µmol/L (normal values: 1.6-11 µmol/L)]. The genotype of the termolabile C677T allele of 5,10 methylene tetrahydrofolate reductase was studied by polymerase chain reaction. The patient was found to be a carrier of the C677T homozygous genotype. She was treated with anticoagulants and showed radiological and clinical improvement. The patient started treatment with folic acid and vitamin B6. Hyperhomocysteinemia was normalized. We performed a retrospective study on 23 patients with clinical and laboratory features of the Hughes syndrome in comparison with 42 patients with unexplained fetal loss (without antiphospholipid antibodies) and with 51 patients with thrombotic events (without antiphospholipid antibodies). Rates of hyperhomocysteinemia were statistically similar in patients with Hughes syndrome and in disease control groups (17.4%, 9.5% and 23.5%, respectively). Using a cut-off point of 11 µmol/l, four patients with Hughes syndrome had hyperhomocysteinemia. Three patients had thrombotic events (including the presented case) and the other patient had 9 consecutive unexplained abortions. Homocysteine levels were similar in patients with Hughes Syndrome and disease controls (10 ± 0.6, 6.9 ± 0.5 and 9.98 ± 0.8 µmol/l, respectively). Patients with thrombosis (without antiphospholipid antibodies) had significantly higher levels of plasma homocysteine than women with abortions (*P* = 0.018).  
**Conclusion.** The results presented in this study suggest that hyperhomocysteinemia is not significantly increased in patients with Hughes syndrome. However, antiphospholipid antibodies may coexist with high levels of plasma homocysteine in individual cases of Hughes Syndrome.

### Sa1.52. Atherosclerosis in Murine SLE.

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**Introduction:** The accelerated development of atherosclerosis and increased risk of cardiovascular disease in young women with systemic lupus erythematosus (SLE) is a disturbing feature of the disease that is not well understood. We have combined mouse models of SLE and atherosclerosis to begin to elucidate the mechanisms of this disease synergy.

**Methods:** Chronic graft-versus-host (cGVH) disease was induced in young apoEKO C57BL/6 mice by injection of 10E8 coisogenic bm12 spleen cells. Mice were maintained on normal chow diet. Mice were sacrificed, and the hearts and aorta were collected at 16 weeks after induction of cGVH for histology. The cholesterol levels were measured with an enzymatic colorimetric method. The frozen heart tissues were stained with Oil red O and hematoxylin, and the aortas were stained with Sudan IV. En face lesion areas were calculated with image-pro 5.0 software system. Serum IgG, anti-dsDNA, anti-chromatin, anti-oxLDL, and anti-cardiolipin levels were measured by ELISA. Proteinuria was detected with Uristix reagent strips at sixteen weeks after induction of GVH. Spleen cells were stained with immunofluorescence and detected with FACS.

**Results:** The plasma cholesterol levels in the apoEKO mice were greatly increased, and this was not significantly changed by cGVH. cGVH induced increased levels of IgG, anti-chromatin, anti-DNA, anti-oxLDL, and anti-cardiolipin, as well as proteinuria, in both C57BL/6 and in apoEKO mice. ApoEKO mice with and without cGVH had substantial lesions in the aortic root and aorta tree, as well as some lesions in the coronary arteries, which were slightly increased by cGVH. ApoEKO mice had increased numbers of splenic marginal zone B cells, which were depleted by cGVH.

**Conclusion:** These results indicate that we can induce cGVH and lupus-like autoimmunity in apoEKO mice, and alter some of the manifestations associated with Atherosclerotic cardiovascular disease (ASCVD). We have extended this approach to other mouse lupus models, such as C57BL/6-lpr/lpr.

### Sa1.53. Association of a Single Nucleotide Polymorphism of PTPN22 (1858C/T) with Rheumatoid Factor Positivity in a Healthy Population.

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**Objective:** It has been demonstrated previously that a single nucleotide polymorphism C1858T in the protein tyrosine phosphatase gene PTPN22 is associated with a rheumatoid factor (RF) positive subset of patients with known rheumatoid arthritis. Our study was performed to investigate the association between this polymorphism and the presence of RF in a healthy population.

**Methods:** Healthy subjects were recruited in Denver, Colorado as part of the ongoing Studies of the Etiologies of Rheumatoid Arthritis (SERA) project. At the time of this interim analysis, 334 subjects were available [mean age of 38 (range 29–56), 88% non-Hispanic white, and 69% female]. Each of these subjects provided

epidemiologic information and underwent an interview and physical examination to ensure that no subjects included in the analysis had evidence of RA. Serum samples were drawn and the presence of RF was determined using nephelometry. The PTPN22 polymorphism (1858C → T) was identified using MGB-Eclipse™ Probe System (Epoch Biosciences, Inc), performed at the Benaroya Research Institute, Seattle, Washington. Statistical analysis was performed using logistic regression (SAS version 8).

**Results:** In this healthy population, 45 out of 334 (13%) subjects had a positive RF. 64 out of 334 (18.9%) had at least one PTPN22 variant allele. After adjusting for age, gender, race, smoking status, and shared epitope status, the PTPN22 polymorphism was marginally associated with the presence RF (OR 2.02, Confidence Limits[CL] 0.92-4.45),  $P = 0.08$ .

**Conclusions:** In this preliminary analysis of healthy subjects, the 1858C → T missense single nucleotide polymorphism in PTPN22 is marginally associated with the presence of RF. The size of the odds ratio is similar to that reported previously for this polymorphism's association with RF positive RA patients and will likely become statistically significant once we complete patient accrual (estimated 450 subjects by Spring 2006). This gene may contribute to pre-clinical immune dysregulation and the initial development of RA-specific autoimmunity.

### Sa1.54. Anti C1q, Anti Histone Antibodies and C1q Deposits in Silent Lupus Nephritis.

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**Specific Objective:** To investigate the presence of Anti C1q, Anti-Histone antibodies and C1q deposits in Silent Lupus Nephritis (SLN).

**Materials and Methods:** 29 SLN (LUPUS 12:26-30, 2003) and 11 SLE patients with Overt Lupus Nephritis (OLN) were included. Urinary and renal function tests were assessed by standard techniques. Renal Biopsies were performed in all the studied SLE patients. Anti C1q (Siegert et.al), Anti Histone(Aesku.diagnostics), Anti ds DNA (Wampole) were detected by ELISA. Immunoglobulins, C1q, C3 and C4 deposits were searched in renal tissues by Immunofluorescence (IF).

**Results:** 18/27 (66.6%) SLN individuals had elevated Anti C1q antibodies (mean titers: 127.3 ± 52.5 EU) while in OLN 4/9 (44.4%) were positive (mean titers: 134 ± 94.8 EU). Anti Histone antibodies were positive in 8 out of 19 (mean titers: 98.5 ± 61.9 U/ml) available SLN sera (42%) while 6 out of 7 (85.7%) OLN showed augmented serum levels (mean titers: 84.5 ± 62.8). Anti dsDNA were elevated in 28/29 (96.5%) SLN patients (mean titers: 98.7 ± 88.3 U/ml) and in 10/11 (91%) of OLN individuals (mean titers: 54.2 ± 30.7 U/ml). Prevalence and mean titers of these three autoantibodies were significantly different than those encountered in 25 control sera ( $P < 0.01$ ). In addition in SLN patients, serum levels of Anti C1q and Anti Histone antibodies significantly correlated with Anti dsDNA levels and with increased activity index in renal tissue ( $P < 0.05$ ). As previously reported, 18/29 (62%) SLN showed WHO Class II kidney lesions. In those SLN with detectable Anti C1q antibodies, IgG (66%), C1q (44%), C3 (77%) and C4 (55%) deposits were found.

**Conclusions:** This is the first report of detectable Anti C1q and Anti Histone autoantibodies in SLN. Furthermore, their significant correlation with Anti dsDNA antibodies and with increased activity index in renal tissue suggest an early and



simultaneous participation of these complexes in Lupus Nephritis.

### **Sa1.55. Modulation of Inflammation in Refractory Rheumatoid Arthritis by Granulocyte Apheresis: First European Prospective Multicenter Open Pilot Study.**

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**Introduction:** Granulocyte apheresis (GCAP) is a novel hemoadsorption treatment that is currently being used for the treatment of some autoimmune diseases. Immunomodulatory properties of GCAP have been reported associated to emerging evidence of clinical improvement in patients.

**Objective:** To assess the efficacy and safety of Adacolumn® GCAP in patients with refractory rheumatoid arthritis (RA).

**Methods:** Patients with active RA who had failed to respond to at least one DMARDs or biologics (TNF-alpha antagonists) were treated with weekly GCAP for five weeks. Clinical assessments and response to therapy were analyzed at weeks 5,7,12 and 20 in an open multicenter pilot trial. The primary outcome measure of therapeutic response was the 20% improvement in the American College of Rheumatology criteria at week 20. EULAR response criteria based in the disease activity score for 28 joints (DAS-28) and disability by the Health Assessment Questionnaire (HAQ) were also analyzed.

**Results:** Twenty seven patients were enrolled: 81.5% were women with mean disease duration of 14.4 years. The mean number of previous DMARDs was 3.7 and 48.1% of them have failed to biologics. On an intent to treat basis analysis 40.7% of patients achieved an ACR20 improvement and 44.4% patients a therapeutic EULAR response at week 20. These percentages were of 50% and 54.5% in the 22 patients who completed the trial. In four out of the 10 patients who completed the trial and previously failed to biologics, an ACR20 response was achieved at week 20. A significant decrease was noted in the different ACR response components, including the tender joint count, swollen joint count, pain score and the patient's and physician's global assessment and also the DAS28 index; most of them improve since week 5. ESR and CRP, but not HAQ, significantly decrease at week 20. The treatment was well tolerated and only one serious adverse event related to study therapy was documented (sepsis due to catheter infection).

**Conclusions:** Treatment with GCAP led to significant clinical improvement in a subset of patients with RA who previously failed to DMARDs or biologics. The therapy was safe and well tolerated. Further large, placebo controlled studies are required to assess the exact role of this therapy in refractory RA.

### **Sa1.56. Interferon-alpha Pathway Activation Identifies a Subgroup of Systemic Lupus Erythematosus Patients with Antibodies against RNA-Binding Proteins, Renal Disease, and Low Complement.**

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Gene expression studies have demonstrated increased interferon (IFN)-inducible gene (IFIG) expression in peripheral blood mononuclear cells (PBMC) of many patients with systemic lupus erythematosus (SLE). Our recent data have implicated a predominant type I IFN effect in the IFIG expression observed in SLE. The objective of this study was to examine the hypothesis that increased disease severity and activity as well distinct autoantibody specificities characterize SLE patients with type I IFN pathway activation. In order to do that, freshly isolated PBMC from 77 SLE patients, 22 disease controls (DC), and 28 healthy donors (HD) were subjected to real-time PCR for 3 IFIG that are preferentially induced by IFN $\alpha$ , and the data were used to derive IFN $\alpha$  scores for all individuals. Expression of IFIG was significantly higher in SLE patients compared to DC or HD. SLE patients with high (H) and low (L) IFN $\alpha$  scores were compared for clinical manifestations of disease, disease severity, disease activity, serologic features and potential confounders by bivariate and multivariate analysis. We found that SLE patients with a H IFN $\alpha$  score had significantly higher prevalence of renal disease, a greater number of ACR criteria for SLE, and a higher SLICC damage index (DI) score than SLE patients with L IFN $\alpha$  scores. Patients with H scores showed increased disease activity, as measured by lower C3, hemoglobin, absolute lymphocyte count, and albumin, and higher anti-dsDNA titer, ESR, and SLEDAI-2K score. The presence of antibodies specific for RNA-binding proteins (RBP: Ro, U1-RNP, Sm), and dsDNA, but not phospholipids, was significantly associated with a H IFN $\alpha$  score. Logistic regression analysis confirmed that renal disease, higher SLICC DI scores, low complement levels, and presence of anti-RNA binding protein (RBP) autoantibodies were independently associated with a H IFN $\alpha$  score, and suggested that the same might be true for the absence of treatment with hydroxychloroquine (HCQ). In conclusion, activation of the IFN $\alpha$  pathway defines a subgroup of SLE patients characterized by increased disease severity, including renal disease, increased serologic disease activity, and autoreactivity to RBP. These data provide support for the further examination of the role of IFN $\alpha$  score as a potential biomarker for lupus disease activity and suggest a pathogenic link between RBP and IFN $\alpha$  production.

### **Sa1.57. The Rheumatic Joint Contains Hyperreactive CD28<sup>null</sup> CD4<sup>+</sup> T Cells.**

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**Background.** A subpopulation of unusual CD4<sup>+</sup> T cells lacking the costimulatory molecule CD28 can be found in a subpopulation of patients with chronic inflammation and to a lesser extent in healthy individuals. These CD28<sup>null</sup>CD4<sup>+</sup> T cells are potent secretors of TNF and IFN- $\gamma$ , and proliferate vigorously upon stimulation. We, and others, have previously demonstrated that circulating CD28<sup>null</sup>CD4<sup>+</sup> T cells can constitute of up to 50% of CD4<sup>+</sup> T cells in peripheral blood (PB) from patients with chronic rheumatic diseases.

**Aim.** Are CD28<sup>null</sup>CD4<sup>+</sup> T cells present also in the inflamed joint of rheumatic patients? And if so, are their functional profile proinflammatory?

**Methods.** Mononuclear cells were isolated from PB and synovial fluid (SF), from inflamed knee joints, of patients with different rheumatic diseases. The cells were sorted into CD28<sup>null</sup> and conventional CD28<sup>+</sup> CD4<sup>+</sup> T cells and stimulated in vitro by anti-CD3 without the presence of antigen presenting cells.

**Results.** CD28<sup>null</sup> CD4<sup>+</sup> T cells could be found in synovial fluid from patients, but only in individuals displaying this population also in their peripheral blood. These CD28-negative cells from the inflamed joints were confirmed to be CD28<sup>null</sup> cells since they had the same restricted TCR Vbeta repertoire as the corresponding cells in the circulation. The joint derived CD28<sup>null</sup> cells were as proliferative and prone to secrete proinflammatory cytokines as the CD28<sup>null</sup> cells in PB.

**Conclusion.** When present in the joint CD28<sup>null</sup> cells is likely to contribute to the inflammation as they easily expand and secrete proinflammatory cytokines. Ongoing efforts include investigations of synovial tissue for CD28<sup>null</sup> cells and linking the presence of these hyperreactive T cells in the joint to a clinical feature.

### Sa1.58. Absence of B Cells Decreases Both Proliferation and Cytokine Secretion.

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The aim is to analyze if the reduced proliferation and cytokine secretion of mononuclear cells in systemic lupus erythematosus (SLE) patients treated with the B cell depleting therapy Rituximab can be explained by the absence of B cells.

**Background and methods:** Rituximab, an anti CD20 antibody therapy, was originally developed against lymphomas, but is now increasingly used in autoimmune diseases. Our earlier studies of Rituximab treated SLE patients have shown that shortly after treatment both proliferation and cytokine secretion decreased in *in vitro* cultures, and increased with the return of B cells in the circulation. To investigate if it is the lack of B cells or the immunosuppressive treatment given together with Rituximab that accounts for this dramatic effect, we established an *in vitro* system. B cells were depleted by anti CD20 magnetic beads from peripheral blood mononuclear cells (PBMC) from three healthy subjects. Cells were stimulated with PHA or anti-CD3 antibodies, with or without anti-CD28 co-stimulation. Cytokine secretion and proliferation were measured with cytometric bead array and <sup>3</sup>H-Thymidine incorporation.

**Results:** Similar to our *ex vivo* patient data, both proliferation and cytokine secretion were reduced in B cell depleted PBMC cultures as compared to intact PBMC cultures. This was true for both PHA and anti-CD3 stimulated cells.

Mainly TNF $\alpha$ , IL-10 and IL-6, but also IL-4 and IL-2, were secreted to a lesser extent.

**Discussion:** Our *in vitro* experiments indicate that it is not the immunosuppressive treatment that accounts for the decreased immune response seen in Rituximab treated SLE patients, but a per se effect of the lacking B cells. Future experiments will delineate if it is their potential to co-stimulate, to present antigens, or to secrete cytokines that is most important for the activation of T cells.

### Sa1.59. Synovial Fluid NKT Cells Display Different Properties Compared to Peripheral Blood NKT Cells in Rheumatoid Arthritis.

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Natural killer T cells (NKT) are a population of regulatory T cells that co-express an invariant T cell receptor as well as NK cell

markers. Several studies have shown that NKT cells are decreased or dysfunctional in autoimmune conditions such as insulin-dependent diabetes mellitus, systemic sclerosis, systemic lupus erythematosus and multiple sclerosis. Significant therapeutic effects of  $\alpha$ -GalactosylCeramide ( $\alpha$ -GalCer), a synthetic antigen of NKT cells, have been demonstrated in animal models of autoimmunity. NKT cells have therefore been implicated to participate in the regulatory immune mechanisms controlling autoimmunity. However, their role in the pathogenesis of rheumatoid arthritis (RA) remains unclear. To this end, we studied the frequency, cytokine profile and heterogeneity of NKT cells in peripheral blood mononuclear cells (PBMC) of 23 RA patients and 22 healthy controls, which included paired PBMC-synovial fluid (SF) samples of 7 and paired PBMC-synovial tissue (ST) samples of 4 RA patients, respectively. Using flow cytometry, a decreased NKT cell frequency was observed in blood of RA patients compared to healthy controls. In addition, direct *ex vivo* ELISPOT analysis revealed a reduced IL-4/IFN- $\gamma$  ratio in NKT cells of RA patients. The invariant T cell receptor sequence was detected in paired SF and ST samples. NKT cells of all healthy controls, but only of 53.8% of the RA patients (responders) expanded upon *in vitro* stimulation. However, reactivity towards  $\alpha$ -GalCer was observed in NKT cells isolated from SF of both responder and non-responder RA patients. Intracellular FACS analysis of the cytokine profile of CD4<sup>+</sup> and CD4<sup>-</sup> PBMC derived NKT cell lines of RA patients revealed that both produced significantly less IL-4 compared to those of healthy controls. In contrast, SF derived NKT cell lines displayed a Th0 phenotype comparable to that of healthy controls. These findings suggest that SF NKT cells are functional, even in patients with non-responding NKT cells in the blood.

In conclusion, our data demonstrate that NKT cells are decreased and biased towards a Th1 phenotype in blood, but are not impaired in SF of RA patients. This indicates NKT cells that might be functionally related to resistance or progression of rheumatoid arthritis.

### Sa1.60. Regulation of Myeloid Cell Function and MHC Class II Expression by TNF.

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Neutralizing agents to TNF are the most successful means to ameliorate systemic autoimmune inflammation. Neutralization of TNF, however, is often associated with the development of autoantibodies, in particular to nuclear antigens, the mechanisms of which are unknown. Here, we analyzed the effect of TNF and its neutralization on MHC class II expression and function of antigen presenting myeloid cells in rheumatoid arthritis (RA). Monocytes were isolated from the peripheral blood of RA patients before and after anti-TNF-mAb treatment and from controls by negative selection, differentiated *in vitro* into macrophages and analyzed by flowcytometry for HLA-DR expression. T cell responses to activation by myeloid cells were assessed in proliferation assays, and mRNA levels of the class II transactivator (CIITA) were determined by semiquantitative RT-PCR. HLA-DR expression was significantly reduced on myeloid cells from RA patients with active disease, but was increased to normal levels after TNF mAb treatment. Concordantly, *in vitro* application of TNF to monocytes from healthy individuals reduced their ability to upregulate HLA-

DR during differentiation to macrophages and, importantly, inhibited their ability to stimulate T cells in mixed lymphocyte reactions. Molecular analysis revealed that the effect of TNF on HLA-DR expression was mediated via suppression of the transcription factor CIITA. The data indicate that TNF decreases HLA-DR expression by reducing CIITA mRNA levels in myeloid cells, functionally resulting in a decreased stimulatory capacity of myeloid cells for T cells. Concordantly, ameliorating disease activity in chronic inflammatory diseases by neutralizing TNF restores HLA-DR expression of myeloid cells and their ability to stimulate T cells. Thus, anti-TNF treatment might lead to augmented T cell activation by myeloid cells, thereby promoting immune responses to (auto)antigens and the development of anti-nuclear antibodies that are frequently associated with anti-TNF therapy.

### Sa1.61. Isolated Type 5 Antimitochondrial Autoantibodies Associated with History of Thrombocytopaenia and Foetal Loss.

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Anti-mitochondrial (AMA) of M5 type antibodies were initially described in patients with autoimmune diseases, and then have been identified in sera of patients with antiphospholipid antibodies and recurrent foetal loss, haemolytic anaemia and thrombocytopaenia, and have controversial association with thrombosis. Up to date, very scarce literature exists regarding M5 type AMA. AMA of M5 type are directed towards an unknown antigen located in the inner membranes of mitochondria (50 kDa). Indeed, M5 type AMA have been always reported in the context of antiphospholipid syndrome strictly linked to antiphospholipid antibodies.

We report here on a 65-years-old Caucasian woman diagnosed as autoimmune polyglandular syndrome (APS) IIIC type, namely autoimmune thyroiditis, pernicious anaemia and recurrent idiopathic thrombocytopaenic purpura. Clinical history was also relevant for two foetal losses at 2 and 4 gestational months, respectively, associated to persistent M5 type AMA at high titre (1/640). Antinuclear, anti-DNA and antiphospholipid antibodies (anticardiolipin, anti-beta-2-glycoprotein-I) were all of them persistently negative through a 10-years follow-up period. Coagulation studies were repeatedly normal. Conclusion: In our patient, type 5 AMA was the only marker of thrombocytopenia and recurrent miscarriages without antiphospholipid antibodies. In isolated cases, M5 Abs appear to be a diagnostic marker for clinical manifestations of antiphospholipid syndrome.

### Sa1.62. FOXP3+ Regulatory T Cells in the Rheumatic Joint: Contribution of Both CD25+ and CD25- T Cells.

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#### Background

Natural regulatory T cells are usually identified in two ways; by mRNA expression of the transcription factor FOXP3 or by the level of surface expression of CD25. Humans are never immunologically naïve, resulting in T cells expressing CD25 also due to activation. Thus, frequency determinations based on CD25 expressing regulatory T cells can never be more than rough estimates.

#### Objective

In this study we investigate the presence of regulatory T cells in different compartments of patients with rheumatic joint disease. We compare CD25 and FOXP3 in peripheral blood and the site of inflammation, by analyzing both synovial fluid and synovial tissue.

#### Materials and methods

FOXP3 mRNA levels were investigated from sorted peripheral blood and synovial fluid CD4 T cells populations. The sorted populations expressed different densities of CD25. RNA was also prepared from synovial tissue biopsies. Additionally, CD25- T cells were activated in vitro to investigate possible induction of FOXP3.

#### Results and discussion

We could find FOXP3 message in all compartments investigated, even in biopsies from synovial tissue. In synovial fluid, the CD25bright cells were markedly enriched for FOXP3 compared to the CD25int, while the difference between the two CD25 populations were less apparent in blood. Interestingly, also cells negative for CD25 could be FOXP3+, and this was more common in synovial fluid than in blood.

Thus, our study shows that FOXP3+ regulatory T cells are not restricted solely to CD25+ T cells. Especially in an inflammatory environment like synovial fluid FOXP3+ CD25- T cells were found. An induction of FOXP3 in CD25- cells could not be mimicked in vitro perhaps suggesting that these regulatory T cells originate from CD25+ cells that have downregulated or shed their CD25 expression. Such a scenario is supported by studies showing the presence of soluble CD25 in both sera and synovial fluid from rheumatic patients.

### Sa1.63. Increased Systemic and Local Expression of Toll-Like Receptor 2 and 4 in Spondyloarthritis Is Downmodulated by Tumour Necrosis Factor alpha Blockade.

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**Objective:** An abnormal host defense against pathogens is implicated in the pathogenesis of spondyloarthritis (SpA), a disease characterized by abundant synovial infiltration with innate immune cells. Considering the role of Toll-like receptors (TLRs) in activation of innate inflammation and occurrence of TLR-dependent infections after TNFalpha blockade, we analyzed TLRs in SpA and their modulation by TNFalpha blockade.

**Methods:** Peripheral blood monocytes were obtained in SpA and rheumatoid arthritis (RA) during infliximab therapy and in healthy controls (HC). Expression of TLR2 and TLR4 and TNFalpha production upon LPS stimulation were analyzed by flowcytometry on different monocyte subsets. Synovial biopsies from 23 SpA before and after infliximab or etanercept treatment and from 15 RA were analyzed by immunohistochemistry.

**Results:** TLR4, but not TLR2, expression was increased on monocytes in SpA, whereas both TLRs were increased in RA. The CD163+ macrophage subset, which is increased at the inflammatory sites in SpA, has a particularly increased TLR expression. Accordingly, expression of both TLRs was significantly higher in SpA than in RA synovium. Infliximab decreased TLR2 and TLR4 expression on monocytes in SpA and RA, leading to lower levels than in HC and to an impaired TNFalpha production upon LPS stimulation. Paralleling the systemic effect, synovial TLRs were downregulated following infliximab as well as etanercept, indicating a class-effect of TNFalpha blockers.

**Conclusions:** SpA inflammation is characterized by increased TLR2 and TLR4 expression which are sharply reduced by TNF $\alpha$  blockade. These data emphasize a central role for innate immune-mediated inflammation in SpA and provide an additional clue for the efficacy as well as the potential side-effects of TNF $\alpha$  blockade.

**Sa1.64. IgM Anti-dsDNA Antibodies Are the Main Anti-Nuclear Reactivity Induced by Infliximab but Not Etanercept Treatment: Biological and Clinical Implications.**

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**Background:** Previously, we demonstrated anti-nuclear antibody (ANA) and anti-dsDNA antibody induction after 30/34 weeks of infliximab therapy in rheumatoid arthritis (RA) and spondyloarthritis (SpA).

**Aim:** To further assess in detail the clinical and biological correlates of autoantibody induction during longer-term TNF $\alpha$  blockade with either the monoclonal antibody infliximab or the soluble receptor etanercept.

**Methods:** 34 SpA and 59 RA patients were treated with infliximab for two years. Additionally, 20 SpA patients were treated with etanercept for one year, providing a unique head-to-head comparison of autoantibody induction during TNF $\alpha$  blockade in a human disease model with low baseline autoimmunity. Sera were blindly analysed for ANA, anti-dsDNA, anti-ENA, anti-histone and anti-cardiolipin antibodies. The anti-dsDNA antibodies were further isotyped with gamma-, mu- and alpha-chain specific conjugates.

**Results:** In the infliximab-treated SpA and RA cohorts, we observed high numbers of newly induced ANA (61.8% and 40.7%) and anti-dsDNA antibodies (70.6% and 49.2%) after one year, but no further increase between year 1 and year 2. In contrast, induction of ANA (10%) or anti-dsDNA antibodies (10%) was only occasionally found in the etanercept-treated SpA cohort. Neither during infliximab nor etanercept, anti-ENA, anti-histone antibodies or clinically relevant lupus-like symptoms were described. Isotyping revealed predominantly IgM and/or IgA anti-dsDNA antibodies. Similarly, infliximab but not etanercept selectively increased the IgM but not the IgG anti-cardiolipin titers.

**Conclusion:** This study indicates that the prominent ANA and anti-dsDNA autoantibody response is not a pure class effect of TNF $\alpha$  blockers, is independent of the disease background and is not associated with clinically relevant lupus-symptoms.

**Sa1.65. High Serum Levels of Chemokines but Low Expression of Chemokine Receptors of Peripheral Blood Mononuclear Cells in Juvenile Systemic Lupus Erythematosus.**

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease and immune function in SLE is paradoxically characterized by active T cell help for autoantibody production, along with impaired T cell proliferative and cytokine responses in vitro. Recently, evidence reveals that chemokines as well as chemokine receptors are closely involved in initiating the hyperreactivity. This study was designed to investigate the expression levels of various chemokines and their receptors in relation to the disease activity of juvenile SLE, and to compare the pattern of chemokine elevations with that in normal individuals. Serum levels of chemokines including CCL-2, CCL-5, CXCL-8, -9 and -10 were analyzed by chemokine cytometric beads arrays (CBA), whereas the expression of chemokine receptors such as CCR-2, -3, -4, and -5 on peripheral blood mononuclear cells (PBMC) were assessed by real-time RT-PCR and/or Western blot. Here we demonstrate the difference of chemokines and their receptors expression in juvenile SLE patients compared with normal individuals. Significantly higher serum levels of CCL-2 (MCP-1), CXCL-8 (IL-8), -9 (MIG) and -10 (IP-10) were found in most SLE patients analyzed, while their chemokine receptors such as CCR-2, -3, -4, and -5 were expressed relatively low in patients' PBMC. Analysis between clinical manifestations such as SLEDAI (Systemic Lupus Erythematosus Disease Activity Index) and levels of the above chemokines expression levels revealed a strong correlation. However, decreased serum levels of CCL-2, CXCL-9 and CXCL-10 appeared in patients with high levels of anti-dsDNA if compared with those in patients with low anti-dsDNA. Overall, four chemokines that were elevated in SLE were proinflammatory, characteristic of activation of the monocyte and macrophage lineage, and in the case of IL-8, also of neutrophils. These data suggest a major role for a cell-mediated immune response occurring in the pathophysiology of SLE.

**Sa1.66. Modulation of Murine Lupus by an Inhibitory GpG Oligonucleotide.**

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Activation of the innate immune system by DNA containing hypomethylated CpG motifs has been implicated in the pathogenesis of systemic lupus erythematosus (SLE). We have previously described an immunomodulatory oligodeoxynucleotide (ODN), containing a single base switch from CpG to GpG, which ameliorated murine experimental autoimmune encephalomyelitis (EAE), a T helper 1-mediated model of human multiple sclerosis. Here, we examined the consequences of immunostimulatory CpG-ODN and inhibitory GpG-ODN treatment in the NZB $\times$ NZW F<sub>1</sub> (NZB/W) murine model of SLE. Beginning at 5 months of age, we administered CpG or GpG ODNs at regular intervals to female NZB/W animals, over a period of 20 weeks. While CpG-ODN treatment did not appear to impact overall disease severity, GpG-ODN treatment significantly delayed the onset of proteinuria, and improved 40-week survival in NZB/W mice. We also determined the effects of ODN administration on NZB/W T lymphocyte cytokine profiles and splenocyte surface marker expression. Interestingly, GpG-ODN treatment enhanced production of TNF- $\alpha$  by NZB/W T cells, and inhibited T cell production of IL-4. Consistent with observations made in the

EAE model, CD11c<sup>+</sup> splenocytes derived from GpG-ODN treated NZB/W mice also displayed reduced surface expression of CD80 and CD86. Taken together, the data indicate that GpG-ODN treatment can modulate immune cell function and ameliorate disease in the NZB/W model of lupus nephritis. The protective mechanism of the GpG-ODN in murine SLE may involve general inhibitory effects on costimulatory molecule expression by antigen presenting cells, as well as alteration of T cell cytokine profiles.

**Sa1.67. The Phosphodiesterase Inhibitor Rolipram Facilitates the Suppression of Antigen-Induced Arthritis in Mice by Oral Administration of the Inducing Antigen.**

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Clinical trials of the oral treatment of patients with autoimmune diseases including rheumatoid arthritis and multiple sclerosis with type II collagen and myelin, respectively, showed disappointing results. This may be in part due to the insufficient induction of oral tolerance to the respective autoantigen in humans. Therefore, we have looked for agents that can facilitate induction of oral tolerance. In the present study, we tested the hypothesis that the phosphodiesterase IV inhibitor rolipram, that was previously reported to produce suppressive cytokines including TGF-beta and IL-10, can facilitate the suppression of antigen-induced arthritis (AIA) in mice by oral administration of the inducing antigen. Such suppressive cytokines have been shown to play a role in oral tolerance, especially induced by low doses of oral antigen. To prove the hypothesis, DBA/1J mice were immunized with ovalbumin (OVA) emulsified with CFA (day 0). AIA was induced by intraarticular injection of OVA in PBS on day 21. Oral tolerance was induced by oral administration of either 0.1 or 10 mg of OVA daily over a period of 5 consecutive days commencing on day -5. Rolipram (1 and 3 mg/kg) was orally given immediately before each administration of OVA. The results showed that oral administration of 0.1 and 10 mg of OVA alone was followed by suppression of AIA, although the extent of suppression of AIA was greater in mice fed 10 than 0.1 mg of the oral antigen. When 0.1 mg of OVA was given together with rolipram, significantly facilitated suppression of AIA was observed. Co-administration of 10 mg of OVA and rolipram failed to modulate the suppression of AIA caused by the oral antigen alone. Secretion of IFN-gamma from spleen cells was suppressed by 0.1mg of oral OVA alone and this suppression was significantly enhanced in mice given both the antigen and rolipram. In contrast, the suppression of IFN-gamma secretion by administration of 20 mg of OVA alone was blocked by the combination of the same dose of the antigen and rolipram. There was no difference in the secretion of IL-10 between either 0.1 or 10 mg of OVA alone- and OVA plus rolipram-treated groups. These results suggest that rolipram appears to facilitate the suppression of AIA by oral administration of low (0.1 mg) but not high (10 mg) doses of OVA. This may be in part explained by significantly accelerated decreases in IFN-gamma in mice treated with the low dose of OVA plus rolipram. In our studies, the facilitated suppression of AIA by the administration of the oral antigen together with the phosphodiesterase IV inhibitor does not appear to be mediated by the modulation of IL-10 secretion. Agents such as rolipram that facilitate induction of oral tolerance might be useful in the treatment of autoimmune diseases in

humans including rheumatoid arthritis with oral pathogenic autoantigens.

**Sa1.68. Therapeutic Response of Rituximab in a Patient with Amyopathic Dermatomyositis Refractory to Methotrexate and Cyclosporin.**

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**Objective:** Rituximab, an anti-CD20 monoclonal antibody, has become a target for immunotherapy of B cell lymphomas and, more recently, B cell-mediated autoimmune diseases. We report a case involving a 45 year old female patient with severe autoimmune disease and B cell immunodeficiency who was treated with rituximab.

**Findings:** The patient initially presented at 42 years of age with Raynaud's phenomenon, positive anti-nuclear antibody (ANA), and positive thyroid peroxidase antibodies. Fever, oral and vaginal ulcers, polyarthritides, digital vasculitis, elevated erythrocyte sedimentation rate, and elevated rheumatoid factor (RF) also developed. The mixed connective tissue disease was treated with azathioprine, hydroxychloroquine, and methylprednisolone. Meanwhile digital infarcts, polychondritis, and a malar facial rash occurred. A classic dermatomyositis rash was confirmed by skin biopsy. Subsequently, her amyopathic dermatomyositis was treated with intravenous immunoglobulin (IVIG) 25 grams in addition to methotrexate 25 mg weekly and cyclosporin resulting in limited improvement. Painful, debilitating digital infarcts, Coombs positive hemolytic anemia, and pulmonary vasculitis evolved. Upon our immune evaluation, lymphocyte studies showed a low B cell percentage of 4%, a low absolute B cell count of 53 cells/ml, increased CD4+CD45RA+ naive T cells at 51%, and low CD4CD45RO+ memory T cells at 27%. Additionally, lymphocytic mitogenic responses were markedly decreased to StaphA, a B cell mitogen. Also, there was an increase in C3D immune complexes. RF was elevated at 58 IU/ml and Epstein Barr Virus (EBV) serology showed a high titer viral capsid antigen IgG and high titer early antigen antibody suggestive of reactivated EBV disease. Treatment of dermatomyositis with an underlying B cell immunodeficiency was started with high dose IVIG at 1 gram/kg of Gammunex combined with cyclophosphamide and steroids. Thereafter, all in vitro markers of autoimmunity including C-reactive protein, ANA, direct Coombs, and RF normalized. However, her pulmonary and upper extremity vasculitis progressed. Rituximab therapy was considered because our immune evaluation revealed a preponderance of CD20+ cells. Five weekly doses of rituximab at 375 mg/m<sup>2</sup> were added to the high-dose IVIG, cyclophosphamide, and steroid therapy. The pulmonary vasculitis improved, digital infarcts and ulcers slowly healed, and all autoimmune markers including RF became normal. Immune studies repeatedly showed <1% CD19+ and CD20+ cells at 24 weeks. The patient was weaned off cyclophosphamide and remains on IVIG and low dose prednisone with no further exacerbations of her autoimmune disease at 24 weeks.

**Conclusion:** Our patient with a severe, refractory autoimmune disease and underlying B cell defect responded successfully to addition of rituximab, specifically targeting a B-cell mediated autoimmune process. The favorable response of rituximab in our patient is supported by recent published reports showing B cell depletion with rituximab led to a sustained clinical response in methotrexate-resistant rheumatoid arthritis.

### Sa1.69. Azathioprine Use but Not Hydroxychloroquine or Prednisone Use Is Associated with Lower Coronary Artery Calcification Determined by Electron Beam Computed Tomography (EBCT) in SLE Patients.

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**Purpose:** To determine if several of the DMARDs, B-blockers, ACE-I, or aspirin are associated with the presence of coronary calcification by Electron Beam Computed Tomography (EBCT) in SLE patients.

**Methods:** One hundred and thirty seven patients with SLE over the age of 18 who fulfilled at least 4 of the American College of Rheumatology criteria for the classification of SLE were recruited for the study. A history, physical exam, EKG and EBCT measuring coronary calcium were performed. Results from the EBCT were used as an independent measure of current cardiovascular disease. The information regarding current, past and number of years on a medication was recorded. Analysis including standard student's t-test was performed on the data.

**Results:** A t-test analysis of the data showed that when comparing patients with SLE who were currently on azathioprine to patients with SLE who were not currently taking the drug, those on the drug had a lower EBCT calcium score, ( $P = 0.03$ ). Results were unchanged when "ever" users were added to the analysis. Comparison of SLE patients taking hydroxychloroquine to those who were never on hydroxychloroquine showed no difference in EBCT calcium score, ( $P = 0.59$ ). Comparison of SLE patients on prednisone to those SLE patients never on prednisone showed no difference in EBCT score, ( $P = 0.35$ ). SLE patients having received intravenous cyclophosphamide also showed no difference in EBCT score compared to those who were never exposed to the drug, ( $P = 0.48$ ). SLE patients taking mycophenolate mofetil and methotrexate similarly showed no difference in EBCT score for those exposed versus not exposed, ( $P = 0.15$  and  $P = 0.24$  respectively). SLE patients on statins had a higher EBCT calcium score than those not on the drugs, ( $P = 0.02$ ) and SLE patients on B-blockers also had a higher EBCT score than those not on the drug, ( $P = .008$ ). Patients on ACE-I and aspirin show no difference in EBCT score compared to those not on these drugs, ( $P = 0.24$  and  $P = 0.22$ , respectively).

**Discussion:** Ever or current azathioprine use is associated with lower EBCT calcium score in SLE patients. Azathioprine is associated with decrease in inflammation in the endothelium and may affect the extent of coronary calcification (Weigel et al, *Thrombosis Research*. 94(2):87-94, 1999 Apr 15. Gao et al, *Circulation*. 80(5 Pt 2):III100-5, 1989 Nov). The other DMARDs studied were not associated with lower coronary calcification. The higher EBCT score associated with B-blocker and statin use is likely associated with previously identified cardiovascular risk factors in these patients.

### Sa1.70. Proteomic Analysis of Secreted Proteins Defines Subtypes of Rheumatoid Arthritis.

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Rheumatoid arthritis (RA) is an autoimmune synovitis characterized by heterogeneity between patients in disease manifesta-

tions, clinical outcomes and therapeutic responses. We applied synovial antigen microarrays and a bead-based multiplex cytokine assay to profile autoantibody and cytokine responses in RA, with the objective of identifying profiles of secreted proteins in blood that provide diagnostic information and delineate disease subtypes. We demonstrate that autoreactive B cell responses targeting deiminated epitopes and elevated serum concentrations of proinflammatory cytokines (TNF $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and IL-15) were present in a subset of early RA patients with features predictive for development of severe RA. In contrast, autoimmune targeting of the native epitopes contained on synovial arrays, including human glycoprotein 39 and collagen types II and V, and low concentrations of serum cytokines were associated with predictors of less-severe RA. Proteomic analysis of secreted proteins enables molecular stratification of patients with early RA into clinically-relevant disease subsets.

### Sa1.71. Death Receptor 5 as a Therapeutic Target: Increased Expression in B and T Cell Subsets in SLE.

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Ligation of Death Receptor 5 (DR5) induces cell death in activated B and T cells. TRAIL, a DR5 ligand, can reduce disease manifestations in animal models. Thus, DR5 is an attractive target for therapeutic elimination of pathogenic lymphocytes in autoimmune diseases. To determine which lymphocytes express DR5, subsets of B and T cells from human blood, tonsil and spleen were identified by surface markers and analyzed for binding of anti-DR5 mAb. Germinal center B cells in the tonsil and spleen expressed DR5, as did plasmablasts in these tissues and in blood. CD38-intermediate cells in the blood did not, consistent with the phenotypic characterization of these as transitional cells. The increase in plasmablasts in the circulation in SLE subjects resulted in an increase in the percentage of blood B cells expressing DR5. A small but reproducible increase in DR5 expression on post-naive subsets was observed in both CD4 and CD8 T cells in healthy subjects. However, DR5 expression was significantly greater in these same subsets of T cells from lupus subjects, compared to the same subsets in healthy controls. Thus, DR5 expression appears to be modulated by both differentiation and by other factors, possibly including disease-associated factors such as type 1 IFN. Therapeutic targeting of DR5-expressing cells would spare resting B cells and naive T cells but has the potential to eliminate activated cells to a degree that would be determined by disease-specific mechanisms.

### Sa1.72. Immune Response to a Citrullinated Peptide of Fibrinogen in DR4 tg Mice Following Intra-Articular Injection of Streptococcal Cell Wall Antigen.

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**Background/Purpose:**

Anti-citrulline antibodies are highly specific serologic markers for RA and the immune response to citrulline is linked to the expression of the RA shared epitope. We have previously demonstrated that DR4 tg mice immunized with citrulline develop arthritis. While citrullination of polypeptides under the influence of peptidyl arginine deiminase (PAD) has been shown to be unregulated in normal mice following the induction of inflamma-

tion (Streptococcal cell wall (SCW) induced), these mice do not develop an immune response to citrulline or chronic arthritis. We sought to investigate the arthritogenic properties of and immune response to citrullinated proteins following acute SCW arthritis induction in DR4-tg mice.

**Methods:**

25 µg of streptococcal cell wall antigen (*Streptococcus pyogenes* Group A PGPS 10S, Lee Laboratories Grayson, Georgia, USA) in 5.0 µl of PBS was injected into one knee joint of DR4 tg and wt mice. The other knee joint of these mice received 5.0 µl of PBS. Mice were sacrificed at various time points to investigate pathological changes, and T and B-cell immune responses.

**Results:**

Pathology of the injected joint from day 2 demonstrated a massive influx of leukocytes and the start of synovial proliferation. Approximately 75% of the leukocyte infiltration had dissipated by day 7. At day 7, synovial hyperplasia, the first signs of erosion at the bone cartilage surface by the proliferating synoviocytes, and depletion of approximately 50% of the proteoglycan normally present in the articular cartilage was observed. Splenic T-cell proliferation was observed in the DR4 tg mice at various time points with a citrullinated peptide of the α chain of fibrinogen (QDF TNCit INK LKN S) but this was not evident in the wt mice. No T-cell proliferation was detected in either group of mice when stimulated with the unmodified version of this peptide.

**Conclusion:**

SCW as expected induced an acute inflammatory arthritis in both normal wt and DR4 tg mice. This was followed by a strong citrulline specific T-cell response only in the DR4 tg mice, presumably by the inflammatory induced expression of PAD and citrullinated fibrinogen. These observations are consistent with our previous studies indicating that the T-cell response to citrulline is restricted by MHC class II molecules expressing the shared epitope. This SCW induced arthritis in DR4 tg mice represents a good model to evaluate the immunogenetic effects of citrulline in-vivo, including the development of citrulline induced arthritis.

**Sa1.73. Homozygous Type I C2 Deficiency and Immunoglobulin Deficiencies in a HLA-B27 Positive Young Girl Presenting with an Antinuclear Antibody-Positive Juvenile Rheumatoid Arthritis.**

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A homozygous type I C2 deficiency was evidenced in a 11-year old girl which was found to be also HLA-B27 positive. This young girl, with antecedents of recurrent otitis media, was admitted to the hospital for a polyarticular arthritis with fever, significant inflammatory syndrome (increased ESR, C-reactive protein and fibrinogen), mild normocytic anaemia, abnormal liver function tests but no eye inflammation. She was found to have antinuclear antibodies at 1:640 on Hep2 cells with a speckled pattern and nuclear dots. Antibodies against extractable nuclear antigens and nDNA were not found. Complement analyses showed the absence of serum hemolytic activity, normal C4, elevated C3 levels and undetectable C2. Homozygous type I C2 deficiency was confirmed by PCR analysis of the C2 gene (28-bp

deletion). Factor B and C4 allotyping showed that the patient was typically homozygous for C2\*Q0, BF\*S, C4A\*4 and C4B\*2 ("S042" complotype). This complotype was linked on one chromosome to the typical HLA-A\*25, B\*18, DRB1\*15(2), DQB1\*06(1) "ancestral" haplotype but on the other chromosome to HLA-A\*2, B\*27, DRB1\*13(6), DQB1\*06(1) which represents a very unusual association with the S042 complotype. Upon serum immunoglobulin determinations, IgD and IgG4 immunoglobulins could not be detected, IgA and IgM levels were close to the lower normal range. Altogether, the clinical syndrome in this patient was related both to homozygous C2 deficiency and positivity for HLA-B27.

**Sa1.74. The Combination of Complement Deficiency and Cigarette Smoking as Risk Factor for Cutaneous Lupus Erythematosus (CLE) in Men; a Focus on Combined C2/ C4 Deficiency.**

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Although deficiencies in the components of the classical pathway of complement activation were among the first identified risk factors for systemic lupus erythematosus (SLE), only a few studies addressed their significance in patients with cutaneous lupus erythematosus (CLE). Among environmental factors, it was postulated that cigarette smoking might intervene in the pathogenesis of LE.

In a retrospective study of 85 patients with CLE, 32 individuals were screened for C4 and/or C2 deficiency. Among them 17 had a C4A deficiency (1 homozygous- 16 heterozygous), five a C4B deficiency (2 homozygous- 3 heterozygous), and two a combined heterozygous C2 and C4A deficiency. The serum level of C4 was decreased in 40 % of patients with C4B deficiency and in only 5 % of patients with C4A deficiency. The C3 level was normal in all patients. A high proportion (58 %) of these complement-deficient patients were male (F/M ratio = 0.70); the mean age at diagnosis was 36 years. Of particular interest was the detection of a combined heterozygous C2 (type I) and heterozygous C4A deficiency in two male patients. This combined deficiency was only rarely reported up to now but its expected frequency in the caucasian population of European descent should approximate 0.1 % and its frequency in patients with LE is unknown. In this series, 82 % of the patients were smokers and 94 % of male patients with CLE were smokers. By comparison, the frequency of smoking in the normal French men and women is about 33 % and 36 %, respectively. It has been recently suggested that smoking behaviour could be related to specific major histocompatibility complex haplotype(s) on chromosome 6 characterized by the presence of a C4A null allele. Our findings seem to corroborate this hypothesis which needs to be confirmed by large prospective studies.

**Sa1.75. Pregnancy Outcomes in Ten Japanese Women with Mixed Connective Tissue Disease.**

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**Objective:** Only a few studies on pregnancy outcome in patients with mixed connective tissue disease(MCTD) are available

and their results are contradictory. The purpose of this study is to examine pregnancy and fetal outcomes in MCTD patients.

**Methods:** A retrospective study we have followed ten mothers with MCTD, during their pregnancies since 1999 to 2003 at Juntendo University Hospital.

**Result:** 1. Of the 10 pregnancies, we observed 5(50%) live birth at term, 2(20%) premature birth, 2(20%) spontaneous abortion, 1(10%) artificial abortion.

2. One case had interstitial pneumonia and high titer of sialyl carbohydrate antigen KL-6 following no therapy, KL-6 level was decreased during pregnancy. At 37 weeks of gestation, the laboratory findings were suggestive of early HELLP syndrome, and the pregnancy was terminated.

3. Two of 10 pregnancies with renal involvement resulted in spontaneous abortion. Serum anti-phospholipid antibodies were not determined either. And active nephritis was recognized at the pregnancy.

**Conclusions:** Since MCTD has a relatively good prognosis, no therapy changes are required during pregnancy. But premature delivery and spontaneous abortion were high rate in this study. It considered that organic involvement and flare are possibility of risk for pregnancy with MCTD women.

#### **Sa1.76. Characteristics of Patients with Clinical Manifestations of APS with Anti-beta<sub>2</sub>-Glycoprotein-I but Not Anticardiolipin Antibodies or Any Other Autoimmune Condition.**

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**Background:** Clinical and immunological abnormalities of patients with clinical manifestations of the antiphospholipid syndrome (APS), with anti-beta<sub>2</sub>-glycoprotein-I antibodies (anti-b<sub>2</sub>-GP-I) but without serum anticardiolipin antibodies (aCL) or any other autoimmune condition are not well documented.

**Materials.** From July 2002 through July 2003, 1179 serum samples obtained in our hospital in the Community of Madrid, were tested for anti-b<sub>2</sub>-GP-I and aCL antibodies by enzyme-linked immunosorbent assay. A total of 56 patients with discordant anti-b<sub>2</sub>-GP-I and aCL antibodies were identified. Fifteen out of 56 patients (26.8%) had positive anti-b<sub>2</sub>-GP-I and negative aCL. The patients had repeated determinations of aCL and lupus anticoagulant, all of which have been negative.

**Results:** A logistic regression analysis, adjusting for the variables age, sex and risk factors of thrombosis showed that the risk for developing APS criteria associated with anti-b<sub>2</sub>-GP-I antibodies was significant [odds ratio 3,88; 95% confidence interval: 1.05-14.27; *P* = 0.04]. Five out of 15 anti-b<sub>2</sub>-GP-I-positive patients (4 female, 1 male) had clinical APS, without serological nor clinical evidence of any autoimmune disease during a mean follow-up of 31.2 months [deep vein thrombosis (DVTs) *n* = 2; DVTs + pulmonary thromboembolism *n* = 2 and retinal thrombosis *n* = 1]. The only abnormal immunological test observed in those five anti-b<sub>2</sub>-GP-I-positive-patients was the findings of IgM hypergammaglobulinemia in one patient. Use of oral anticoagulants were given to only one anti-b<sub>2</sub>-GP-I-patient. **Conclusion:** Not rarely clinicians might be in front of patients that accomplish clinical criteria of APS but they do not have the autoantibodies listed in the classification

criteria set of APS. Determination of anti-b<sub>2</sub>-GP-I should be taken into account in the evaluation of individual cases with clinical characteristics of the APS and repeated negative results on conventional antiphospholipid antibody tests.

#### **Sa1.77. Transcriptional Regulation of Fli1 and Lupus.**

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The Ets transcription factor Fli1 functions as a regulator of hematopoiesis and hemostasis and is required for early development. Fli1 also has been implicated in the regulation of the immune system and autoimmunity. Fli1 is expressed in the thymus and spleen and its overexpression in mice results in the development of immunological renal disease similar to that observed in systemic lupus erythematosus. Elevated expression of Fli1 has been observed in the spleen of lupus mouse models NZB/NZW f1 and MRL/lpr. Furthermore, elevated levels of Fli1 in peripheral blood monocytes from lupus patients correlates with disease activity. Interestingly, MRL/lpr mice that have a heterozygous knockout of Fli1, and a subsequent 50% reduction in Fli1 expression, have significantly reduced renal disease and prolonged survival. These studies strongly indicate that Fli1 plays an important role in the pathogenesis of lupus. To further understand the role of Fli1 in the immune system we are examining the regulation of Fli1 in normal and lupus mice. Our preliminary results show that Fli1 expression is higher in CD19+ and CD8+ cells from predisease NZM2410 and MRL/lpr lupus prone mice and in CD8+ cells from late disease MRL/lpr mice compared to BALB/c mice. Transient transfections of Fli1 promoter/reporter constructs into a B cell line indicate that the highest level of expression is driven by a 400 bp region encompassing most of exon 1 and that most of the positive regulatory elements necessary for this expression are localized within a 200 bp region. Furthermore, we have examined the promoter and upstream regulatory regions of Fli1 from BALB/c, NZM2410 and MRL/lpr spleen and identified a polymorphism in exon 1. Transient transfection analyses indicate this polymorphic region contributes to the positive regulation of Fli1 promoter/reporter constructs. Interestingly, this region is highly homologous to the human Fli1 sequence and is located adjacent to a regulatory element found to be necessary for Fli1 expression in a leukemia cell line. Further analyses of the regulatory region, including the polymorphism, will allow further insight into what elements and binding factors are necessary for normal expression, as well as aberrant expression in lupus prone mice.

#### **Sa1.78. Chronic GVH-Like Disease in a Man Exposed to Organic Solvents: Evidence for a Role of Microchimeric Cells of Maternal Origin.**

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Chronic GVHD (cGVHD) shares features with systemic sclerosis (SSc), including T cell activation and excess collagen deposition in tissues. Increased foeto-maternal microchimerism has



been shown in SSc, suggesting that allo-immunity could contribute to its pathogenesis. Exposure of breeder mice to vinyl chloride has been shown to result in proliferation of microchimeric cells and parallel development of skin fibrosis. Herein, we describe immunological findings in a male patient with lymphocytic infiltrates and fibrosis involving skin, lungs and the digestive tract highly reminiscent of cGVHD, following exposure to organic solvents. We provide evidence that maternal microchimerism could be involved in the disease process.

The patient was lymphopenic with a CD4/CD8 ratio of 0.33. Lymphocyte phenotyping revealed a high proportion of circulating T cells bearing activation markers, among both CD4 (81% CD25+, including 42% CD25high cells) and CD8 (23% CD25+) populations. Oligoclonal expansion of T cells was demonstrated by flow cytometry and immunoscope analysis, involving CD8+ cells belonging to V $\beta$ 7 (32% of CD8 cells) and V $\beta$ 17 (24% of CD8 cells) families. Both V $\beta$ 7+CD8+ and V $\beta$ 17+CD8+ cell populations stained negatively for CD27, CD28 and perforin. High resolution HLA typing of the patient and his mother demonstrated that they were nearly identical for MHC class II (both were DRB1\*1104 DRB1\*1302 and DQB1\*0301 DQB1\*0604, patient DPB1\*0301 DPB1\*0401, mother DPB1\*0301 DPB1\*0402), but not for MHC class I molecules. Search for microchimeric cells of maternal origin by FISH in peripheral blood revealed the presence of 7 XX cells among a total of 40600 analysed cells (0.017%) from the patient.

Bidirectional mixed lymphocyte cultures (MLCs) were performed using T cells from the patient and irradiated non-T cells from his mother, and vice versa, to explore the potential functional consequences of maternal microchimerism in this patient. In presence of patient non-T cells, maternal CD8+ cells showed an increase of HLA-DR expression (24% vs 8.4% when cultured alone) but neither proliferation nor IFN-gamma production, whereas CD4 cells remained quiescent. In contrast, both CD4+ and CD8+ T cells derived from the patient were activated in presence of maternal non-T cells, as shown by increased HLA-DR expression (41.6% vs 16.4% in absence of maternal non-T cells for CD4+ cells, and 32% vs 11% for CD8+ cells) and secretion of IFN-gamma. Removal of patient CD4+CD25high cells from cultures resulted in decreased overall activation of patient T cells in response to maternal non-T cells, indicating that they were effector and not regulatory T cells.

This observation suggests that chronic activation of T lymphocytes related to long term persistence of maternal cells and exposure to organic solvents might lead to a GVH-like disease reminiscent of SSc.

#### Sa1.79. IgG Fc Receptor Polymorphisms Influence Susceptibility to Collagen-Induced Arthritis.

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SWR mice are resistant to collagen-induced arthritis (CIA) despite carrying the arthritis susceptible haplotype H-2q and being able to produce of anti-collagen type II (CII) antibodies following CII-immunization. These IgG anti-CII antibodies are generally pathogenic, as they can trigger joint inflammation via interactions with IgG Fc receptors (Fc $\gamma$ R), notably Fc $\gamma$ RIII. However, SWR mice are resistant to the arthritogenic properties of these antibodies. Considering this, we have in the present study investigated if

possible Fc $\gamma$ R polymorphisms are involved in the susceptibility to CIA. This was studied by generating mice carrying the Fc $\gamma$ RIII gene from the arthritis susceptible DBA/1 mouse (F4.D +/+) or from the SWR mouse (F4.S +/+). After CII-immunization, F4.D +/+ mice, but not F4.S +/+ mice, developed a progressively severe arthritis. In addition, the direct effect of IgG anti-CII antibodies on arthritis development was studied by passive transfer of a cocktail of monoclonal anti-CII antibodies to F4.D +/+ and F4.S +/+ mice. Like in actively induced arthritis, F4.D +/+ mice developed a severe arthritis in contrast to F4.S +/+ mice, which were almost protected from disease. Consequently the gene for Fc $\gamma$ RIII was sequenced in DBA/1 and SWR mice, as well as in 9 additional mouse strains; C57BL/6, C57BL/10, BALB/c, CBA, NZW, NZB, BXSB, NOD and MRL. We found that Fc $\gamma$ RIII exhibits three different haplotypes in mice, Fc $\gamma$ RIII:V, Fc $\gamma$ RIII:H and Fc $\gamma$ RIII:T, and that SWR (Fc $\gamma$ RIII:V) and DBA/1 mice (Fc $\gamma$ RIII:H) indeed differ in the Fc $\gamma$ RIII gene. Interestingly, the DBA/1 mouse shared the Fc $\gamma$ RIII:H haplotype with the autoimmune-prone strains, NZW, NZB, BXSB, NOD and MRL. We also demonstrate that SWR and DBA/1 mice differ at the level of Fc $\gamma$ RIIB, displaying the Ly-17.1 or the Ly-17.2 haplotype respectively. These results suggest that polymorphisms in Fc $\gamma$ Rs may form the basis of one aspect of susceptibility to autoimmune arthritis.

#### Sa1.80. Outcome of a National Israeli Cohort of Pediatric Systemic Lupus Erythematosus.

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**INTRODUCTION:** About 10–20% of systemic lupus erythematosus SLE develops during childhood. Its clinical manifestations range from mild constitutional symptoms to progressive involvement of all target organs. The aim of this study was to describe the clinical manifestations and outcomes of a national cohort of pediatric patients with SLE.

**PATIENTS / METHODS:** We have collected retrospective data on all cases meeting the ACR diagnostic criteria of childhood onset SLE, registered in the Israeli national registry of children with rheumatic diseases, who were diagnosed and followed between 1987–2003. We examined disease activity and damage by using SLE disease activity index (SLEDAI), and SLE collaborating clinics/ACR (SLICC/ACR) disease damage.

**RESULTS:** 102 patients were identified. 81% were females. The mean age at diagnosis was 13.3  $\pm$  2.6 years (range 6.9–17.7). Initial clinical manifestations included renal involvement in 41%, CNS in 7%, hematological in 94%, malar rash in 49%, oral or nasal ulcerations in 21%, musculoskeletal in 45%, and serositis in 16%. The mean SLEDAI was 17.2  $\pm$  9.0 (range 2–60). 80 children (80%) started therapy with corticosteroids, and 19 (19) with immunosuppressive drug.

83 children with 1 year of follow up had a mean SLEDAI of  $8.2 \pm 8.0$  (range 0–46). 55 (66%) were still on corticosteroids and 27 (32%) were on immunosuppressive drugs. 60 children had 3 years of follow up with a mean SLEDAI of  $9.5 \pm 7.3$  (range 0–36). 44 (73%) were on corticosteroids and 23 (38%) were on immunosuppressive drugs. Their mean SLICC/ACR damage index was  $0.4 \pm 1.1$  (0–7). 44 children had 5 years of follow up with a mean SLEDAI of  $6.7 \pm 5.2$  (0–21). 28 (64%) of them were on steroids, 22 (50%) on immunosuppressive drugs. Their mean SLICC/ACR damage index was  $0.5 \pm 1.2$  (0–7). Five patients developed chronic renal failure, one died.

**CONCLUSIONS:** In our national cohort the 5-year outcome of pediatric SLE was good; the damage index was very low with relatively low activity in most patients. It is possible that relatively early and prolonged use of immunosuppressive medications in many patients led to the good outcome.

### **Sa1.81. Dyslipoproteinemia in the Active Course of SLE: A Contributory Role for Anti-Double Stranded DNA Antibodies.**

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Dyslipoproteinemia is common in lupus patients. In this study, we investigated dyslipoproteinemia in the course of active SLE with focus on the role of anti-dsDNA antibodies as a possible contributory factor. Forty-six lupus patients under 45 years old who fulfilled the American College of Rheumatology revised criteria for the classification of SLE were selected. The exclusion criteria consisted of: renal failure, nephrotic syndrome, thyroid and liver disease, diabetes mellitus, obesity, pregnancy, taking drugs that induce dyslipidemia. Disease activity was measured by systemic lupus erythematosus disease activity index criteria. The controls were forty-one healthy individuals matched for age ( $\pm 3$  years) and sex. According to the lipid profiles, in active, inactive and control groups, we found the high level of serum triglycerides and VLDL and low level of serum HDL in active group compared with inactive group ( $P < 0.05$ ). This pattern of dyslipoproteinemia was observed in patients with positive anti-dsDNA antibodies when compared with patients with negative anti-dsDNA antibodies ( $P < 0.05$ ). This pattern of dyslipoproteinemia in active SLE is attributable to autoimmune mechanisms especially in relation to the presence of anti-dsDNA antibodies.

### **Sa1.82. Mutations in Gene Transcripts of Systemic Lupus Erythematosus (SLE) T Lymphocytes.**

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RNA editing is the co- or post-transcriptional modification of RNA which results in the insertion, deletion or substitution of nucleotides. RNA editing correct, extend or diversify the information encoded within the corresponding genomic sequence, and frequently alter the function of the affected RNAs. Therefore, RNA editing plays an important role in the regulation of gene expression

and in the induction of phenotypic variability. The occurrence of high circulating levels of type I interferons (IFNs) in SLE has been well documented. Our previous experiments demonstrated up-regulation of type I IFN inducible RNA editing gene, 150-kDa ADAR1 expression in SLE T cells. Goal of these experiments is to identify the role of type I IFN inducible ADAR1 in editing of protein kinase A (PKA) and ADAR2 gene transcripts of normal and SLE patients. cDNAs synthesized from T cells of SLE and normal control groups were amplified using PKA and ADAR specific primers. The amplified products of the PKA and ADAR2 transcripts were cloned into pCR2.1-TOPO vectors. Sequence analysis of the PKA and ADAR2 transcripts demonstrated 3 to 5-fold increase of A  $\rightarrow$  G transcript mutations in SLE compared to controls. Novel A  $\rightarrow$  G editing sites were identified in the PKA and ADAR2 gene transcripts of SLE T lymphocytes. The ADAR1 gene up-regulation suggests a possible cause for PKA and ADAR2 gene transcript editing in SLE T cells. In addition to A  $\rightarrow$  G, novel T (U)  $\rightarrow$  C editing was also observed in PKA and ADAR2 gene transcripts of normal and SLE T cells. The enzyme responsible for such editing and the mechanisms underlying such editing are unknown. Sequence analysis of the PKA and ADAR2 transcripts demonstrated 2.5 to 5.4-fold increase of T (U)  $\rightarrow$  C transcript mutations in SLE compared to controls. Taken together, these results clearly indicate the increased occurrence of mRNA editing in the PKA and ADAR2 gene transcripts of SLE T lymphocytes. Mutant gene transcripts are pathophysiologically significant, for they can encode diverse, aberrant forms, including truncated, dominant-negatives, resulting in abnormal gene function. Therefore, it is proposed that deficient and/or abnormal activity of genes such as PKA and ADAR2 will contribute to the pathogenesis of SLE by impairing T cell functions.

### **Sa1.83. The Mechanism of Nasal Tolerance in Lupus Prone Mice Is T Cell Anergy Induced by Immature B Cells That Lack B7 Expression.**

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To determine if B cells of lupus prone NZB mice possess intrinsic defects that directly lead or contribute to T cell hyperresponsiveness, we injected age, sex and MHC II matched NZB and Balb/c mice with histone peptide H471 representing a dominant Th cell epitope in histone H4 of the nucleosome. We cocultured purified CD4+ T and B220+ B cells of naïve or peptide primed NZB and Balb/c mice in the presence of the peptide. We found that B220+ B cells of NZB mice express high levels of surface CD86 following antigen priming. Antigen presentation exclusively by autoimmune B cells of NZB mice induced hyperresponsiveness from normal CD4+ T cells of Balb/c mice. T cell hyperresponsiveness is a result of CD86 costimulation by B cells of NZB mice. Induction of nasal tolerance to H471 in NZB mice suppressed CD86 surface expression and led to downregulation of T cell proliferative response and cytokine production. More interestingly, B220+ B cells purified from nasally tolerized NZB mice induced T cell anergy to anti-CD3 and anti-CD28 antibody stimulation in vitro. The anergic T cells do not possess suppressive function in coculture with naïve T cells nor produce suppressive cytokines interleukin 10 (IL-10) and transforming growth factor-beta (TGF- $\beta$ ) upon anti-CD3 and anti-CD28 antibody stimulation in vitro.

### Sa1.84. Synovial Fluid and Inflammatory Response in Rheumatoid Arthritis.

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The TH1 immunologic reaction is the major amplification factor in pathogenesis of the rheumatoid arthritis (RA). Rheumatoid arthritis is destructive synovitis of autoimmune nature. Cytokines TH1 lymphocytes with products of synoviocyte disrupt natural balance in cytokine network inside synovial tissue, which leads to inflammatory reaction and joint damage. Experimental researches in vitro and in vivo on mice model have proved that cytokines of interleukine-12 (IL-12), interleukin-15 (IL-15) and interleukin-18 (IL-18) participate in pathogenesis of erosive inflammatory arthritis. The research has the aim to find out relation between concentrations of TNF-alpha, IL-18, IL-15 and IL-12 in serum samples (S) and synovial fluid (SF) in patients with active RA. Concentrations of cytokines in serum samples and SF are measured in 64 patients with high (HiA), modest (MoA) and mild active (MiA) disease, according to the Disease Activity Score 28 (DAS 28). The comparison of concentrations between S and SF showed that patients with active RA have considerably increased ( $P < 0,01$ ) concentrations of TNF-alpha, IL-18 and IL-15 in SF samples, while values of IL12 were higher in S without significant difference in accordance with SF. Significant increase of TNF-alpha, IL-15 and IL-18 in SF compared to S existed in HiA group. In groups MoA and MiA there was significant increase of TNF-alpha, IL-15 in SF, while concentrations of IL-18 and IL-12 were not different between samples ( $p > 0,01$ ). The conclusion is that patients with active RA have higher concentrations of TNF-alpha, IL-18 and IL-15 in SF than in S, which proves the proposition that those cytokines are produced in joint and could be good indicators of local disease activity. Concentration of IL-18 in SF reflects local activity in patients with the most severe form of the disease.

### Sa1.85. Glycopeptides from Type II Collagen Incorporating Galactosylated Hydroxylysine Mimetics To Study the Fine Specificity of Arthritogenic T Cells.

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Five analogues of the bovine type II collagen (bCII) immunodominant glycopeptide [ $\beta$ -D-Gal-(5R)-5-Hyl<sup>264</sup>]CII(256-270) (**1**), carrying diverse modifications at the hydroxylysine (Hyl) side chain were designed and synthesized to explore the fine specificity of bCII-reactive T cells involved in the initiation and/or regulation of collagen-induced arthritis (CIA), a mouse model for rheumatoid arthritis (RA). The required  $\beta$ -D-Galactosyl-(5R)-5-Hydroxy-L-lysine and corresponding mimetics conveniently protected for solid phase synthesis were all obtained by a divergent route featuring enantiopure 5-hydroxylated 6-oxo-1,2-piperidinedicarboxylates as key intermediates. All three bCII-specific T hybridomas used in this study as well as a recurrent pathogenic CD4<sup>+</sup> T cell clone isolated from bCII-immunized DBA/1 mice recognized the galactosylated form **1** of the immunodominant bCII (256-270) epitope. These cells were extremely sensitive to changes at the  $\epsilon$ -amino group but differ

in their pattern of recognition of analogues with Hyl side chain modified at C-5 (i.e. inversion of stereochemistry, methylation). These data further document the importance of collagen posttranslational modifications in autoimmunity and in the CIA model in particular and provide new insight on the molecular interaction between glycopeptide **1** and the TCR of pathogenic T cells.

### Sa1.86. CD8 $\alpha$ on Monocytes May Aggravate Immune-Complex Mediated Disease by Binding MHC Class I and Enhancing TNF Production.

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CD8 $\alpha$  is expressed by monocytes and macrophages (Mo and M) in rats, but according to available evidence not in mice. While a few previous studies, most dating from the 1980's, suggested human Mo or M express CD8 $\alpha$  this was not convincingly demonstrated. CD8<sup>+</sup> Mo and M are present in several rat models of diseases involving immune complexes, such as glomerulonephritis, arthritis, and ischaemia. Depletion of CD8<sup>+</sup> cells mitigates pathology in these disease models. If Mo and M express CD8, they may be partially responsible for pathology in some of these diseases in rats and humans previously ascribed to CD8<sup>+</sup> T cells. TNF is therapeutically targeted in many of these diseases, and released in large quantity by Mo. We hypothesized CD8 on human Mo may be involved in TNF-mediated pathology in immune complex diseases. Six anti-CD8 $\alpha$  mAb recognized human monocytes (CD14<sup>hi</sup>) by flow cytometry. Ten to 25 percent of monocytes expressed high amounts of CD8 $\alpha$ , while the remaining 75–90% of monocytes expressed low amounts of CD8 $\alpha$ . A proportion of Mo and lymphocytes can be difficult to distinguish by some methods including cell morphology, flow cytometry (FSC-SSC gating), and potentially anti-CD3 mAb labelling. Moreover, because many of the anti-CD8 $\alpha$  mAb used here are sold for clinical evaluation (e.g. OKT8, B9.11, LT8, 51.1), to avoid confusion between CD8<sup>hi</sup> Mo with CD8<sup>hi</sup> lymphocytes in clinical and research settings, careful definition of T cells (e.g. anti-TCR mAb) may be necessary. CD8 $\alpha$  protein was found on the surface of a Mo cell line (THP-1) in continuous culture that also expressed CD8 $\alpha$  mRNA. In the absence of another source of CD8 $\alpha$ , THP-1 and likely other Mo transcribe and translate CD8 $\alpha$ . Functionally, Mo may use both CD8 $\alpha$  and Fc $\gamma$ R to bind tissues containing immune complexes. We established that Mo can bind tetramers of MHC class I (HLA-A2), independent of bound peptide. CD8 $\alpha$  accounted for some MHC class I tetramer binding to Mo, as this was partially inhibited by some anti-CD8 $\alpha$  mAb. In agreement with literature, not all CD8 $\alpha$  mAb blocked binding of MHC class I, demonstrating that mAb binding to the surface of Mo does not indiscriminately block binding of MHC class I tetramers. Select anti-CD8 $\alpha$  mAb but not non-specific mAb imbedded in immune complexes enhanced Mo TNF production. Similar studies with other anti-CD8 $\alpha$  mAb did not induce TNF production, suggesting that particular epitopes of CD8 $\alpha$  activate Mo, and mAb specific for surface proteins of Mo do not indiscriminately enhance TNF production. The ability of Mo to bind MHC class I and release TNF through CD8 $\alpha$  shows that Mo CD8 $\alpha$  could be involved in initiating and aggravating pathology induced by immune-complexes. It is possible that some effects attributed to CD8<sup>+</sup> T cells, where T cells have been inadequately characterized, are due in part to CD8<sup>+</sup> Mo.

### Sa1.87. Anti-Cyclic Citrullinated Peptide and Anti-Sa Antibodies for the Diagnosis of Rheumatoid Arthritis in an Out-Patient Clinic of Chronic Inflammatory Connective Tissue Diseases (CICTD).

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**Introduction:** Several studies have shown the diagnostic usefulness of anti-cyclic citrullinated peptide (CCP) and anti-Sa antibodies in rheumatoid arthritis (RA), but up to now there is no study that has assessed both autoantibodies simultaneously in a cohort of patients.

**Objective:** To determine the sensitivity, the specificity, the positive (PPV) and negative (NPV) predictive values of anti-CCP and anti-Sa in a monographic out-patient clinic of CICTD.

**Methods:** Cross-sectional study. We studied 250 patients: 87 RA, 90 CICTD, 50 espondyloarthritides, 19 polymyalgia rheumatic (PMR) and 4 juvenile idiopathic arthritis. Anti-CCP and anti-Sa antibodies were identified by ELISA and immunoblotting techniques, respectively.

**Results:** Anti-CCP antibodies were detected in 63/87 RA (sensitivity: 72,4% specificity: 94,4%, PPV: 87,5%, NPV: 81,9%), 3/19 PMR, 2 palindromic rheumatism (PR), 1 systemic lupus eritematosus, 1 undifferentiated connective tissue disease (UCTD), 1 ankylosing spondylitis (AS) and 1 undifferentiated espondyloarthritides (sensitivity 72.4%, specificity 94.4%, positive predictive value 87.5% and negative predictive value 86.5%). Anti-Sa antibodies were detected in 38/87 patients with RA (Sensitivity: 43,6%, specificity: 96,3%, PPV: 86,3%, NPV: 76,2%), 2 UCTD, 1 Sjögren's syndrome, 1 PR, 1 AS and 1 juvenile idiopathic arthritis.

**Conclusions:** The specificity and the predictive values of anti-CCP and anti-Sa antibodies for the diagnosis of RA are comparable. However, the sensitivity of anti-CCP antibodies is higher, given the higher sensitivity of the ELISA technique when compared with immunoblotting.

### Sa1.88. Evaluation of the Effect of Different Modified Extracellular Matrix Proteins in the Inflammation and Extracellular Matrix Turnover in Cartilage and Synovial Tissue Co-Cultures from Patients with Osteoarthritis (OA) and Rheumatoid Arthritis (RA).

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**Introduction:** Modified extracellular matrix (ECM) proteins such as hydrolyzed (elastin, collagen fibronectin, thrombospondin, etc.) or polymerized proteins (collagen-PVP), has been shown to regulate inflammatory processes. Due to, the evaluation of the effect in osteoarthritis (OA) and rheumatoid arthritis (RA), diseases related to chronic inflammation, results of a special interest. **Objectives:** To evaluate the effect of the hydrolyzed collagen and elastin, (b) collagen-PVP, and (c) the mixture of the hydrolyzed collagen and elastin plus collagen-PVP in co-cultures from

cartilage and synovial tissue from RA or OA knee or hip. **Material and Methods:** Cartilage and synovial tissue co-cultures from 5 patients with RA (ACR) or 5 patients with OA (ACR) were performed. All of them were prescribed for total knee or hip replacement surgery. Each tissue was fragmented into 96 segments of approximately 5mm<sup>3</sup>. Tissues were cultured with RPMI-1640, 10% SFB, antibiotics and antimicrobics during 7 days under the following conditions: a) RPMI (control), b) 1% collagen-PVP, c) 1% hydrolyzed collagen and elastin and d) 1% hydrolyzed collagen and elastin + 1% collagen-PVP. In order to determine the effect of the different culture conditions on the ECM turnover (elastin, collagen and sulfate proteoglycans and hyaluronic acid) tissues were stained with Hematoxylin and Eosin, Verhoeff and Alcian Blue staining techniques. Proinflammatory cytokines (IL-1b, IL-8, IL-10, IL-12, TNF-a, and IFN-g) in supernatants were quantified by ELISA. Data were normalized by total protein concentration evaluated by the Folin-Lowry micro-method. IL-1b, TNF-a and Ki-67 expression was determined by histochemistry. The statistical analysis was made by *t*-Student test and U Mann-Whitney. **Results:** The histological analysis showed a remodeling tissue, related to an increase of highly sulphated proteoglycans, sialomucin and hyaluronic acid. A scarce increment of elastin fibers in tissues treated with the mixture of hydrolyzed and collagen-PVP vs. control cultures were observed. A 2-3-fold increment of Ki-67 was determined in the tissues treated vs. control cultures. IL-1b and TNF-a were determined 1.5-2-fold lower levels in treated cultures vs. controls. IL-8 levels were decreased in all supernatants from treated co-cultures from RA patients. However, only in supernatants from OA tissue treated with hydrolyzed proteins statistically decreased vs. the controls was determined. **Conclusions:** Modified proteins induce a tissue remodeling, promoting the recovery of cartilage proteoglycans, down-regulating the expression from some proinflammatory cytokines and promoting the chondroid cells proliferation.

### Sa1.89. Anti-alpha-Fodrin Antibodies of IgA Isotype in Patients with Sjögren's Syndrome (SS).

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**Introduction:** The  $\alpha$ fodrine is localised in the plasma membrane of most mammals' cells. After the cell break-down during apoptosis by the action of the caspase-3, a cleavage-product of the alpha-fodrin of 120 kD acts as a neoantigen, being recognised by sera of patients with SS. 90-60% of patients with SS presented positive IgA anti- $\alpha$ fodrin antibodies.

**Objective:** To assess the frequency and the clinical associations of IgA anti-alpha-fodrin antibodies in patients with primary and secondary SS in our environment.

**Materials and Methods:** We studied 491 patients diagnosed of SS, 209 primary SS and 282 secondary SS: 190 rheumatoid arthritis (RA), 59 systemic lupus eritematosus (LES), 13 scleroderma and 19 polymyositis. IgA anti- $\alpha$ fodrin antibodies were tested by ELISA.

**Results:** Anti- $\alpha$ fodrin antibodies were detected in 29 patients (5.9%): 6/209 primary SS (2,7%) and 23/282 secondary SS (8,2%), 13/190 RA (6.8%), 6/60 SLE (10%) and 2/19 Polymyositis (10,5%). No significant differences were observed when we compared patients with and without anti- $\alpha$ fodrin antibodies,

neither in epidemiological data, in time of disease evolution, nor in clinical manifestations.

**Conclusions:** In our environment, IgA anti-alpha-fodrin antibodies are not common in patients with SS (5.9% vs 90–60% of the literature), and are not associated with any specific clinical manifestation. Sensitivity of IgA anti-alpha-fodrin antibodies is higher in secondary SS.

### Sa1.90. Tumor Necrosis Factor Genetic Polymorphism as Part of Rheumatoid Arthritis Evolution.

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**RATIONALE:** Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by chronic synovial joint inflammation leading to cartilage and bone destruction. The etiology and pathogenesis of the disease is still unsolved, although evidences reveal that TNF plays a central role. The pathogenic role of TNF can be evidenced by: It has been observed that in plasma, synovial fluid and tissue of patients with RA high concentrations of TNF are found; transgenic mice that over-express the human TNF gene develop a polyarthritis similar to RA, while the administration of human TNF monoclonal antibodies from their birth, prevent the articular lesions and diminish the incidence of murine arthritis and, probably the most notable piece of evidence, comes from studies in which it has been demonstrated the clinical benefit of patients with RA treated with anti-TNF monoclonal antibodies or with soluble receptors of TNF. **OBJECTIVE:** The purpose of this work is to study the historic evolution of RA, which it would be considered an increasing disease, relatively new, favored by a specific mutation at position-308 in the promoter region of the TNF gene. **METHODS:** 308 single nucleotide polymorphism (SNP) was determined by polymerase-chain reaction (PCR)-restriction fragment length polymorphism. **RESULTS:** The-308 SNP determines a higher expression of this cytokine. The frequency of this polymorphism is 43.5% in Caucasian population. In studies of association of de SNP-308 and RA, the positive findings have been: in Caucasian population the allele *TNF2* is 3 times higher in patients with RA, than in healthy controls; a relation between the SNP-308 and the presence of extra-articular manifestations with rheumatic nodules; in Swedish patients it has been demonstrated that individuals bearing the heterozygous form, develop a more severe disease and at an earlier age, and a significant association with bad prognosis was found in Turkish patients with RA. **CONCLUSION:** 308 SNP seems to participate in the evolution of RA. **Financed by Fondecyt-Chile 1040860 and Fondef-Chile D03I1055.**

### Sa1.91. Mannan Binding Lectin and Complement C4A in Icelandic Multicase Families with Systemic Lupus Erythematosus.

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**Objective:** Low mannan binding lectin (MBL) and C4AQ0 has been associated with systemic lupus erythematosus (SLE). We asked whether low MBL might predispose to SLE in members of multicase SLE families, where there is an overall increased frequency of C4AQ0. **Methods:** Low MBL was detected by measuring serum levels (ELISA) and genotyping for mutant structural (O) and promoter (LX) alleles (RT-PCR). C4AQ0 was detected by protein electrophoresis. Twenty-four SLE patients from nine Icelandic families were compared to 83 first-degree and 23 second-degree non-SLE relatives, and 24 unrelated family members served as controls. **Results:** MBL-low (wild-type/O) and MBL-deficiency (O/O, LX/O) genotypes were associated with MBL levels below 1000 µg/L, and low MBL was observed in five of the nine families ( $n = 86$ ). In these five families, 64% of SLE patients, 38% of their first-degree and no second-degree relatives carried MBL-low/deficiency genotypes ( $P < 0.001$ ) compared to 29% of the controls ( $P = 0.007$ ). All patients carried a MBL-low allele (O, LX) and/or C4AQ0 ( $P < 0.001$ ). The SLE patients also had C4AQ0 combined with MBL-low/deficiency genotypes more often than their non-SLE relatives ( $P = 0.01$ ) and controls ( $P = 0.015$ ). In accordance with MBL genotypes, patients from these families also had lower MBL levels than their relatives ( $P < 0.001$ ) and controls ( $P = 0.02$ ). Low MBL predisposed to SLE independently of C4A status. There was no evidence of MBL consumption. **Conclusion:** MBL-low/deficiency genotypes and low MBL serum levels predispose to SLE independently of C4AQ0. Low MBL was absent in four of the nine families, highlighting the heterogeneity of SLE.

### Sa1.92. Abnormal Dendritic Cell Activation in SLE.

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Circulating peripheral blood mononuclear cells (PBMCs) from SLE patients express high levels of Type I interferon (IFN-I) inducible genes. IFN-I is produced by plasmacytoid dendritic cells (PDCs) and promotes the maturation of myeloid dendritic cells (MDCs), which play a key role in antigen presentation. We investigated the interrelationships between IFN-I production and circulating PDCs and MDCs in subjects with SLE ( $n = 88$ ), other autoimmune diseases ( $n = 82$ ), and healthy controls ( $n = 57$ ). Expression of the IFN-inducible genes Mx1 and OAS (real-time PCR) was increased in SLE PBMCs vs. the other groups ( $P < 0.0001$ , ANOVA). High Mx1 expression was seen in ~20% of lupus patients (compared to the mean + 2 S.D. of controls). In contrast, circulating PDC and MDC counts (flow cytometry) were decreased in ~50% of SLE patients compared with controls ( $P < 0.0001$ , ANOVA). Production of autoantibodies against dsDNA and snRNPs (Sm/nRNP, Ro, and La) was positively associated with high IFN-I production and negatively with the numbers of circulating PDCs and MDCs, whereas anti-phospholipid antibody production was negatively associated with IFN-I production. Patients with low PDC/MDC counts fulfilled a greater number of ACR criteria and had a higher prevalence of renal disease. To better understand the basis for the low numbers of circulating dendritic cell precursors in SLE, we asked whether the PDC/MDC counts were persistently or intermittently low. Longitudinal studies showed that in individual SLE patients

IFN-I inducible gene expression can fluctuate as much as 100-fold. These differences were independent of respiratory infections, SLEDAI, and medication use. However, PDC and MDC counts tended to remain low in SLE regardless of the IFN-I levels. Healthy subjects exhibited a different pattern: IFN-I expression was generally much lower at baseline, increased dramatically within 1–2 days in response to viral upper respiratory infections, and returned to baseline within 10 days; interestingly, the numbers of circulating PDCs/MDCs did not decrease during viral infections. Studies in a mouse model suggested that the low circulating PDC/MDC counts in lupus are due to enhanced dendritic cell maturation (increased CD86 expression) and migration from the circulating pool to sites of inflammation. We propose that IFN-I produced by abnormally activated PDCs stimulates MDC maturation and the presentation of self-antigens, culminating in autoimmunity. The abnormal dendritic activation in SLE could reflect either an intrinsic dendritic cell defect or an exaggerated response to extrinsic (microbial?) activators.

### Sa1.93. Marginal Zone Lymphoma with Autoantibodies to RO52 in Sjogren's Syndrome.

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Marginal zone (MALT) lymphomas arising in the stomach sometimes bear immunoglobulin receptors specific for *Helicobacter pylori* antigens and eradication of the infection may lead to tumor regression. Marginal zone lymphomas also arise in the salivary glands of patients with Sjogren's syndrome (SS). It is not known if these SS-associated tumors also have antigen specificity. We studied two SS patients with B cell neoplasms consistent with marginal zone lymphomas originating within the parotid gland. Both patients were positive for anti-Ro52 autoantibodies using a recombinant human Ro52 based ELISA. The first patient's lymphoma exhibited typical lymphoepithelial lesions on H&E staining and was k L-chain<sup>+</sup>, CD5-, CD10-, and CD23- by flow cytometry. The solitary tumor was excised surgically after which her anti-Ro52 IgG autoantibody level fell from 735 units to 414 units. In contrast to the total IgG, which exhibited a k/l ratio of 1.5:1, the k/l ratio of her anti-Ro52 antibodies was ~60:1. The k L-chain<sup>+</sup> anti-Ro52 antibody level decreased by more than 40% after surgery whereas the l L-chain<sup>+</sup> anti-Ro52 remained unchanged indicating that excision of the tumor specifically decreased the k L-chain<sup>+</sup> anti-Ro52 antibodies. The second patient's lymphoma was l L-chain<sup>+</sup> with surface markers compatible with a marginal zone lymphoma. The neoplasm was too widespread at the time of diagnosis to permit local excision. The ratio of k/l L-chains in total immunoglobulin from Patient 2 was 8:1, whereas the k/l ratio of her anti-Ro52 autoantibodies was 0.5:1. Thus, both the tumor and most of her anti-Ro52 antibodies were l L-chain<sup>+</sup>. These data suggest that the production of anti-Ro52 antibodies was dependent on the presence of tumor cells (Patient 1) and that the anti-Ro52 antibodies exhibited non-random usage of L-chains (Patients 1 and 2), consistent with the possibility that the neoplastic B cells produced autoantibodies against Ro52. By analogy with marginal zone lymphomas arising in the stomach that are specific for *H. pylori* antigens, we speculate that some marginal zone lymphomas originating in the salivary glands may be stimulated by the self-antigen Ro52. These cases raise the

possibility that anti-Ro52 autoantibodies, a specificity characteristic of Sjogren's syndrome, can be produced by marginal zone B cells and that these cells may on occasion undergo neoplastic transformation.

### Sa1.94. IL-1 Receptor Expression in Muscle Fibre Membrane and Muscle Cell Nuclei in Patients with Polymyositis or Dermatomyositis.

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**Background:** Interleukin-1 (IL-1) is a prototype of a pro-inflammatory cytokine in that it induces expression of a variety of genes and synthesis of several proteins that, in turn, induce acute and chronic inflammatory changes. Polymyositis and dermatomyositis are chronic inflammatory muscle disorders, characterized by proximal muscle weakness and by inflammatory cell infiltrates in skeletal muscle. Increased expression of IL-1alpha in endothelial cells of capillaries and IL-1beta in mononuclear inflammatory cells, is a consistent finding in muscle tissue from myositis patients. The pathophysiologic role of these cytokines in myositis has not yet been clarified. Our hypothesis is that IL-1 could have a negative effect on muscle fibre metabolism and regeneration and contribute to the persisting muscle weakness and muscle fatigue often seen in these patients.

**Aim:** To investigate if muscle fibres express IL-1 receptors (IL-1R) in healthy or inflamed muscle tissue and if so, if there was any difference of IL-1 receptor expression between symptomatic and non-symptomatic muscle tissue.

**Method:** Muscle biopsies from 8 polymyositis patients, 3 dermatomyositis patients, and 6 healthy controls were included in this study. The muscle biopsies from the patients were taken from two different sites, one from a symptomatic muscle and the other from a non-symptomatic muscle. IL-1alpha, IL-1RI, and IL-1RII expression was investigated by immunohistochemistry. Localization of IL-1RI and IL-1RII expression was also investigated by double staining and confocal microscopy with laminin to identify muscle fibre membrane and a the marker BOBO3 to identify nuclei.

**Results:** IL-1RI and IL-1RII were expressed, in the membrane and in the nuclei of muscle fibres as well as in inflammatory cells and endothelial cells in muscle biopsies from myositis patients. Healthy controls only had a scattered pattern of IL-1RI and IL-1RII expression in a few endothelial cells and in a few nuclei of the muscle fibres. There was no difference between symptomatic and asymptomatic muscles. The membrane and nuclear expression of IL-1RI and II was confirmed with double staining and confocal microscopy. IL-1a was expressed in endothelial cells and inflammatory cells in the patients.

**Conclusion:** This is the first time that IL-1RI and IL-1RII have been described in the membrane of human muscle fibers. Furthermore, IL-1Rs were localized to cell nuclei. The implication of this is not known, but IL-1alpha is known to have an intracrine pathway and this could possibly be mediated through nuclear receptors. The observed expression of IL-1RI and IL-1RII in muscle fiber membranes supports our hypothesis that IL-1 could

have a direct effect on muscle fibres and thereby affect muscle function.

**Sa1.95. Increased Expression and Production of Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL) by T Cells from Lupus Patients Is a Feature of Active Disease.**

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TRAIL is a member of the TNF family with proapoptotic activity. Increased T cell associated and soluble TRAIL (sTRAIL) levels in serum from patients with systemic lupus erythematosus (SLE) have been previously reported. In this study we set to characterize the upregulation of both T cell associated and sTRAIL in vivo and the modulation of TRAIL expression and soluble protein release following T cell activation and IFN $\alpha$  exposure in vitro. Lastly, the functional ability of the two forms of TRAIL to mediate apoptosis was compared. Using flow cytometry the ex vivo expression of membrane bound TRAIL was higher on CD4+ and CD8+ T cells from ten lupus patients compared to ten healthy controls, particularly on activated CD69+CD8+ T cells. sTRAIL levels determined by ELISA in sera from 34 lupus patients and 26 controls were significantly elevated in patients with active SLE and correlated with disease activity and with levels of IFN  $\alpha$  but not with any particular clinical feature. In vitro, both T cell associated and sTRAIL were maximally induced by T cell activation plus IFN  $\alpha$  in patients and controls. Western blot analysis of immunoprecipitated sera demonstrated the presence of the 21 kD monomeric forms but not of multimeric forms of sTRAIL. Although both T-cell associated and sTRAIL were functional in vitro in inducing apoptosis of TRAIL sensitive Jurkat cells as determined by Annexin V staining and 51Cr release assay, the apoptotic activity of membrane TRAIL was 2.5 fold higher compared to that of sTRAIL. In conclusion, increased T cell associated and sTRAIL is a feature of active disease in lupus patients and likely reflects the in vivo T cell activation and IFN  $\alpha$  production. IFN  $\alpha$  induced enhancement of TRAIL expression and of TRAIL mediated apoptosis may amplify the abnormal apoptotic process in SLE.

**Sa1.96. Intramuscular Administration of Polymerized Type I Collagen for the Treatment of Patients with Rheumatoid Arthritis. A Double Blind Placebo-Controlled Clinical Trial.**

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**RATIONALE:** Collagen-polyvinylpyrrolidone (Collagen-PVP) has been shown to down-modulate some proinflammatory mediators expression on synovial tissue from rheumatoid arthritis (RA) patients. Collagen-PVP subcutaneous administration to RA patients was safe and well-tolerated drug for the short term-treatment.

**AIM:** To determine the efficacy, tolerance and safety of intramuscular injections of porcine type I collagen-PVP in patients with RA in a long term-therapy.

**METHODS:** The protocol was approved by the Committee of Medical Ethics of the National Institute of Medical Sciences and Nutrition. Only patients who gave informed consent to participate

were recruited. The study was double blind placebo-controlled and included 30 patients who fulfilled the 1987 American Rheumatism Association (ACR) criteria for active RA. Patients on stable therapy with methotrexate and/or non-steroidal antiinflammatory drugs (NSAIDs) were enrolled in a 1 year prospective, comparative and longitudinal study. Patients were treated in accordance to Freyberg scheme with intramuscular injections of 2 ml of collagen-PVP (3.4 mg of collagen) or 2 ml of placebo during 6 months. The primary endpoints were done according to Ritchie index (RI, 72-joint count), 72-swollen joint count, disease activity score (DAS), erythrocyte sedimentation rate (ESR), and C-reactive protein (CRP). The secondary endpoints included morning stiffness, pain intensity on a visual analog scale (VAS), and spanish-health assessment questionnaire (HAQ-DI). The improvement was determined using American College of Rheumatology response criteria (ACR20, 50 and 70). Statistical analysis was performed by the non-parametric double tailed Mann-Whitney U test. Data were expressed as the mean  $\pm$  SD. The *p* values smaller than or equal to 0.05 were considered as significant.

**RESULTS:** Collagen-PVP was safe and well tolerated. There were no adverse events. Patients had a statistically significant improvement (*P* < 0.05) in collagen-PVP-treated vs. placebo at 6 months of treatment in: swollen joint count (8.2  $\pm$  0.8 vs. 14.9  $\pm$  1.6;  $\Delta$ -14, -55% vs.  $\Delta$ -10, -41%), RI (10.6  $\pm$  0.8 vs. 15.2  $\pm$  1.5;  $\Delta$ -19.1, -60% vs.  $\Delta$ -13.6, -45%), morning stiffness (9.6  $\pm$  3.1 vs. 27.1  $\pm$  5.9 min;  $\Delta$ -84, -67% vs.  $\Delta$ -84, -57%), HAQ-DI (47.0  $\pm$  10.8 vs. 25.2  $\pm$  10.3;  $\Delta$ -0.7, -47% vs.  $\Delta$ -0.3, -25%), DAS (3.5  $\pm$  0.2 vs. 4.3  $\pm$  0.3;  $\Delta$ -2.4, -36%,  $\Delta$ -1.4, -25%), ACR20 (78.6 vs. 71.4%), ACR50 (57.1 vs. 0%) and ACR70 (7.14 vs. 0%), CRP (1.1  $\pm$  0.4 vs. 2.5  $\pm$  0.7;  $\Delta$ -0.8, -20% vs.  $\Delta$ 0.6, 126%) and rheumatoid factor (243.9  $\pm$  57.3 vs. 490.4  $\pm$  74.9;  $\Delta$ -128, -20% vs.  $\Delta$ -80, -0.9%). Patients treated with collagen-PVP required lower doses of methotrexate vs. placebo (12.6  $\pm$  0.6 vs. 14.2  $\pm$  0.7 at 6 months and 12.3  $\pm$  0.8 vs. 15.4  $\pm$  0.6 at 12 months; *p*<0.05). Serological or hematological parameters remained unchanged.

**CONCLUSION:** Collagen-PVP has been shown to be a safe and well-tolerated drug for the long-term treatment of RA. Combination of collagen-PVP plus methotrexate was more efficacious than methotrexate alone. This drug could be useful in the treatment of RA.

**Sa1.97. Clinical Characteristics of 88 Patients with Juvenile or Adult Dermatomyositis.**

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**Introduction**

Dermatomyositis (DM), belonging to the group of the idiopathic inflammatory myopathies, is characterized by a bimodal pattern of age-specific incidence of rates, with peaks in the age group from 5 to 16 years (juvenile DM) and in the age group from 45 to 65 years (adult DM). The aim of this study is to evaluate the clinical characteristics of 22 patients with juvenile DM.

**Methods**

A national registry of patients with juvenile dermatomyositis (JDM) was elaborated by the authors in Hungary. We summarize

data of the register according signs and symptoms, disease course, frequency of relapses and survival of patients with JDM. Analysis was performed using data for 22 patients diagnosed between 1976 and 2003 according to Bohan and Peter's criteria. Survival probability was calculated by Kaplan-Meier method. Data of juvenile patients were compared with data of 66 patients with adult DM.

### Results

All children had symmetrical weakness of the proximal muscles. The most frequent cutaneous features were facial erythema and Gottron's papules (19/22). Extramuscular and extraskeletal manifestations of the disease were more frequent in adult patients. The most common extramuscular feature was arthralgia (7/22). Only one patient with juvenile DM had interstitial lung disease (ILD). Cardiac manifestation of the disease or respiratory muscle involvement was not observed in juvenile patients. Respiratory muscle involvement (12/66) and ILD (11/39) were more frequent among adult DM patients than the cardiac manifestation of the myositis (6/55). In view of the disease course, the authors found that frequency of polycyclic and monophasic subtypes of the disease were similar. The hazard of the relapse was found higher during the first year after the remission. None of the juvenile patients died. Among adult patients 4 disease-specific deaths occurred.

### Conclusion

Patients with JDM are usually admitted to Dermatology departments, therefore paediatric dermatologists should be familiar with the clinical presentation of JDM. We report the first study on clinical characteristics and disease course of patients with juvenile DM who were diagnosed, treated and followed-up in Hungary. To our best knowledge, this is one of the largest studies on comparing clinical data of juvenile and adult patients.

### Sa1.98. Pre-B Cell Colony Enhancing Factor Stimulates Production of Proinflammatory Mediators by Human Monocytes and Is Overexpressed in Systemic Autoimmune Disease.

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Studies of early vertebrates have provided clues to the development of the adaptive immune system, and even more primitive animal phyla, the Porifera, express molecules that are precursors of mammalian innate immunity. To gain new insight into potential mechanisms of autoimmunity in humans, we have studied a cytokine-like molecule, pre-B cell colony enhancing factor (PBEF), a protein produced by sponges in response to xenogenic cells and with nicotinamide (Nam) phosphoribosyl transferase activity in both bacteria and mammals. PBEF was induced in B lymphocytes by activation of surface immunoglobulin receptors and IL-4 and stimulated production of IL-8 and other proinflammatory mediators by monocytes through an NF- $\kappa$ B dependent pathway. Consistent with its homology to Nam phosphoribosyl transferase, PBEF induction of IL-8 was inhibited by Nam. To determine whether PBEF might be overexpressed in patients with autoimmune disease, PBEF mRNA was measured by quantitative real-time PCR in PBMC from 89 patients with systemic lupus erythematosus (SLE), 22 patients with rheumatoid arthritis (RA), and 28 healthy donors (HD). The mean PBEF expression was significantly increased in SLE (relative expression

compared with housekeeping control =  $3.95 \pm 5.61$ ) and RA ( $3.31 \pm 3.8$ ) compared with HD ( $0.87 \pm 0.48$ ), with p for both comparisons  $<0.001$ . Consistent with the in vitro induction of IL-8 by PBEF, expression of PBEF was positively correlated with expression of IL-8 in SLE PBMC ( $r^2 = 0.589$ ,  $P < 0.0001$ ). In summary, our data demonstrate that PBEF is a cytokine-like inducer of proinflammatory mediators that utilizes the nicotinamide adenine dinucleotide and NF- $\kappa$ B pathways to stimulate innate immune system activation. Increased production of PBEF in autoimmune diseases may represent a mechanism that promotes inflammation and tissue damage and may be a rational target for therapeutic inhibition.

### Sa1.99. Mannose-Binding Lectin Polymorphisms in Rheumatoid Arthritis and the Associations with Radiological Progression Rate and Serological Markers.

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**Background:** Mannose-binding lectin (MBL) is a pattern recognition molecule encoded by the *MBL2* gene. A role for MBL in susceptibility or severity of disease has been found in a variety of pathologies, such as infectious, autoimmune, and cardiovascular diseases. Previously, the role of MBL in patients with rheumatoid arthritis (RA) has been reported, however, without concordant results. Several DNA polymorphisms have been characterized in the *MBL2* gene, resulting in considerable variations in quantity and/or functionality of the soluble MBL protein. Due to a strong linkage disequilibrium, only 7 common haplotypes are detected in humans. These are the following, arranged according to decreasing expression levels and/or functionality of soluble MBL: HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, and LYQC. The genotypes, taking into account only one promoter polymorphism and expressed as a two-letter code, are YA/YA, YA/YO, YA/XA, YO/YO, YO/XA, and XA/XA.

**Objectives:** 1) to compare the *MBL2* genotypes in RA versus healthy controls, and 2) to investigate the associations with radiological progression rate and serological markers.

**Patients and methods:** *MBL2* genotyping (INNO-LiPA *MBL2*, Innogenetics, Ghent, Belgium) was performed in 166 RA patients and 172 healthy controls. The radiological progression rate in RA was assessed as the modified Larsen score divided by disease duration. All RA patients were tested for anti-cyclic citrullinated peptide antibodies (anti-CCP2 antibodies, Eurodiagnostica, Arnhem, The Netherlands), rheumatoid factor (RF, Latex Fixation assay, DifcoLaboratories, Detroit, MI) and shared epitope (INNO-LiPA HLA-DRB1 or-DRB decoder amplification kits, Innogenetics, Ghent, Belgium). The Mann-Whitney U test was used for comparing the different combined haplotypes. *P*-values  $<0.05$  were considered significant.

**Results:** No differences were detected in *MBL2* genotype frequencies between RA patients (YA/YA: 31%; YA/YO: 30%; YA/XA: 25%; YO/YO: 6%; YO/XA: 5%; XA/XA: 3%) and healthy controls (YA/YA: 35%; YA/YO: 22%; YA/XA: 25%; YO/YO: 6%; YO/XA: 7%; XA/XA: 5%). The median radiological progression rate in RA was similar for the different *MBL2* genotypes: YA/YA: 3.5; YA/YO: 3.4; YA/XA: 3.3; YO/YO: 2.4; YO/XA: 3.2; XA/XA: 2.6. Bad radiological prognosis ( $\geq 5.45$ , defined as the 25% highest radiological progression rate) was not linked with a specific *MBL2* genotype. There were no differ-



ences in median anti-CCP2 concentrations between the *MBL2* genotypes: YA/YA: 140 U/ml; YA/YO: 233 U/ml; YA/XA: 149 U/ml; YO/YO: 153 U/ml; YO/XA: 170 U/ml; XA/XA: 176 U/ml. The median RF titers were 50 U/ml for all *MBL2* genotypes. Also the percentage of shared epitope positive patients was comparable in the different *MBL2* genotypes: YA/YA: 78%; YA/YO: 73%; YA/XA: 70%; YO/YO: 78%; YO/XA: 83%; XA/XA: 75%.

**Conclusions:** In our study, neither RA susceptibility nor radiological prognosis was associated with a specific *MBL2* genotype. Furthermore, RA-associated serological markers were equally found in the different *MBL2* genotypes.

### **Sa1.100. Interferon Inducible Proteins Are Novel Autoantigens in Systemic Lupus Erythematosus.**

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Many studies have indicated a role for the type I interferon system in both human and murine systemic lupus erythematosus (SLE). In the anti-CD1 T cell receptor transgenic lupus mouse model (CD1 SLE model), single positive (CD4+ or CD8+) T cells from transgenic BALB/c donors induce a lupus-like syndrome when transferred to irradiated BALB/c nu/nu recipients. Disease in these mice is characterized by the presence of anti-dsDNA antibodies, proteinuria, and immune complex glomerulonephritis. Immunoprecipitation studies have revealed that a prominent target of serum autoantibodies in CD1 SLE mice is an interferon-inducible antigen that has been identified as a member of the interferon-inducible 200 (Ifi200) family. We therefore hypothesized that IFN-inducible proteins are also targeted by serum autoantibodies derived from human lupus patients. We screened a panel of monoclonal and polyclonal antibodies directed against well-characterized lupus autoantigens by Western blot and found that the expression of Ifi16 and Ro52 proteins is inducible in HT1080 cells upon treatment with IFN- $\alpha$  and that the expression of both proteins peaks at 24 hours following treatment. Ifi16 is the human homolog of mouse Ifi202 and has previously been shown to be an IFN- $\gamma$ -inducible target of antinuclear antibodies in 29% of SLE patients. The expression of both Ifi16 and Ro52 is also induced following treatment with IFN- $\beta$ , another type I IFN. HT1080 cells were treated with IFN- $\alpha$  for 24 hours, lysed, and probed by Western blot with sera derived from 15 SLE patients. Serum from a single patient uniquely targeted an unidentified 32 kD-interferon inducible protein. Finally, the expression of Stat1, an inducible protein involved in both type I and type II IFN signaling, was elevated in PBMCs derived from SLE patients as compared to normal controls. Taken together, the data suggest that IFN-inducible proteins represent a novel class of autoantigens and reveal a potential link between the type I IFN system and autoimmunity.

### **Sa1.101. Estrogen Receptor Effects on the Anti-Inflammatory Action of PPARs in Lupus Mice.**

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Nuclear receptors are recognized as regulators of inflammation. PPAR $\gamma$  is a nuclear receptor that was previously considered an orphan receptor but is now studied for its role in many biological processes. PPAR $\gamma$  agonists are inhibitors of inflammatory mediators including nitric oxide (NO), TNF-alpha, and

interleukin 6. Because of these early reports, the thiazolidinediones (TZDs) and other synthetic PPAR $\gamma$  agonists are being studied in both animal models and human patients for their effects on inflammatory diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis. We recently reported that PPAR $\gamma$  is not necessary for the reduction of the inflammatory mediator iNOS or NO by synthetic PPAR $\gamma$  agonists. However, intrinsic PPAR $\gamma$  function in the absence of synthetic agonists appears to play an endogenous role in inflammatory modulation. It is thought that PPAR $\gamma$  competes with other nuclear factors, like NF $\kappa$ B for common cofactors and activator molecules by a mechanism called transrepression. Our interest is in determining if such transrepression occurs between nuclear hormone receptors, specifically estrogen receptors (ER) and PPARs in inflammatory conditions. Such competition between the estrogen receptors and PPARs may partially account for gender differences in inflammatory diseases. Our current studies demonstrate that like activation of PPAR $\gamma$ , activation of PPAR $\delta$  with GW610742X at 10–20 $\mu$ M is effective at significantly reducing NO production by 50% in LPS stimulated RAW246.7 macrophages. Since RAW246.7 macrophages are reported not to express PPAR $\gamma$ , this suggests that PPAR $\delta$  may be an effective regulator of inflammation. We elected to use a murine model of lupus for our studies due to 10 fold higher incidence of lupus in females compared to males. We bred the ER $\beta$  -/- genotype onto the MRL/lpr lupus mouse background for 8 generations. We collected peritoneal macrophages and stimulated them with LPS to induce NO production. After treatment with the PPAR $\delta$  agonist GW610742X, we measured effects on NO production. GW610742X reduced NO production by macrophages at 10–20 $\mu$ M concentrations. NO reduction was most effective in macrophages from ER $\beta$  -/- mice at 55% and least effective in ER $\beta$  +/- mice at 30%. Our results suggest that nuclear receptors like ER $\beta$  may interfere with the anti-inflammatory properties exhibited by some of the PPARs. In addition these results demonstrate the anti-inflammatory properties of PPAR $\delta$ . Competitive nuclear receptors may be useful pharmacological targets for the treatment of inflammatory diseases like SLE.

Supported by VA Medical Research Service and NIH grant AR047451.

### **Sa1.102. Pathogenetic Value of the Disbalance Trace Elements under Systemic Sclerosis.**

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Heavy metals can induce autoimmune reactions by direct linkage with MHC molecules, by modifying membrane proteins, presentation of cryptopeptides, "crushing" autoantigen by toxic radicals of oxygene, increase activity of enzymes of nucleic exchange. These statements have formed the basis for studying concentration of Fe, Cu, Zn, Co, Mn, Cr, Sr, Pb, Ba in blood serum of 116 patients with systemic sclerosis by inductive coupled plasma(ICP) method.

It was established, that in patients with systemic sclerosis deficiency of Zn, Fe and Mn is observed, increased value of Cu, Co and Cr, indicates clear correlations between the disease duration, level of activity, character of clinical symphomes and some immunological parameters. Copper concentration is mostly expressed (increase in 5 times in comparison with norm) and zinc (decrease in 3 times). Methods of treatment used in systemic

sclerosis cases (in particular, penicillamine) promotes further zinc exhaustion on blood serum and cellular levels.

The received results point to trace elements disbalance as pathogenetic basis of this disease.

### Sa1.103. Increased Granulopoiesis in Active SLE Blood: New Insights into SLE Pathogenesis.

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Gene expression profiling of active Systemic Lupus Erythematosus (SLE) mononuclear cells show a significant granulopoiesis signature that can be traced down to the presence of immature blood neutrophil precursors. Indeed, using antibodies against CD16 and CD11b, neutrophils can be separated into three stages: immature neutrophil (IN) stage I (CD16-/CD11b-); IN stage II (CD16-/CD11b+), and stage III or mature neutrophil (CD16+/CD11b+). While in healthy individuals cells negative for either CD16 or CD11b are not found in the blood, blood neutrophils in SLE patients contain cells belonging to all three stages. We have successfully obtained RNA from highly pure populations of immature (stage I) and mature (stage III) SLE as well as mature healthy blood neutrophils. After RNA amplification, labeling and hybridization to Affymetrix U133 gene chip arrays, we have detected i) genes that are specifically transcribed in immature versus mature neutrophils, ii) neutrophil genes that contribute to the SLE-specific gene signatures. Furthermore, we have found that the presence of immature neutrophils correlates with SLE disease activity and with the development of renal disease, suggesting that they are relevant to disease pathogenesis and severity.

The presence of immature neutrophils in the SLE blood could be a reactive process due to the apoptosis of mature cells. Indeed, we have found that the number of immature neutrophils in the SLE blood mononuclear fraction correlates with the ability of the patients's serum to induce apoptosis of healthy mature neutrophils. Whether anti-neutrophil antibodies and/or death-inducing molecules like TRAIL are responsible for the pro-apoptotic effect of SLE serum on healthy neutrophils is currently under investigation.

We have also found that mature SLE neutrophils display accelerated spontaneous apoptosis when compared with healthy mature neutrophils in culture. Despite an increased apoptosis rate, mature SLE neutrophils promptly release as much IL-8 and MIP1-alpha as healthy cells when stimulated with the TLR2 agonist lipopeptide and TLR7/8 agonist R848, implying that these cells are functional. The release of cytokines by pro-apoptotic SLE neutrophils could be contributing to an inflammatory environment which would facilitate the maturation of antigen-presenting cells and the processing of apoptotic material in an immunogenic manner, explaining some of the key pathogenic events in SLE.

### Sa1.104. Rheumatoid Factor Seropositivity in a Normal Population Is Associated with the Use of Oral Contraceptives and Cigarette Smoking.

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**Objective:** To determine whether oral contraceptive (OCP) use and cigarette smoking are associated with the presence of rheumatoid factor (RF) in a cohort of reproductive age women without rheumatoid arthritis (RA).

**Materials/Methods:** Subjects were 297 women who were the parents of children enrolled in a cohort originally established for the prospective study of the development of type I diabetes mellitus-related autoimmunity. This parental population was selected because it is enriched with HLA-DR4, a susceptibility marker for both type I diabetes mellitus and RA. Subjects were interviewed and examined to rule out a current diagnosis of RA. When exam or history was consistent with a possible diagnosis of RA, the subject's medical record was reviewed. Subjects who met 1987 American College of Rheumatology criteria for RA were excluded from the analyses. A questionnaire was administered to obtain data on current and past history of tobacco and OCP use. Serum samples obtained from the adults at the time of the interview were tested for RF by nephelometry. Logistic regression models were performed to determine the association between RF and pack years of smoking as well as OCP use. Adjusted odds ratios (OR) and 95% confidence intervals (CI) were used as the measures of association.

**Results:** Subject age range was 23.1-53.7 years (mean: 38.1, median: 38.3). 89.6% of women reported ever using OCP's, and the mean duration of OCP use was 7.3 years. Categories of smoking were Never ( $n = 204$ ), 1-19 pack-years ( $n = 88$ ), and  $\geq 20$  pack-years ( $n = 5$ ). 10.4% of the subjects had a positive RF. After adjusting for age, ethnicity, presence of the shared epitope, and OCP use, smoking  $\geq 20$  pack-years was associated with a positive RF, OR: 18.94 (95% CI: 2.74, 130.85),  $P = 0.0017$ , compared with Never smoking. Smoking 1-19 pack-years [OR: 0.72 (95% CI: 0.27, 1.90)] was not associated with a positive RF compared with Never Smoking. Ever use of OCP's was negatively associated with the presence of RF, OR: 0.20 (95% CI: 0.08, 0.50),  $P = 0.0006$ , independent of age, ethnicity, presence of the shared epitope, and smoking status.

**Conclusions:** These data suggest that OCP use may protect against the development of RF in individuals without RA. Additionally, heavy cigarette smoking may be a risk factor for RF seropositivity in individuals without RA. Previous studies have proposed that smoking is a risk factor for RA development, and OCP use is inversely associated with RA development. Our results suggest that both of these environmental exposures may act very early in the development of clinically apparent disease.

### Sa1.105. Efficacy of Apratastat, a Novel Dual Inhibitor of TNF- $\alpha$ Converting Enzyme/Metalloproteinase, in Murine Collagen-Induced Arthritis Models.

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**Background:** Tumor necrosis factor (TNF)- $\alpha$  is an established target for the treatment of rheumatoid arthritis (RA). Pro-TNF- $\alpha$  is proteolytically processed to a soluble form by TNF- $\alpha$  converting

enzyme (TACE). Orally bioavailable, small molecule inhibitors that block release of TNF- $\alpha$  present a highly desirable strategy for treating RA.

**Objectives:** 1) To determine the effects of Apratastat, a novel dual TACE/metalloproteinase inhibitor in two CIA models. 2) To compare Apratastat in one CIA model with a broad-spectrum metalloproteinase inhibitor, having no significant TACE activity (1).

**Methods:** Collagen-induced arthritis (CIA) was induced in DBA/1 mice by immunizing at the base of the tail with bovine type II collagen (CII) emulsified in complete Freund's adjuvant, and boosting with either lipopolysaccharide (LPS) or CII emulsified in incomplete Freund's adjuvant 21 days later.

**Results:** Apratastat was evaluated in CIA mouse models with either an LPS boost or a collagen boost. In a prophylactic regimen with an LPS boost, Apratastat was administered orally at doses of 5, 10, or 20 mg/kg BID from days 18 through 35 post inoculation. The minimum efficacious dose was 10 mg/kg BID. In a second CIA model with a collagen boost, therapeutic treatment was initiated in each mouse when the disease severity score was at least 1. Apratastat (10, 25, 50, or 100 mg/kg BID, and 200 mg/kg QD, PO), or MPI-369 (100 mg/kg BID, PO), a broad-spectrum metalloproteinase inhibitor having no significant TACE activity, were evaluated in the therapeutic regimen. Apratastat at 100 mg/kg BID or 200 mg/kg QD produced a significant reduction in clinical and microscopic disease severity scores. In contrast, MPI-369 did not show activity. The lower doses of Apratastat were similar to the vehicle control.

**Conclusion:** These data show that a potent TACE/metalloproteinase inhibitor, Apratastat, reduces the clinical and histological manifestations of joint destruction in 2 murine CIA models of immune-mediated arthritis.

**Reference:**

1. J. Med. Chem. 2003, 46, 2361-75.

**Sa1.106. Marginal Zone (MZ) B Cells Are Most Susceptible to Loss of Tolerance in the Chronic GVH Model of SLE.**

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Systemic lupus erythematosus (SLE) is characterized by the production of autoantibodies and glomerulonephritis. The chronic GVH (cGVH) model of SLE is induced by allo-recognition of foreign major histocompatibility complex (MHC) Class II determinants. Our studies using CD4KO mice showed that endogenous CD4 T cells are required for emergence of autoreactive B cells during cGVH. In new studies we have attempted to characterize which subsets of B cells are prone to losing tolerance in cGVH. We have used the 3H9 "knockin" tg model, in which the chromosomal JH locus has been replaced with the rearranged V(D)J-3H9tg. B cell subsets were characterized phenotypically by expression of specific cell surface proteins. Thus, immature B cells were sorted as HSA<sup>hi</sup>IgM<sup>hi</sup>AA4.1<sup>+</sup>IgD<sup>-</sup> while mature B cells were sorted as HSA<sup>lo</sup>IgM<sup>lo</sup>AA4.1-IgD<sup>+</sup>. These different B cell subsets were adoptively transferred into irradiated (300 rads) JHT recipients, which lack endogenous B cells because of a targeted deletion in their JH segment. cGVH was induced by challenging the recipients with bm12 spleen cells. Our data showed that mature

B cells lose tolerance and produce anti-dsDNA autoantibodies sooner than immature B cells. To determine which subset of the transferred mature B cell population was susceptible to loss of peripheral tolerance during GVH, we utilized additional cell surface markers. IgM<sup>int</sup>IgD<sup>hi</sup>CD21<sup>int</sup>CD23<sup>hi</sup>CD1-CD9<sup>lo</sup> were sorted as follicular (FO) recirculating B cells, while IgM<sup>hi</sup>IgD<sup>lo</sup>CD21<sup>+</sup>CD23-CD1<sup>hi</sup>CD9<sup>hi</sup> were considered as MZ B cells, which are normally located at the junction of white and red pulps. In similar adoptive transfer experiments followed by induction of GVH, our data indicated that MZ B cells lost tolerance and secreted autoantibodies sooner than FO B cells. MZ B cells have recently been proposed to play a critical role in host defense against T-independent blood-borne pathogens and in spontaneous development of autoantibodies, and expanded MZ B cell populations have been characterized in several lupus-prone strains of mice. Our data now show that MZ B cells are particularly vulnerable to loss of tolerance in the cGVH model of SLE and thus, may be an attractive target for therapeutic interventions.

**Sa1.107. Elevated Levels of Anti-Oxidized Low Density Lipoprotein Autoantibodies Is Correlated with Disease Activity of Systemic Lupus Erythematosus.**

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Systemic lupus erythematosus (SLE) is a multi-systemic autoimmune disease characterized by a great diversity of clinical manifestations accompanied by a large number of autoantibodies. In the present study we investigated the change of anti-dsDNA autoantibodies and anti-oxLDL autoantibodies in the progression of SLE. Twenty-seven patients with SLE were defined according to the American College of Rheumatology criteria and were monitored over 2 years for disease activity. The anti-dsDNA autoantibody was measured by Farr assay and the anti-oxLDL autoantibody was determined by a standardized EIA. Both the antibodies were measured twice a year over the time. The results showed that 18 subjects were positive (> 5 IU/mL), 8 subjects were negative and 1 subject varied in the borderline for levels of anti-dsDNA autoantibody; and 11 subjects were positive (> 100 unit/mL), 10 subjects were negative and 6 subjects varied between positive and negative for levels of anti-oxLDL autoantibody in the all detection during the monitored period. Six out of 27 patients were all-negative levels of both the autoantibodies. The levels of both autoantibodies substantially decreased in one patient and markedly increased in 4 patients over the time. The levels of anti-oxLDL autoantibody were significantly higher ( $P < 0.001$ ) in the anti-dsDNA autoantibody positive group ( $119.4 \pm 49.2$  unit/mL) compared to the anti-dsDNA autoantibodies negative group ( $82.9 \pm 37.7$  unit/mL). There was a very significant correlation between the levels of anti-dsDNA autoantibody and the levels of anti-oxLDL autoantibody in the anti-dsDNA antibody positive group ( $R = 0.5856$ ;  $P < 0.0001$ ). There was no any correlation between both the autoantibodies in the anti-dsDNA antibody negative group ( $R = 0.0619$ ). This study further suggests that oxidative damage is involved in the pathogenesis of SLE. The anti-oxLDL autoantibodies are as similar as anti-dsDNA autoantibodies to be relevant to SLE progression. It could be used as an associating marker in the monitoring of disease activity in SLE. The mechanism of elevated the levels of anti-

oxLDL autoantibodies in active SLE subjects should be further study.

**Sa1.108. Pediatric Systemic Lupus Erythematosus (SLE)-Loss of Tolerance to Maternal Microchimerism?**

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**Objective:** Pregnancy-derived microchimerism has been implicated in the pathogenesis of autoimmune diseases that resemble graft-versus-host disease (GVHD). Evidence for fetal microchimerism (FMc) has been found in the blood and organs of women with systemic sclerosis and SLE. The mouse model for GVHD in which parental lymphocytes induce SLE-like glomerulonephritis and autoantibodies suggests that maternal microchimerism (MMc) may also be involved in SLE. We found maternal cells in the tissues of patients with autoimmune diseases and controls in the form of T lymphocytes, renal tubular cells, hepatocytes, and cardiac myocytes. Thus, persistence of MMc implies normal immune tolerance to maternal antigens, and loss of tolerance could lead to inflammation directed at MMc within tissues. Alternatively, clonal proliferation of maternal T lymphocytes reactive to host antigens could lead to elevated MMc in the peripheral blood. This study investigated MMc levels as well as tolerance to maternal antigens in childhood onset SLE.

**Methods:** Pediatric subjects were studied to avoid the potentially confounding factor of FMc. We used Real-Time Quantitative PCR specific for non-shared maternal HLA alleles to quantify maternal DNA in peripheral blood from 38 subjects: 14 with SLE and 24 age-matched, healthy controls. Lymphocyte reactivity was evaluated by intracellular cytokine assay and flow cytometry after stimulation of the proband's peripheral blood mononuclear cells with CD14+ macrophages from the mother or an unrelated donor (URD).

**Results:** Maternal DNA was detected in 21% of SLE patients and 38% of controls (OR 0.45,  $P = 0.17$ ). The mean level of MMc in SLE was 1.7 genome equivalents (gEq)/million, (range 0–12) compared to 20.5 gEq/million, (range 0–319) in controls. After stimulation by URD macrophages 14% of CD4+ T lymphocytes from a healthy child produced interferon- $\gamma$ , whereas only 2% responded to maternal; 25% produced IL-4 in response to URD and 2% to maternal. In contrast, tolerance to maternal cells was not observed in a pediatric SLE patient: 10% of CD4+ lymphocytes produced IFN- $\gamma$  in response to URD macrophages compared to 20% to maternal, and 16% produced IL-4 to URD compared to 31% to maternal.

**Conclusions:** MMc was common in the healthy subjects tested. In contrast to previous studies in which FMc and MMc was increased in autoimmune disease, there was a trend toward decreased MMc in pediatric SLE patients. In one SLE patient increased reactivity to maternal antigen presenting cells was observed, suggesting that MMc could be cleared from the circulation by host T cells. These preliminary results suggest the hypothesis that immunological tolerance to MMc is intrinsic to normal biology but may be lost in chronic inflammatory disease, leading to tissue-specific inflammation. Additional studies are needed to investigate MMc in tissues for frequency,

morphology and phenotype and to extend initial observations suggesting a loss of tolerance to MMc in SLE.

**Sa1.109. Hypogammaglobulinemia in a Pediatric Patient with Idiopathic Retroperitoneal Fibrosis Treated with Tamoxifen.**

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We present a 16 year old male with a history of retroperitoneal and mediastinal fibrosis. His case was complicated by renal and ureteral obstruction, and superior and inferior venae cavae obstruction with significant collateral flow. This child's symptoms began at the age of 10, when he noticed increased fatigue with activity. At that time renal function tests were abnormal, with ultrasound showing right hydronephrosis and CT scan showing stenosis of the inferior vena cava (IVC) between the infrarenal portion of the IVC and the hepatic portion, with extensive collaterals circulation. Ureteral stents were placed, and pulse doses of solumedrol were administered but the obstruction persisted. Previous reports have shown that the drug tamoxifen promotes activation-independent shedding of L-selectin (CD62-L) from neutrophils and lymphocytes, and it has been used in adults to treat this disease. It was decided to add this medication to his treatment regimen along with prednisone. The tamoxifen did result in resolution of his ureteral obstruction and removal of the stents. His course was complicated by a large deep venous thrombosis of the lower extremity and thrombosis of the SVC for which he continues to take warfarin prophylactically. Our patient has not had evidence of recurrence in the second month since discontinuation of tamoxifen, but he continues to be hypoxemic and oxygen dependent with physical activity. Pulmonary function testing is indicative of severe restrictive disease. What makes this case unique is hypogammaglobulinemia (with an IgG in the range 244–473 mg/dL) both before and during the treatment course. Although his absolute B cell count is low, the percentage from the total counts is normal. Additionally, he has no identifiable source of protein loss from his body. The lack of immunoglobulin production in this patient points towards a possible early diagnosis of combined variable immunodeficiency (CVI). We will be treating him with intravenous immunoglobulin (IVIG) in order to replace the deficiency and prevent infection. This is the first report of CVI in retroperitoneal fibrosis.

**Sa1.110. Dendritic Cells Pulsed with Type II Bovine Collagen Modulate the Murine Collagen Induced Arthritis.**

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Dendritic cells (DCs) are antigen presenting cells that play an important role in the immunopathogenesis of rheumatoid arthritis. DCs genetically modified have shown to reduce the severity of arthritis in the murine model of collagen induced arthritis (CIA). In addition, DCs expressing differential levels of MCH class II and co-stimulator molecules have demonstrated

to interfere with the induction of CIA by a deficient presentation of antigen to T cells. *Aim:* To assess the capacity of DCs pulsed with peptides derived from type II bovine collagen (CII) to modulate the antigen-specific immune response in mice with active arthritis. *Methods:* After five days of culture with GM-CSF, bone marrow-derived DCs were incubated with LPS and then pulsed for 4 h with CII. DBA1/lacj mice with active arthritis were subcutaneously inoculated either with CII-pulsed DCs or DCs alone. Both clinical and histopathological parameters were followed up for 70 days after the first CII administration. *Results:* Previous the immunization procedure, DCs were phenotypically and functionally characterized by MHC class II and co-stimulator markers and by a phagocytosis assay, respectively. In both analyses DCs displayed a semi-mature pattern. We observed that the group of mice with CIA and immunized with DCs alone showed a higher score of clinical signs than the control group of CIA ( $P < 0.05$ ). However, the group of CIA mice immunized with CII-pulsed DCs showed a significant lower clinical score than CIA controls ( $P < 0.005$ ). *Conclusion:* The immunization with semi-mature DCs pulsed with type II bovine collagen interferes with the immunological course of murine arthritis in an antigen-specific manner.

Financed by Fondecyt-Chile 1040860, Fondef-Chile D03I1055 and Mecesup UCH0115.

#### **Sa1.111. IL-12 Regulates Collagen-Induced Arthritis in DQ8 Mice through STAT4-Dependent Mechanisms.**

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IL-12 signaling through STAT-dependent pathway is essential for induction of IFN $\gamma$  and differentiation of Th1 cells. Mice expressing HLA-DQ8 in the absence of endogenous class II molecules develop severe collagen-induced arthritis (CIA), an animal model for rheumatoid arthritis. The development of arthritis in DQ8 mice is T cell dependent with a Th1 profile. To understand the role of IL-12 in collagen-induced arthritis, we have generated Abo.DQ8 transgenic mice lacking STAT4. Both STAT4 $^{-/-}$  and STAT4 $^{+/+}$  mice could mount response to collagen in vitro. Immunization of both strains of mice with type II collagen led to development of arthritis. However incidence of CIA was significantly higher in DQ8.STAT4 $^{+/+}$  mice compared to DQ8.STAT4 $^{-/-}$  mice. The development of arthritis was dependent on Th1 cytokines as both transgenic mice produced IL-18 and TNF- $\alpha$ . DQ8.STAT4 $^{-/-}$  mice produced very low levels of IFN $\gamma$  suggesting IL-12 controls the production of IFN $\gamma$  through a STAT4-dependent pathway. STAT4 $^{-/-}$  mice developed milder arthritis with delayed onset than that observed in DQ8.STAT4 $^{+/+}$  mice. CIA in the absence of IFN $\gamma$  and IL-12, suggests that these cytokines might regulate the severity of disease. Mice carrying non-susceptible HLA-DQ6 transgene produce lower amounts of Th1 cytokines and develop milder CIA with lower incidence compared to DQ8 mice. This suggests that while MHC is the major predisposing factor, cytokines produced in response to antigen presented by the MHC molecule may determine severity of disease.

#### **Sa1.112. Identification of Genes Participating in Suppression of Murine SLE by CD8<sup>+</sup> T<sub>H</sub>1 Cells.**

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Systemic lupus erythematosus (SLE) is caused by autoantibodies (e.g. anti DNA), and immune complexes causing organ damage. NZB/NZW F1 female (BWF1) mice tolerized with an artificial peptide (pCONSENSUS, pCONS) based on anti-DNA IgG sequences containing MHC Class I and Class II T cell determinants, develop regulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells and inhibitory CD8<sup>+</sup> T cells, both of which suppress autoAb production. In the present study, using the Affymetrix Gene Chip array 430, 2.0, we analyzed 45,000 murine genes. One to one comparison of white blood cells (WBC), CD4, and CD8 cell subsets from tolerated vs. non-tolerized mice showed 448, 174 and 60 genes that are differentially expressed by at least two-fold, respectively. From the CD8<sup>+</sup> T cell arrays, we confirmed upregulation of several genes using real-time PCR. Increased expression pattern (more than two fold) with real-time PCR of 6 genes in CD8<sup>+</sup>T cells from tolerized mice was repeatedly found (IFI202B, Trp-53, Foxp3, CCR7, bcl2, and IFN $\gamma$ 1). Immunophenotyping and cell sorting revealed significant expansion of CD3<sup>+</sup>CD8<sup>+</sup>, after pCONS treatment in BWF1 mice. Significant increased mRNA expression of TGF $\beta$  and Foxp3 was found in CD8<sup>+</sup>CD28<sup>+</sup>Ti stimulated with anti-CD3/CD28 after pCONS treatment, suggesting a role of TGF $\beta$  and Foxp3 in the suppressor function of these CD8<sup>+</sup> T cells. Anti-TGF $\beta$  abrogated suppression of anti-DNA production in vitro by CD8<sup>+</sup> T cells cocultured with syngeneic CD4<sup>+</sup> T helpers and B cells. Intracellular staining revealed increased expression of IFN- $\gamma$  and Foxp3 in splenic CD8 T cells from tolerized mice compared to those from untreated mice. Furthermore, Annexin V and 7AAD staining showed significantly less apoptosis in tolerized CD8<sup>+</sup>Ti cells than in naive. CD8<sup>+</sup> Ti from tolerized mice exposed to si RNA of Foxp3 lost their ability to suppress anti-DNA production in vitro. This confirms the role of Foxp3 in the suppression associated with this model of immune tolerance. The role of other upregulated molecules are being studied in a similar fashion. We conclude that the suppressive function of tolerized CD8<sup>+</sup> T cells relates to increased survival and secretion of TGF $\beta$ , and to upregulation of Foxp3. Other gene products upregulated in these cells may also relate to their ability to survive and suppress autoimmunity. These studies on the molecular mechanisms of suppression of lupus in BWF1 mice may have potential therapeutic value in human SLE.

#### **Sa1.113. Impact of Gender on Immune Nephritis.**

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**Background:** We have recently reported that the co-administration of anti-glomerular sera with LPS leads to severe nephritis, as gauged by proteinuria, azotemia, glomerular crescent formation, and proliferative lesions. The focus of this work is to ascertain how gender might impact nephritis in this model.

**Methods:** Male and female C57BL/6 mice (some of which were deficient in estrogen receptors) were challenged with anti-glomerular serum plus LPS, and monitored for evidence of renal disease. In addition, some mice were subcutaneously implanted with 40-day release estradiol pellets (0.5mg), and then subjected to the nephrotoxic insult.

**Results:** LPS and anti-glomerular antibody challenged female C57BL/6 mice exhibited significantly worse proteinuria (10.9 vs. 4.9 mg/24h at peak of disease,  $P < 0.007$ ) and blood urea nitrogen (120.3 vs. 55.8 mg/dL at peak of disease,  $P < 0.029$ ,  $N = 5$  mice per group), compared to age-matched C57BL/6 male mice. Moreover estradiol-treated C57BL/6 males exhibited significantly worse proteinuria as early as D8 (16.7 vs. 11.8 mg/24h,  $P < 0.0005$ ,  $N = 5-6$  mice per group), compared to placebo-treated C57BL/6 males. Importantly, estradiol-treated C57BL/6 males that were deficient in ESR1 estrogen receptors exhibited significantly lower proteinuria (5.7 mg/24h), compared to estradiol treated C57BL/6 males (13.3 mg/24h,  $P < 0.004$ ,  $N = 3-12$  per group).

**Conclusion:** It is clear that gender can profoundly influence immune-mediated nephritis, through the action of estrogens. Studies are in progress to determine the cellular and molecular bases for the differences.

#### **Sa1.114. Use of Gene-Expression Profiling in Labial Salivary Glands Using cDNA Microarrays To Identify New Pathogenic Pathways in Primary Sjögren's Syndrome.**

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**Objective.** The pathogeny of primary Sjögren's syndrome (pSS) is still poorly understood. Since pSS is a multifactorial disease, an approach focused on a single gene or gene product could be too restrictive. No comprehensive, systematic study of genes expressed or repressed in salivary gland, one of the main target-organ of patients with pSS, has ever been reported. We aimed to compare the gene-expression profiling in salivary glands of patients with pSS with that of subjects without any auto-immune features.

**Patients and Methods.** Patients with pSS according to European-American consensus group criteria were included in the study, as well as patients with SS and rheumatoid arthritis and subjects without any feature of autoimmunity (no autoantibody, focus score  $< 1$ ). Total RNA was isolated from labial salivary glands and RNA levels, quality and purity were assessed with the use of the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer. RNA was amplified (one-round amplification, Ampliamp, Ambion, UK), controlled on the BioAnalyzer, and c-DNA fluorescent probes were prepared from 250 ng of amplified RNA. CDNA fluorescent probes were hybridized to custom chips, containing 11000 cDNA spots (manufactured by the Commissariat à l'Energie Atomique, CEA, France). RNA from a pool of 4 normal parotid samples was amplified and used as a common reference. Each sample was hybridized twice, with a dye-swap. All intensity values were normalized using LOWESS. The whole procedure of gene-expression profiling fulfilled the MIAME standards for microarray data. P values were corrected for multiple comparisons according to Benjamini-Hochberg False Discovery Rate procedure.

**Results.** Thirty-two microarrays were hybridized, using salivary glands of 7 patients with pSS, 7 subjects without any feature of autoimmunity and 2 patients with SS and rheumatoid arthritis. Taking into account features present in all microarrays, 169 genes (93 genes up-regulated, 76 genes downregulated) were differentially expressed between patients with pSS and subjects without any feature of autoimmunity. 612 genes were differentially expressed between these 2 groups after algorithmic evaluation of missing values. Hierarchical clustering showed that 6/7 patients with pSS clustered tightly together, as well as 6/7 subjects without any feature of autoimmunity. The 169 genes mainly belonged to the cell-cycle, oxidative burst, apoptosis, protein metabolism and chemokine families. Validation of these results using quantitative RT-PCR and immunohistochemistry are currently under way.

**Conclusion.** This gene-expression profiling study in the target organ of pSS, which fulfilled the best standards for microarray data, demonstrates that the transcriptomic approach could be very helpful to unravel new pathogenic pathways in autoimmunity.

#### **Sa1.115. Four Systemic Lupus Erythematosus Autoantigens Identified by HEp-2 Library Screening.**

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**Objective:** To determine and characterize autoantigens that stimulate autoantibody production in systemic lupus erythematosus (SLE) by screening human epithelioma-2 (HEp-2) expression library with patient sera.

**Materials and Methods:** Forty pediatric SLE patients with nephritis were screened for high titer anti-nuclear antibodies (ANA) by immunofluorescence and Western blot using HEp-2, leukemia (acute lymphocytic and myelocytic leukemia), and normal cells. Two sera yielded extremely high-titer ANA. Column-purified immunoglobulin G (IgG) samples from these two pediatric patients (both with diffuse proliferative glomerulonephritis) were used to screen a HEp-2 cell line expression library made with a Zap II cDNA synthesis kit by Stratagene. After immunoscreening, phagemids from the lambda ZAP-II vector were excised, amplified by PCR, characterized by agarose gel electrophoresis, and sequenced. Sequenced samples were identified by the National Center for Biotechnology Information (NCBI) BLAST program.

**Results:** Out of the seventeen clones that were isolated, seven were identified by both sera. After screening out vector and repeat sequences, four unique antigens were identified: melanoma antigen gene Xp-2 (MAGE Xp-2), ribosomal protein P0, ribosomal protein P1, and ribosomal protein S6.

**Conclusion:** Four autoantigens in systemic lupus erythematosus were identified by HEp-2 expression library screen. Screening different tissue expression libraries with patient sera may further characterize SLE autoantibody antigen specificities, improve our understanding of the pathogenesis of autoantibody production and end-organ damage in SLE, and may lead to the development of novel therapeutic interventions.

#### **Sa1.116. Exploration of a Burn Medication for Potential Anti-Inflammatory Agent through External Application.**

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**Objective:** External application of the extract of two herbs from two genera, *Axonopus* and *Ludwigia*, abbreviated as A/L extract, has been used to treat patients with second to third degree of burn injuries with high efficacy for decades. How A/L extract works to enhance the healing of burn injuries was unknown. The main symptoms after severe burn are redness, pain, blister, and swelling due to inflammation; therefore, anti-inflammatory medication is a basic and critical treatment. Hence, the aim of our studies is to determine whether A/L extract has anti-inflammatory activities.

**Methods:** Non-adherent peripheral blood leukocytes (NA-PBL) and monocytes were isolated from human whole blood collected from three healthy donors. Human NA-PBL and monocytes were then stimulated with PHA with or without the presence of filter-sterilized A/L extract. Supernatant was collected at 48hr and 72 hr for the measurement of TNF-alpha and IL-2 by ELISA. The cells were also stained with MTT at 72hr for proliferation assay.

Three groups of mice after 2<sup>nd</sup> or 3<sup>rd</sup> degree of burn by boiled water were treated with placebo, 0.1% A/L extract, or 0.5% A/L extract daily.

**Results:** In vitro studies from human NA-PBL and monocytes stimulated with PHA show that the incubation of the cells with A/L extract reduced the production of IL-2 (30% for NA-PBL, 48% for monocytes at 48 hrs) and TNF-alpha (~15% for both NA-PBL and monocytes at 48 hours and 35% for NA-PBL and 20% for monocytes at 72 hours). The proliferation assay shows that the cell proliferation stimulated by PHA was inhibited by 25% with the presence of A/L extract. The results from the mice with burn injuries show a dose response: it took the mice ( $n = 7$ ) treated with placebo about a month to heal, 3 weeks for the group ( $n = 6$ ) treated with 0.1% A/L extract and 15 days for the group ( $n = 7$ ) with 0.5% A/L extract. In independent experiments, the treatment of A/L extract on the mice with severe inflammation on whole back and whole limb also reduced the symptoms of inflammation, such as redness and swelling, within an hour; so did the results from the volunteers with pain on joint.

**Conclusion:** The results from both in vitro and in vivo studies suggest that external application of A/L extract is able to reduce both superficial and sub-dermal inflammation. The external application to treat sub-dermal inflammation, such as Rheumatoid arthritis, will greatly reduce any risk of side effects. Further efforts will be made to study the effect of A/L extract on synoviocytes and the safety of oral usage to treat systemic inflammation.

#### **Sa1.117. Regulation of Autoimmune Arthritis by the Homologous Dominant Self and Cryptic Mycobacterial Hsp65 Epitopes.**

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Identification of the disease-regulating T cell determinants within an antigen targeted in an autoimmune disease would be of significance both in better understanding of the mechanism of natural remission/recovery from acute phase of the disease and in developing antigen-specific immunotherapeutic approaches for that disease. Although pathogenic epitopes have been identified in several self and foreign antigens, there is barely any information regarding bona fide regulatory T cell epitopes. Using the Lewis rat adjuvant-induced arthritis (AA) model of human rheumatoid arthritis (RA), we have defined

disease-regulating T cell epitopes within the AA-related antigen, mycobacterial heat-shock protein 65 (Bhsp65), and in its self homolog, rat hsp65 (Rhsp65). These epitopes reside within the C-terminal region of hsp65. Interestingly, despite sequence differences between the corresponding homologous C-terminal epitopes of the foreign (mycobacterial) and self hsp65, these epitopes are not only immunogenic and crossreactive with each other, but they also possess comparable AA-regulating properties. Intriguingly, the C-terminal epitopes of the self hsp65 are well processed and presented (dominant) from the native antigen, whereas those of the foreign hsp65 are poorly processed and presented (cryptic or hidden). However, like Bhsp65, Rhsp65 is also a good immunogen, showing that Lewis rats are not tolerant to either hsp65. Our results support a model for immunoregulation of autoimmune arthritis in which arthritic Lewis rats having inflammatory acute AA upregulate the expression as well as presentation of endogenous self hsp65 leading to priming of ambient C-terminal epitope-reactive T cells. These T cells then contribute to the downregulation of acute AA. Concurrently, the cryptic epitopes of Bhsp65 are efficiently processed under the inflammatory milieu of acute AA, and thereby, activate subsets of T cells directed against C-terminal epitopes of Bhsp65. These T cells are then activated and expanded by C-terminal epitopes of Rhsp65, and vice versa, resulting in a reinforcement of the regulatory T cell activity against AA. These results describe a novel disease-regulating feature of cryptic antigenic determinants contrary to their well-known pathogenic attribute observed in other antigens. Thus, cryptic epitopes of autoimmune diseases-related antigens can be exploited for immunotherapeutic purposes in RA and other autoimmune diseases. (Supported by grants from the NIH, Arthritis Foundation- National Office and Maryland Chapter, and the Maryland Arthritis Research Center.)

#### **Sa1.118. Both Chlordecone and Estrogen Enhance the Germinal Center B Cell Reaction and Reduce the Splenic B Cell Apoptosis in Ovariectomized (NZB × NZW) F1 Mice.**

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Systemic lupus erythematosus (SLE) is a debilitating autoimmune disease that affects women nearly nine times as often as men. Estrogen has been implicated as an important risk factor. The weakly estrogenic organochlorine pesticide chlordecone can accelerate the onset and severity of immune complex glomerulonephritis in female (NZB × NZW) F1 (NZB/WF1) mouse model. Moreover, chlordecone exposure caused the earlier onset of serum IgG anti-dsDNA and anti-chromatin in ovariectomized NZB/WF1 mice, similar to the effects caused by exogenous exposure to 17-beta estradiol. This murine model of SLE is characterized by the spontaneous development of germinal centers (GCs), which play a key role in the maturation of the humoral immune response. Chemokine receptors CXCR4 and CXCR5 are important for GC dark and light zone organization, and cell adhesion molecules ICAM and VCAM-1 may play an important role in reducing GC B cell apoptosis. We therefore examined the effects of chlordecone and 17-beta estradiol on

germinal center B cells using two-month old ovariectomized NZB/WF1 mice. Mice were subcutaneously implanted with sustained-release pellets containing 17-beta estradiol (0.025mg/kg/day) or chlordecone (0.5 and 2.5mg/kg/day). Control mice received pellets with matrix only. Mice were sacrificed six weeks after implantation, and spleen cells were studied by flow cytometric analysis. Splenic B cells were also purified for proliferation assays using <sup>3</sup>H-thymidine and apoptosis tests with annexin-V and 7-AAD. Both estradiol and chlordecone caused a significant increase in spleen size/weight ( $P < 0.01$  by estradiol and  $P < 0.05$  by chlordecone, ANOVA). We also found that both chlordecone and estradiol increased the percentage of B cells expressing the GC marker GL7 ( $P < 0.01$  by ANOVA), the B cell co-stimulatory molecule B7-2 ( $P < 0.01$ ), and the activation marker CD44 ( $P < 0.01$  by estradiol and  $P < 0.05$  by chlordecone). The expression of CXCR4 ( $P < 0.01$ ), CXCR5 ( $P < 0.01$  by estradiol and  $P < 0.05$  by chlordecone), ICAM ( $P < 0.01$ ), and VCAM-1 ( $P < 0.01$  by estradiol and  $P < 0.05$  by chlordecone) on splenic B cells was upregulated as well. On the other hand, neither chlordecone nor estradiol produced a significant increase in B cell proliferation, but they both significantly reduced splenic B cell apoptosis. These findings suggest that both chlordecone and estradiol may enhance autoimmunity through effects on the germinal center response.

#### **Sa1.119. IL10 Promoter Polymorphisms in Mexican Patients with Spondyloarthropathies.**

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The spondyloarthropathies (SpA) are a heterogeneous group of diseases characterized by axial as well as peripheral enthesitis and arthritis and less frequently by a range of extra-articular manifestations. The strong association between HLA-B27 and the SpA, particularly ankylosing spondylitis (AS) in the general population and in first degree relatives of affected individuals confers this allele a significant role in disease susceptibility. Yet, the fact that only approximately 2% of HLA-B27 individuals develop AS and that a small proportion of patients with AS does not carry the B27 allele suggests other genes participate in the disease susceptibility. Of special interest is the IL-10 gene that codified a potent immune modulator. Thus, the aim of the present study was to investigate the allele distribution of IL10 promoter polymorphisms in SpA Mexican patients. One hundred and twenty-six patients with SpA (55 with undifferentiated SpA (U-SpA), 55 with AS, and 16 with reactive arthritis (ReA)) and 91 healthy controls were studied. The IL-10 promoter polymorphisms (positions-592, -819 and-1082) were determined by polymerase chain reaction-restriction fragment length polymorphism. Statistical methods included the Mantel-Haenzel,  $\chi^2$ , Fisher's exact test, and Woolf method for odds ratio (OR). The allele and genotype frequencies of the three polymorphic sites were similar in the whole group of SpA patients and healthy controls. When clinical subgroups (U-SpA, AS and ReA) were compared to healthy controls, the results did not reveal significant differences in allele or genotype frequencies. These data suggest that IL10 promoter polymorphisms are not involved in the genetic susceptibility to SpA in Mexican patients.

#### **Sa1.120. Genetic Dissection of Congenital Heart Block in a Rat Model.**

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Congenital heart block is a passively transferred autoimmune disease where Ro52 antibodies are transported from the mother to the fetus in Ro/SSA positive women. The diagnosis of the mother may be SLE or Sjögren's syndrome. The fetus can be affected with mild symptoms or they may develop a life-threatening heart block. It has been shown that the Ro52 antibodies are necessary for disease development, and immunization of rat mothers with Ro52 derived peptides results in heart block in the pups. However, the fact that mothers of affected children can later give birth to healthy children despite persisting Ro52 autoantibodies indicates that another factor such as genes are involved in the penetrance of the disease. The aim of this study was to investigate this by ascertaining the MHC and non-MHC genetic influence in heart block development in an immunization-induced rat model of congenital heart block.

Four rat strains, 3 sharing the same MHC region (RT1) haplotype (DA, PVG.AV1, LEW.AV1), and 2 with similar background genes but different MHC genes (LEW.L and LEW.AV1) were studied. Female rats were immunized with Ro52 or control protein and mated. The resulting pups (208 from Ro52 immunized mothers, and 105 from the control immunized mothers) were analysed for congenital heart block with extremity-lead electrocardiography of conscious pups. Serum samples were taken throughout the study from both mothers and pups and epitope mapping of the antibody specificities using recombinant Ro52 and a panel of overlapping and mutated Ro52 peptides was performed.

Analysis of the ECGs of the 313 born pups from the four strains showed that in the 3 strains sharing the same MHC region haplotype (DA, PVG.AV1, LEW.AV1) AV block I developed in 45%, 35% and 44% of the pups, respectively. In the Lew.L strain only 14% of pups were affected by AV block I. These results indicate that genes in the MHC region are important for penetrance of the disease ( $P < 0.001$ ). There was no significant difference in generated Ro52 antibody epitope specificity between the rat strains. The hearts of all 44 rat mothers and 24 of the pup hearts were collected for immunohistochemical investigation of inflammatory markers.

Our study is the first to explore genetic influence in development of congenital heart block in animal models, and results show that genes found in the rat MHC region are of significant importance for the penetrance of congenital heart block, while non-MHC genes appear to have little or no influence on the disease.

#### **Sa1.121. Identification of Disease Profiles for Rheumatoid Arthritis Using Antibody and Antigen Arrays.**

*David L. Hirschberg,<sup>1</sup> Anya Tsalenko,<sup>1</sup> Mark Westall,<sup>1</sup> Bill Fisher,<sup>1</sup> Bo Curry,<sup>1</sup> Wolfgang Hueber, Orr Sharpe,<sup>2</sup> Beren Toomoka,<sup>2</sup> Dorothy Yang,<sup>1</sup> Artie Schleifer,<sup>1</sup> Willy McAllister,<sup>1</sup> William H. Robinson,<sup>2</sup> Viorica Lopez-Avila.<sup>1</sup>* <sup>1</sup>Agilent Technologies Inc., Palo Alto, USA; <sup>2</sup>Stanford University School of Medicine, Division of Immunology & Rheumatology, Stanford, CA, USA.



Autoimmune maladies, such as, rheumatoid arthritis are difficult to diagnose and most likely represent a collection of pathologies that give rise to the symptoms of each disease. Furthermore, there is no definitive set of markers for these diseases that can be monitored in real time to predict or assess the effect of any treatment. Simultaneous and serial measurements of hundreds of proteins in the blood and synovial fluid will be needed to make a differential diagnosis and to discover novel patterns that would stratify the diseases. Protein microarrays are well suited for this type of proteomic approach despite the fact that they are limited by the specificity of the affinity reagents (i.e., antibodies), and the measurement accuracy is dependent on the array performance, sample preparation, and assay reproducibility. We demonstrate an improved method for fabrication of antibody and antigen microarrays using a thermal inkjet printer and functionalized glass slides, and have addressed quality issues such as checking purity and integrity of proteins prior to deposition, printed array quality, cross-reactivity of the affinity reagents, and need for removal of the high abundant proteins. Using our optimized array platform we developed antibody arrays for detection of over 60 markers of inflammation, including autoantibodies, cytokines, chemokines, soluble receptors and enzymes. Forty samples of synovial fluid and serum from RA patients and controls were run on the arrays. The arrays demonstrated increased levels of known inflammatory markers in RA samples among them matrix metalloproteases and cytokines.

#### **Sa1.123. Identification of disease profiles for rheumatoid arthritis using antibody and antigen arrays.**

David L. Hirschberg,<sup>1</sup> Anya Tsalenko,<sup>1</sup> Mark Westall,<sup>1</sup> Bill Fisher,<sup>1</sup> Bo Curry,<sup>1</sup> Wolfgang Hueber, Orr Sharpe,<sup>2</sup> Beren Toomoka,<sup>2</sup> Dorothy Yang,<sup>1</sup> Artie Schleifer,<sup>1</sup> Willy McAllister,<sup>1</sup> William H. Robinson,<sup>2</sup> Viorica Lopez-Avila.<sup>1</sup> <sup>1</sup>Agilent Technologies Inc., Palo Alto, USA; <sup>2</sup>Stanford University School of Medicine, Division of Immunology and Rheumatology, Stanford, CA, USA.

Autoimmune maladies, such as, rheumatoid arthritis are difficult to diagnose and most likely represent a collection of pathologies that give rise to the symptoms of each disease. Furthermore, there is no definitive set of markers for these diseases that can be monitored in real time to predict or assess the effect of any treatment. Simultaneous and serial measurements of hundreds of proteins in the blood and synovial fluid will be needed to make a differential diagnosis and to discover novel patterns that would stratify the diseases. Protein microarrays are well suited for this type of proteomic approach despite the fact that they are limited by the specificity of the affinity reagents (i.e., antibodies), and the measurement accuracy is dependent on the array performance, sample preparation, and assay reproducibility. We demonstrate an improved method for fabrication of antibody and antigen microarrays using a thermal inkjet printer and functionalized glass slides, and have addressed quality issues such as checking purity and integrity of proteins prior to deposition, printed array quality, cross-reactivity of the affinity reagents, and need for removal of the high abundant proteins. Using our optimized array platform we developed antibody arrays for detection of over 60 markers of inflammation, including autoantibodies, cytokines, chemokines, soluble receptors and

enzymes. Forty samples of synovial fluid and serum from RA patients and controls were run on the arrays. The arrays demonstrated increased levels of known inflammatory markers in RA samples among them matrix metalloproteases and cytokines.

#### **Sa1.124. Relationship of school attendance with quality of life, physical function, disease activity and damage in pediatric systemic lupus erythematosus.**

L N Moorthy,<sup>1</sup> M G Peterson,<sup>2</sup> M J Harrison,<sup>2</sup> K B Onel,<sup>3</sup> D R Mohan,<sup>1</sup> Thomas Lehman.<sup>2</sup> <sup>1</sup>Robert Wood Johnson University, New Brunswick, NJ, USA; <sup>2</sup>Hospital for Special Surgery, New York, NY, USA; <sup>3</sup>La Rabida Children's Hospital, Chicago, IL, USA.

**Background:** Pediatric systemic lupus erythematosus (SLE) is a multisystem disease that significantly impacts quality of life (QOL) of children. SLE's impact on school attendance, an important outcome and potential predictor of other outcomes, has received little attention.

**Objective:** Examine the relationship of school attendance with QOL, physical function, SLE activity and damage in children with SLE.

**Design/Methods:** In this cross-sectional study, children with SLE (6–18 years) and parents completed child/parent versions of: Childhood Health Assessment Questionnaire (CHAQ), Pediatric QOL Inventory (PedsQL Generic 4.0 and Rheumatology 3.0 modules). Physician completed: SLE Disease Activity Index (SLEDAI) and Systemic Lupus International Collaboration Clinics/ACR Damage Index (SLICC). Number of days over the prior 30 the child missed school due to physical/mental health reasons was recorded using PedsQL family information form. Spearman correlations were determined between number of missed school days and other variables.

**Results:** 24 children (23 girls) with SLE (mean age 15 ± 3 years, mean education 9<sup>th</sup> grade, mean SLE duration 46 ± 30 months, SLEDAI 0–24, SLICC 0–6, median CHAQ 0.3, mean PedsQL–Generic 67 ± 20), and 19 parents (median CHAQ 0, mean PedsQL–Generic 69 ± 18) participated. 4 children were excluded because school attendance was inapplicable. In 30 days prior to participation, 10 children (50%) missed school with a mean of 3 ± 7 days. Mean number of days too ill to play = 3 ± 7 and mean number of days needed someone = 3 ± 6 days. The number of missed school days moderately correlated with decreased QOL as reported by parents and as measured by the PedsQL–Generic module (r 0.56, p 0.02), but did not correlate significantly with Rheumatology module, CHAQ, SLEDAI, SLICC, or any of the child reported scores.

**Conclusions:** Number of missed school days is correlated with decreased general QOL in children with SLE as perceived by their parents. However, parallel correlation between missed school days and overall QOL as perceived by children was not identified. Discrepant perception between parents and children warrant further investigation in a larger cohort. Lack of correlation of school attendance with other scales suggests that generic scale captures a less tangible element of SLE.

**Category:** Autoimmune Rheumatologic Diseases

**Keywords:** quality of life; systemic lupus erythematosus (SLE); childhood diseases

**Theme:** Other; Related to quality of life of autoimmune diseases

### Sa1.122. Development and Validation of the Simple Measures of Impact of Lupus Erythematosus in Youngsters (SMILEY©) Questionnaire.

L N Moorthy,<sup>1</sup> M G Peterson,<sup>2</sup> M J Harrison,<sup>2</sup> K B Onel,<sup>3</sup> M J Baratelli,<sup>1</sup> D R Mohan,<sup>1</sup> T Lehman.<sup>2</sup> <sup>1</sup>Robert Wood Johnson University, New Brunswick, NJ, USA; <sup>2</sup>Hospital for Special Surgery, New York, NY, USA; <sup>3</sup>La Rabida Children's Hospital, Chicago, IL, USA.

**Background:** Pediatric systemic lupus erythematosus (SLE) is a chronic fluctuating disease significantly impacting quality of life (QOL). There is no pediatric SLE-specific health-related QOL tool. SLE heterogeneity, children's evolving needs and expectations, and parent-proxy respondents complicate QOL measurement.

**Objective:** Develop a brief, valid and easily understood SLE-specific pediatric QOL tool.

**Design/Methods:** We developed Simple Measure of Impact of Lupus Erythematosus in Youngsters (SMILEY©) based on qualitative research on pediatric SLE- a 31 item tool, with parallel child/parent versions and 5-faces scale items. Children 2–18 years and parents completed child–parent versions of the SMILEY©, Pediatric Quality of Life Inventory (PedsQL) Generic 4.0 scale, and Childhood Health Assessment Questionnaire (CHAQ). Children also completed the Piers–Harris Self–Concept Scale. Correlations of child/parent SMILEY© versions with the corresponding versions of the above scales were determined by Spearman rank test.

**Results:** 15 children (12 girls) with SLE (mean age  $14 \pm 2$  years, mean SLE duration  $44 \pm 37$  months, SLE disease activity index 0–20, mean self–concept  $50 \pm 9$ ) participated with mean SMILEY© score of  $106 \pm 20$ . 14 parents had a mean SMILEY© score of  $98 \pm 24$ . Significant correlations are indicated by asterisks (table). Subjects completed SMILEY© in <10 minutes. Correlation of child/parent SMILEY© ( $n = 14$ ) was not significant ( $r = 0.4$ ,  $P = 0.1$ ).

Table 1  
Correlation of child/parent SMILEY with corresponding child/parent version of the scales

SMILEY	CHAQ	PedsQL Generic
SMILEY child (n 15)	0.8**	0.7**
SMILEY parent (n 13)	0.5	0.5

\*\* $P < 0.01$ ; \* $P < 0.05$ .

**Conclusions:** Our results demonstrate that SMILEY© is a brief, easily understood and valid pediatric SLE-specific QOL scale. Lack of significant correlation between child/parent reports suggests that we may be measuring different information from children/parents; children's perception of their QOL may be different from their parent's perceptions. Accrual of additional subjects is necessary to confirm results. [Table 1].

**Category:** Autoimmune Rheumatologic Diseases.

**Keywords:** Quality of life; systemic lupus erythematosus (SLE); children

**Theme:** Other; Related to quality of life of autoimmune diseases

## Diabetes & Other Autoimmune Endocrine Diseases

### Sa1.125. A Fractal Analysis of Binding and Dissociation Kinetics of Connective Tissue Interstitial Glucose, Adipose Tissue Interstitial Glucose and Related Analytes on Biosensor Surfaces.

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A fractal analysis is used to model the binding and dissociation kinetics of biomedical analytes like connective tissue interstitial glucose, adipose tissue interstitial glucose, insulin and other related analytes. The analysis provides insights into diffusion-limited analyte-receptor reactions occurring on heterogeneous biosensor surfaces.

The fractal analysis is applied to the binding of glucose and other related analytes in solution to glucose derivatives immobilized on a biosensor chip. The fractal analysis provides a useful lumped parameter(s) analysis for the diffusion-limited reaction occurring on a heterogeneous surface via the fractal dimension and the rate coefficient. To demonstrate fractality a log-log plot is made with a large amount of available data. It is a convenient means to make the degree of heterogeneity that exists on the surface more quantitative. The fractal approach provides additional information about interactions that may not be obtained by a conventional analysis of biosensor data.

#### Results and Conclusions:

Numerical values obtained for the binding and the dissociation rate coefficients are linked to the degree of heterogeneity or roughness (fractal dimension,  $D_f$ ) present on the biosensor chip surface. The binding and the dissociation rate coefficients are sensitive to the degree of heterogeneity on the surface. For example, for the binding of adipose tissue interstitial glucose, as the fractal dimension value increases by a factor of 3.31 from  $D_{f1}$  equal to 0.5720 to  $D_{f2}$  equal to 1.891, the binding rate coefficient increases by factor of 8.88 from  $k_1$  equal to 0.0545 to  $k_2$  equal to 0.4841. An increase in the degree of heterogeneity on the probe surface leads to an increase in the binding rate coefficient. A dual fractal analysis gives a better fit in most cases for the binding kinetics. A single fractal analysis is adequate to describe the dissociation kinetics. Affinity (ratio of the binding to the dissociation rate coefficient) values are also presented.

The values of binding rate coefficient,  $k$ , linked with the degree of heterogeneity existing on the biosensor surface provide a complete picture of the reaction kinetics occurring on the sensor chip surface. Dual fractal analysis is used only when the single fractal analysis did not provide an adequate fit. This was done by regression analysis provided by Quattro Pro 8.0.

It is suggested that roughness on surface leads to turbulence which enhances mixing of glucose and decreases diffusional limitations leading to an increase in the binding rate coefficients for glucose.

### Sa1.126. Serum Levels of Interleukin-16 in Patients with Newly Diagnosed Type 1 Diabetes Mellitus.

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Type 1 diabetes mellitus is considered to be the typical autoimmune disorder resulting in the loss of pancreatic beta-cells

with the consequent development of absolute insulin deficiency. However, the exact changes of the components of the immune system including impairments of different cytokines levels preceding the development of clinically overt type 1 diabetes mellitus remain not fully understood. Therefore, the aim of this study was to investigate the serum levels of interleukin-16 in patients with newly diagnosed type 1 diabetes mellitus. We studied 10 patients with clinically overt type 1 diabetes mellitus aged 8–15 years old before initiating of insulin therapy and 10 aged-matched healthy control subjects. Serum interleukin-16 levels were measured by specific immunoenzyme assay. We found that serum levels of interleukin-16 were significantly decreased in patients with type 1 diabetes mellitus compared to control subjects  $94 \pm 9.99$  pg/ml vs.  $270 \pm 34.78$  pg/ml (mean  $\pm$  SEM), respectively,  $P < 0.001$ . These data could explain the decrease of the number CD4<sup>+</sup> T-lymphocytes in subjects with newly diagnosed type 1 diabetes mellitus which was revealed in our earlier studies as these lymphocytes represent the target cells for interleukin-16. We may hypothesize that revealed significant decrease of the interleukin-16 production could play a role in the pathogenesis of autoimmune insulinitis and consequent clinical manifestation of type 1 diabetes mellitus.

#### **Sa1.127. Antigen-Based Therapies Utilizing Ignored Determinants of $\beta$ -Cell Antigens Can More Effectively Slow Late-Stage Autoimmune Disease in Diabetes-Prone Mice.**

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As organ-specific autoimmune diseases do not become manifest until well-advanced, interventional therapies must inhibit late-stage disease processes. Using a panel of immunogenic peptides from various  $\beta$ -cell antigens, we evaluated the factors influencing the efficacy of antigen-based therapies in diabetes-prone NOD mice with advanced disease. The ability of the major  $\beta$ -cell autoantigen target determinants (TDs) to prime Th2 responses declined by ~80% between 6 to 12 weeks of age, while the ability of immunogenic ignored determinants (IDs) of  $\beta$ -cell antigens to prime Th2 responses was unaffected by the disease process. The different patterns of TD and ID immunogenicity (even from the same  $\beta$ -cell antigen) may be due to the exhaustion of uncommitted TD, but not ID-reactive, T cell pools by recruitment into the autoimmune cascade. Therapeutic efficacy was associated with a peptide's immunogenicity and ability to promote Th2 spreading late in the disease process, but not its affinity for I-Ag7 or its expression pattern ( $\beta$ -cell specific/non-specific, or rare/abundant). Characterization of some IDs revealed them to be "absolute" cryptic determinants. Such determinants have little impact on T cell selection, leaving large precursor T cell pools available for priming by synthetic peptides. Traditional antigen-based therapeutics using whole autoantigens or their TDs cannot prime responses to such determinants. These findings suggest a new strategy for designing more efficacious antigen-based therapeutics for late-stage autoimmune diseases.

#### **Sa1.128. NKT Cells May Contribute to the Immunopathogenesis of Non-Alcoholic Steatohepatitis.**

Kazuto Tajiri,<sup>1</sup> Yukihiko Shimizu,<sup>1</sup> Yoshiharu Tokimitsu,<sup>1</sup> Yasuhiro Nakayama,<sup>1</sup> Katsuharu Hirano,<sup>1</sup> Masami Minemura,<sup>1</sup> Kazumi Ebata,<sup>1</sup> Toshiro Sugiyama,<sup>1</sup> Koichi Tsuneyama.<sup>2</sup> <sup>1</sup>Third

Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan; <sup>2</sup>First Department of Pathology, Toyama Medical and Pharmaceutical University, Toyama, Japan.

**Objectives:** Non-alcoholic steatohepatitis (NASH) is a common chronic liver disease, leading to cirrhosis and hepatocellular carcinoma. It has been considered tumor necrosis factor  $\alpha$  or adipocytokine is important in the pathogenesis of NASH, which has a feature of metabolic syndrome. Although NASH is characterized by necro-inflammatory changes, an essential role of the inflammatory cells has not yet been identified. To clarify the role of inflammatory reactions in the pathogenesis of NASH, we analyzed the composition of liver-infiltrating cells isolated from liver biopsy specimens of NASH. **Methods:** 26 patients with NASH and 23 with fatty liver (FL) were analyzed. We performed immunohistochemical staining using antibodies against CD3, CD4, CD8, CD56, CD68, and CD15. Oxidative stress was assessed by the expression of 4-hydroxy-2-nonenal (HNE). We counted the numbers of each population of liver-infiltrating cells (/mm<sup>3</sup>). We diagnosed the liver histology using scoring system proposed by Brunt et al., and examined the correlations between the histological scores and the number of each population of liver-infiltrating cells. Moreover, we analyzed the surface markers of isolated liver-infiltrating cells by flow cytometry with antibodies against CD3, CD56, CD11b, lineage markers, HLA-DR, and CD1d in 5 cases. In addition, localization of CD1d expression was also examined, and analyzed the association with that of CD56<sup>+</sup> cells by an immunohistochemical method. **Results:** Among various populations, only the numbers of CD56<sup>+</sup> cells were significantly higher in NASH than those in FL (NASH: FL =  $57.8 \pm 48.5$ :  $15.4 \pm 15.2$ ,  $P = 0.02$ ). In NASH, the numbers of CD56<sup>+</sup> cells tended to be decreased as fibrosis progresses ( $r = -0.55$ ,  $P = 0.039$ ), and the expression of HNE or the number of CD68<sup>+</sup> cells showed a positive correlation with that of CD56<sup>+</sup> cells (HNE;  $r = 0.56$ ,  $P = 0.036$ , CD68;  $r = 0.55$ ,  $P = 0.041$ ). Moreover, flow cytometric analysis showed that NKT cells (CD3<sup>+</sup>CD56<sup>+</sup>), rather than NK cells (CD3-CD56<sup>+</sup>), are increased in the livers with NASH. Furthermore, flow cytometric analysis showed that CD1d molecules are found to be expressed on antigen presenting cells such as macrophages (CD11b<sup>+</sup>) and dendritic cells (lineage- DR<sup>+</sup>). In addition, CD1d expression was significantly increased in the liver with NASH compared with FL or normal livers. Importantly, a part of CD1d expression was recognized around CD56<sup>+</sup> cells. **Conclusion:** We showed that NKT cells are proliferated and activated in an early stage of NASH. In the liver with NASH, hepatocytes contain a lot of fat with the lipid being peroxidated, which generates oxidative stress. Antigen presenting cells might uptake peroxidated lipid derived from degenerated hepatocytes, and present processed lipid antigen on CD1d. NKT cells would recognize those lipid antigens through CD1d, leading to the release of various cytokines. These processes could contribute to the pathogenesis of NASH.

#### **Sa1.129. Pro-Apoptotic DNA Vaccination as a Therapy for Type 1 Diabetes.**

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Type 1 diabetes is in most cases the result of cell-mediated autodestruction of pancreatic  $\beta$ -cells. We have shown previously that DNA coding for the pro-apoptotic BAX protein promotes diabetes prevention in pre-diabetic NOD mice when co-delivered

intramuscularly with DNA coding for a secreted form of the  $\beta$ -cell antigen glutamic acid decarboxylase (GAD). Furthermore, co-delivery of BAX recruits dendritic cells containing plasmid-encoded protein in peripheral lymphoid organs. Here, we report that the same DNA vaccine reverses new onset diabetes when delivered intradermally and induces immunosuppressive CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg). Female NOD mice received the vaccine at the time of diabetes onset and a second time in case of relapse. A variety of responses to the treatment were observed. Mice responded either to both the initial and relapse treatment, to the initial treatment only, or not at all. Fifty percent of the treated mice were overtly diabetic (fasting blood glucose > 300 mg/dL) by the age of 40 weeks. By contrast, more than 90% of untreated mice and of mice treated with plasmid vector alone were overtly diabetic by that age. Immunological analysis revealed that draining lymph nodes of mice treated with the pro-apoptotic DNA vaccine contained higher numbers of Treg with enhanced immunosuppressive function compared to control animals. Our results indicate that delivery of a DNA vaccine alone can reverse the symptoms of an autoimmune disease, and suggest a real clinical potential for pro-apoptotic DNA vaccines in the treatment of immune-mediated inflammatory disorders.

### **Sa1.130. Increased $\beta$ Cell Replication with Onset of Diabetes and after Induction of Immune Tolerance in the NOD Mouse.**

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Recent studies concerning  $\beta$  cell turnover suggest that there is a continual process of  $\beta$  cell death and renewal. The effects of autoimmunity on this process have not been well studied but the regenerative potential of islet cells has been suggested by recent studies in animal models. We have examined changes in  $\beta$  cell mass and replication in the presence of islet inflammation, and during tolerance induction with CD4+CD25+ regulatory T cells.

The mean percentage of replicating  $\beta$  cells was higher in female pre-diabetic NOD mice (age = 9–14 wks) compared to aged matched NOD/Scid mice ( $1.62 \pm 0.9$ ,  $n = 6$  vs.  $0.19 \pm 0.1\%$ ,  $n = 5$ ;  $P < 0.01$ ). There was no significant difference in  $\beta$  cell mass between the two groups ( $0.07 \pm 0.04$  mg,  $n = 4$  vs.  $0.09 \pm 0.05$ ,  $n = 4$ ;  $P = \text{NS}$ ). Upon diabetes onset in female NOD mice, the percentage of replicating  $\beta$  cells increased to  $3.56 \pm 0.9\%$ ,  $n = 3$ . These data are consistent with a compensatory response to inflammation.

To further demonstrate that the increase in  $\beta$  cell replication was in response to the anti-islet autoimmune process,  $\beta$  cell replication was measured in NOD/Scid mice 2 and 4–5 weeks post-transfer of diabetogenic splenocytes. Our data show an increase in the mean percentage of replicating  $\beta$  cells with time ( $0.90 \pm 0.1\%$ ,  $n = 2$ , and  $1.93 \pm 0.4\%$ ,  $n = 3$ ;  $P < 0.05$  at 2 and 4–5 weeks post transfer, respectively). In contrast, no change in  $\beta$  cell replication was seen in non-treated age-matched NOD/Scid mice ( $0.26 \pm 0.1\%$ ,  $n = 2$  and  $0.15 \pm 0.1\%$ ,  $n = 3$ ;  $P = \text{NS}$ ).

Cotransfer of CD4+CD25+ regulatory T cells (Tregs), harvested from NOD mice treated with anti-CD3 mAb, prevented the development of diabetes in NOD/Scid mice by adoptive transfer of diabetogenic splenocytes. Four weeks after adoptive transfer of diabetogenic cells and Tregs, only 1 out of 5 NOD/Scid mice developed diabetes. In contrast, 5 out of 5 NOD/Scid mice that received CD4+CD25- T cells and diabetogenic cells developed diabetes ( $P = 0.05$ ). In mice protected by Tregs,  $\beta$  cell mass was

significantly greater than in those that received CD4+CD25- cells ( $0.02 \pm 0.02$  mg,  $n = 5$  vs.  $0.00011 \pm 0.00001$  mg,  $n = 5$ , respectively;  $P = 0.03$ ). The percent of replicating  $\beta$  cells in mice that received Tregs was  $1.97 \pm 0.6\%$ ;  $n = 5$ , whereas  $\beta$  cell mass was too small to determine replication rates in recipients of CD4+CD25- cells.

These findings suggest that in NOD mice, islet inflammatory lesions can play a role in stimulating  $\beta$  cell replication. Nevertheless, heightened  $\beta$  cell replication, initially in response to autoimmunity, is insufficient to overcome the rate of autoimmune-mediated beta cell destruction. Treatment with Tregs leads to improved preservation of  $\beta$  cell mass with maintenance of heightened  $\beta$  cell replication and prevents the development of diabetes in NOD mice.

### **Sa1.131. A Subset of Insulin and Glucagon Positive Cells Survives Destruction in “Older” Female NOD Mice.**

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The NOD mouse is a key animal model of Type 1A diabetes. The majority of female NOD mouse develops immune mediated diabetes between 15 and 35 weeks of age but a subset remain non-diabetic after 40 weeks of age. Lack of diabetes development in these mice could be due to a failure of the immune system to completely target and destroy beta cells or to the presence of insulin producing cells resistant to destruction or both. In order to distinguish between these two hypotheses, we studied the pancreatic histology of 11 non-diabetic female NOD mice that remained non-diabetic after 40 weeks of age. These mice range in age between 42 and 96 weeks. From each pancreas we obtained H&E sections as well double immunofluorescence staining with antibodies to insulin and glucagon. Each of the eleven mice had severe islet lymphocytic infiltrates, which affected the majority of the islets. In 5/11 mice there was no insulin staining while in 5 mice there were islet cells that exhibited double positivity for insulin and glucagon. In one mouse, we could detect both single insulin positive cells (negative for glucagon) and cells with double positivity. In all mice studied, glucagon positive cells were preserved and in mice with insulin positivity only a subset of the glucagon positive cells expressed positivity for insulin. Transfer of diabetes was analyzed for six of these NOD mice by injecting  $3 \times 10^7$  splenocytes in 8–10 week old SCID- NOD recipients. These splenocytes rapidly induced diabetes (< 5 weeks).

These data show that older non-diabetic NOD females show insulinitis with loss of beta cells and that their splenocytes are capable of transferring diabetes. Furthermore, the surviving insulin positive cells show an unusual phenotype characterized by positivity for glucagon. Further studies will elucidate the origin of these cells and we hypothesized that double positive cells resist immune mediated beta cell destruction though alternatively they may represent unusual recently developed insulin expressing cells.

### **Sa1.132. PDL1 Regulates Autoimmunity by Limiting Expansion of Autoreactive Th1 Cells and Mediates Resistance to Diabetes in NOD Mice.**

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We have previously demonstrated that the Programmed Death-1 (PD1) pathway plays a critical role in regulating the development of diabetes in NOD mice. In the present study, we explored the mechanisms involved in this regulation utilizing various knockout, transgenic and NOD congenic strains of mice. We observed that B cell deficient NOD mice, which are normally resistant to diabetes, develop the disease following PDL1 blockade whereas CD4 deficient NOD mice were resistant to PDL1 blockade; thereby suggesting CD4+ T cells play an important role in anti-PDL1 mediated acceleration of diabetes. We then utilized BDC2.5 TCR Tg mice to more clearly dissect the role of PDL1 in regulating autoreactive CD4+ T cells. PDL1 blockade precipitated early onset diabetes, an expansion of BDC2.5 TCR Tg cells, and decreased apoptosis. NOD congenic mice that have a variable degree of resistance to autoimmune diabetes (NOD.Idd5, NOD.Idd3, NOD.Idd3/10/18, and NOD.Idd9 usually develop diabetes with an incidence of 40%, 20%, 8% and 3%, respectively) were then utilized to study the role of PDL1 in diabetes resistance. Anti-PDL1 mAb treatment accelerated diabetes in all the congenic strains albeit with different acceleration patterns. Interestingly, in NOD.Idd9 congenic mice, PDL1 blockade both accelerated the time of onset as well as increased the incidence of diabetes to 50%. Utilizing Idd9 mice, we have shown that blockade of PDL1 results in precipitation of diabetes by changing the local cytokine milieu from Th2 to Th1 cells, with upregulation of IFN-gamma and TNF-alpha. We conclude that PDL1 regulates autoimmune diabetes by limiting the expansion of autoreactive Th1 cells and thus plays a critical role in mediating disease resistance. These results provide the rationale for developing novel therapies to re-establish tolerance in autoimmune diabetes.

### Sa1.133. Human HLA-A2 MHC Class 1 Molecule Is Permissive for the Development of Diabetes in Experimental Autoimmune Diabetes.

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Type 1 diabetes is an immune-mediated disease characterized by the autoimmune destruction of pancreatic  $\beta$ -cells. A variety of environmental and genetic factors are involved in the development of the disease. We have previously developed an experimental autoimmune diabetes (EAD) model with insulin peptide B:9-23 immunization and/or polyinosinic-polycytidylic acid (PolyI:C), as a viral RNA mimic Toll-like receptor (TLR) activator, in transgenic mice expressing the costimulatory molecule B7.1 in their islets (driven by the Rat Insulin Promotor, RIP). B:9-23 peptide immunization induces diabetes and insulin autoantibodies (IAA) in I-A<sup>d</sup> mice expressing B7.1. A major goal is the development of "humanized" mice that can develop immune mediated diabetes with known native autoantigen stimulus. As reported by Marron et al., human leukocyte antigen (HLA)-A2.1 transgenic NOD mice progress to diabetes more rapidly than A2 negative mice. We have combined transgenes for human A2.1 with B7.1 transgenic (BALB/c B7.1x C57Bl/6 A2.1 mice, original C57Bl/6 B7.1 mice were a gift from Dr L. Wen and C57Bl/6 A2.1 mice were provided from Dr B.L. Kotzin). Mice were immunized with B:9-23 with or without PolyI:C. Overall 29% of the mice by 41 weeks ( $n = 7$ ,

range diabetes onset 22–43 weeks) developed diabetes following PolyI:C injection alone. Forty-three percent developed diabetes by 26 weeks ( $n = 7$ ; range 21–26 weeks,  $P < 0.54$ ) with B:9-23 immunization and PolyI:C injection. Our studies indicate that human A2 is permissive for the development of diabetes in EAD but does not accelerate disease in the model following either TLR3 activation or peptide B:9-23 immunization. Studies are underway to define potential islet peptide A2 restricted antigen presentation using ELISPOT analysis in this model of immune mediated  $\beta$ -cells destruction.

### Sa1.134. Similarities and Differences in Autoimmune Responses between Type 1 and Type 1.5 Diabetes Patients.

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Type 1.5 diabetes or LADA comprises approximately 10% of Caucasian adult phenotypic type 2 diabetes patients. The islet reactive autoantibodies and T cells found in type 1.5 diabetes patients suggest an autoimmune pathogenesis. In this study, we asked how T cell reactivity and phenotype compared in type 1.5 versus type 1 diabetes. We identified type 1.5 diabetes patients ( $n = 12$ ) through autoantibody and T cell responses to islet proteins. T cell reactivity to islet proteins was measured by cellular immunoblotting and peripheral T cell populations were measured by FACS and compared between type 1.5 patients versus type 1, type 2, and normal controls. T cells from both type 1 and type 1.5 diabetes patients respond similarly to islet proteins in the molecular weight regions of 116kDa, 97kDa, 60kDa. In contrast, islet proteins in the molecular weight regions of 65-90kDa and 21-38kDa were significantly ( $P < 0.05$ ) less stimulatory to T cell responses from type 1.5 diabetes patients versus type 1 patients. The number of CD4+CD25+ cells was significantly lower ( $P < 0.05$ ) in the peripheral blood of type 1 patients compared to type 1.5 patients, type 2 patients and normal controls. CD4+CD38+ cells were significantly lower in the peripheral blood of type 1.5 patients compared to the other subject groups and T cell receptor positive gd cells were significantly ( $P < 0.05$ ) decreased in the peripheral blood of type 1 and type 1.5 patients. These results suggest that type 1 and type 1.5 diabetes are immunologically similar in some respects but in other ways are different. These differences may be important in the slower disease progression of the type 1.5 diabetes disease process.

### Sa1.135. The Role of Cytomegalovirus Infection in the Development of Type 1 Diabetes-Associated Autoimmunity in HLA-Susceptible Children.

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Type 1 diabetes is an autoimmune disease resulting from the destruction of pancreatic beta cells. The HLA genotype influences the disease susceptibility. However, only a small proportion of genetically susceptible subjects progress to type 1 diabetes, which suggests that also environmental factors play a major role in the

disease process. Several viral infections including cytomegalovirus (CMV) infections have been implicated to be associated with the development of type 1 diabetes-related autoimmunity. The aim of this study was to explore whether there is any correlation between CMV infections and type 1 diabetes-associated autoimmunity in young children with HLA-conferred disease susceptibility. The cases with diabetes-associated autoantibodies and their HLA, age and sex-matched controls were participants in the Diabetes Prediction and Prevention (DIPP) Study running at the Universities of Turku, Tampere and Oulu in Finland. According to the study protocol, newborn infants carrying susceptible HLA genotypes are observed at 3 to 6 months intervals and regularly tested primarily for ICA and if positive also for GADA, IA-2A and IAA. Forty-one prospectively followed autoantibody positive subjects (21 girls, 20 boys, age 3 to 48 months, median 18 months) and 190 sex, age and HLA-matched controls were analyzed for CMV IgG class antibodies by EIA at the time of seroconversion to autoantibody positivity or within the next 6 months. No significant difference was seen in the prevalence of CMV antibodies. At the time of seroconversion 10 (24%) of the autoantibody positive subjects and 60 (32%) of their controls were positive for CMV IgG class antibodies ( $P = 0.47$ , Chi-square test). No differences were either found between ICA, GADA, IAA or IA-2 positive subjects and control subjects when each autoantibody was analyzed separately. In conclusion, these data suggest that early cytomegalovirus infections do not increase the probability to develop type 1 diabetes-associated autoimmunity.

#### **Sa1.136. Rotavirus-Specific T-Cell Responses and Type 1 Diabetes (T1D): No Evidence for Interrelationship.**

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Rotavirus infections have been implicated as a possible trigger of T1D. We elucidated this connection by comparing peripheral blood T-cell responses to rotavirus between children with newly diagnosed T1D ( $n = 37$ ), children with T1D-related autoantibodies ( $n = 34$ ) and control children carrying HLA-conferred susceptibility to T1D but without T1D-related autoantibodies ( $n = 104$ ). Lymphocyte proliferation assays based on stimulation with an antigen were performed using freshly isolated peripheral blood mononuclear cells. We measured also children's IgG and IgA class rotavirus antibodies in plasma samples drawn at the same time as the T-cell samples. No differences were observed in the strength of T-cell responses to rotavirus between the children with overt T1D or multiple autoantibodies, or the control children. Furthermore, also the frequencies of positive T-cell responses to rotavirus were closely similar in all three groups. The result remained similar, when only the children with serological evidence of earlier rotavirus infections were studied. Further, no differences were observed in the responses to the control antigens PPD and tetanus toxoid, but T-cell responses to purified coxsackie B4 virus were stronger in the children with autoantibodies than in the control children. In conclusion, our cellular immunity studies provided no evidence supporting association of rotavirus infections with T1D or presence of T1D-related autoantibodies in young children.

#### **Sa1.137. The Possible Biochemical Targets of Altered Opioid Mediated Nociception at Diabetes Conditions.**

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Diabetes is associated with infringement in endogenous opioid system and opposite alterations in various type opioid receptor sensitivity. However, it is not clear whether membrane lipid modification processes take part in development of such disorder as well, as their possible role in etiology of neurological complications in these patients. In order to evaluate processes mentioned involvement we suppose to investigate effects of selective delta-opioid receptor agonist Deltorphine II, delta-1-receptor antagonist 7-Benzylidennaltrexone and delta-2 receptor antagonist Naltriben on some aspects of phosphoinositide cycle functioning in lymphocytes. The regulator role of processes mentioned at the initial membrane-bound step of studied opioid agonist and antagonists information translocation has been demonstrated in both diabetic and non-diabetic cells. The evidence exists that alterations in lipid-mediated signal transduction pathways and receptors lipid microenvironment may be responsible for altered nociception at diabetes conditions. The amplification of free radical processes is known to be one of pathogenic mechanisms to form a basis for neuropathological complications at diabetes conditions. However, it is not clear whether membrane lipid free radical oxidation processes and enzymes of antiradical defense system take part in such disorders. In order to evaluate the involvement of above mentioned processes in altered nociception at diabetes conditions the intensity of lipid peroxidation processes have investigated as well, as activity of enzymes of cell antiradical protection, superoxid glutathione reductase dismutase in diabetic patients erythrocyte membranes at the conditions of opioid receptors blockade and sensitization. Some correlation between intensity of enzymatic lipid peroxidation and opposite changes of receptor sensitivity as well, as possible participation of cell antiradical protection enzymes have been demonstrated. The evidence exists, that studied lipid mediated transduction pathway together with receptor microenvironment modifications appear to be possible biochemical targets of altered nociception at diabetes conditions.

#### **Sa1.138. Combinatorial treatment of recent-onset type 1 diabetes by induction of islet-antigen specific Tregs and anti-CD3.**

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**Rationale:** A central issue when using immune-suppressive therapy in transplantation and autoimmunity is to balance benefits with systemic side effects. Administration of non Fc-binding anti-CD3 has shown good efficacy in temporarily maintaining C-peptide levels in recent-onset diabetes over a 1–2 year timeframe, but immunosuppressive side effects limit higher-dose regimens or continuous administration. One novel attractive avenue in type 1 diabetes (T1D) is the islet autoantigen-specific induction of adaptive regulatory T cells (Tregs) that can act *in vivo* as bystander suppressors of autoaggressive responses selectively within the pancreatic draining lymph node (PDLN). In our hands, such cells can be induced via oral or intranasal administration of insulin, proinsulin peptides and DNA vaccines expressing islet antigens and efficacy is associated with IL-4 and IL-10 production. Their site

specificity is a strong clinical advantage and can be attributed to the fact that autoantigens leading to their expansion and activation are only presented in the PDLN. Since anti-CD3 is known to increase numbers of intrinsic CD25<sup>+</sup> Tregs as well as TGF- $\beta$  and IL-10 production, we reasoned that it would create a favorable systemic milieu for islet-antigen specific induction of autoreactive, adaptive Tregs and synergy could be expected.

**Approach:** Combinatorial therapy of recent-onset diabetes in NOD and RIP-LCMV mouse models using anti-CD3 Fab<sup>2</sup> and intranasal proinsulin.

**Results:** At least 50% increased reversion of recent-onset diabetes was observed in NOD and RIP-NP mice treated with a combination of anti-CD3 and proinsulin compared to each intervention given alone. In particular, mice with more rapid beta cell loss benefited from this synergistic effect. Mechanistically, we observed increased numbers of proinsulin specific antigen-induced Tregs producing IL-4 and IL-10 in mice that received combinatorial therapy compared to those receiving anti-CD3 alone. Furthermore, we found much higher numbers of TGF-beta producing CD25<sup>+</sup> Tregs in mice treated with both, compared to mice that received anti-CD3 alone.

**Conclusion:** Antigen-specific induction of Tregs can synergize with systemic immune modulatory approaches to revert recent-onset T1D. Synergy was evidenced by enhanced clinical efficacy and increased numbers of intrinsic TGF-beta producing CD4<sup>+</sup>CD25<sup>+</sup> as well as adaptive autoantigen-specific IL-4/IL-10 producing Treg populations. This strategy should be considered for the clinic and maybe also for transplantation.

D.B. is supported by a JDRF postdoctoral fellowship and MGVIH is supported by DK51091, A144451 and a U-19 prevention center grant.

### Sa1.139. De Novo Generation of Antigen-Specific CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells from Human CD4<sup>+</sup>CD25<sup>-</sup> Cells.

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CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells are thought to be essential in maintaining tolerance to self antigens. These cells, known as regulatory T cells (T<sub>R</sub>), have been classified in two main categories: natural T<sub>R</sub>, which are derived from the thymus, and adaptive T<sub>R</sub>, which arise in the periphery. The importance of these cells in controlling responses to foreign antigens has just begun to be described. Previously, we have reported that activation of human CD4<sup>+</sup>CD25<sup>-</sup> T cells led to expression of FoxP3 in CD25<sup>+</sup> cells and acquisition of cell contact-dependent, cytokine-independent regulatory activity. Results in our laboratory reveal that these in-vitro derived T<sub>R</sub> could be generated from both memory and naive cells and by activation of CD4<sup>+</sup>CD25<sup>-</sup> T cells with alloantigen or a foreign antigen, HA (307-319). In the HA system, MHC class II tetramers were used to identify antigen-specific T cells. Antigen activation of CD4<sup>+</sup>CD25<sup>-</sup> led to two populations of CD4<sup>+</sup>CD25<sup>+</sup> cells, Tetramer<sup>+</sup> and Tetramer<sup>-</sup> cells, with antigen specific suppression occurring only in the Tetramer<sup>+</sup> cells. HA generated T<sub>R</sub> required cognate antigen for activation, but once activated subsequently suppressed noncognate bystander T cell responses as well. For this reason, we have started testing the ability of a diabetes

autoantigen, GAD65, to generate antigen-specific T<sub>R</sub>. GAD-specific T<sub>R</sub> can be generated from both normal and diabetic subjects, and once activated these T<sub>R</sub> also suppress bystander T cell responses. This raises the possibility that antigen-specific T<sub>R</sub> may be useful therapeutically in autoimmune diseases by localizing generalized suppressive activity to tissues expressing select target antigens.

### Sa1.140. TGF- $\beta$ Enhances Autoimmune Diabetes by Increasing Survival of Memory Effector CD8 Lymphocytes.

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TGF- $\beta$  is produced by a variety of cell types and can exhibit strong immune dampening functions. Surprisingly, in a model of virally-induced autoimmune diabetes, local expression of TGF- $\beta$  in  $\beta$ -cells under the control of a doxycycline-dependent promoter resulted in increased islet infiltration and disease incidence. This was attributed to a selective anti-apoptotic effect of TGF- $\beta$  on differentiated memory-effector CTL. Conversely, mice lacking functional TGF $\beta$ -receptors on T cells exhibited enhanced apoptosis and fewer memory CD8 lymphocytes. The effects of TGF- $\beta$  were clearly dependent on the T cell differentiation status, as it effectively suppressed activation of naive anti-viral CTL. Our results highlight a novel aspect of the pleiotropic nature of TGF- $\beta$ , and have implications for the design of immuno-therapies involving this cytokine.

### Sa1.141. Protection from Type 1 Diabetes by iNKT Cells May Require Interactions with CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells.

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Invariant natural killer T (iNKT) cells comprise a subset of regulatory T cells characterized by their co-expression of NK cell markers and an invariant TCR that recognizes a lipid antigen in a CD1d-restricted manner. Previously, we reported that activation of iNKT cells by the sphingoglycolipid alpha-galactosylceramide ( $\alpha$ -GalCer) protects against type 1 diabetes (T1D) in NOD mice. This protection involves the polarization towards a Th2-like immune response accompanied by increased IL-4. As potent activation of iNKT cells also trans-activates other immune cells, we further analyzed whether iNKT cells influence the function of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells. We found that CD4<sup>+</sup>CD25<sup>+</sup> T cells from NOD.CD1d<sup>-/-</sup> mice deficient in iNKT cells functioned similarly in vitro to CD4<sup>+</sup>CD25<sup>+</sup> T cells from wild-type NOD mice and suppressed the proliferation of NOD T responder cells upon stimulation with  $\alpha$ -GalCer. Adoptive transfer of NOD diabetogenic T cells and NOD CD4<sup>+</sup>CD25<sup>+</sup> T cells pretreated in vivo with multiple low doses of  $\alpha$ -GalCer further indicated that activation of iNKT cells do not influence the regulatory capacity of CD4<sup>+</sup>CD25<sup>+</sup> T cells to inhibit the transfer of T1D. In contrast, protection from T1D

mediated by adoptive transfer of activated iNKT cells requires the presence of CD4+CD25+ T cells, as splenocytes pretreated with  $\alpha$ -GalCer and depleted of CD25+ cells were unable to confer protection from T1D. These data suggest that even though iNKT cells may not influence CD4+CD25+ T cell activity, iNKT cell-mediated protection appears to require the presence of CD4+CD25+ T cells. (Supported by CIHR grant MOP 64386 to TLD).

**Sa1.142. Immunomodulation in Type 1 Diabetes by NBI-6024, An Altered Peptide Ligand of the Insulin B<sub>(9-23)</sub> Epitope.**

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NBI-6024 is an altered peptide ligand (APL) corresponding to the 9–23 amino acid region of the insulin B chain (B<sub>(9-23)</sub>), which is an epitope recognized by interferon (IFN)- $\gamma$ -producing T lymphocytes in type 1 diabetic patients. Immunomodulatory effects of NBI-6024 in recent-onset type 1 diabetic patients in a phase I clinical trial (NBI-6024-0003) were measured using the ELISPOT assay in peripheral blood mononuclear cells. IFN- $\gamma$  responses to B<sub>(9-23)</sub> were observed in 62.5% (5 of 8) of patients receiving placebo and in only 8% (1 of 13) of non-diabetic untreated control subjects. NBI-6024 administration (five biweekly or biweekly-monthly s.c. injections) led to a dose-dependent reduction in the percentage of patients with IFN- $\gamma$  responses to B<sub>(9-23)</sub> and a concomitant increase in the percentage with IL-5 responses to B<sub>(9-23)</sub>. Similar trends were observed with NBI-6024-specific ELISPOT responses. This Phase I clinical study demonstrated that NBI-6024 treatment did not enhance, but rather suppressed, the pathogenic IFN- $\gamma$  response of recent-onset type 1 diabetic patients in a dose-dependent fashion. Enhanced IL-5 responsiveness after NBI-6024 treatment is consistent with induction of a T helper (Th) 2-like immunological phenotype. The significance of these findings on the clinical outcome of disease is under investigation in a Phase II multi-dose study.

**Sa1.143. A Single Explanation for Three Puzzles in Type 1 Diabetes (T1D).**

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There is an excess of HLA-DR3/DR4 heterozygous individuals among Caucasian patients with T1D. The incidence of T1D is

rising in many countries and the MHC haplotype composition of T1D patients has also changed over the past several decades. Earlier, we proposed that, for any polygenic disease, if there is mixing of two populations with reciprocally different frequencies of susceptibility genes at different loci, the incidence in the mixed offspring will be much higher than in the original populations because of susceptibility gene complementation at those different loci. We propose that the apparent increased risk for HLA-DR3/DR4 heterozygotes as compared with homozygotes for either HLA-DR3 or DR4 and the heterozygote overrepresentation among patients is because their parents appear to come from different, previously isolated populations. Thus, HLA-DR3 and DR4 are both disease and population markers. Our evidence is that (a) HLA-DR parental genotype distribution deviated from the Hardy-Weinberg equilibrium in a way opposite to the patients: the parents had a paucity of HLA-DR3/DR4 heterozygotes, (b) DR3-transmitting parents had different HLA-A2 frequencies on their untransmitted HLA haplotypes than those who transmitted DR4, (c) DR3-positive parents had different *INS* allele frequencies than DR4-positive parents, and (d) parents of T1D patients had greater self-reported ethnic mixing than control parents. Thus, a specific kind of intrafamilial population admixture explains all three phenomena.

**Sa1.144. The (Relatively) Simple Genetics of a Polygenic Disease: Type 1 Diabetes (T1D).**

Chester A. Alper,<sup>1,2</sup> Charles E. Larsen,<sup>1,2</sup> Edmond J. Yunis,<sup>2,3</sup> Zaheed Husain,<sup>1,2</sup> Zuheir L. Awdeh.<sup>1</sup> <sup>1</sup>Immunogenetics Division, The CBR Institute for Biomedical Research, Boston, MA, USA; <sup>2</sup>Departments of Pediatrics, Medicine and Pathology, Harvard Medical School, Boston, MA, USA; <sup>3</sup>Department of Cancer Immunology and AIDS, The Dana-Farber Cancer Institute, Boston, MA, USA.

Concordance for T1D is approximately 40% for monozygotic twins, approximately 12% for MHC-identical sibs, and approximately 6% for sibs in general. Thus, there is an MHC T1D susceptibility gene, but non-MHC genes are also required for susceptibility. From the population distribution of certain MHC markers and from analysis of affected sib pairs, the MHC susceptibility gene(s) must be expressed recessively and have a frequency in the general population of approximately 0.53. Because all of the MHC markers for T1D are embedded within conserved extended haplotypes with fixed DNA over at least the *HLA-B* to *HLA-DRB1*/*-DQB1* interval (around 1 megabase in length), identification of the true T1D susceptibility locus has been difficult. The presence at low frequency of so-called protective HLA-DR/DQ haplotypes in patients strongly suggests that *HLA-DRB1*\*0301, *DQB1*\*0201 (DR3) and *HLA-DRB1*\*04, *DQB1*\*0302 (DR4) are markers for susceptibility but not themselves the true MHC susceptibility gene(s), which remain(s) to be discovered. Intrinsic penetrance is the concordance rate in monozygotic twins and is the rate at which all completely susceptible persons in the population (who have all necessary susceptibility genes) have T1D. Penetrance of susceptibility genes is not likely due to differential environmental effects since the concordance rate in dizygotic twins is the same as the rate in all sibs. From the fact that homozygotes for MHC-determined dominantly expressed IgD deficiency have about twice the frequency of the deficiency as heterozygotes, we concluded that penetrance in that situation is stochastic and a property of the MHC susceptibility gene. It seems likely that a similar mechanism is operative in T1D.



### Sa1.145. An Atypical Case of IPEX Syndrome with Multiple *FOXP3* Mutations.

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The rare X-linked syndrome known as IPEX (Immune dysregulation, Polyendocrinopathy, Enteropathy and X-linked inheritance) is characterized by early-onset IDDM (type 1 diabetes), severe enteropathy, eczema, and variable autoimmune phenomena. The IPEX gene has been mapped to chromosome Xp11.23-Xq13.3 and encodes a putative DNA-binding protein, *FOXP3*, which has a significant homology to forkhead/winged-helix transcription factor family. We have observed the IPEX phenotype in one patient, at the age of one month, with intractable diarrhea, dermatitis and elevated IgE. Autoantibodies against pancreas, thyroid and gut were negative. The mutation analysis of the *FOXP3* gene has revealed three distinct nucleotide substitutions. The mutations consist of a known splice-site mutation (exon 4 and 5 boundary), and two other aberrations (within intron 8 and the second within exon 9), previously not described. Interestingly, expression studies of *FOXP3* mRNA detected a product of about 100bp smaller compared to the normal control. The sequence analysis of this product revealed the absence of exon 2. Molecular analysis extended to other members of the family showed the same *FOXP3* mutations in one of the two healthy brothers of the patient; in addition the mother was identified as a carrier for the same mutations. At present, the patient is 17 months old and his diarrhea is under remission without treatment, as he is routinely monitored in order to control the disease progression. IPEX is a syndrome usually associated with overwhelming autoimmunity and severe phenotype. Here we present an atypical case of IPEX manifesting in unusually mild clinical features corresponding to a novel set of *FOXP3* mutations. Further studies will be important to clarify the link between the phenotype of this patient and the specific molecular aberrations in the *FOXP3* gene. Moreover, whether the gene product without *FOXP3* exon 2 is a normal splicing variant of *FOXP3* mRNA, or is the result of multiple mutations found in the patient is under investigation.

## Immunology of the Eye

### Sa1.146. Increase of Serum KL-6 Levels In Sera of Uveitis Patients with Sarcoidosis.

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**Purpose:** KL-6 is a human glycoprotein secreted by type II alveolar cells in lung, and its serum levels increase in pneumonia of various causes. KL-6 is a member of the MUC-1 family, which is expressed in cornea and conjunctiva as well as lung. The purpose of the present study is to investigate the clinical usefulness of quantifying serum KL-6 levels for diagnosing and following up sarcoidosis in patients with uveitis.

**Patients & Methods:** Sera were obtained from 24 uveitis patients diagnosed as sarcoidosis, 37 uveitis patients with other etiologies, and 138 healthy control subjects. Patients were considered to have indication for sarcoidosis when their KL-6 concentrations exceeded 370 U/ml. Within this criterion, 137 of 138 (>99%) of healthy subjects were negative for sarcoidosis.

Serum KL-6 concentration was determined by a human KL-6 electrochemiluminescence immunoassay (ECLIA).

**Results:** The average level of KL-6 in sera of uveitis patients with sarcoidosis, other etiologies, and healthy controls were  $387 \pm 52$  (Mean  $\pm$  SD) U/ml,  $266 \pm 23$ , and  $183 \pm 6$ , respectively. The level of KL-6 in uveitis patients with sarcoidosis was significantly higher than in patients with other etiologies of uveitis. The KL-6 measurement identified 45.8 % of sarcoidosis-positive patients. When the KL-6 results were combined with serum angiotensin-converting enzyme (ACE) concentrations, 87.5 % of sarcoidosis patients were identified, compared to 66.7 % using ACE results alone. The combined measurement identified 10.8 % of non-sarcoid patients and 0.72 % of healthy subjects as positive (false positive). And there were significant correlations between serum KL-6 and ACE levels in the patients with sarcoidosis. Moreover, serum KL-6 concentrations were less affected by systemic corticosteroid administration than ACE, and never affected by ACE inhibitory drugs for systemic hypertension.

**Conclusions:** Combined measurements of serum KL-6 and ACE may be useful as a screening for sarcoidosis in uveitic patients. And also, it may be valuable to follow up the diagnosed sarcoidosis, because the concentration of serum KL-6 less fluctuates than that of ACE in patients treated with systemic corticosteroids and/or anti-hypertensive drugs.

Supported by JSPS Research Fellowship for Young Scientists, Japan Society for the Promotion of Science, Tokyo.

### Sa1.147. Cytotoxic CD8<sup>bright</sup>CD56<sup>+</sup> T Cells are Immunopathogenic Effectors in Patients with Active Behcet's Uveitis.

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Recent our report demonstrated that the intraocular infiltration of CD8<sup>bright</sup>CD56<sup>+</sup> T cells was a distinct feature in active Behcet's uveitis (BD) from other etiologies of endogenous uveitis. However, the phenotypic natures and effector functions of CD8<sup>bright</sup>CD56<sup>+</sup> T cells in active BD have remained elusive. This study was conducted to determine the phenotypic and functional characteristics of CD8<sup>bright</sup>CD56<sup>+</sup> T cells and to investigate the cytotoxic mechanisms of these subsets. Forty five patients with BD (active: 24, inactive: 21) and 20 healthy controls were recruited in this study. Phenotypic analysis of fresh PBMCs were performed using anti-CD8 mAb and anti-CD56 mAb in conjunction with a three- or four-color immunofluorescence tests for the expression levels of the following molecules: CD11b, CD27, CD45RA, CD45RO, CD62L, CD94, NKG2D, HLA-DR. Flow cytometric measurements of intracellular cytokines (IFN- $\gamma$  and IL-4) and cytotoxic molecules (intracellular perforin and surface FasL) were performed by in vitro PMA and ionomycin (PI) stimulation. Ex vivo cytolytic capacities of purified CD8<sup>bright</sup>CD56<sup>+</sup> T cells against K562, Raji, and human umbilical vein endothelial cell line (HUVEC) were measured by standard <sup>51</sup>Cr release assay. Modulation of cytotoxicity was done using the treatment of HUVEC by rhIFN- $\gamma$  and the treatment of effector cells by concanamycin A (CMA) or brefeldin A (BFA). CD27 and CD62L were down-regulated on peripheral CD8<sup>bright</sup>CD56<sup>+</sup> T cells in patients with active BD in contrast to the up-regulation of CD11b and HLA-DR. Interestingly, CD94/NKG2A was up-regulated on peripheral CD8<sup>bright</sup>CD56<sup>+</sup> T cells in BD in contrast to the down-regulation of NKG2D. Furthermore, in patients with active uveitis, these subsets were polarized to produce

IFN- $\gamma$ , contained high amounts of preformed intracellular perforin, and exclusively expressed surface FasL upon stimulation by PI. Moreover, in vitro cytolytic functions of CD8<sup>bright</sup>CD56+ T cells in active BD were up-regulated against both K562 and Raji, which were effectively inhibited by CMA. Interestingly, in vitro cytolytic activity of these subsets against HUVEC was also up-regulated, which was effectively suppressed by BFA rather than by CMA. Cytolytic functions of PI-stimulated CD8<sup>bright</sup>CD56+ T cells were greatly enhanced against HUVEC, which was augmented by pretreatment of IFN- $\gamma$  on HUVEC. CD8<sup>bright</sup>CD56+ T cells, characterized by cytotoxic effector memory Tc1 phenotypes with functional NK receptors, play immunopathogenic roles in BD and exhibit strong cytolytic functions against vascular endothelial cells through FasL-dependent pathway.

**Sa1.148. Flt3L-Elicited, In Vitro-Matured Splenic Dendritic Cells (DC) Induce Autoimmune Disease in the Retina.**

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Experimental autoimmune uveitis (EAU), a model for human uveitis, is induced in mice by immunization with a retinal antigen in complete Freund's adjuvant and pertussis toxin. Here we describe a new EAU model induced with in vitro-matured, retinal antigen-pulsed DC. DC were expanded in vivo by hydrodynamic injection of 50  $\mu$ g of Flt-3 ligand DNA. On day 5, the size of the spleen was doubled and the CD11c+ population tripled from an average of 4.9% to 15.0%, for a total increase of 6-fold over background. Combined stimulation of CD11c+ DC with LPS and anti-CD40 enhanced expression of costimulatory molecules and secretion of IL-1, IL-6, IL-10, IL-12, IFN- $\gamma$  and TNF- $\alpha$ . When pulsed with a uveitogenic peptide, these cells induced vigorous immune responses in vivo. Furthermore, 2 injections of DC 4 days apart plus pertussis toxin elicited a typical EAU-like inflammation in eyes of susceptible B10RIII mice, with an incidence of up to 87%. Sorted CD8 $\alpha$ + and CD8 $\alpha$ - DC subpopulations exhibited differential cytokine production when stimulated as above, with the CD8 $\alpha$ - population releasing more IL-1 $\beta$ , IL-2, IL-6, IL-10 and TNF- $\alpha$  than the CD8 $\alpha$ + population, whereas CD8 $\alpha$ + DC produced more IL-12 and IFN- $\gamma$ . Expression of CD80 and CD86 was similar. Current work is aimed at examining differences in the ability of these splenic DC subpopulations to induce EAU. This alternative EAU model, which allows direct manipulation of DC in vitro, will permit better characterization of the role of these cells in autoimmunity to the retina and may help to devise new approaches to therapy.

**Sa1.149. Thrombospondin 1 Mediates a Contact Dependent Mechanism by Which Retinal Pigment Epithelial (RPE) Cells Regulate T Cell Function.**

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Soluble factors released by cultured RPE cells were previously shown to suppress T cell proliferation, and to confer T cells with regulatory properties. This study was performed to investigate the

possibility that molecules on the surface of RPE cells can exert similar effects on T cell function. T cells activated via soluble  $\alpha$ -CD3 were cultured in the presence or absence of fixed RPE cells for 72h in serum free medium, and their proliferation, as well as their ability to suppress the proliferation of bystander T cells, was assessed via 3H thymidine incorporation. For the latter, T cells were irradiated at the end of the 72h co-culture period with RPE cells, and were then washed and re-plated with freshly isolated T cells activated with  $\alpha$ -CD3 antibodies. T cell viability was quantified using the alamar blue bioassay. In order to investigate whether surface TSP-1 played a role in mediating the contact-dependent effect of RPE cells on T cell function, the above experiments were repeated using fixed RPE cells recovered from the eyes of TSP-1 knockout mice. These studies were complemented with immunohistochemistry and western blotting in order to confirm the expression of TSP-1 by cultured RPE cells. Finally, to begin to address the potential TSP-1 receptors on T cells involved in mediating this effect, we examined the importance of the integrin  $\alpha$ 4 $\beta$ 1 (VLA-4), used by naive and activated T cells for attachment to the TSP-1 molecule. This was done by preventing the binding of TSP-1, on RPE cells, to VLA-4 on T cells, with blocking antibodies, and by blocking the function of its associated potassium channel, Kv1.3, with margatoxin (MgTx). Our results show that fixed RPE cells suppress the proliferation of activated T cells, and in turn confer them with a similar ability to suppress bystander proliferation. Further, cultured RPE cells expressed TSP-1 homogeneously throughout their cell surface, and fixed RPE cells lacking the TSP-1 gene, failed to suppress T cell proliferation. Similarly, inhibiting TSP-1 binding to its receptor VLA-4 on T cells, or blocking the function VLA-4's associated potassium channel, Kv1.3, prevented T cells from becoming regulatory. We conclude that aside from soluble factors, RPE cells also exhibit a contact-dependent mechanism, mediated by TSP-1/VLA-4 interactions, by which they suppress the proliferation of activated T cells, and confer them with regulatory properties.

**Sa1.150. Altered Peptide Ligands of a Retinal Antigen Protect from Anti-Retinal Autoimmunity by Eliciting Active Regulatory Mechanisms.**

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We identified altered peptide ligands (APL), capable of immunomodulating experimental autoimmune uveitis (EAU), a Th1-driven disease induced in B10.RIII mice by immunization with the retinal antigen IRBP in complete Freund's adjuvant. Alanine-substituted peptides of the major pathogenic epitope, residues 161-180, were synthesized. They were tested for immunogenicity, crossreactivity with the native 161-180 epitope, pathogenicity, and ability to prevent EAU when given in incomplete Freund's adjuvant (IFA) 2 weeks before EAU challenge with native p161-180. Two peptides, 169A and 171A, were unable to elicit disease and crossreacted with p161-180 by lymphocyte proliferation. Mice pre-treated with either of the putative APL failed to develop EAU and had reduced cellular responses to p161-180 by lymphocyte proliferation and by delayed hypersensitivity. Their cytokine response profile to p161-180 showed reduced IFN- $\gamma$  and enhanced IL-4, and serum antibody titers to p161-180 revealed reduced IgG2a and elevated IgG1 isotypes, suggesting a Th2 shift in the response. Protection was transferable with lymphoid cells from protected donors to naive

recipients who were subsequently immunized for EAU. Thus, APL pretreatment appears to prevent induction of EAU by skewing the subsequent response towards a non-pathogenic effector phenotype, as well as by eliciting regulatory cells.

### Sa1.151. Modulation of Innate Immune Response with Granulocyteapheresis in Behçet's Disease: 1 Year Experience.

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**Introduction:** Granulocyteapheresis (GCAP) is a novel treatment that has the capability of modulating innate immune response in some autoimmune diseases such as Ulcerative Colitis, Crohn's Disease, Rheumatoid Arthritis, and Behçet's Disease. We present our results with this treatment in a period of 1 year in 3 cases of ocular Behçet's Disease.

**Objective:** to evaluate GCAP efficacy to control uveitis activity, number of relapses and steroid sparing effect in a 1 year period.

**Material and Methods:** 3 patients with ocular Behçet disease resistant to conventional medical treatment were selected for GCAP treatment. One patient presented active uveitis and the others were clinically inactive. Patients demographics were: age  $25,7 \pm 3,1$ ; 2 female/1 male; time from diagnose  $66,7 \pm 45,7$  months; average of uveitis relapses/year  $5,38 \pm 3,6$ ; all patients were steroid dependant and needed cyclosporine A (2/3), azathioprine (2/3) and/or micophenolate mofetil (1/3) to control the disease. Informed consent was obtained from all patients. GCAP treatment was approved by Spanish Ministry of Health for Compassionate Use. We performed an induction treatment of 1 GCAP session/week during 10 consecutive weeks. Patients were maintained with 1 GCAP session each month during 1 year. The GCAP procedure consists in an extracorporeal blood circulation through a column filled with cellulose diacetate beads. Each procedure lasts 1 hour and 1.8 liters of blood are processed at 30 ml/h. Visual acuity, intraocular inflammation degree, and prednisone requirements were assessed during the study period. No adverse event were reported.

**Results:** During induction treatment uveitis was controlled in all patients and none of them presented any relapse. Visual acuity was stabilized in 3 eyes and improved in the other 3. Prednisone dose was tapered down from  $35,8 \pm 13,7$  to  $15 \pm 5$  mg/day. After completion of 1 year maintenance treatment, mean number of uveitis relapses were  $1 \pm 1$ , and daily prednisone dose was reduced to  $21,6 \pm 7,6$ . Visual acuity was stable during maintenance treatment.

**Conclusion:** GCAP treatment is a safe and effective treatment for ocular Behçet's Disease refractory to conventional medical treatment. A one year maintenance regimen can reduce uveitis relapses, preserve visual acuity and reduce prednisone requirements.

### Sa1.152. Systemic Autoimmune Disease in Patients with Uveitis.

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Rodriguez-Molina,<sup>1</sup> E. Fernandez-Cruz.<sup>1</sup> <sup>1</sup>Clinical Immunology Unit, Immunology Department, University Hospital Gregorio Marañon, Madrid, Spain; <sup>2</sup>Ophthalmology Department, University Hospital Gregorio Marañon, Madrid, Spain; <sup>3</sup>Transfusion Center, Community of Madrid.

**Background.** The eye (and especially the highly vascular tissues such as the uvea and the conjunctiva) is considered a special "target" for immunopathologic reactions. Uveitis refers to inflammation of the uveal tract, which includes the iris, ciliary body, and choroid. Uveitis is a potentially blinding condition predominantly occurring in the working age group. Although the aetiology is unknown in most cases, many patients have an associated underlying systemic disease. Uveitis can be the initial manifestation of an autoimmune systemic disease, and may appear years before the diagnosis of the primary disease. A retrospective study was conducted of patients with uveitis to determine the frequency of associated autoimmune systemic diseases and to assess the value of limited laboratory screening of these patients. **Materials.** 64 patients (33 male, 31 female) with uveitis (38 anterior, 14 posterior, 4 intermedia, 8 panuveitis) were studied. All patients underwent a standard diagnostic protocol including the following immunological tests: serum immunoglobulins, complement components, circulating immune complexes (CIC), antinuclear antibodies (ANA), antineutrophil cytoplasmic antibodies (ANCA), anticardiolipin antibodies (ACA) and major histocompatibility complex antigens. **Results:** Overall 87.5% of patients had at least one detectable immunological abnormality. 14/64 patients had detectable levels of ANA (titer 1/40-1/320) (21.9%), 8/64 IgM ACA (12.5%), 7/64 IgG ACA (10.9%), 5/64 raised ANCA (7.8%) and 4/64 positive rheumatoid factor (6.3%). A relationship with a subclinical autoimmune systemic disorder could be presumed in 11/64 cases (17.2%) defined as the presence of autoantibodies (ANA, ANCA or ACA) in the presence of complement consumption, hypergammaglobulinemia or increased CIC. HLA-B27-associated anterior uveitis was observed in 8/64 patients (12.5%), HLA-DR52-associated posterior uveitis in 2/64 (3.1%), HLA-DR53-associated Vogt-Koyanagi-Harada syndrome in 1/64 (1.6%) and HLA-A29-associated birdshot retinochoroidopathy in 1/64 (1.6%). A definite association with a systemic autoimmune disease was determined for 8/64 patients (12.5%) most of them with lupus like disease (LLD) (4), LLD plus antiphospholipid syndrome (1), Sjögren syndrome (2) and systemic vasculitis (1). The presence of the systemic autoimmune disease was not suspected prior to eye involvement and was only recognised after the subsequent diagnostic procedures. **Conclusion.** In a proportion of patients with uveitis an autoimmune systemic disorder may be present. The systemic autoimmune diseases were frequently undiagnosed before the onset of the ocular disease and before the uveitis consultation. Studies of the immunological profile can therefore help in further assessment of patients with uveitis.

### Sa1.153. In Silico Prediction of Binding of Auto-Antigenic Peptides to HLA-DRB1 in Vogt-Koyanagi-Harada Disease.

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**Purpose:** To evaluate the ability of computer-based algorithms (in silico) to identify putative auto-antigenic peptides and to evaluate predicted binding of putative auto-antigenic peptides in different populations with the same disease. **Methods:** Three

internet-accessible computer-based algorithms were used to predict binding of tyrosinase (TYR) and tyrosinase-related protein-1 (TRP-1)-derived peptides to HLA-DRB1\*0405. These results were compared to published studies of in vitro immunogenic responses to TYR and TRP-1-derived peptides by HLA-DRB1\*0405-restricted T cells from patients with Vogt-Koyanagi-Harada (VKH) disease. Three websites (SYFPEITHI, MHC-Thread, and Propred) were used to determine relative likelihood of binding scores for the immunogenic TYR and TRP-1 peptides determined in vitro to HLA molecules that confer risk for VKH disease in different populations, HLA-DRB1\*0101, DRB1\*0404, and DRB1\*0405. Results: We found that using all three websites together, at least one in silico fragment overlapped with each of the in vitro peptides by at least nine amino acids with the exception of TRP-1 p243-254, which overlapped with a fragment predicted by Propred by seven amino acids. The peptide that was immunogenic in vitro in the most patients (TYR p426-437) was identified by all three websites. TYR p134-146, TYR p423-434 and TYR p426-437 were found by Propred to have a high likelihood of binding to HLA-DRB1\*0101, DRB1\*0404, and DRB1\*0405, while TYR p193-203 and TYR p429-440 were predicted by Propred to have a high likelihood of binding only to HLA-DRB1\*0405. Conclusions: In silico-predicted binding of putative immunogenic peptides to specific HLA-DR alleles holds promise for high throughput evaluation of putative auto-antigens and suggests a correlation between HLA binding and immunogenicity. Predicted binding of immunogenic peptides to HLA-DRB1\*0101 and DRB1\*0404 is consistent with clinical studies that have found that these alleles confer risk for VKH disease in Mestizo individuals. Conversely, differences between likelihood of binding of immunogenic peptides to relevant HLA molecules suggest that there may be differences as well between peptides that induce disease in Mestizo and Asian populations.

#### **Sa1.154. Increased Ratio of Activating:Inhibitory Killer Immunoglobulin-Like Receptor Genes in Uveitis.**

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Purpose: To evaluate Killer Immunoglobulin-like receptor (KIR) genes in patients with uveitis. Methods: Individuals diagnosed with birdshot chorioretinopathy (BCR) ( $n = 19$ ) and Vogt-Koyanagi-Harada (VKH) disease ( $n = 25$ ) were evaluated for both activating and inhibitory KIR genes as well as Class I human leukocyte antigen (HLA) specificities using polymerase chain reaction protocols. The presence or absence of the appropriate HLA ligands for inhibitory genes was established. The individual genotypes, the patterns of gene inheritance, and the ratio of activating:inhibitory genes with their HLA ligands were compared to local and published Caucasian (BCR) or Mestizo (VKH) controls. Results: The ratio of activating:inhibitory KIR genes with their HLA ligands was elevated compared to controls both forms of uveitis. In addition, patterns of activating genotype not found in any controls were found. Conclusion: The ratio of activating:inhibitory KIR genes is increased in individuals with BCR and VKH disease. The fact that a similar pattern was found in two different forms of uveitis with distinct clinical syndromes and known HLA associations implies that increased activating:inhibitory KIR genes may be a marker of risk for uveitis.

## **Poster Session 2** 3:30 PM–5:30 PM, 5/14/2005 **Immunity and Infection**

### **Sa2.01. Quality Assurance Survey (QAS) in the Detection of Infectious Diseases with Outbreak Potential.**

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Objectives: 1. That a system in monitoring 14 infectious diseases with outbreak potential is essential for its containment, thus control if not eradication.

2. That the National Reference Laboratory (NRL)-Philippines shall establish partnership with National Epidemic Sentinel Surveillance System (NESSS)- National Epidemiology Center-Department of Health, Philippines.

Materials and Methods: A network of hospital sentinel are to the Regional Epidemiology and Surveillance Units. Sentinel sites are hospitals which have at least 250 bed capacity. The hospital have a laboratory capacity to do malarial smears, blood and stool cultures and hepatitis serology, participating authorities, surveillance personnel and availability of communication means between sentinel sites and regional epidemiology and surveillance units.

Hospital admission are the basis in monitoring occurrences of diseases, thus, a rapid, timely, accurate information and early warning on the disease outbreak.

Quality Assurance procedures are ensured in each sample collection and during transport to NRLs. laboratory results are the sole basis to confirm clinical diagnosis.

laboratory management includes Quality Control sampling to monitor accuracy and precision of existing procedures.

Results: Sixteen (16) RESU were involved nationwide and all the Five (5) designated NRLs-Philippines conducted the screening and specific/confirmatory tests.

All data from these surveillance and quality evaluation were extensively used utilized by the Department of Health for policy formulation and program evaluation, thus were able to contain the infectious disease identified.

CONCLUSION: A quality assurance survey which is essential in the containment of infectious disease with outbreak potential has been established by the National Reference Laboratory-Philippines. This system will enable monitoring of precise and accurate screening and confirmatory tests of the disease and therefore a ready detection and containment if not eradication.

### **Sa2.02. Lack of Association between Interferon-Gamma Receptor-1 Polymorphism and Pulmonary Tuberculosis in Iranian Population Sample.**

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**Background:** Tuberculosis is one of the most common infectious diseases in the world. In recent years, genetically approach has been developed. One of the interesting gene for investigator is IFN- $\gamma$ R1.

**Aim:** Determination of susceptibility to tuberculosis with polymorphism of IFN- $\gamma$ R1 gene.

**Material and Method:** Study was prospective case-control. Fifty patients with smear & Culture positive tuberculosis have been chosen randomly. They were matched with 54 healthy controls with no history of TB. Polymorphism at 395 codon of IFN- $\gamma$ R1 gene was detected with Newport method. Data were analyzed with SPSS version 11.

**Results:** Mean age of patients and control were  $55 \pm 20$  and  $53 \pm 13.5$  years respectively. Demographic characteristic had no difference within two groups. ( $P$ -value  $> .05$ ) one patient in case group had heterozygote mutation at IFN- $\gamma$ R1 gene. In control group there were no mutations.

**Conclusion:** Genetically susceptibility to TB is not in 395 codon of IFN- $\gamma$ R1 in Iranian TB sample and polymorphism of this loci has occurred in 2% of TB patients and 0.96% of total study population.

### Sa2.03. The Functional Activity of Specific Antibodies in Progression of HIV-Infection.

Irina I. Andreeva.<sup>1</sup> <sup>1</sup>Clinical of Immunology, Rostov State Medical University, Rostov-on Don, Russian Federation.

**Background.** Polyclonal activation of B-lymphocytes is one of the basic mechanisms of the HIV pathogenesis, however, the resulting anti-HIV antibodies show no protective effects. In this connection, it is expedient to study the functional properties of antibodies, in particular, their affinity.

**Methods.** We observed 88 patients aged 25–45 and infected with HIV-1, of which 42 patients were at the stage of generalized lymphadenopathy (LAP), 34 persons at the stage of pre-AIDS, 12- at the stage of AIDS. ELISA was used to determine the anti-HIV antibodies titer, the degree of their affinity (AK).

**Results.** It was found that at the LAP stage the content of immunoglobulins at this stage of disease was IgA- $1.59 \pm 0.42$ g/l; IgM-1,  $0.2 \pm 0.20$ g/l; IgG-10,  $90 \pm 1.26$ g/l, but the anti-HIV antibody titer was Ig  $4.30 \pm 0.14$ , and the degree of their affinity (AK) was  $30 \pm 6$  units. As the HIV infection progressed at the pre-AIDS stage the content of immunoglobulins was IgA- $1.85 \pm 0.54$ g/l; IgM- $1.11 \pm 0.12$ g/l; IgG- $13.28 \pm 1.28$ g/l, as well as increase of the degree of the anti-HIV specific antibodies Ig.  $4.94 \pm 0.11$  accompanied by reduction of the degree of their affinity AK was  $50 \pm 9$  units. As the HIV infection progressed at the AIDS stage the content of immunoglobulins was IgA- $1.05 \pm 0.34$ g/l; IgM- $1.51 \pm 0.14$ g/l; IgG- $10.15 \pm 1.16$ g/l, the anti-HIV specific antibodies was Ig.  $4.00 \pm 0.05$ , accompanied by reduction of the degree of their affinity- AK was  $60 \pm 8$  units.

**Conclusions.** Thus, the development of the infection process is characterized by aggravation of the insufficiency of the functional activity of the anti-HIV specific antibodies, confirmed by the marked reduction of their affinity.

### Sa2.04. Impairment of Recent Thymic Emigrants in HCV Infection.

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**Object:** Virus C hepatitis (HCV) often has a more favorable course in younger patients. Considering the involution of the

thymic function with age, we investigated the output of recent thymic emigrants in HCV patients.

**Materials and methods:** To evaluate recent thymic emigrants, we used a competitive quantitative PCR in order to determine the percentages of cells with Cj-T cell receptor excision circles (TREC). This study was performed in 13 HCV patients at diagnosis and before any anti-HCV treatment. The results obtained in this group were compared to those obtained in a group of 17 age-matched controls.

**Results:** We found that in the 13 HCV patients naive for anti-HCV treatment percentage of TREC was 3%. We could not detect a correlation between the percentages of TREC and the patients' viremia. In contrast, in the 17 age-matched controls the percentage of TREC detected by us was 6% ( $P = 0.02$ ).

**Conclusions:** Our study describes a novel immune defect in HCV patients. Additional studies are needed to get further insight in the possible role of TREC defect in the pathogenesis and prognosis of the disease.

### Sa2.05. The Persistence of Allergen-Specific IgE and Long-Lived Plasma Cells.

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**Background:** IgE antibodies play a major role in the pathogenesis of type I allergies. As the half life of serum IgE is short, plasma cells continuously have to secrete large amounts of IgE to maintain the serum titers over long periods of time. It is currently debated, whether IgE-secreting plasma cells are short-lived end products of a chronic activation of B cells, or long-lived, if maintained in supportive niches of the bone marrow or in inflamed tissue. **Method:** We have analyzed proliferation and lifetime of IgE-secreting plasma cells in an ovalbumin (OVA)-specific, murine allergy model. The time point of origin and the plasma cell turnover in the spleens, lungs, lymph nodes and the bone marrow of OVA allergic mice were determined according to incorporation of BrdU into DNA of proliferating cells. Organs and sera were analysed using ELISPOT, ELISA, fluorescence microscopy and flow cytometry.

**Results:** 4–6 weeks old mice were sensitized with OVA and then continuously fed BrdU for 2 weeks, supplied via their drinking water. 25% of IgE-secreting plasma cells in spleens of the OVA allergic mice were BrdU-positive, indicating that they had proliferated within the time of BrdU-feeding. 75% of the IgE-secreting splenic plasma cells had been generated before that time period and thus had a lifetime of more than 2 weeks. Anti-proliferative, immunosuppressive therapy (cyclophosphamid) did not eliminate the cells producing OVA-specific IgE antibodies, indicating that the respective plasma cells are not dividing and long-lived. **Conclusion:** IgE-secreting plasma cells can be long-lived. These long-lived, IgE-secreting plasma cells provide allergen-specific IgE independent of the presence of allergen and are resistant to immunosuppression.

### Sa2.06. Transmission Hepatic Electron Microscopic Findings in Chronic Experimental Schistosomiasis Mansoni after Praziquantel and an Antifibrotic.

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Univeristy, Cairo, Egypt; <sup>2</sup>Parasitology Department, Ein Shams University, Cairo, Egypt; <sup>3</sup>Parasitology Department, Theodore Bilharz Research Institute, Guiza, Cairo, Egypt.

This study aims to evaluate the effect of a drug combination: praziquantel (CAS 55268-74-1 EMBAY 8440, Biltricide), and an antifibrotic agent (B\_Amino Propionitrile Mono fumarate salt (2079-89-2), upon the transmission hepatic Electron microscopic findings in chronic experimental murine schistosomiasis mansoni. In this study, a group of 40 Swiss albino mice was used. This group was further subdivided into four small subgroups. Subgroup I: constituted infected untreated challenged contol mice. Sacrifice was done 13 weeks later, a time needed for the infection to develop into a chronic one. Subgroup II: infected mice treated with praziquantel 500mg/Kg body weight, orally for two successive days, 13 weeks post primary infection. Sacrifice was done 5 weeks later. Subgroup III: infected mice given B-Amino Propionitrile daily as 5mg powder in 0.5ml saline for 14 successive days. Sacrifice was done 18 weeks post primary infection. Subgroup IV: mice given both PZQ + BAPN. Sacrifice was done 18 weeks post primary infection.

Mice given Beta Aminopropionitrile (BAPN) alone, compared to those given praziquantel solely, or in combination, revealed amelioration in the mitochondrial changes, reappearance of the cristae and absence of already deposited collagen. The nucleus regained its regular nuclear membrane and condensed chromatin. These changes were less evident in the other previously mentioned groups.

**Key words:** Chronic Schistosomiasis mansoni, Primary infection, challenge or secondary infection, Beta amino propionitrile, Praziquantel., Transmission Electron Microscopy.

#### **Sa2.07. Transmission Hepatic Electron Microscopic Findings in Acute Experimental Schistosomiasis Mansoni after Praziquantel and an Antifibrotic.**

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This study aims to explore the repercussions of a drug combination: praziquantel (CAS 55268-74-1 EMBAY 8440, Biltricide), and an antifibrotic agent (B\_Amino Propionitrile Mono fumarate salt (2079-89-2), upon the transmission hepatic Electron microscopic findings in acute experimental murine schistosomiasis mansoni.

In this study, a group of 100 Swiss albino mice was used. This group was further subdivided into four small subgroups. Subgroup I: infected untreated challenged contol mice Subgroup II: infected mice treated with praziquantel 500mg/Kg b. weight, orally for two successive days. Subgroup III: infected mice given B-Amino Propionitrile daily as 5mg powder in 0.5ml saline for 14 successive days. Subgroup IV: mice given both PZQ + BAPN. Sacrifice was done twelve weeks post primary infection.

Mice given the combination regimen Praziquantel + Beta Aminopropionitrile (PZQ + BAPN), compared to those given each drug solely, revealed amelioration in some of the previously swollen mitochondria with roughening of the endoplasmic reticulum. Agaim, there was resorption of the previously deposited collagen fibres in the intercellular matrix. These findings are the main stigmata of hepatic cellular regeneration. These data were less salient in mice given Praziquantel or Beta aminopropionitrile alone.

**Key words:** Acute Schistosomiasis mansoni, Primary infection, challenge or secondary infection, Beta amino propionitrile, Praziquantel., Transmission Electron Microscopy

#### **Sa2.08. Serum Transaminase Levels in Murine Schistosomiasis Mansoni after Giving Praziquantel and an Antifibrotic Agent.**

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This study is a trial to evaluate the effect of a combination between an anthelmintic drug praziquantel (CAS 55268-74-1 EMBAY 8440, Biltricide), and an antifibrotic agent (B\_Amino Propionitrile Mono fumarate salt (2079-89-2). It is also a trial to elucidate the repercussions of this drug combination upon worm load and percent resistance to reinfection. Moreover, it aims to study liver enzymes level (Alanine aminotransferase and Aspartate amino transferase ALT & AST) in experimental murine schistosomiasis mansoni.

In this study, a group of 100 Swiss albino mice was used. This group was further subdivided into six small subgroups. Subgroup I: constituted infected untreated control mice. Subgroup II: infected untreated challenged contol mice. Subgroup III: challenged control mice. Subgroup IV: infected mice treated with praziquantel 500mg/Kg b. weight, orally for two successive days. Subgroup V: infected mice given B-Amino Propionitrile daily as 5mg powder in 0.5ml saline for 14 successive days. Subgroup VI: mice given both PZQ + BAPN. Sacrifice was done 112–124 days post primary infection.

Mice given the combination regimen Praziquantel + Beta Aminopropionitrile (PZQ + BAPN), compared to those given each drug solely, revealed absence of worm recovery at perfusion, the highest score of percent resistance to reinfection, and a 19.6+0.9 & 18.3+0.9 IU/L serum alanine aminotransferase & aspartate aminotransferase levels respectively. These data were less salient in mice given Praziquantel or Beta aminopropionitrile alone.

**Key words:** Chronic Schistosomiasis mansoni, primary infection, challenge or secondary infection, Beta amino propionitrile, Praziquantel. Serum transaminase levels (ALT &AST).

#### **Sa2.09. Effect of a Novel Muramyl Dipeptide Derivative Together with Praziquantel in Experimental Schistosoma Mansoni Infection.**

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The goal of this study, is to evaluate the effect of a combination between an anthelmintic drug praziquantel (CAS 55268-74-1 EMBAY 8440, Biltricide), and a muramyl dipeptide derivative Adamantylamide Dipeptide (AdDP) {CAS 768-94-5 (Amantadine)} in potentially tolerized Schistosoma mansoni infected, egg-injected C57BL/6 mice. It is also a trial to elucidate the repercussions of this drug combination upon worm and tissue egg loads and oogram pattern. A group of 120 C57BL/6 mice was used in the experiment. This group was further subdivided into five small subgroups. Subgroup I constituted infected control

mice. Subgroup II: received four intravenous doses (10ug each via the tail vein) of soluble egg antigen (SEA) on days-7, -5, -3 and-2 before infection. Subgroup III: included infected mice given AdDP 12 mg subcutaneously in 0.2 ml saline. Subgroup IV: infected mice given Antigen (SEA) + AdDP. Subgroup V: included mice given Antigen (SEA) + AdDP + PZQ(500 mg/Kg for two successive days). Sacrifice was done 10 weeks post infection.

Egg-injected mice given the combination regimen Praziquantel + Adamantylamide Dipeptide (PZQ + AdDP), compared to infected untreated control, revealed absence of worm recovery at perfusion and 100 % dead ova in the oogram. Again an evident reduction in the hepatic and intestinal tissue egg loads was recorded in this group compared to infected untreated (wether egg injected or not) control mice.

Key words: Schistosomiasis mansoni infection, Soluble Egg Antigen, Adamantylamide Dipeptide, Praziquantel.

### Sa2.10. Discovery of Neutralizing CpG ODN from Serotype 2 and 5 Adenoviruse Based on the Relationship between Free Energy and Bioactivity.

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Large amount of CpG-S ODN as well as bacterial DNA could trigger over inflammatory response, even sepsis. Therefore, it is important to balance the activity of CpG-S ODN and reduce the release of cytokines induced by CpG-S ODN. Despite comparable levels of unmethylated CpG dinucleotides, DNA from serotype 12 adenovirus (Adv12 DNA) is immunostimulatory, but DNA from serotype 2 and 5 (Adv2 DNA, Adv5 DNA) are nonstimulatory and can even inhibit activation by bacterial DNA. Based on the above difference, Krieg found some neutralizing CpG ODN (CpG-N ODNs). However, Zhao found above CpG-N ODN had no inhibitory effects even when the concentration ration reached to 10:1. Therefore, it seems the CpG-N ODN's sequences should be investigated further. In the present experiments, we searched for CpG-N ODN in Adv2 and Adv5 DNA, DNA after comparing the sequence differences between Adv2 DNA, Adv5 DNA and Adv12 DNA, Escherichia coli DNA (EC DNA). Nineteen specific CpG motifs and 12-nucleotide sequences in Adv2, 5 DNA were ascertained after numbers and frequencies of 256 kinds of CpG motifs in Adv2, Adv5, Adv12 or EC DNA were calculated. However, none had been found to have the properties of CpG-N ODN after their assays on TNF- $\alpha$  release induced by CpG-S ODN. Accidentally, we found there existed a relationship between free energy and bioactivity. Therefore, putative CpG-N ODN were re-discovered and investigated. We got six CpG-N ODNs with activity to inhibit TNF- $\alpha$  release induced by CpG-S ODN. Among them, CpG-N ODN208 was the strongest one. In our *in vitro* experiments, CpG ODN208, without cellular toxicity, inhibited TNF- $\alpha$  release from hPBMC or RAW264.7 induced by CpG-S ODN in a dose- and time-dependent manner. In our *in vivo* experiments, CpG-N ODN208 could markedly protect mice from lethal challenge by CpG-S ODN and significantly decreased TNF- $\alpha$  release in mice. Above results suggested that there existed a relationship between free energy and bioactivity of CpG-N ODN, and strong inhibitory CpG-N ODN could be screened based on this kind of relationship.

### Sa2.11. Evaluation of IL-10 Serum Level in Visceral Leishmaniasis.

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**Background:** Visceral leishmaniasis is a fatal disease, which is caused by *Leishmania* spp. After inoculation of promastigotes by sand fly, some individuals can develop type-1 immune response and control the infection, while others may be involved with acute form of the disease. It seems that IL-10 can inhibit type-1 immune response and cause more severe form of the disease. The aim of the this study was to evaluate the IL-10 serum levels in the different courses of the disease.

**Methods:** Thirty two patients with visceral leishmaniasis were included in this study. Blood samples were collected from the patients in three phases: at the time of diagnosis, after medical therapy (when the patients became afebrile), and at the time of discharge. Sera were separated and stored at -70°C until cytokine assay. IL-10 level was determined using ELISA method.

**Results:** The mean IL-10 serum levels were significantly different in three phases of sampling ( $P = 0.002$ ). The mean  $\pm$  SE of IL-10 serum levels in three phases were  $77.4 \pm 15.6$  pg/ml,  $55.8 \pm 18.6$  pg/ml and  $17.7 \pm 4.9$  pg/ml, respectively.

**Conclusion:** According to our results higher levels of IL-10 at the time of diagnosis may act as an inhibitory factor for cellular immunity and have a role in the development of visceral leishmaniasis. It seems that after medical therapy, IL-10 serum level decreases significantly, which may be associated with recovery.

### Sa2.12. IL-10 Gene Polymorphisms and Susceptibility to Brucellosis.

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**Background:** *Brucella* spp. Is a gram-negative facultative intracellular bacterium and causative agent of brucellosis. It is clarified that type-1 immunity is important to control *Brucella* infection. In this regard, macrophages have critical role. IL-10 is a Th2-type cytokine that inhibit macrophage activation. It is known that production of IL-10 is affected by its gene promoter polymorphisms. In this study we investigated the relationship between IL-10 gene promoter polymorphisms and susceptibility to brucellosis.

**Methods:** One hundred and ninety patients with brucellosis, 186 healthy individuals who were members of patients' family and 82

healthy animal husbandmen who had infected animals with *Brucella* were included in this study. All individuals were genotyped for three bi-allelic IL-10 gene promoter polymorphisms at positions -1082(G/A), -819(T/C), and -592(A/C) using PCR-RFLP.

**Results:** Genotype and allele frequencies of -592(A/C) and -819(T/C) were significantly different between patients and animal husbandmen groups ( $P < 0.05$ ).

**Conclusion:** There are some reports showed that A allele at position -592 of IL-10 gene is associated with lower IL-10 production in-vitro or in-vivo. According to the results, higher frequency of A allele at position -592 in animal husbandmen may cause these individuals more resistant to disease.

### Sa2.13. Investigation of IFN- $\gamma$ Gene Polymorphism in Visceral Leishmaniasis.

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**Background:** Although correlation between kala-azar disease and several factors have been determined, some unknown factors also exist that need to be recognized. Protective immunologic response against leishmaniasis are characterized by a strong cell-mediated immune response. IFN- $\gamma$  is an important component of type-1 immunity. Since cytokine gene polymorphisms may associated with different ability for cytokine production, the aim of this study was to investigate the relationship between IFN- $\gamma$  gene polymorphism and kala-azar.

**Methods:** We genotyped 122 patients with kala-azar, 63 patients' siblings who were healthy, and 103 healthy individuals who were resident in endemic area and had positive Leishmanin skin test. Genomic DNA was extracted from blood samples and IFN- $\gamma$  gene polymorphism at position +874 (T/A) was determined by allele specific polymerase chain reaction (ASPCR) method.

**Results:** The frequency of TT genotype in patients was significantly less than their siblings (22.1% and 30% respectively) [ $P = 0.021$ ], while no significant difference was detected between patients and healthy individuals resident in endemic area ( $P = 0.35$ ).

**Discussion:** Cell-mediated immune response is an effective immunity against *Leishmania* and IFN- $\gamma$  play a fundamental role in cellular immunity induction. The results showed that TT genotype was increased in patients compared to their siblings. Some researchers reported the association of IFN- $\gamma$  +874 TT genotype with higher IFN- $\gamma$  production. Therefore, it seems that higher production of IFN- $\gamma$  in patients' siblings may help them to be more resistant to infection.

### Sa2.14. Polymorphisms of IL-10 Gene Promoter in Patients with Kala-azar.

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**Background:** Visceral leishmaniasis is caused mostly by *Leishmania infantum* in south of Iran. Manifestations range from asymptomatic infection to fatal disseminated visceral disease. Protective immune response against *Leishmania* is cell-mediated immunity and it is known that IL-10 can down-regulate this kind of response. Researchers showed that polymorphisms in IL-10 gene promoter can regulate IL-10 production. The aim of this study was to determine the relationship between IL-10 gene polymorphisms and outcome of the disease.

**Methods:** One hundred and twenty pediatric patients involved with kala-azar, 57 healthy individuals who were patients' siblings and 102 healthy individual who lived in endemic area without any history of kala-azar or cutaneous leishmaniasis and with positive Leishmanin skin test were included in this study. Polymorphisms of IL-10 gene promoter (-1082G/A, -819T/C, -592A/C) were determined using PCR-RFLP.

**Results:** There were no significant differences in genotype and allele frequencies of investigated IL-10 gene polymorphisms between the groups.

**Conclusion:** It is documented that protective immunity against leishmaniasis is cell-mediated immunity. Therefore the presence of Th2-type cytokines during the disease can worsen the condition of the patients. Since the results showed no significant differences in genotype and allele distributions between the groups, study of the cytokine profiles and other cytokine gene polymorphisms are recommended.

### Sa2.15. Role of Regulatory CD25+CD4+ Cells during Infection with *Trypanosoma cruzi*.

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During infection by *Trypanosoma cruzi*, the etiological agent of Chagas disease, both cellular and humoral immune responses are essential in controlling the parasitemia. While this immune response is necessary for protection it is also responsible for the morbidity caused by the infection. CD25+CD4+ regulatory cells (Tregs) represent a unique lineage of T cells that have an important role in controlling immune responses against self and foreign antigens. We wanted to investigate if Tregs played any role in controlling immune responses during *T.cruzi* infection. C57BL/6 mice were depleted of CD25+ cells (injected with monoclonal antibody PC61) or were injected with an isotype matched control (GL113) and infected with parasites of the strain Colombiana (strain that elicits an intense myocarditis). Both groups were evaluated for the amount of circulating parasites, mortality rate, immunological parameters and histological analysis of the heart throughout the course of the infection. Our results show that Tregs have a role during infection by *T.cruzi*. Animals depleted of



CD25+ cells had decreased levels of parasites in the bloodstream and decreased mortality rate. This is associated with an augmentation of activated T cells and better production of pro-inflammatory cytokines. CD25+ depleted animals also display a more severe inflammation of the cardiac tissue when compared to control animals.

### Sa2.16. Dendritic Cell Mediated Immune Response Is Impaired by the *Mycobacterium tuberculosis* Mannosylated-LipoArabinoMannan.

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Both dendritic cells (DC) and natural killer (NK) cells have a role in innate immunity against tuberculosis. Interactions between *M. tuberculosis* and DCs have demonstrated either a constrained survival or slight growth of the intracellular bacteria. Data of DC maturation during *M. tuberculosis* infection are discordant. Similar discrepancies were observed when studying NK interactions with *M. tuberculosis* infected DCs, with either a killing of *M. tuberculosis* H37Ra (Brill et al. Infect. Immun. 2001) or an absence of cytolysis of the infected DCs mainly due to a fully mature phenotype (Ferlazzo et al. Eur. J. Immunol. 2003). One of the main component playing a key role in DC-*M. tuberculosis* interaction is the mannosylated-lipoarabinomannan (ManLAM), present at the surface of the bacteria (Tailleux et al. J. Immunol. 2003). ManLAM is one of the candidate ligand to DC-SIGN, a DC specific lectin binding receptor, allowing *M. tuberculosis* entry inside DCs. ManLAM may have a counteractive effect on DCs towards a fully protective inflammatory response. Here, we investigated the impact of ManLAM or PiLAM (LAM from *M. smegmatis* capped with phosphoinositide residues) on DC maturation and function using combined approaches of phenotyping, cytokines release, NK cell activity and T-cell priming. In contact with ManLAM, DCs displayed a pattern of partial maturation including the intermediate expression of MHC class I and class II molecules and a low expression of the costimulatory molecules CD80 and CD86 compared to fully LPS-matured DCs. They cannot be considered as immature DCs because they lost their FITC-dextran phagocytic activity. At the opposite, they do not present a fully matured phenotype. Indeed, ManLAM-incubated DCs are still sensitive to autologous NK lysis and are not able to prime naive T-cell responses. Absence of NK lysis was noticed when ManLAM-DCs were incubated with CD40 antigen, confirming a partial maturation of DCs with ManLAM only and the need of a second signal to complete maturation. Altogether, the overall effect could be an impaired innate immune response towards *M. tuberculosis*.

### Sa2.17. Inflammatory Response and Apoptosis of Polymorphonuclear Neutrophils by Prevalent Strains of *Mycobacterium tuberculosis*.

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**Background:** Macrophages and Polymorphonuclear Neutrophils (PMN) are the professional phagocytes involved in anti-bacterial defense. The PMN influx, the first line of defense, occurs

as the early response to curtail the mycobacterial infection. Chemokines stimulate the migration of PMN from circulation to the site of infection. *Mycobacterium tuberculosis* (M.tb) induced activation leads to proinflammatory response and apoptosis of PMN. **Objective:** We characterised two prevalent strains of Mycobacteria (S7 and S10) showing differential immune response in PPD positive population. Here, we aimed to study the efficacy of these strains to induce apoptosis and modulate the expression of surface molecules and cytokine secretion in PMN of TB patients. **Methods:** PMN were isolated from RBC pellet obtained from Ficol-Hypaque gradient centrifugation and further subjected to sedimentation in 3% Dextran. PMN were infected with various mycobacterial strains (S7, S10, and H37Rv) at Multiplicity Of Infection (MOI) of 3:1 and incubated for 3 and 18hrs. The Phagocytic index, percentage of apoptotic Neutrophils (Annexin V-FITC positive by FACS), cell phenotypes (CD16 and CD69 by FACS) and cytokines (TNF-a and IL-1b by ELISA) were assessed. **Results:** A significant increase in Annexin V positive cells with corresponding decrease in CD16 and CD69 expression was observed with S7 and S10 strains when compared to uninfected control after 3hrs of infection. Further decrease in CD16 expression was observed at 18hrs but no significant change in Annexin V positivity. When compared to H37Rv, S7 showed high CD16 expression at both the time points but high CD69 expression only at 3hrs. **Conclusions:** Clinical strains down-regulated CD16 expression and inhibited *de novo* synthesis of an early activation marker, CD69 on TB-PMN. These strains also showed a significant increase in apoptosis after infection thereby reducing the number of phagocytes and escaping from the intracellular lytic microenvironment. Thus clinical isolates were able to inhibit the early activation of Neutrophils and thin out the killing mechanisms for their own survival.

### Sa2.18. Comparison of Regional and Systemic Humoral Immune Response to a Parasitic Infection.

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The kinetics of humoral immune response against *Trichinella spiralis* (TS) were characterized with immunofluorescence assay. The mesenteric lymph nodes (MLN) and the spleen of infected rats were examined for concurrent expression of multiple antibody (Ab) isotypes from day 1 to day 15 after infection. The tissues were processed and stained with either a pan-B cell marker (OX33) conjugated with rhodamine (XRITC) or combinations of dual monoclonal Ab probes plus secondary Ab conjugated with XRITC or fluorescein (FITC). As compared to the uninfected controls, the MLN and the spleen showed significant proliferation of dual-Ab expressing B cells (Debc) on days 7 and 10 respectively, with the regional immune response proceeding ahead of the systemic response. During the immune response, only minimal numbers of B cells expressed single Ab isotype while most B cells expressed more than one isotypes of Ab. When combining all the numbers of Debc within each tissue for each of the respective days, and comparing those numbers with the total numbers of B cells that were OX33+ in the serial sections of the same tissue specimens, the combined Debc in the spleen were >6 times higher than OX33 labeled B cells on day 10, and the Debc in MLN were > 3 times higher than OX33+ B cells on day 10. Our results thus indicate that the Debc were most likely expressing more than two isotypes on the surface during the peak days of the humoral

immune response to the pathogen and such phenomenon occurred in both immunologic tissues.

### Sa2.19. Co-Administration of Corticosteroids (CS) with Anti-Tuberculous Drugs (ATD) in Tuberculous Meningitis Had Not Only Reduced the Intra-Cranial Pressure Symptoms, Also Had Increased Patient's Compliance.

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**Purpose:** Patients with tuberculous meningitis, under ATD regimen alone though had been associated with an improvement in the clinical manifestations, but still there been sporadic incidences of residual manifestations on completion of ATD, concerning cerebral edema from chronic inflammatory changes of tuberculosis. Concomitant administration of (CS) had significantly improved the clinical outlook.

**Methods:** Patients with tuberculous meningitis, complaining of evening rise of temperature & manifestations from raised intracranial pressure i.e. confusion, anorexia, diffuse head ache, irritability lethargy, on ophthalmoscope there had edema of the optic disc, then one group of ( $n = 8$ ) patients had been medicated with (ATD) alone (ATD-) another group of ( $n = 10$ ) patients with (ATD) & (CS) (ATD+). For adult's prednisolone 40mg/day in divided dose for 7–10 days followed by 20mg/day in divided dose, then tapering the dose according to the therapeutic response by 5 mg weekly for 8–9 weeks. For children prednisolone 1–2mg/kg body weight for 7–9 weeks. In another trials Dexamethasone in dose of 0.15mg/kg body weight four times/day for 1–2 weeks, then discontinued in a tapering fashion over 4 weeks had been also found beneficial. On completion of the treatment, Patients with (ATD+) had a uniform therapeutic response with almost insignificant incidence of residual symptoms, where as Patients with (ATD-) alone comparatively been associated with occasional head ache, altered sensorium etc.

**Results:** (CS) in the therapeutic trials had the beneficial role of resolving portentous CSF resulting from inflammatory changes. also had improved patients compliance concerning intake of (ATD).

**Conclusions:** From the later on follow up of patients, there was no incidence of relapse in either group, but ( $n = 2$ ) patients treated with (ATD-) still had been medication for head ache & convulsion etc.

**Clinical Implications:** Patients with (ATD+) had been provided with steroid medication card on completion of treatment.

### Sa2.20. Relationship Study between the Status of Innate Immunology and the Infection of SARS.

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**Objectives:** To investigate the relationship between the status of innate immunology and the infection of SARS. **Methods** The persons including of following six groups have been studied. Group A, 4 cases of the doctors or nurses who contacted closely with SARS patients without careful prevention but not infected. Group B, 3 cases of the doctors or nurses who were infected by SARS in the working despite with careful prevention. Group C, 8 cases of pneumonia who was treated in hospital with SARS patients but no SARS; Group D, 22 intensive SARS patients; Group E, 36 mild SARS patients and Group F, 21 cases of healthy children who have no antibody responses with HBsAg injection. The machine of Array 360 has been used to detect the

gross of IgM, IgG and IgA in plasma. The lymphocytes subtype of CD3, CD4, CD8 and CD56 have been assayed by flow cytometer. The number of CR1 on erythrocyte was detected by cell-ELISA. PCR and RFLP have been used to analysis the genomic density polymorphism of ECR1. The immune reactivity of lymphocyte to foreign antigen will be observed by dynamic microscope. **Results:** The gross of IgM, IgA and IgG Group A, C and F are all in lower level than that of normal individuals, especially in IgM ( $P = 0.0014$ ), and the reactivity of their lymphocyte to antigen is also in lower levels. The percent of NK cells is significantly higher than that of normal. By contrary, the levels of IgM in group B, D and E are significantly higher than normal individuals, and their lymphocytes are easily destroyed by antigen stimuli. The numbers of CD3/CD4/CD8 are all in low levels. The number of ECR1 in intensive SARS patients are significantly lower than that in mild SARS patients but the former CR1 are most in HH genotypes. **Conclusion:** There is significant relationship between the higher immune responses and the SARS infection. The immune destroy may be the main pathogenesis of SARS.

### Sa2.21. A Safe Nanoemulsion Adjuvant Produces a Killed-Virus Nasal Vaccine for Smallpox.

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**Rationale:** Current, live virus vaccines for smallpox have unacceptable side effects. We use nanoemulsions (NEs), a soy-oil based antimicrobial with virucidal activity toward Vaccinia Virus (VV), as an adjuvant for a killed virus nasal vaccine. The nanoemulsion works by first removing viral envelope proteins then triggering phagocytosis of the proteins into antigen presenting cells in the mucosa.

**Methods:** A NE approved for human use was mixed with VV (Western Reserve serotype). Mixtures of NE and VV were applied to the nares of mice. Systemic and mucosal antibody and cellular immune responses were then evaluated, and animal sera were tested for the ability to neutralize virus.

**Results:** A brief incubation with 10% nanoemulsion led to greater than a six-log reduction of virus titer. EM analysis suggested the NE disrupted the viral lipid membrane. Anti-VV mucosal IgA and serum IgG were detected three weeks after a single intranasal administration of NE/vaccinia mixture, and the immune response was optimized in animals vaccinated 2–3 times. High titers of VV neutralizing antibodies were detected in mice immunized with NE-killed virus. Virus-specific Th1 immunity (INF $\gamma$  production) was observed in splenocytes from immunized animals. No evidence of protective immunity was observed in control animals immunized with formalin-killed virus. No animal had evidence of viral replication after vaccination, documenting the complete inactivation of the virus by NE.

**Conclusions:** VV inactivated by NE is immunogenic and can serve as a killed virus mucosal vaccine for small pox. The presence of NE is necessary for the development of robust protective mucosal and systemic immunity.

### Sa2.22. Novel Pan-DR Binding T-Cell Epitopes of Adenovirus Induce a Mixed Profile in Healthy Donors.

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### Background

In the immunocompromised host adenovirus can cause fatal infections, especially after stem cell transplantation. No specific antiviral therapy of proven value currently exists for severe adenoviral infection. A potential form of treatment is adoptive therapy infusing adenovirus specific T-cells from the donor. The aim of the study was to identify target epitopes of adenovirus and to capture and characterize adenovirus specific T-cells.

### Methods

Eleven proteins of adenovirus type 5 were selected. By using a computer algorithm designed to predict HLA pan-DR binding T-cell epitopes, we selected 19 peptides based on predicted high affinity to multiple HLA-DR alleles and a high degree of homology with other adenovirus serotypes. Peripheral blood mononuclear cells (PBMCs) from 26 healthy adults were isolated and incubated with these peptides. Proliferation expressed as Stimulation Index (SI) was determined. Six peptides with highest SI were selected. In ten subjects the cytokine and chemokine profile induced by these epitopes was determined with Multiplex Immuno-assay (MIA). In addition PBMCs were cultured with complete inactivated adenovirus and restimulated with the different peptides. Subsequently, with MIA cytokine production was measured. Moreover, with the use of T-cell capture method<sup>1</sup> and FACS-sorting it was possible to induce and capture adenovirus specific T-cells. With polymerase chain reaction (PCR) characterization of adenovirus specific T-cells was performed.

### Results

Six pan-DR binding epitopes of adenovirus were selected based on a positive proliferation response in a large percentage of donors, namely E1B protein (65% of donors), 2 peptides of hexon protein (58% and 73%), DNA-polymerase (57%), E3A-glycoprotein (46%) and fiber protein (34%). These epitopes induced a predominant pro-inflammatory cytokine and chemokine profile. Also after culturing with complete inactivated adenovirus and restimulation with the adenoviral peptides, cytokine profile showed a pro-inflammatory pattern, suggesting that peptides are naturally processed. By using T-cell capture method adenoviral peptide specific CD4+ T-cells were identified and sorted. Preliminary data show that such adenovirus specific T-cells display a high expression of TGF-1 beta, IFN gamma and Tbet (Th1 response), but remarkably also expression of GATA3 and IL10 (Th2 response).

### Conclusion

With a pan-DR binding computer algorithm it was possible to identify HLA-class II restricted T-cell epitopes of adenovirus type 5. These peptides induce a predominant pro-inflammatory cytokine profile and also seem to be naturally processed. With T-cell capture method it was possible to capture and characterize the adenovirus specific T-cells. This is an important step towards adoptive immunotherapy by infusion of adenovirus specific T-cells.

<sup>1</sup>Prakken, B. et al. Artificial antigen-presenting cells as a tool to exploit the immune 'synapse'. 2000 Nat.Med 6.12:1406-10.

### Sa2.23. Naturally Occurring Breakdown Products of Inflammation, Generated in Inflammatory Sites, Functioned as Down-Regulator of Inflammation.

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Inflammation usually occurs in extravascular tissues following the invasion of micro-organisms, such as bacteria, to the body. These sites also contain immuno-modulators (e.g., chemokines, cytokines, acute phase proteins), and leukocyte-derived extracellular matrix-specific enzymes, such as heparanase and elastase, which can modify the composition of the tissue, and probably also degrade certain inflammatory mediators, thus yielding putative bioactive products. We postulate that these new small molecular weight mediators can exert effector functions needed to evoke or terminate inflammation.

Herein, we identified novel immunoregulatory compounds in the degraded products of human body fluids, especially in wound fluids of chronic leg ulcers of diabetic patients. Thus far we were able to isolate and identify several amino acid sequences and synthesized, and examined them in vitro and in vivo. Among these peptides, two are derived from apolipoprotein A-1 and two from fibrinogen. Treatment of purified human T cells with these peptides down-regulated nuclear factor-κB activity and reduced the secretion of TNF-α and interferon-γ. In vivo, these peptides markedly inhibited DTH reaction and ConA-induced hepatitis in mice. We suggested that these peptides may terminate inflammatory reactions by transmitting negative signals to the inflammation-inducing leukocytes.

Our results indicate that by using this approach we may have found a first set of such novel anti-inflammatory peptides. We hope that such peptides can be used to down-regulate inflammatory reactions in vivo in human patients suffering from chronic inflammatory diseases.

### Sa2.24. The Inflammatory & Immunological Complex Clinical Presentation of Mycoplasmal Infection in School Children.

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**Purpose:** Outbreaks of mycoplasma infection had been associated in school children/camps at early and late winter seasons, sometimes with a considerable number of hospital admissions & loss of school days amongst children & adolescents.

**Methods:** In an under developed community with bunch of schools, children & adolescents age 5–16 years mean age  $10 \pm .5$  years. Presenting features i.e. raised temperature 95% cases, malaise 85–90%, cough 80–85%, headache 80%, medium to fine rales 60–65%. On Laboratory investigation were leucocytosis (10,000–20,000/m<sup>3</sup>) 20–30% cases, raised ESR, variable Pulmonary segmental/lobar consolidations seen, cold agglutinin level 1:4-1:256 titer appears in blood of 50% of children, most of the cases had raised indirect of fluorescent antibody in the range of 1:10–1:320. Also isolation of organisms i.e. M. Pneumoniae.

**Results:** Ignored/late treated cases from the distant locations, almost 0.1–0.9% had transverse myelitis, encephalitis meningo-encephalitis proved by CSF, PCR, culture studies. Hematological complications, 0.5–1% had thrombocytopenia, hemolytic anemia, and renal failure. Cardiac complications i.e. myopericarditis, haemopericardium heart block, 0.02–0.1% etc; 2–4% with mucocutaneous manifestations, i.e. erythematous maculopappular eruptions, stevens-johnson's syndrome, 4–5% with joint manifestation i.e. mimicking rheumatic arthritis. 10–20% of complicated cases had nausea, vomiting, altered bowel habits, etc;

**Conclusions:** Mycoplasma infection is a contagious disorder resulting from substandard hygienic living, poor school health (failure of provision of well ventilated classrooms/Isolation of infected cases) etc;

**Clinical Implications:** Mycoplasma pneumonia is held responsible for 20% cases of community acquired pneumonia.

### Sa2.25. Yeast Cells Activation Method of Hemaimmune Reaction Road Map Experimental System.

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**Objective:** To establish a experimental system of war against yeast cells in hemaimmune reaction road map.

**Methods:** 0.2ml suspension of yeast cells ( $5 \times 10^8$ /ml) (or 0.2ml NS as control) were added into 0.2ml fresh anticoagulant (citric acid) whole blood or 0.2ml white blood cells, added 0.3ml plasma (or 0.3ml NS as control), and incubated for 1h at 37°. Further more, we added 0.2 ml red blood cells into the tube above-mentioned respectively, and incubated for 1h at 37°. The hemaimmune reactionary activity was assessed by checking blood cell adhering rate to yeast cells, CD35, DARC (Fy6), CXCR4, IL-8, IL-6, CD25, gene activation (Fy6 gene) et al.

**Results:** Yeast cells can activate hemaimmune reaction road map experimental system showing change of various indexes. Yeast cell activation method will be a useful method to study the relationship between whole blood cells and plasma and the relationship between red blood cells and white blood cells in hemaimmune reaction.

**Conclusion:** It can provide a useful method for innate and adaptive immunity study and for immune regulation study and for the theory study of hemaimmune reaction road map in clinic.

### Sa2.26. Phage Display Epitope Study of Two Toxins Produced by Enteroaggregative *E. coli* That Cause Pediatric Diarrhea: Identification and Characterization of Antigenic and Immunogenic Mimotopes.

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In earlier publications from this laboratory, two toxins a of 104kDa (Pet) and other of 114 kDa (Pic), members of the SPATES (serine-protease autotransporters from enterobacteriaceae) family were described as molecular agents associated with pediatric diarrhea caused by *Enteroaggregative E. coli*, especially in developing countries. Despite different location of their genes, in the chromosome (Pic) and in a plasmid (Pet), the proteins showed a high level of sequence identity. Both proteins have been identified in the same patient and are immunogenic, however, neither their epitopes nor immunological relationships with each other and with the disease are known. Collections of peptides with properties of immunodominant epitopes that elicit the immune responses in patients, would be very beneficial for the research advancing into the immunology of the proteins and their relationships with the disease. To the identification of such peptides we screened gp3 random phage-display linear and constrained libraries with sera of patients and with rabbit antisera obtained against Pet and Pic proteins. The screening of two highly complex phage libraries resulted in collections of peptides sharing well-defined consensus

motifs. Although the motifs did not show similarity to the proteins, the carrying those peptides phage were reactive with sera and induced in mice and rabbits antibodies reactive against Pet and Pic in ELISA and in denaturing Western blot. The results indicate that they mimic epitopes containing immunogenic determinants that do not loss their antigenic activity upon denaturation. In addition, the anti-sera against Pet-related peptides showed a cross-reaction with the Pic, and *vice versa*, i.e. both toxins are not only structurally but also immunologically similar. We conclude that the peptides from phage libraries specific to epitopes of these enterotoxins allow further elucidation of their roles in the pathogenesis of diarrhea and are useful in laboratory diagnosis of patient sera. In the ongoing experiments the peptides with best immunogenic and antigenic potentials are used in serological studies of patients and for their ability of inducing neutralization-effective responses in animal models.

### Sa2.27. The Combined Modulation of Nicotine and *Chlamydia* Heat Shock Protein on Cell Proliferation and Apoptosis in HEp-2 Cells.

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Nicotine, the major active component of tobacco, has been reported to modulate the process of inflammation and apoptosis. *Chlamydia pneumoniae* (Cpn), an obligate intracellular pathogen, is a common cause of respiratory tract infections worldwide and has been considered among other risk factors involved in coronary artery disease. Cpn- infected cells have been reported to be resistant to apoptosis, but it is not clear what component of Cpn is responsible for this action. The present study investigated the role of both nicotine and *Chlamydia* heat shock protein 60 (cHsp-60) individually and in combination on viability, proliferation and apoptosis in human epithelial cell line (HEp-2). The results of these studies showed that treatment of HEp-2 cells with nicotine significantly increased cell count and viability compared to the untreated controls. Treatment of HEp-2 cells with cHsp-60 did not result in significant changes in cell count and viability. Interestingly, when apoptosis was induced in HEp-2 cells with TNF-alpha, cHsp-60 did in fact significantly increase cell count and viability. In order to ascertain whether this increase reflected a protection against apoptosis, caspase activity was assessed. Results showed that active caspases were down-regulated in cells treated with either nicotine or with cHsp-60. Combined treatment with both nicotine and cHsp-60 resulted in even further down-regulation of active caspases. The anti-apoptotic action of nicotine was blocked by D-tubocurarine chloride, a nicotinic receptor antagonist. The high prevalence of Cpn infection and the ready availability of nicotine in the population lead to concerns about possible combined exposure to both agents. The impact of the combined exposure to nicotine and cHsp-60 on parameters of immune status, in both normal and immunocompromised hosts, warrants further investigation.

Key Words: Nicotine, cHsp-60, HEp-2 cells, apoptosis

### Sa2.28. Circulating Vδ1 and Vδ2 T Cells in HIV-1-Infected Patients: Response to CXCR3 and CXCR4 Ligands and to HIV-1 Tat.

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Twentyeight HIV-1-infected patients, in the stage A of the disease, according to the CDC (Center for Disease Control, Atlanta) criteria, have been studied. We found in 18 out of 28 patients an increase in the number of circulating V $\delta$ 1 T cells (3–9%) of T lymphocytes (healthy donors: 1–3%). Three of these patients also displayed an increased number of peripheral V $\delta$ 2 T cells (4–6% vs 2–3% in healthy donors). Moreover, V $\delta$ 2 T cells were CXCR3<sup>bright</sup> and CXCR4<sup>dull</sup>, while among the V $\delta$ 1 subset 50% of the cells were CXCR3- and CXCR4<sup>+</sup>. V $\delta$ 1 and V $\delta$ 2 T cell clones transmigrate across endothelial monolayers in response to interferon- $\gamma$  inducing protein-10 (IP-10/CXCL10) and stromal-derived factor-1 (SDF-1/CXCL12) according to the expression of the specific receptors CXCR3 and CXCR4. In a fraction (10%) of V $\delta$ 1 T cell clones coexpressing CXCR3 and CXCR4, the homeostatic chemokine 6Ckine/SLC (CCL21) was more effective than IP-10/CXCL10 in driving transendothelial migration of V $\delta$ 1 CXCR3<sup>+</sup> cells, while V $\delta$ 2 CXCR3<sup>+</sup> cells were driven more efficiently by IP-10/CXCL10.

IP-10/CXCL10 or 6Ckine/SLC/CCL21 and SDF-1/CXCL12-induced transmigration were inhibited by the phosphoinositide-3 kinase (PI-3K) blockers wortmannin and LY294002, supporting that CXCR3 and CXCR4 are coupled to PI-3K. This was further confirmed by the activation of the PI-3K-dependent serine kinase Akt/PKB obtained upon ligation of CXCR3 and CXCR4. In addition, occupancy of CXCR3, but not of CXCR4, led to CAMKII activation; accordingly, the CAMKII inhibitors KN62 and KN93 could decrease IP-10/CXCL10 and 6Ckine/SLC/CCL21-driven transmigration. Finally, we show that HIV-1 protein Tat, which can be found in the sera of these patients, interferes with the chemotactic activity of IP-10/CXCL10, 6Ckine/SLC/CCL21 and SDF-1/CXCL12 and that the inhibition is due to the cystein-rich domain of the protein, which contains CXC and CXC chemokine-like sequences. This mechanism may contribute to the redistribution of the two  $\gamma\delta$  T cells subset, the resident increasing in peripheral blood in early AIDS and removed from intestine in the advanced disease, supporting the importance of  $\gamma\delta$  T cells in the first defence against HIV-1 infection.

### **Sa2.29. Adenovirus Capsid Hexon Is the Main Target Protein of Adenovirus-Specific CD4<sup>+</sup> T-Cells That Display a Th1 like Cytokine Profile in Healthy Adults.**

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Adenovirus infections are usually harmless in healthy children and adults but are serious and potentially fatal in immunocompromised individuals. Like for CMV and EBV-infections, adoptive transfer of specific T-cells is also discussed for adenoviral infections. However, the main adenovirus derived antigenic target of adenovirus-specific T-cell responses has yet to be defined. We here identified the main immunodominant protein of the adenovi-

rus-specific CD4<sup>+</sup> T-cell response and functionally characterized adenovirus-specific CD4<sup>+</sup> T-cell with respect to their cytokine profile. Three major adenoviral proteins including two structural capsid proteins (hexon, penton, polymerase) were recombinantly expressed. CD4<sup>+</sup> T-cell-lines specific for the entire set of adenovirus-derived proteins were generated after stimulation of PBMC from healthy adults with adenovirus-lysate 3 (Ad-Lys3) according to antigen-induced CD154 surface expression. Specificity of adenovirus-specific CD4<sup>+</sup> T-cell-lines was evaluated after restimulation with Ad-hexon, Ad-penton, Ad-polymerase according to intracellular expression of CD154. Direct qualitative cytokine profile of Ad-specific CD4<sup>+</sup> T-cells was assessed after short-term stimulation of whole blood of healthy adults with Ad-hexon and co-expression analysis of CD154 with IL-2, IL-4, IL-10, TNF $\alpha$  and IFN $\gamma$ . Ad-Lys3-specific CD4<sup>+</sup> T-cell-lines can easily be generated according to CD154-expression and showed high specificity for the adenovirus derived hexon capsid protein. Only a few Ad-Lys3 specific CD4<sup>+</sup> T-cells responded to Ad-penton and none to Ad-polymerase. Qualitative assessment of the cytokine profile of Ad-hexon specific Th-cells proved a Th1-like profile with no IL-4 expressed and dominant expression of TNF $\alpha$  and IFN $\gamma$ . Hexon capsid protein is the main target protein of the adenovirus-specific CD4<sup>+</sup> T-cell response in adults that is characterized by a Th1-like cytokine profile. Our results envisage that Adenovirus-derived hexon protein is a candidate antigen for *ex vivo* generation of adenovirus-specific T-cells in cellular therapies.

### **Sa2.30. Identification of SARS T Cell Epitopes Using the iTopia<sup>TM</sup> Epitope Discovery System.**

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**Objective:** SARS is a severe infectious disease caused by a virus identified through gene sequencing and serological analysis as a new strain of human coronavirus. SARS coronavirus (SARS-CoV) causes severe acute respiratory symptoms in patients and results in a high mortality rate. Antigenic peptides recognized by SARS virus specific CTL's are useful tools for studying the CTL responses during infection enabling researchers to better monitor disease and develop T-cell mediated vaccines. In order to identify potential immunogenic epitopes from the SARS N-protein, we used Beckman Coulter's iTopia Epitope Discovery System\* to analyze the protein across 8 MHC Class I alleles.

**Materials and Methods:** The iTopia Epitope Discovery System was used to screen the SARS N protein by analyzing all overlapping nonamers (414 peptides) across 8 different HLA-A/B alleles: HLA\*A0101, HLA\*A0201, HLA\*A0301, HLA\*A1101, HLA\*A2402, HLA\*B0702, HLA\*B0801, HLA\*B1501. The peptide binding assay is the first assay in the system and is a rapid screening of all test peptides across all 8 alleles. All identified binders are further characterized with the ED50 Affinity and Off Rate dissociation assays. The previously identified binders are serially diluted and analyzed in the Affinity assay. An ED 50 value is determined and represents the concentration of peptide needed to achieve 50% binding. These same binders are also analyzed in the Off Rate assay. Peptides are incubated for up to 8 hrs and time points are taken at specified intervals. Results are analyzed using a one phase exponential decay curve and are expressed as t 1/2 value in hrs representing the amount of time needed to achieve 50% dissociation of the peptide from the MHC complex. The Off Rate

and Affinity values for each test peptide are evaluated using a multiparametric calculation to generate an iScore. This iScore allows peptides to be systematically ranked for subsequent functional studies. These newly identified peptides can be used to manufacture HLA Class I iTag MHC tetramers which are an important tool to visualize and quantify the precise in vivo SARS CTL response.

**Results:** Identification of SARS candidate epitopes are as follows: 6 epitopes for HLA\*A0101, 30 epitopes for HLA\*A0201, 18 epitopes for HLA\*A0301, 28 epitopes for HLA\*A1101, 37 epitopes for HLA\*A2402, 18 epitopes for HLA\*B0702, 7 epitopes for HLA\*B0801 and 27 epitopes for HLA\*B1501. Based on iTopia rankings of candidate epitopes, SARS iTag™ MHC Tetramers were manufactured and used to stain cryopreserved PBMC's from SARS infected donors.

**Conclusion:** Using the iTopia Epitope Discovery System, the SARS N protein (422 amino acids) was screened and potential immunogenic epitopes were identified based on experimental binding, affinity and off rate determinations. SARS results demonstrate the capacity of the iTopia system to screen large number of MHC Class I restricted peptides and prioritize the epitopes with greatest potential for producing an immune response.

\* For Research Use only; not for use in diagnostic procedures

### **Sa2.31. *Leishmania* Infection in Two Twins, Association to Perforin Defect.**

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**Introduction:** The underlying pathology of haemophagocytic lymphohistiocytosis (HLH) relates to inherited defects on natural killer cell and cytotoxic T-cell activity. Mutations on perforin gene in familial-HLH forms and infections in acquired-HLH forms have been published.

**Objective:** To study a family with two twins with suspect symptom and bone marrow aspiration signs of HLH and; negative serology for bacteria, virus or parasites in both twins. These twins presented a different degree of clinical severity (**Twin 1** had more severity than **Twin 2**).

**Methods:** Family study perforin expression was analysed by four-color multiparametric flow cytometry COULTER<sup>®</sup> EPICS<sup>®</sup> XL™ Flow Cytometer of Beckman-Coulter (Coulter Corporation). Perforin expression was studied in CD56+CD3- cells, CD56+CD3+ cells, CD8+ CD3+ cells. Genetic analyses were carried out to identify the putative perforin mutation. Genomic DNA was prepared from PBMC using standard protocols, after informed consent was obtained. Exons 2 and 3 of the perforin gene (prf1) were amplified using polymerase chain reaction in the family and in several controls. Quantification of plasma sIL-2R levels was performed in triplicate using commercially-available ELISA kits (Bender MedSystems, Viena, Austria).

**Results:** The family study showed decreased perforin expression in CD 56+CD3- NK cells, CD8+CD3+ cytotoxic T cells in the father and **Twin 1**, and a normal expression in the mother and **Twin 2** when we compared it with healthy controls of similar age. Sequencing of prf1 disclosed one mutation, previously described (Clementi, Blood 2002; 100 (6):2266-7.), in **Twin 1** and father in the codon 91 of the exon 2 (GCG-GTG that changes Ala91 to Val). Both were hetero-

zygous for this mutation. In all others samples, including **Twin 2**, no mutations were detected. Although, we did not sequence the entire prf1 gene, it is unlikely that the **Twin 2** have another perforin mutation since their expression was normal by flow cytometry.

Both twins had higher levels of sIL-2R than healthy controls of similar age; **Twin 1** showing a higher level (18.55 ng/ml) than **Twin 2** (14.38 ng/ml). In contrast, sIL-2R levels of both progenitors were normal.

While, *Leishmania* parasites were visualized by electron microscope examination on the liver tissue of both twins. Finally Treatment began with antimonials and both patients recovered completely but more quickly **Twin 2**.

**Conclusion:** Visceral Leishmaniasis associated to HLH syndrome can cause additional and considerable etiologic diagnosis difficulties. However, our results indicate that could be convenience of analysis of perforin defects and concomitant infections in all types of HLH. *Leishmania* infection is not only able to trigger haemophagocytic syndrome but perforin defects may worsen eradication of leishmania infection.

### **Sa2.32. The Calcium-Promoted Ras Inactivator (CAPRI) Links Fcg Receptor to Cdc42 and Rac1 and Is Essential for Host Innate Defense.**

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Fc receptor (FcR)-mediated phagocytosis requires the activation of the Rho GTPases Cdc42 and Rac1. However, how Cdc42 and Rac1 are recruited to the FcR is unknown. Here we show that the Ca<sup>2+</sup>-promoted Ras inactivator (CAPRI), a Ras GTPase-activating protein, functions as an adaptor for Cdc42 and Rac1 during FcR-mediated phagocytosis. CAPRI-deficient macrophages exhibit impaired FcgR-mediated phagocytosis and oxidative burst, as well as a defective activation of Cdc42 and Rac1. CAPRI interacts constitutively with both Cdc42 and Rac1, and translocates to phagocytic cups during FcgR-mediated phagocytosis. Importantly, CAPRI-deficient mice have impaired innate immune response to bacterial infection. These results suggest that CAPRI provides a link between FcgR and Cdc42 and Rac1 and is essential for host innate defense.

### **Sa2.33. An 8 Year Old Girl with Recurrent Dermatomal Herpes Outbreaks.**

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**Case Report:** An 8 year old Caucasian girl presented with a five year history of recurrent dermatomal vesicular lesions affecting her face and back. At 2 1/2 years of age, the patient developed primary gingivostomatitis with painful, vesicular lesions over her entire oral mucosal surface. She continued to have mucosal outbreaks every other month until 3 1/2 years of age when she complained of facial irritation, soreness, and fevers with subsequent vesicular lesions originating at the corner of her mouth and following the V3 dermatome. Her lesions never cross the midline and occur several times a year triggered by respiratory infections or stress. She continues to have acute outbreaks despite prophylactic acyclovir dosed at 45 mg/kg/day. The patient also has a history of recurrent respiratory tract

infections since six weeks of life. These infections resolve after standard courses of oral antibiotics. She has no history of lower respiratory tract, bone, joint, deep tissue, or fungal infections. The family history is remarkable for recurrent viral infections in her father and two siblings. Her physical examination was unremarkable with no evidence of lymphadenopathy, hepatosplenomegaly, or chronic rash. A direct fluorescent antibody test performed during a subsequent outbreak was positive for herpes simplex virus and negative for varicella virus in perilesional skin. Multiple viral cultures of lesions at different times were positive for herpes simplex virus. An evaluation for a possible defect in the innate and cell-mediated immune system revealed decreased natural killer cell lysis, an intracellular cytokine assay (ICC) for interferon-gamma production was negative for varicella, cytomegalovirus, Epstein Barr virus, and influenza, a delayed type hypersensitivity (DTH) skin test was reactive to tetanus and mumps, and a lymphoproliferation assay (LPA) for mitogens and tetanus was normal. DTH skin test reactivity and LPA to candida were nonreactive. An HIV test was negative. Evaluation for STAT 1 deficiency was negative. Flow cytometry revealed an elevated CD4/CD8 ratio. Humoral immune function was evaluated and immunoglobulin levels of IgG, IgA, IgM, and IgE were normal and IgG2 was mildly decreased. *Diphtheria*, *Tetanus*, and *Haemophilus* antibody titers were protective. Zero of twelve Pneumococcal antibody levels were >1.0 ug/ml before and after vaccination. HSV IgG titers were positive while varicella virus IgG titers were equivocal. Flow cytometry revealed an elevated number of CD 19+ cells.

**Discussion:** The patient presented with recurrent dermatomal vesicular lesions which were caused by herpes simplex virus. Health care providers who typically associate recurrent dermatomal outbreaks with herpes zoster should consider culturing for HSV or other viral etiologies. Although rare, recurrent dermatomal HSV lesions may be a hallmark of an as yet undefined immunodeficiency that may be elucidated as the field of clinical immunology matures.

### Sa2.34. Reconstitution of Genetically Determined Deficiency of the Innate Immune Defence with Recombinant Mannan-Binding Lectin (MBL).

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The innate immune defence encompasses a number of cellular and humoral components. Among the latter the complement system plays important roles, and is also involved in establishing an adequate specific clonal immune response. The recently established mannan-binding lectin (MBL, or mannose-binding lectin) complement pathway relies on the recognition of the microorganisms by their pathogen-associated molecular patterns (PAMPs). The oligomeric structure of MBL provides for 12 or more clustered C-type lectin domains, which allows for high avidity binding to suitably spaced sugar residues. This binding triggers the activation of the MBL-associated serine protease, MASP-2, which in turn activates C4 and C2 to generate the C3 convertase, C4bC2b. The level of MBL in plasma varies from less than 10 ng to 5 µg/ml and is determined by polymorphisms in exon one and in the promoter region. Numerous studies have found association between MBL deficiency and increased susceptibility to infections. It appears that alone MBL deficiency does not

predispose to infections, but only when also other elements of the immune system are suboptimal. Thus, e.g., leukaemia patients in chemotherapy are at high risk of serious infections when MBL deficient (Peterslund et al. *Lancet*, 358, 637-8, 2001), MBL deficient colorectal cancer patients have increased risk of post-operative infections (Ytting H et al. *Cancer Immunol Immunotherapy*, online pub, 2004). MBL deficiency has also been reported to be associated with autoimmune manifestations, and surprisingly, with increased risk of atherosclerotic diseases. Recombinant MBL is now being produced in a human cell line by NatImmune A/S, Copenhagen by methodologies modified from Vorup-Jensen et al. (*Int. Immunopharmacology*, 1, 677-87, 2001; Jensenius et al. *Biochem Soc Trans.* 2003 31, 763-7, 2003) and purified to yield a preparation certified for clinical trials. The rMBL shows physical and biological characteristics similar to those of plasma-derived MBL. Preclinical and phase I trials have been conducted and are currently being evaluated. No safety concerns were observed.

### Sa2.35. An Active Role for Complement Regulator CD46 in Signal Transduction upon Infection.

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The renal epithelium is the initial site of contact between pathogens and their hosts. Through this interaction epithelial cells may have the opportunity to detect and respond to pathogens, which leads to activation of inflammatory responses and recruitment of leukocytes. This initial phase of inflammatory response to infection often involves activation of innate immune system in particular the complement. A series of fluid phase and cell surface inhibitors, including **Membrane Cofactor Protein (CD46)**, exist to prevent excessive complement activation. CD46 is also known to be the receptor for various types of pathogens. Here we have investigated the potential of CD46 to mediate signal transduction and subsequent internalisation in human renal epithelial cells. CD46 was found on both apical and basolateral surfaces of cells by staining with anti-CD46 antibody and a fluorochrome. Introduction of a secondary antibody brought clustering of receptors. Incubating at 37 °C led to internalisation of antibody, which was not seen when incubating at 4 °C. Extracellular antibody was detected by a green-labelled fluorochrome. Cells were then fixed and permeabilised, and a red-labelled fluorochrome was used to detect intracellular and extracellular antibody. We have previously shown that src kinases are activated on CD46 cross-linking. However, internalisation was independent of src kinase activity. Here we present data demonstrating that antibody can be internalised specifically through its binding to CD46 receptor, which suggests that CD46 may play an active role in mediating signalling events. It is our interest to investigate whether CD46 can serve as a receptor for mediating internalisation of opsonised pathogens upon infection.

### Sa2.36. Access to the Entire Human Antigen-Specific CD4+ T-Cell Response According to Antigen-Reactive CD154 Expression.

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Current techniques to assess antigen-specific T-cells suffer from various limitations: Either the access is restricted to activated cells that reacted with the expression and secretion of cytokines or could only be performed with a so far rare selection of specific peptide MHC-II multimers. We report here on a new method to assess the entire fraction of CD4<sup>+</sup> T-cells specific for a particular antigen independent of their cytokine memory.

For this we have employed here the intracellular and extracellular detection of antigen-induced CD154 expression after in vitro stimulation with various antigens such as CMV, TT, Adenovirus, birch allergen, SEB and Mycobacterium tuberculosis ESAT-6. In the course of antigen-driven in vitro activation of CD4<sup>+</sup> T-cells CD154 is specifically expressed by antigen-specific CD4<sup>+</sup> T-cells. Antigen-specific CD154 expression is detectable intracellularly in fixed CD4<sup>+</sup> T-cells when stimulations are performed in the presence of Brefeldin A facilitating co-expression analysis of cytokines. Moreover, we developed a strategy to circumvent the rapid internalisation of reactive surface CD154 expression after interaction with its counterpart CD40. This enabled us to isolate a live antigen-specific CD4<sup>+</sup> T-cells with a simple single cell surface staining after in vitro stimulation for the fast generation of highly specific CD4<sup>+</sup> T-cell lines.

Our approach offers the striking option to assess the entire pool of CD4<sup>+</sup> T-cells with a defined specificity allowing for combined quantitative and qualitative analysis of Th-cell immunity and for isolation of specific Th-cells for targeted cellular immunotherapies.

### Sa2.37. Excessive Innate Immune Responses Could Prime Massive Hepatocyte Apoptosis Induced by Lipopolysaccharide.

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**Background/Aims:** Although liver diseases are known to be exacerbated by bacterial infections, the mechanism is still unclear. In a mice model, *P. acnes*-primed mice show granuloma formation in response to the bacteria, and massive hemorrhagic liver injury is observed after lipopolysaccharide (LPS) injection. However, whether antigen-specific response or granuloma formation is requisite for the injury is unknown. We, therefore, examined whether antigen-nonspecific accumulation of DCs and macrophages in the liver by the overexpression of GM-CSF could prime severe liver injury after LPS injection.

**Methods:** We injected a recombinant adenovirus encoding GM-CSF (AdGM) containing  $1 \times 10^8$  plaque-forming units, and one mg per mouse of LPS was administered seven days later into C57BL/6 (B6) mice via a tail vein. The composition of hepatic mononuclear cells were analyzed by flow cytometry. The concentrations of GM-CSF, TNF- $\alpha$  and IFN- $\gamma$  in the serum of mice were measured by ELISA. Liver histology, serum alanine aminotransferase (ALT) levels and apoptosis of hepatocytes were also examined. To further examine the role of Fas pathway and TNF- $\alpha$  in the apoptosis of hepatocytes, we used FasL-deficient mice B6-*gld/gld*. In both B6-wild type and B6-*gld/gld* mice 100 $\mu$ g per mouse of a neutralizing anti-TNF- $\alpha$  antibody was intravenously injected 30 minutes before LPS injection.

**Results:** Liver histology of the AdGM-primed mice showed marked infiltrates of mononuclear cells (CD11b<sup>+</sup>macrophages and CD11c<sup>+</sup>I-A<sup>b</sup><sup>+</sup>CD11b<sup>+</sup>myeloid DCs) without granuloma

formation on day 7. Expression of toll-like receptor-4 on intrahepatic mononuclear cells isolated from AdGM-primed mice was up-regulated. Although serum ALT levels were within normal range before LPS injection, the levels were elevated as early as 6 hours after LPS injection only in AdGM-primed mice. The peak levels were  $5922 \pm 3678$  IU/L reached at 12 hours after LPS injection, and all those mice died within 24 hours. Serum TNF- $\alpha$  concentrations were elevated after LPS injection, and peaked at 2–6 hours after LPS injection. Hemorrhagic liver injury was histologically recognized, In AdGM-primed mice, TUNEL-positive hepatocyte nuclei were already observed one hour after LPS injection when liver injury was not histologically apparent, and the rates of TUNEL-positive apoptotic hepatocytes were increased to about 80% three hours after LPS injection. When AdGM and LPS were injected in FasL-deficient B6-*gld/gld* mice, serum ALT levels were not elevated by the pretreatment with a neutralizing anti-TNF- $\alpha$  antibody.

**Conclusions;** Our present study provides a new model of severe liver injury, in which massive accumulation of innate immune cells such as DCs and macrophages in the liver by overexpressing GM-CSF enhances the susceptibility to LPS, leading to hemorrhagic liver injury with massive hepatocyte apoptosis after LPS injection. Both TNF- $\alpha$  and Fas-mediated signaling were thought to be important for the hepatocyte apoptosis.

### Sa2.38. Evaluation CD64 Marker in Neonatal Sepsis for Rapid Diagnosis.

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**Introduction:** Neonatal sepsis is a life-threatening disease with an incidence of 1 to 10 per 1000 live births and a mortality rate of 15% to 50%. The clinical signs are nonspecific and indistinguishable from those caused by a variety of neonatal non-infective disorders. The aim of this study was to determine sensitivity, specificity, positive and negative predictive value of neutrophil markers, in early diagnosis of neonatal sepsis.

**Methods:** In this study were comprised 65 neonates with a gestational age of 27 to 38 weeks who suspected for sepsis within 28 days of life. 1 ml of whole blood was obtained from neonates to determine CD64 expression of peripheral blood neutrophils by flow cytometry. Neonates were classified into two groups. Classification was based on positive blood culture. In the sepsis group ( $n = 8$ ) all of neonates were positive blood culture and had clinical symptoms. In the suspected group ( $n = 57$ ), each neonate had at least 1 clinical sign but negative blood culture. 12 healthy term neonates with physiologic hyperbilirubinemia classified in control group.

**Results:** CD64 was elevated in sepsis group and this increase was significant ( $p < 0.001$ ). specificity and sensitivity of CD64 were 100% and 92% respectively. The negative and positive predictive value of CD64 for identifying sepsis were 100% and 88% respectively.

**Discussion:** High sensitivity of CD64 (100%) and specificity of 92.3% indicates that evaluation of CD64 as neutrophil marker can help clinicians for early diagnosis of neonatal sepsis.



### Sa2.39. *T. Whipplei* Specific Immune Reactions Are Significantly Reduced in Patients with Whipple's Disease.

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**Objective:** Whipple's Disease (WD) is a rare systemic infectious disease prefaced by replication of the actinomycete *Tropheryma (T.) whipplei* in macrophages of the intestinal mucosa. The possibility of a predisposing immune deficiency is discussed for WD and a reduced TH1 reactivity that could explain the rarity of the disease despite the ubiquitous existence of the pathogen, was already shown for WD patients. However, due to a lack of bacterial preparations *T. whipplei*-specific intestinal or peripheral immune reactions of WD patients were not analysed till now.

**Methods:** *T. whipplei*-specific stimulations were performed in peripheral blood and lamina propria lymphocytes (LPL) with sonicated *T. whipplei* cultures, followed by FACS-analysis of expression of cytokines (IFN $\gamma$ , IL-4, IL12) and activation markers (CD69, CD154) of CD4+ T-cells. Several common bacterial and viral antigens (Tetanus Toxoid, Cytomegalovirus and Tuberculin) were used to control general antigen-specific reactivity.

**Results:** We established specific stimulations with recently evolved laboratory strains of *T. whipplei* and compared for the first time immune reactions of WD patients, healthy controls (age-matched and young), and patients with Tuberculosis as a mycobacterial model infection. In every control and patient with Tuberculosis analysed so far, we were able to detect *T. whipplei*-specific CD4+ T-cells in the blood as well as in the duodenal mucosa. However, WD patients showed a significantly reduced reactivity against the pathogen. The reactivity against SEB of WD patients was slightly reduced compared to controls, whereas the reactivity to common antigens was comparable. Additionally, we used a system with *in vitro* generated autologous dendritic cells loaded with *T. whipplei* antigens that were able to enhance reactivity in healthy controls but still could not induce antigen-specific reactions in WD patients.

**Conclusion:** Hence we can conclude that *T. whipplei*-specific reactivity occurs in healthy controls and that frequent exposure to the bacteria seems to activate immune reactions, whereas our results hint at a significantly reduced antigen-specific reaction of WD patients resulting in insufficient protection against the pathogen and onset of WD.

### Sa2.40. Adoptive Transfer of CFSE-Labeled Autologous PBMC for the Visualization of T Cell Migration in a Non-Human Primate Model.

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**Background:** Several inflammatory pathological conditions at mucosal surfaces are thought to be the consequence of altered T cell homing. Our aim was to study the migration of T cells under such conditions in the non-human primate system. The transfer of allo- or congenic fluorescently labeled cells has provided a useful technique for the visualization of T cell activation, proliferation and migration *in vivo* and is a well established application in the mouse model. However, until now there is no equivalent technique in the

non-human primate system, which for several diseases is the only available animal model where the pathologic development is similar to that in humans.

**Methods:** Here we describe the establishment of an autologous transfer system in the rhesus macaque model. Mononuclear cells were isolated from peripheral blood, labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE) and tracked after re-injection.

**Results:** Flowcytometric analysis after transfer revealed a distinct population of labeled lymphocytes in peripheral blood and lymph nodes as well as in the intestinal mucosa of all animals. The percentage of labeled T cells in peripheral blood remained almost stable for up to 4 weeks, whereas the fluorescence intensity of the whole population decreased slightly, probably due to physiological turnover of intracellular proteins. Preliminary results indicate an increased frequency of CD4+ T cells migrating to the mucosa after transfer of *in vitro* activated cells compared to the migration of non-activated cells.

**Conclusions:** The well established transfer of CFSE labeled cells in the mouse system has been successfully adapted to the rhesus macaque system and allows us to analyse the migratory behaviour of T cells in diseases where the only available animal model is the non-human primate system, e.g. HIV/SIV infection.

### Sa2.41. The Effect of Post-Traumatic Stress Syndrome on the Outcome of Influenza Vaccination.

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Psychological stress is known to affect immune function and to influence on infectious disease susceptibility. Previous studies have demonstrated that chronic stress can impair humoral immune response to influenza vaccination but no data is available on post-traumatic stress disorder (PTSD). PTSD, according to Diagnostic and Statistical Manual of Mental Disorders (DSM-IV), is a condition (or anxiety disorder) that can occur after exposure to extreme traumatic experience and is accompanied by intense fear, helplessness or horror. Exposure to trauma can result in immune deregulation, and increasing number of evidence suggests that there are immune alterations associated with PTSD.

The aim of this study was to determine the effect of psychological stress on the immune response to influenza vaccination in war related PTSD patients ( $n = 32$ ). Peripheral blood mononuclear cells (PBMC) and sera were obtained before and 14 days after vaccination (Agrippal, Chiron, Italy) from patients and control subjects during 2003/2004 winter season. Detection of specific antiviral antibody titre in sera for all viral strains contained in the vaccine was performed with inhibition of hemagglutination (IH) assay. *Ex vivo* tetramer staining of recently activated CD8+ T lymphocytes was used for monitoring of T cell response specific for HLA-A\*0201-restricted influenza A matrix antigen (M1<sub>58-66</sub>) and haemagglutinin antigens (A/New Caledonia/H1N1, HA<sub>345-354</sub>, HA<sub>542-550</sub>) before and after influenza vaccination. Sixteen patients showed 4-fold

increase of H1N1 antibody titre 14 days after vaccination. In four of total ten HLA-A\*0201+ patients 2- to 4-fold increase of frequency of recently activated influenza-specific T cells was observed after vaccination. However, PTSD patients showed diminished frequencies of influenza-specific CD8+ T cells after vaccination compared to healthy controls. Generated humoral response in our patients argues against the hypothesis that post-traumatic stress might influence the protection following vaccination. Diminished cellular response in PTSD patients could indicate that immune dysregulation observed earlier in these patients selectively affects cellular immune response.

#### **Sa2.42. Cholesterol Loading of T Cells as a Model of T Cell Aging.**

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Lymphocytes functions are impaired in human aging explaining the increased susceptibility to diseases. It was observed that defects in T-cell receptor signal transduction could explain this age-related immune senescence. Lipid rafts are critical components of the membranes that serve for signal transduction enhancement. Our hypothesis is that changes in lipid rafts properties could explain defects in signal transduction. Cholesterol is a major component of the cell membrane and especially of lipid rafts. In this connection, we found an elevation by two folds of lipid rafts associated-cholesterol in T-cells with aging. This increase was associated to defects in lipid rafts coalescence, impairments in Lck and LAT association to lipid rafts. We sought to determine the role of cholesterol in immune senescence. We modulated cholesterol content in peripheral T-cells from normolipemic young donors using a mixture of cyclodextrin and cholesterol. Cholesterol content in whole cell extracts as well as in lipid rafts was measured by HPLC. We were able to increase cellular cholesterol content by 1.5 fold to up to 7 folds. We observed changes in cell shape and size (microscopy, flow cytometry) as well as a dramatic loss of membrane fluidity. We analyzed T-cell proliferation and protein phosphorylation (western-blotting) following CD3 and CD28 stimulation. We found that an *in vitro* increase in membrane cholesterol by 2-folds have similar consequence on signal transduction as observed in human aging. We could here establish a T-cell model of aging by cholesterol modulation. Altogether, these data suggested that signal transduction critically depends on membrane cholesterol content and that changes in lipid rafts properties may be taken into account to explain immune senescence as well as in other pathological diseases.

#### **Sa2.43. The Role of Programmed Death-1 Pathway in a Neutrophil Mediated Shock Syndrome.**

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Programmed death-1 (PD-1) and its ligands PD-L1 and PD-L2 are members of B7/CD28 superfamily, and play important roles in modulating the adaptive immunity in experimental autoimmune encephalomyelitis (EAE), type-1 diabetes, asthma and transplantation. In the study of PD-1 pathway in EAE, we found that

multiple injections of either PD-1 or its ligand blocking antibody after standard EAE induction caused a shock syndrome in multiple mouse strains, which included 129X1/SvJ, C57BL/6, and SJL/j mice. Animals showed piloerection, cyanosis, drop in body temperature and were eventually dead within 2 hours of antibody injection. Interestingly, BALB/c mice, a strain known to be TH2 prone, are relatively resistant to the development of shock syndrome. In addition, the blockade of PD-1 pathway did not enhance OVA peptide induced anaphylaxis in BALB/c mice. Pathological study in shock mice found massive neutrophil infiltration in the lung, liver and skin, suggesting PD-1 pathway blockade directly or indirectly activated neutrophils in this model. Consistently, freshly isolated neutrophils from normal mice were found to express PD-1 and PD-L1, and neutrophils isolated from anti-PD-L1 treated mice showed enhanced and accelerated superoxide production upon PMA stimulation. We are currently further studying the role of PD-1 pathway in animal models known to involve neutrophils in pathogenesis, such as sepsis and ischemia-reperfusion injury.

#### **Sa2.44. Immune Response Balance on Human Cutaneous Leishmaniasis.**

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T helper lymphocytes play a major role on infectious diseases and these cells can be driven into at least two different subpopulations according to their cytokine production pattern. Th1 lymphocytes are implicated on the resolution of infections caused by intracellular pathogens such as *Leishmania* species, while Th2 cells are thought to be important to the establishment of these infections. This Th1/Th2 balance was previously showed to be able to drive antibody isotype switching. Based on this knowledge ELISA tests were performed to measure the plasma levels of anti-*Leishmania* specific IgG1, IgG3, IgG4 and IgE antibody isotypes. Production of Interleukine-4 and IFN- $\gamma$  by peripheral blood mononuclear cells (PBMC) from subjects living in an endemic area of American Cutaneous Leishmaniasis (Bahia State, Brazil) was analysed. Patients were grouped on active lesions ( $n = 20$ ), cured lesions (scars) ( $n = 40$ ) and asymptomatic ( $n = 34$ ). PBMC were cultured for 24 and 96 hours stimulated with *Leishmania braziliensis* antigens. The analysis of IgG isotypes response showed that both IgG1 and IgG3 were able to differentiate the group of patients presenting active lesions from the others (Kruskal Wallis;  $P = 0.0004$  and  $P = 0.0004$ , respectively). Moreover, the analysis of cytokine production revealed that IFN- $\gamma$  level was significantly higher on patients with active or cured leishmaniasis than on the asymptomatic group (Kruskal Wallis;  $p < 0.0001$ ). There was a correlation between the production of IFN- $\gamma$  by PBMC stimulated with *L. braziliensis* antigens and the plasma levels of IgG1 and IgG3 antibody isotypes in patients presenting active lesions (Spearman Correlation;  $P = 0.0065$  and  $P = 0,0107$ , respectively). The level of IL-4 was significantly higher on patients presenting active lesions than on the others (Kruskal Wallis;  $P < 0.0001$ ). The results confirm that IFN- $\gamma$  is produced during human Cutaneous Leishmaniasis and that, during active lesions, a concomitant high production of IL-4 is observed. Our results also point out that IgG1 and IgG3 are useful antibodies to differentiate patients with active lesions.

### Sa2.45. Investigatin of IL-4 Gene Polymorphism in Patients with Brucellosis.

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Brucellosis is endemic in many Middle East countries including Iran, where it undermines animals and human health. The Immune response against *Brucella* involves both humoral (Th2) and cell-mediated (Th1) immunity. The Th1/Th2 cytokine ratio seems to be involved in the susceptibility or resistance to *Brucella* infection. Th1 cytokines confer resistance were as Th2 cytokines predispose to brucellosis. IL-4 is a Th2 cytokine which its production can be affected by its gene polymorphism at position -590(C/T). The aim of this study was to investigate the association between IL-4 gene promoter polymorphism and susceptibility to brucellosis.

One hundred and ninety seven patients with brucellosis, 186 healthy individuals who were members of patients family and 82 healthy animal husbandmen who had animals involved with brucellosis, were included in this study. All individuals were genotyped for IL-4 gene promoter polymorphism at position -590(C/T) using PCR-RFLP.

The results showed the distribution of CC genotype is significantly more in animal husbandmen than the patient group ( $P = 0.034$ ). No statistical significant differences in genotypes distribution were shown between patients and their healthy family, but there was a trend toward increased frequency of CC genotype in family group ( $P = 0.063$ ).

As data shown, the distribution of CT and TT genotype which were associated with more production of IL-4 were increased in patients (29.3%) compare to animal husbandmen (17.4%,  $P = 0.034$ ) and patients' family members (21%,  $P = 0.063$ ). We suggest that individuals who carry T allele can produce more IL-4 and this cytokine induce Th2-type immune response which could be important to develop a full-pictured brucellosis. While, individuals with CC genotype can probably induce a Th1-type immune response more effectively and control the *Brucella* before developing the disease.

### Sa2.46. Immunophenotype Characterization of Peripheral Blood T Lymphocytes before and after Treatment in Tuberculosis Patients.

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**Introduction:** Tuberculosis is a chronic mycobacterial infection. The main effector cells against mycobacterium tuberculosis are CD4<sup>+</sup> T lymphocytes.

**Objective:** Our objective in this research was to evaluate the quantity of T lymphocytes and their subpopulation before and after treatment with combination of 4 drugs recommended

by WHO (DOTS Protocol) in sputum-positive tuberculosis patients.

**Materials and Methods:** 20 patients as cases and 20 healthy people were selected as controls. Flow cytometry was done for TCD3+, TCD4+ and TCD8+ lymphocytes by use of monoclonal antibodies.

**Result:** Our results showed that there was a defect in cell mediated immunity during tuberculosis showing itself as decrease in TCD3+ and TCD4+ lymphocytes and increase in TCD8+ lymphocytes. The changes in TCD3+ and TCD4+ but not in TCD8+ were reversible after 2 months of treatment.

**Conclusion:** The result of our study and other studies confirm defect in cell mediated immunity during infection with mycobacterium tuberculosis. Although decrease in CD4<sup>+</sup> lymphocytes that are main cells in defense against tuberculosis, has been established, there is no consensus about decreasing or increasing of CD8+ lymphocytes in tuberculosis.

### Sa2.47. Class I Restricted Epitopes Highly Represented in the *Trypanosoma cruzi* Genome Are Recognized by CD8+ T Cells from Individuals with Chronic *T. cruzi* Infection.

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Immunity to *T. cruzi* is complex, minimally involving a substantial antibody response and the activation of CD4+ and CD8+ T cell responses.

We have recently shown that chronic chagasic patients with mild clinical disease have a significantly higher frequency of IFN- $\gamma$  producing T cells specific for a parasite lysate than do individuals with severe disease. However, it has been difficult to identify the specific epitopes recognized by *T. cruzi*-specific T cells, and thus to quantify the response of CD8+ T cells from infected individuals. In *T. cruzi*-infected mice, peptides from transsialidase family proteins (ts), are dominant targets of the CD8+ T cell response. In the present study, we have investigated the response of CD8+ T cells from HLA-A2.1+, *T. cruzi*-infected individuals to ts family, HLA-A2.1-binding epitopes.

The 1294 genes annotated as encoding ts family proteins were screened for homologues of two ts-derived, HLA-A2.1-binding peptides that we had previously found to be recognized by a low frequency of CD8+ T cells in HLA-A2.1+ infected subjects. This screening yielded a total of 845 homologues, 71 of which were encoded by >50 ts. Thirty-two of the 71 peptides elicited IFN- $\gamma$  production by CD8+ T cells from HLA-A2.1 transgenic mice chronically infected with *T. cruzi*, with peptides with the highest frequency of occurrence and the highest predicted HLA-A2.1-binding affinity stimulating the greatest response. Antigenicity of ts peptides was fairly associated with the capacity to bind multiple molecules from the A2 supertype. To ascertain whether these epitopes were recognized by CD8+ T lymphocytes from *T. cruzi*-infected subjects. IFN- $\gamma$  ELISPOT responses were evaluated with PBMC from chronic chagasic patients.

IFN- $\gamma$  secretion was demonstrated in 10 out of 17 patients (59%) with mild disease, with 24 out of the 28 peptides assayed recognized by at least one patient. For each responder, the

frequency of peptides that were capable of eliciting recall IFN- $\gamma$  responses varied between 11% (3 of 28 tested) and 100% (5 of 5 tested); peptides ts3, ts37, ts38, ts44 and ts66 being the most frequently recognized. By contrast only 2 out of 10 patients with severe clinical stages showed positive responses. These data demonstrate that the frequency of IFN- $\gamma$  producing T cells in chronic chagasic patients is determined in part by the clinical status of the patient and also by the frequency of occurrence of the epitopes in the *T. cruzi* genome. We have identified a very specific set of class I MHC restricted peptides target of immune responses in patients capable of controlling *T. cruzi* infection. The identification of CD8+ T cell targets in the natural infection with *T. cruzi* may be applicable to the development of vaccines against *T. cruzi*.

#### **Sa2.48. Sustained Expansion, Activation and Maturation of Virus-Specific CD8+ T Cells after Acute Parvovirus B19 Infection.**

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Human Parvovirus B19 ('B19') is a ubiquitous DNA virus that causes erythema infectiosum, polyarthropathy, transient aplastic crisis and fetal death. IFN-gamma elispots, intracellular cytokine staining, and HLA-peptide tetrameric complex ('tetramer') staining were used to identify CD8+ T lymphocyte responses to the non-structural protein NS1. To understand the origins of such responses and their potential role in disease we prospectively analysed the evolution of virus-specific CD8+ T cell responses, in five individuals, during and after acute infection. Individuals responded to B19 tetramers at frequencies up to 2% CD8+ T cells. Surprisingly their responses increased over the first year post infection despite resolution of clinical symptoms and control of viremia. Parvovirus-specific CD8+ T cells rapidly developed and maintained an activated CD38+ phenotype, with strong expression of perforin and CD57 and downregulation of CD28 and CD27. These cells possessed strong effector function and intact proliferative capacity (demonstrated by IFN-gamma elispots, Chromium-51 release assays and tetramer staining of B19-specific CD8+ T cell lines). **In** remotely infected individuals lower frequencies of cells specific for individual epitopes were observed, typically <0.5% of CD8+ T cells, which were CD38 low although also CCR7 low. The likely explanation for the striking persistent expansion of such activated mature effector cells after acute resolution of infection- analogous to CMV infection- is through low level antigen exposure. Such cells may contribute to the long term control of this significant pathogen.

#### **Sa2.49. Defining Putative T Cell Epitopes from PE and PPE Families of Proteins of *Mycobacterium tuberculosis* with Vaccine Potential.**

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There is substantial evidence that BCG provides partial protection against pulmonary tuberculosis. But this evidence does

not convincingly demonstrate that BCG prevents tuberculosis (TB) development. The availability of *Mycobacterium tuberculosis* H37Rv genome sequence has thrown open new opportunities for designing a rational vaccine for TB. The identification of T cell epitopes from immune relevant antigens remains a critical step in the development of vaccines. The discovery of two multigene families, PE and PPE, which make up to 8% of *Mycobacterium tuberculosis* H37Rv genome has kindled an interest in using these proteins as potential vaccine candidates. All possible nonameric peptide sequences from PE and PPE proteins were analysed computationally, for their ability to bind to 33 alleles of class I HLA. Of all PE and PPE proteins, a significant number of these peptides are predicted to be high affinity HLA binders, irrespective of the length of the protein. Structural basis for peptide binding to HLA was carried out by molecular modeling studies. The structural studies correlated well with the binding prediction. Two proteins from PE and PPE family of *Mycobacterium tuberculosis* genome have been selected for further study as these proteins show high percentage of binding peptides. The recombinant proteins were tested for their ability to elicit immune response in mouse model. The recombinant Rv1818c is good inducer of antibody response but very poor inducer of T cell response, where as Rv3812 is shown to induce good T cell response in terms of invitro T cell proliferation, IFN $\gamma$  secretion and DTH response. The predicted epitopes are experimentally verified by identification of T cells, which specifically recognize the naturally processed epitope in an HLA-restricted fashion. These peptides may be used in a vaccine cocktail keeping in mind the HLA haplotype profile of the population.

#### **Sa2.50. The Study between the Changes of CD35 Expressed on Erythrocyte in Patients with Hepatocirrhosis and the Severity of Liver Function Destroying.**

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**[Objective]** To study the relationship between the changes of CD35 expressed on erythrocyte (ECD35) in patients with hepatocirrhosis and the severity of liver function destroying. **[Methods]** One-step detection method of ECD35 has been used 108 cases in hepatocirrhosis as followed, 2 percent of Glutaraldehyde-fixed red blood cells (RBC) are analyzed in V well microstate plates. To the cell buttons in each well, the compound of b-CR1Ab+SA\*AP was added and mixed thoroughly. The microtitre was incubated at 37°C for 45min, followed by three washes in 1% S/BSA. Substrate was added and incubated at 37°C for 20min. The supernatant was transferred to a clean U microtitre plate to facilitate reading in a plate reader at 405nm (A<sub>405</sub>). The number of CD35 was calculated by the standard of red cell. Another detection of ALT, CHE, ALB, GLO, TBIL, and GAM were assayed with the machine of AU 5400. **[Results]** The number of ECD35 in patients with Hepatocirrhosis was significantly lower than that in healthy individuals. The patients were divided in to two groups according to the levels of ECD35 by 200/cell. The results showed that the patients with low activity of CHE and decreased ALB were almost in low CD35 number groups, the levels of GLO, TBIL and GAM are increased accordingly. There is no obviously relationship between the changes of ALT and ECD35. **[Conclusions]** The results suggest that the quantity of CD35 on erythrocytes may be involved in pathogeny, development and

prognosis of the hepatocirrhosis. The detection of the Quantity of ECD35 maybe used as an important assay in liver functions

### Sa2.51. Deficient IgM to IgG Antibody Switch in Patients Unresponsive To Conjugate Pneumococcal Polysaccharides.

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**Introduction:** We have identified a group of patients with recurrent respiratory infections who have normal total immunoglobulins and normal response to protein antigens but who failed to develop IgG antibodies to the Pneumococcal 7-valent conjugate vaccine (PCV7) serotypes. **Rationale:** To investigate if the mechanism of unresponsiveness to the PCV7 vaccine could be due to a defect in isotype switching. **Methods:** This is a retrospective study of 60 patients referred to our clinic for evaluation of recurrent respiratory infections who received complete immunization with the PCV7 vaccine for age, according to ACIP guidelines. All patients were evaluated with total immunoglobulin and IgG subclass concentrations and their immunization history was documented. We measured IgG and IgM anti-pneumococcal polysaccharide (PP) antibody titers against serotypes 1, 4, 6B, 9V, 14, 18C, 19F and 23F by a standardized ELISA test. The data were analyzed using Epi Info and SPSS. Samples were obtained from patients who were already immunized by their primary care physicians. The patients were assembled into 3 groups, an unimmunized group with laboratory data prior to the vaccine and two immunized groups of responders and non-responders according to their IgG anti-PP antibody titers. Non-responder patients were defined as those with IgG anti-PP antibody titers to all 7 serotypes included in the PCV7 vaccine not statistically different from unimmunized controls. **Results:** Despite differences in IgG anti-PP antibody titers, both responder and non-responder patients had IgM antibody titers to all 7 serotypes significantly higher than those of unimmunized controls. **Conclusions:** These results suggest that a deficient IgM to IgG antibody switch may be the explanation for the failure of patients to respond to conjugate pneumococcal polysaccharides. The cellular and molecular mechanisms underlying this defect are under investigation.

### Sa2.52. A Delayed Diagnosis of Cystic Fibrosis in a Patient with Asthma and Allergic Bronchopulmonary Aspergillosis.

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#### INTRODUCTION:

Allergic Bronchopulmonary Aspergillosis (ABPA) is a complex hypersensitivity reaction including both IgE- and IgG-mediated immune responses following *Aspergillus* colonization of an asthmatic airway. Cystic Fibrosis (CF) is a multisystem disease characterized by disordered exocrine gland function. Disease manifestations include respiratory, gastrointestinal, hepatobiliary, pancreatic, and reproductive dysfunction. ABPA is extremely difficult to recognize in the context of CF, as clinical, radiographic, and immunologic features frequently overlap.

#### CASE REPORT:

A 49 year old Caucasian female with a past medical history of steroid-dependent asthma, allergic rhinitis, ABPA, and frequent pneumonias presented with acute maxillary sinusitis. Pediatric

medical history was unremarkable. Asthma was first diagnosed in the patient's third decade of life. Past medical history was additionally remarkable for infertility and intermittent episodes of hemoptysis. At 26 years of age *Mycobacterium intracellulare* pneumonia was diagnosed with symptoms of respiratory distress and hemoptysis. Two years later, ABPA was diagnosed. In the subsequent 23 years, the patient had multiple hospitalizations for "asthma" exacerbations, hemoptysis, and pneumonia. Daily oral steroids and intermittent courses of antibiotics were given to control asthma symptoms and infections. At 43 years of age, the patient was hospitalized for *Pseudomonas* pneumonia with symptoms of respiratory distress and hemoptysis. At 49 years of age, while presenting with acute sinusitis, a chest and sinus CT were performed. Sinus CT was remarkable for chronic sinusitis changes, and chest CT demonstrated multifocal bilateral cylindrical bronchiectasis, with multiple mucous-impacted dilated bronchi. Sweat chloride testing and genetic testing were consistent with the diagnosis of CF with the  $\Delta F508$  and L206W mutations.

#### CONCLUSIONS:

This case illustrates a delayed diagnosis of CF, in the setting of an adult patient with recurrent infections, atypical pneumonias, asthma, and ABPA. It further demonstrates the importance of clinical suspicion for CF in patients of all ages who present with a history of asthma and frequent sinopulmonary infections. Sweat chloride testing is a non-invasive testing modality that may dramatically alter both management and prognosis for patients with CF.

### Sa2.53. Determination of *Borrelia burgdorferi* Outer-Surface Protein A<sub>161-175</sub> Peptide Binding to HLA-DR Molecules Associated with Antibiotic-Refractory or Antibiotic-Responsive Lyme Arthritis.

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**Objective:** Clinical correlations have linked antibiotic-refractory Lyme arthritis with certain HLA-DR molecules and the *B. burgdorferi* T cell epitope, outer surface protein A<sub>161-175</sub> (OspA<sub>161-175</sub>). Previously, HLA-DRB1\*0401, 0101, and 0404 molecules, which are associated with antibiotic-refractory arthritis were shown to bind this peptide, whereas the DRB1\*0801 and 1101 molecules, which are associated with antibiotic-responsive patients, did not. To further these studies, we determined the relative binding affinity of OspA<sub>161-175</sub> to 14 HLA-DR molecules, and correlated peptide binding with MHC frequency in antibiotic-refractory or antibiotic-responsive arthritis patients.

**Methods:** The cDNAs of the extracellular domains of DR $\alpha$  and DR $\beta$  attached to the leucine zipper sequences were cotransfected and purified in *Drosophila* cells. DR molecules were purified by affinity chromatography. MHC-peptide binding was detected by the capture of HLA-DR/Eu-OspA<sub>161-175</sub> complexes on DR antibody coated plates. Dilutions of the peptide (0.0001-50  $\mu$ M) were tested, and the half maximal binding concentration (1/2 max) was determined. Binding affinity was correlated with the frequency of each MHC molecule in 71 antibiotic-refractory and 50 antibiotic-responsive patients.

**Results:** Of the 14 MHC molecules tested, 7 bound the OspA<sub>161-175</sub> peptide. The DRB1\*0401 molecule bound the peptide

strongly ( $1/2 \text{ max} = 0.003 \mu\text{M}$ ), the DRB1\*0101, 0404, or 0405 molecule bound it moderately well ( $1/2 \text{ max} = 0.2\text{--}0.4 \mu\text{M}$ ), and the DRB1\*0402 or 0102 molecule bound it weakly ( $1/2 \text{ max} = 4\text{--}6 \mu\text{M}$ ). In addition, testing of the linked and equally expressed DRB1\*1501/DRB5\*0101 molecules showed that this DRB5 molecule, but not the DRB1 molecule, bound the peptide moderately well. Altogether, 79% of patients with antibiotic-refractory arthritis had at least one of these 7 OspA peptide-binding HLA-DR molecules compared with 46% with antibiotic-responsive arthritis (odds ratio = 4.4,  $P < 0.001$ ). In contrast, the DRB1\*0301, 0801, 1101, 1104, 0701 and DRB4\*0101 molecules showed no binding of the OspA peptide. Only 21% of the antibiotic-refractory patients had one of these alleles compared with 54% of the antibiotic-responsive patients (odds ratio = 0.2,  $P < 0.001$ ). Moreover, patients with two OspA<sub>161-175</sub>-binding alleles were 9.6 times more likely (CI 1.9-46.9) to have an antibiotic-refractory course than patients with two non-OspA<sub>161-175</sub> binding alleles.

Conclusion: The highly significant correlations of OspA<sub>161-175</sub> peptide binding to implicated HLA-DR molecules further supports the hypothesis that T cell recognition of this bacterial epitope is important in the autoimmune pathogenesis of antibiotic-refractory Lyme arthritis. This is the only form of chronic inflammatory arthritis in which the identity of each component of the disease-associated tri-molecular, MHC-peptide-TCR complex has been deciphered.

#### **Sa2.54. Mycobacteria Directly Induces Cytoskeletal Rearrangements for Macrophage Motility through TLR2-Dependent PI3. K Signaling.**

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Cell migration and adhesion are both important for controlling mycobacterial infection and are critically dependent on the reorganization of the cytoskeleton. Mycobacteria elicits rapid morphological changes such as cell spreading the process relevant to *in vivo* changes of macrophage shape during extravasation and migration. In this study we investigated the BCG mycobacteria-induced signaling events leading to macrophage cytoskeletal rearrangements employing specific pharmacological inhibitors to suppress distinct kinase pathways known to be elicited by infection. Viable or lysed mycobacteria, as well as purified cell wall lipoprotein p19, TLR2 agonist, induced RAW264.7 cells to extend actin-rich pseudopods which imparts circular spreading within 30 min that was substituted by persistent cell polarization 24h post-treatment. BCG induced rapid phosphatidylinositol 3- kinase, PI3-K, activation which become recruited to the activated TLR2 receptor. Suppression of PI3-K with LY294002 inhibitor abrogated generation of cell protrusions and polarity demonstrating the involvement of PI3-kinase pathway in actin reorganization essential for cell motility. Inhibition of MEK1/ERK kinases with PD98059 reduced the number of polarized cells but did not prevent filopodia or lamellopodia formation suggesting the role of this pathway in stabilization of leading lamellopodia. Nor NF- $\kappa$ B nor p38MAPK activation by BCG were important for cytoskeletal rearrangements observed although their suppression inhibited chemokine and growth factors secretion by activated macrophages that could promote the cell motility in an autocrine manner.  $\beta$ 1-integrins blockade with a corresponding antibody

inhibited macrophage spreading and polarization but had no effect on pseudopod protrusions or PI3-K activation demonstrating the down-stream position of integrin-mediated adhesion in signaling pathway leading to motility phenotype. The data obtained demonstrate that direct effect of mycobacteria on macrophage shape at least in part is mediated through TLR2-dependent PI3-K activation.

Financial support: CNPq, FAPERJ, CAPES.

#### **Sa2.55. Chlamydia pneumoniae Infection of Human Immune Cells Modulates Cytokine Production.**

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*Chlamydia pneumoniae* (Cpn) is an obligate intracellular bacterium known to cause acute upper respiratory tract infections, but recent studies show Cpn is also involved in a variety of chronic inflammatory diseases, including atherosclerosis and possibly autoimmune diseases like multiple sclerosis as well as arthritis and asthma. In fact, we have reported that this organism can be detected by PCR for Cpn specific 16s rRNA in human peripheral blood mononuclear cells associated with subclinical chronic bacterial infection. Even though this organism grows preferentially in epithelium cells in the respiratory tract, in the present study we show that these bacteria can infect and replicate in the human monocytic cell line (THP-1) and the human T cell line (Molt-4) and also human peripheral blood mononuclear cells *in vitro* as detected by FITC-conjugated anti-Chlamydia monoclonal antibody (specific to Chlamydia LPS) staining or PCR for Cpn 16s rRNA. The Cpn mRNA level was upregulated at 48h as compared to 24h after infection assessed by real-time PCR for Cpn 16s rRNA ( $p < 0.05$ ). We also found that Cpn infected cells evinced marked alteration of cytokine production important in immune responses to microbial infection. Cpn infection of human immune cells modulated a variety of cytokines. In particular, Cpn infected MOLT-4 and THP-1 cells suppressed TGF  $\beta$ 1 production as compared to uninfected cell assessed by ELISA ( $p < 0.05$ ). In contrast, Cpn infection of the human cell lines *in vitro* markedly stimulated production of proinflammatory cytokines, including TNF  $\alpha$  and IL 10. Interestingly Cpn infected PBMCs enhanced production of TNF  $\alpha$ , IFN  $\gamma$ , IL 4 (PHA induced), IL 10 and IL 12 as compared to lower TGF  $\beta$ 1. Infection with viable Cpn stimulated more IL 12 and IFN  $\gamma$  (Th1 cytokines) and less IL 4 (Th2 cytokines) as well as TGF  $\beta$ 1 (Th3 cytokines) compared to stimulation with heat killed Cpn in PBMCs. This may be related to antibacterial immunity by this common opportunistic bacterium. Further studies are warranted to assess the role of Chlamydia on immune responsiveness, since Cpn are considered important in induction and progression of atherosclerosis, neurologic or autoimmune diseases. The cytokine profile differences observed appeared related to antibacterial immunity and perhaps autoimmunity. Thus, infection of human immune cells with Cpn may result in significant immune deregulation.

#### **Sa2.56. Homeostatic Chemokines: Organizers of Cellular Interactions Required for T Cell Priming in the Pulmonary Environment during the Influenza Infection.**

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**Background:** Homeostatic chemokines (HCs) play an important role in the compartmentalization of B- and T cell environments

in secondary lymphoid organs (SLOs). Additionally, at early embryonic steps of the development of these tissues, they organize the recruitment of inducer cells (CD3-CD4+CD45+), facilitating their interaction with organizer populations (VCAM+ICAM+). This cellular event promotes a second wave of chemokine production, responsible for the latter recruitment of immune cells to their future homes in B- and T cell zones. Additionally, HCs have been also detected at sites of inflammation, where they contribute to the formation of ectopic tertiary lymphoid structures. **Rationale:** in our murine model of influenza infection, we detected HC local production by Northern blot, Taqman-PCR and immunohistochemistry. This finding encourages us to explore the participation of these molecules during the pulmonary immune response against influenza viruses. **Methods:** C57BL/6-, cxcl13 -/-, plt/plt -/- and It alpha -/- mice were infected with PR8 influenza virus and sacrificed at different time points, collecting spleens and lungs for the analysis of the immune response and the course of the infection. Cellular interactions and distribution of immune cells was evaluated by immunohistochemistry and immunofluorescence. Taqman PCR was a useful tool for measuring chemokine production. Finally, by using flow cytometry, we determined the phenotype of immune cells, the functionality of CD8 T cells and the migration of T and B-lymphocytes to the spleen of uninfected mice. **Results:** we found that the absence of homeostatic chemokines is highly associated to the generation of altered cellular interactions in the local environment, which modified the course of the infection. It appears that their production is playing an important role in the organization of the B- and T cell microenvironments in the lung, in a similar way than they do in SLOs. Interestingly, it was appreciated that in the absence of the draining lymph node (cxcl13 -/-) or adequate APC-T cell interactions (plt/plt -/-), influenza-infected mice were still able to mount an adequate primary T cell response, killing the vast majority of viruses. Even though, the viral clearance was a little bit delayed in Knockout mice, compared with the fast killing of virus found in wild type mice. **Conclusions:** Our findings reinforce the idea that the lung can work as an alternative place for T cell priming and that local cellular interactions could be enough for the initiation of the T cell response against Influenza viruses.

### Sa2.57. Human 'Memory' CD4+ T Cells Are Distributed between Five Phenotypically and Functionally Distinct Subsets.

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T cells are heterogeneous, varying in terms of their phenotype, functional capabilities and history of antigen-encounter. The derivation of a functionally relevant model for classifying CD4+ T cells has been hampered by limitations on the numbers of parameters that may be measured using classical 4-colour flow cytometry. The purpose of this study was to develop a detailed, functionally meaningful scheme for classifying human CD4+ T cells. To achieve this we have taken advantage of the introduction of reagents for 5-colour flow cytometry and have performed multi-colour FACS analysis on resting, polyclonally-activated and antigen-stimulated preparations of peripheral blood mononuclear cells taken from healthy individuals. We show that CD4+ T cells are predominantly distributed between six of eight possible subsets identified by expression of CCR7, CD45RA and CD28.

One subset corresponds to the previously described naive compartment and one has the phenotypic properties of the described central memory compartment. Two of the remaining four subsets include antigen-experienced CD4+ T cells that express CD45RA. We find clear differences between CD4+ T cells within the different subsets in terms of their expression of cytolytic mediators, their capacity to secrete cytokines and their ability to proliferate. Furthermore, we find that T cells specific for different virus infections accumulate within different subsets, implying that protective immunity against different pathogens involves CD4+ T cells with different phenotypic and functional properties. On the basis of these results we propose a cross-sectional model for classification of peripheral CD4+ T cells. Knowledge of where T cells lie on this model informs about their functional capacity and can reflect their history of antigen-exposure.

### Sa2.58. Characterization of the Th Cellular Immune Response Induced by the Diphtheria Toxin Cry1A Mutants in Spleen Cells from Intranasally Primed BALB/c Mice.

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The objective of this work was to characterize directly the Th1 (IL12p70, IFN-g), Th2 (IL4, IL10) cytokine patterns induced by spleen cells from BALB/c mice presensitized by intranasal route and restimulated with diphtheria toxin Cry1A mutants. Differential cytokines induced by spleen cells were measured and quantified by ELISA. We found that each set of toxins are priming different effectors T cells, measured by. Wild type Cry1A toxins are priming mostly Th1 cytokine producers whereas DTB quimeric toxins are priming both Th1-Th2 cytokine producers cells, suggesting that eight B fragment diphtheria toxin motif exchanged in domain I from Cry1A toxins are influencing the capacity of these toxins to modulate the Th cellular immune response. In addition the data support the assumption that helical topology from domain I contribute to the cellular properties of the Cry proteins by acting like epitopes for T cells subsets and this in agreement with theoretical predictions, using ANTHEPROT program. By other hand it was observed that Cry1A toxins have the ability to induce regulatory T cells. In this aspect Cry1A toxins represent a good model to study several aspects of immunoregulation at the molecular and cellular level in mice.

### Sa2.59. Intranasal Vaccination with Chlamydial Protease-Like Activity Factor and Interleukin-12 Promotes the Clearance of Pulmonary *C. trachomatis* Infection.

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*Chlamydia trachomatis* is a gram-negative bacterium that infects the human conjunctiva, respiratory and genital tract, and is a cause of significant morbidity worldwide. Chlamydia has evolved various immune evasion mechanisms that have posed complex problems in the development of an efficacious anti-chlamydial vaccine. HeLa cells infected with *C. trachomatis* display a progressive reduction in immunostaining for  $\beta$ 2-microglobulin (MHC class I), which parallels the accumulation of intracellular Chlamydial protease-like

activity factor (CPAF). Therefore, neutralization of CPAF activity in vivo may be a viable vaccine approach against *C. trachomatis* infection. To this end, we have shown the efficacy of interleukin-12 (IL-12) as a potent mucosal adjuvant. BALB/c mice were immunized intranasally (i.n.) with CPAF + IL-12 and challenged i.n. with *C. trachomatis*. CPAF + IL-12 immunized animals displayed significantly greater reduction (>95%) in lung bacterial load on day 10 after *C. trachomatis* challenge as compared to mice immunized with CPAF, IL-12 or PBS alone. CPAF + IL-12 immunization induced significantly higher titers of serum anti-CPAF total Ab, IgG2a and IgG2b antibodies as compared to animals immunized with CPAF, IL-12 or PBS alone. Furthermore, pulmonary anti-CPAF total Ab, IgG2a and IgA were induced to higher levels in animals receiving CPAF + IL-12 as compared to other immunization groups. In addition, CPAF + IL-12 immunized MHC class I deficient mice displayed reduced bacterial clearance after pulmonary *C. trachomatis* challenge as compared to vaccinated wild type animals. Together, these results demonstrate that i.n. CPAF + IL-12 immunization induces robust systemic and mucosal antibody responses and may enhance the clearance of *C. trachomatis* from the lungs via a CD8+ T cell mediated mechanism. (Supported by National Institutes of Health grants AR048973-02 and SO6GM008194-24)

#### **Sa2.60. Identification of ssRNA Sequences That Stimulate Innate Immunity through TLRs.**

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Viral infection of the mammalian host triggers the immune system to innate and adaptive immune responses. Recognition of these conserved pathogen-associated molecular patterns (PAMP) is directed mostly by Toll-like receptors (TLR). Ten TLR family members have been reported in human where TLR9, 8, 7 and 3 respond to nucleic acid derivatives and are intra-endosomal in location, potentially allowing for viral PAMP recognition.

Recently, we have discovered that single-stranded RNA (ssRNA) stimulates both human and mouse immune cells to secrete cytokines and chemokines. In response to ssRNA human PBMC release IFN-alpha, IFN-gamma, TNF-alpha, IL-6, IL-8, IL-10, IL-12p40, IP-10 and MCP-1 while Th2 cytokines and chemokines were not detected. Monocytes were the main source for TNF production, pDC the main source for IFN-alpha and NK-, NK/T-cells the main source for IFN-gamma production upon stimulation with viral derived ssRNA sequences. We have identified genomic regions and sequences from ssRNA viruses that are stimulatory and can show that these responses are sequence specific. ssRNA sequences are currently under pre-clinical investigation and may serve as potential vaccine adjuvants and immune modulators.

#### **Sa2.61. Mechanism of In Vivo Cytotoxicity Mediated by CFP10-Specific CD8+ T Cells.**

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The human pathogen *M. tuberculosis* is responsible for more deaths than any other infectious disease. The public health problems posed by tuberculosis have grown more serious as a

consequence of the global AIDS epidemic and the emergence of multidrug resistant strains of *M. tuberculosis*. To combat this ongoing worldwide scourge, vaccine development for tuberculosis continues to be a global priority. Most infected individuals develop long-lived protective immunity, which is able to control and contain *M. tuberculosis* in a manner that is T cell-dependent. Thus, there has been considerable interest in understanding how different T cell subsets contribute to the primary immune response following infection, and whether those T cells can be elicited by vaccination and can mediate protection against *M. tuberculosis*. This information will be required rationally design the best vaccine to advance into human trials. There is excellent experimental evidence that CD8+ T cells participate in the immune response to tuberculosis, which has generated interest in vaccine strategies that elicit *M. tuberculosis*-specific CD8+ T cells. To activate CD8+ T cells, mycobacterial antigens need to enter and be processed by the MHC class I pathway, which can be targeted by using DNA vaccines or on live attenuated vaccines strains. Unfortunately, evaluating the role of CD8+ T cells in host resistance and determining whether vaccines elicit protective CD8+ T cells has been hampered because few mycobacterial antigens have been identified that are recognized by CD8+ T cells. Thus, even basic questions concerning the function of CD8+ T cells during *M. tuberculosis* infection remain unanswered.

We have identified an epitope of the CFP10 protein that is recognized by up to 30% of the CD8+ T cells in the lungs of mice following *M. tuberculosis* infection. Interestingly, deletion of the region of the mycobacterial genome that encodes CFP10 was the original event that resulted in the attenuation of *M. bovis* BCG and also attenuates *M. tuberculosis*. Furthermore, most *M. tuberculosis* infected people have evidence of immunity to the CFP10 antigen. As predicted, CFP10-specific CD8+ T cells were detected in mice infected with the Erdman and H37Rv *M. tuberculosis* strains, but not in mice infected with H37Ra or BCG. Importantly, CFP10-specific CD8+ T cells recognized *M. tuberculosis* infected macrophages. We have previously shown that in vivo cytolytic activity of CD8+ T cells specific for mycobacterial antigens could be detected in the spleen, pulmonary LN and lungs of infected mice. We now present our studies on the how the cytolytic activity is mediated including the finding that it is independent of the CD95/95L pathway. We now in the process of determining whether perforin and Rab27a are required for in vivo cytotoxicity following infection. These studies highlight the cytolytic function of antigen-specific CD8+ T cells elicited by *M. tuberculosis* infection and enable us to determine whether the cytotoxic function of CD8+ T cells is required for optimum pulmonary immunity to *M. tuberculosis* infection.

#### **Sa2.62. Characterization of an Lck-Independent Pathway of T-Cell Activation Used by Bacterial Superantigens: Mechanistic and Therapeutic Implications on Autoimmunity.**

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The current paradigm to explain activation of mature T cells following engagement of their T-cell antigen receptor (TCR) with peptide:MHC molecules relies on early activation of the src family kinase Lck. Lck phosphorylates tyrosine-based activation motifs on the subunits of the TCR complex, providing sites for recruitment and activation of the syk family kinase ZAP-70. ZAP-70 phosphorylates the transmembrane adapter LAT providing docking sites for the assembly of multimolecular complexes. These signalosomes trigger several signaling cascades that cause translocation / activation of transcription factors leading to changes in gene expression. Despite the requirement for Lck under most conditions, T cell activation can proceed in the absence of Lck under some circumstances. One of these involves the activation of T cells by superantigens (SAG), a type of stimuli implicated in the development of autoimmunity. Also, we and others have shown that TCR partial agonists with the capacity to induce T cell tolerance also use an Lck-independent TCR signaling pathway. We have dissected Lck-independent TCR signaling on human T cells upon stimulation with SAG. In contrast to Lck-dependent TCR signaling, early TCR signaling in the absence of Lck was characterized by lack of phosphorylation of ZAP-70, LAT and phospholipase (PLC) C $\gamma$ -1. We found that SAG induced significant activation of the mitogen-activated protein kinases (MAPK) ERK-1/-2, Ca<sup>2+</sup> influx, and translocation of NF-AT and NF- $\kappa$ B transcription factors in the lck-deficient T cells, leading to production of interleukin-2 (IL-2). The Lck-independent T cell response was blocked with protein kinase C (PKC) and MAPK inhibitors, but not by inhibitors of PI-3 kinase. In addition, we observed that IL-2 production by Lck-deficient T cells was enhanced by lithium chloride (LiCl) and minimally inhibited by pertussis toxin. Since LiCl stabilizes IP3 generation, which is involved in Ca<sup>2+</sup> influx, we explored the involvement of alternative PLCs, in particular PLC- $\beta$ . We found that inhibition of PLC- $\beta$  blocked Lck-independent activation of ERK-1/-2 suggesting that this enzyme is involved in TCR signaling. In conclusion, our data provide initial characterization of the Lck-independent TCR signaling induced by SAG. This pathway is dependent on activation of PLC- $\beta$  and PKC. Since the activity of PLC- $\beta$  is regulated by heterotrimeric G-proteins, our data suggest that SAG-induced T cell activation involves a G protein-dependent pathway. These findings have implications to design inhibitors of T cell activation in the context of SAG-induced diseases.

### Sa2.63. HLA Phenotype and the Production of IFN- $\gamma$ and Interleukin-10 in Patients with Chronic Urogenital Chlamydia.

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**INTRODUCTION:** The intensity of immune response as well as the predisposition to many diseases, including infectious illness, has been associated with specific HLA phenotypes.

**OBJECTIVE:** To investigate the relationship between specific HLA phenotypes of patients and the predisposition to a chronic course of urogenital chlamydia in addition to *in vitro* production of IFN- $\gamma$  and interleukin (IL)-10 by peripheral blood mononuclear cells (PBMC).

**STUDY DESIGN:** Seventy-eight patients with urogenital chlamydia were included in this study. HLA phenotype was

determined by the standard Terasaki microlymphocytotoxic test utilizing a panel of anti-HLA-A and -B sera. Cytokine expression was assessed by commercial ELISA (Immunotec, France) of supernatants following *in vitro* culturing of PBMC with or without mitogen.

**RESULTS:** Patients with chronic urogenital chlamydia were found to have significantly greater prevalence of the HLA antigens A10, B27, and B51. Individual analysis of HLA phenotypes demonstrated that IFN- $\gamma$  production was independent of the presence of these HLA risk-associated antigens. Conversely, significantly greater *in vitro* levels of IL-10 were observed in 83% of patients with the HLA risk-associated antigens for chronic urogenital chlamydia when compared to the study control subjects.

**CONCLUSIONS:** HLA antigens A10, B27, and B51 can be considered as risk antigens associated with development of a chronic course of urogenital chlamydia. *In vitro* production of IL-10 by PBMC from patients with chronic urogenital chlamydia and these HLA risk-associated antigens is significantly greater than in control subjects. In contrast, IFN- $\gamma$  production is not dependent upon HLA phenotype. The chronic course of urogenital chlamydia in some patients may be associated with an immunosuppressive effect induced by interleukin-10.

### Sa2.64. Are Probiotics Essential Adjunctive Therapies for *C. difficile* Enteritis?

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Recurrent *C. difficile* gastroenteritis is increasing in both incidence and severity, especially in the young and elderly. Although the antibiotics flagyl and vancomycin are effective in ameliorating acute symptoms, both are often followed by recurrences after they are discontinued. Other antibiotics, which also kill normal flora in addition to the intended pathogen, also exacerbate or induce relapses in *C. difficile* carriers who are otherwise asymptomatic. The following cases demonstrate the need to replace normal flora with a suitable probiotic, not only to hasten remission but to prevent recurrences and to eliminate the carrier state.

Case 1) MG, age 85, was admitted for the third time from assisted care to Intensive Care for intravenous fluids and parenteral antibiotics. In 10 months she suffered 8 relapses, 6 requiring extended hospitalization and rehabilitation. Confined to isolation, she had severe abdominal pain, suicidal depression, thrush, and persistent diarrhea, only temporarily relieved by flagyl or vancomycin therapy. Stools were persistently positive for *C. difficile*. As an alternative to long-term antibiotic therapy, the family investigated probiotics and asked her doctors to start it. They declined.

After obtaining legal control of the patient, we discontinued both vancomycin and flagyl and started probiotics. Four enteric coated capsules (T Trio, Natren Co) containing 3 live organisms, *L. acidophilus*, DDS-1 strain, *B. bifidum*, Myeloff strain, and *L. bulgaricus* were given q.i.d. The stools gradually became formed and the patient's symptoms disappeared. Unexpected was the remission of arthritis, allowing both prednisone and methotrexate to be discontinued. The dose was gradually reduced to 1 capsule b.i.d. Diarrhea recurred once when probiotics were inadvertently discontinued. *C. difficile* was eliminated from the feces after 3 months and remains so with monthly testing for the past year.

Case 2) BP, age 74, was admitted to a nursing home for a diabetic leg ulcer. He soon developed recurrent *C difficile* diarrhea treated with flagyl or vancomycin, which recurred whenever therapy was discontinued. For the next year, long after his leg ulcer had healed, he was in and out of the nursing home for retreatment of *C diff*. Finally probiotics were added, symptoms abated, and he was able to be discharged home. He also continues to take prophylactic probiotics, 1 or 2 caps/day and has remained well for the past year. Other similar cases, one a child, will be presented.

We suggest that *C difficile* is an increasing problem not only because of immune deficiencies, but also because of the increase in use of antibiotics which kill the normal bowel commensal bacteria. These normal gut bacteria perform vital immunoregulatory functions and also close the tight junctions between endothelial cells which *C difficile* toxins open, thus allowing unregulated absorption of both toxins and allergens. The remarkable improvement of arthritis in case 1 fits the theory that certain antigen epitopes on gut bacteria may cross-react with synovial cells as the gut becomes more permeable. Probiotics should be considered essential for recurrent *C difficile*, either used alone or as adjunctive therapy.

#### Sa2.65. Alterations in Th1 Subpopulations Correspond to Clinical Pathology in Severe Trauma Patients.

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Post-trauma alterations in T-cell surface markers and functions are suggested to correlate with increased time to recovery and development of multiple organ dysfunction syndrome (MODS). Recently, dysfunctional co-stimulation, shifts in T-cell subset ratios and emergence of regulatory T-cells have all been suggested as contributors to immunological dysfunction. However, a definitive link between trauma patients' T-cell immune alterations and clinical outcome is still undefined. Here, 31 trauma patients were divided into two groups based on the severity of their clinical course (average of the injury score and time to recovery from MODS; <30 versus >30). We assessed the surface expression of the co-stimulatory ligand-CD28 and the subset markers-CXCR3 (Th0/Th1), CCR5 (Th1) and CCR4 (Th2) to reflect shifts in T-cell populations or decreased activation. Regulatory T cell receptor levels-dual CD4CD25 and CTLA4, were also assessed to indicate any evidence of immunosuppression. These parameters were correlated to severity of clinical outcome <30 versus >30. Additionally, T cell intracellular cytokine (IL-2, IFN $\gamma$ , IL-4, GM-CSF and IL-10) levels in response to ex vivo PMA/Ionomycin stimulation were assessed. Patients ( $n = 31$ ) from three different clinical centers were followed over 28 days post-injury (sampled at <12hrs and days 1,4,7,14,21 and 28). Samples from normal subjects ( $n = 22$ ) were simultaneously processed. Surface expression of CCR5 and CXCR3 were significantly decreased while CCR4 and dual CD4CD25 expression were significantly increased in T-cells from all trauma patients as compared to normal subjects. CTLA4 was unchanged. Similarly, stimulated intracellular cytokine (IL-2 and IFN $\gamma$ ) levels were significantly reduced

in T-cells from all trauma patients as compared to normal subjects. Some adaptive T cell post-injury alterations may occur in all patients while other selective aberrant alterations may be contributing to clinical pathology. Consequently, we compared alterations in these immune parameters between the patients' groups with the most severe (>30) versus less severely (<30) clinical courses. Only IFN $\gamma$  and CXCR3 ( $p < 0.05$  at 14 days post-injury) expression were significantly reduced in patients with a poor clinical course, when compared to the less severely injured subjects. However, there was no significant difference in CCR5 or IL-2 between the two patient groups. These data suggest a selective defect in the Th1 populations from the severely injured patients, rather than a simple expansion of their Th2 cells. Such selective Th1 defects in severely injured patients could contribute to their increased clinical pathology.

#### Sa2.66. The Effect of Immunotherapies on In Vivo Antiviral Responses to HIV.

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To determine the capacity of immune-based therapies (IBT) to boost HIV-specific immunity, a randomized, controlled, phase II trial tested IM ALVAC (vCP1452)  $\pm$  daily, low-dose SQ interleukin-2 (IL2) (1.2 mU/M<sup>2</sup> BSA). Subjects enrolled had [HIV] < 50 & [CD4+T] > 400. There were 3-steps of 12-wks each: Step 1, IBT, ALVAC/IL2; Step 2, "Diagnostic (HAART) Treatment Interruption" (DTI); Step 3, extension of DTI; if [HIV] < 30,000 (< 4.48 log<sub>10</sub>) and [CD4+T] > 50% of baseline. Endpoints = mean trough plasma [HIV] wks 21–25 of Step 2, and % of subjects to enter Step 3=Responders (R). 28/42 subjects completed the trial; 14 were Rs and entered Step 3. The mean  $\pm$  SEM trough [HIV] of  $r = 3.73 \pm 0.15$  log<sub>10</sub> and is significantly lower ( $p < 10^{-4}$ ) than Nonresponders (NR) =  $4.88 \pm 0.08$ . In Step 2, [CD8+T] increased 2-fold in Rs compared to NRs. R [CD4+T] remained at 100% of baseline through steps 2 & 3, while NRs lost 20% of [CD4+T]. Analyzed according to IBT regimens, the highest proportion of Rs was observed in the vaccine groups: placebo = 33%; vaccine = 78%; placebo + IL2 = 33%; vaccine + IL2 = 50%. These data indicate that a DTI is a valid clinical trial design to test IBTs, & support the notion that therapeutic immunization may improve the *in vivo* antiviral host response.

#### Sa2.67. An Automated Methodology for Evaluation of Dendritic Cells in Whole Blood.

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Dendritic cells (DCs) are potent antigen presenting cells that play important roles in the induction of immune responses against microorganisms and tumors and are also vital players in the induction of tolerance and autoimmunity. Due to these unique features, DCs are very promising therapeutic tools being used to manipulate the immune system and form the basis of a number of ongoing clinical trials as biological adjuvants and modulators in cancer, infectious disease and autoimmunity. In order to further study the biology of these relatively rare DCs, as well as evaluate their efficacy in the various clinical trials, the easy identification and enumeration of these cells in whole blood, preferably in an automated and standardized format is pivotal.

The current study was designed to develop an automated and standardized methodology for the phenotypic identification and enumeration of DCs in whole blood. The Beckman Coulter, Inc. 3-color DC phenotyping reagents were used in conjunction with the COULTER® PrepPlus 2 automated pipetting instrument, the TQ-Prep automated lysing and fixing instrument, the CellPrep automated non-centrifugal washing instrument, and the Cytomics FC500 flow cytometer with totally automated instrument setup, data acquisition, and data analysis. Normal and abnormal whole blood samples were evaluated for identification and enumeration of DCs and results compared to the recommended validated manual techniques.

The automated methodology enabled a more precise, and standardized “hands off” identification and enumeration of DCs in whole blood. Signal to noise ratios were improved while maintaining similar cell recoveries compared to the manual methods in both normal and abnormal blood specimens. Therefore, the use of validated reagents, precise pipetting, automated preparation, automated cytometer setup, defined gating strategies, and automated data analysis and data reduction techniques enables a standardized phenotypic evaluation and enumeration of rare dendritic cell events in whole blood.

#### **Sa2.68. Ozonotherapy and Quality of Life in HIV Positive Patients.**

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We have been done a prospective longitudinal study of positive HIV patients admitted at the sanatory living in Guantanamo province in a period of two years. The sample includes 17 patients which were divided into three aleatory groups. The first one was treated with Ozone, the second with a conventional treatment and the third with Ozone therapy-conventional treatment. Then we did an evaluation of some immunohematological and biochemist parameters and clinical success. At the beginning, in 32 % of the patients the Eritro was high, lymphocytes subsets were within the normal limits and 69,2 % had an oxidative stress from moderate to severe and with a good evolution without opportunistic infections.

#### **Sa2.69. Characteristics of Fas Ligand Expression by Human Cytomegalovirus Infection.**

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The immune response is regulated not only by cell proliferation and differentiation, but also by apoptosis. Malfunctions of apoptosis have been implicated in many forms of human diseases. Many viral infection disturb the normal regulation of apoptosis in the various cells. Although Fas-mediated apoptosis is one of the immune effector pathways leading to the elimination of virus-infected cells, viruses may escape immune surveillance by expressing Fas ligand (FasL) on the infected cells. Human cytomegalovirus (HCMV) infection was reported to induce FasL on retinal epithelial cells. This study was performed to find out the induction of FasL in various cell lines and to elucidate the characteristics of FasL expression in human fibroblasts upon HCMV infection. The constitutive FasL expression was found on T cell lines (Jurkat, H9 and CEM), but not on B cell line (Nalm6),

osteogenic sarcoma cell lines (Saos-2, G292, HOS and MG63), astrocytoma cell line (U373MG), lung cancer cell line (A549), human fetal lung fibroblasts (FLF), Epstein Barr virus-transformed lymphoid cell line (LCL) and human kidney cell line (293) by flow cytometry analysis. Upon HCMV infection with 2 multiplicity of infection, FasL expression was found only in Saos-2, FLF and LCL although some cell lines such as G292 and MG63 could be infected with HCMV. Time-course analysis of FasL expression with flow cytometry showed that FasL expression was found on the fibroblast surface immediately after HCMV infection. FasL expression was down-regulated at 12 hr and then up-regulated at 24 hr and thereafter, but down-regulated after 96 hr. FasL expression rate in FLF was increased dependent on the increasing titer of HCMV. Apoptotic death of Jurkat cells were measured by propidium iodide staining and flow cytometry for the detection of FasL expression on the cell surface of FLF after Jurkat cells were co-cultured with FLF infected by HCMV with various infectious doses. It was found that the apoptotic death rate of Jurkat cells on the HCMV-infected FLF was proportional to the infectious doses of HCMV to FLF. Relative changes of mRNA expression of FasL and HCMV immediate early (IE) and early proteins were measured by real time polymerase chain reaction in FLF after HCMV infection at sequential time points. HCMV infection up-regulated FasL mRNA in less than 6 hr but down-regulated to the basal level from 12 hr to 24 hr and then up-regulate FasL mRNA thereafter, but down-regulated after 96 hr. It was similar to the changes of FasL expression. It was reported that only HCMV IE2 activated the promoter of FasL, but in this experiment HCMV IE1 and IE2 mRNA were increased and persisted continuously except in 72 hr, and HCMV UL44 mRNA were increased and persistent. These results mean that other factors than HCMV IE2 involve in the regulation of FasL expression. Conclusively the functionally active FasL could be induced in the restricted cell types by HCMV infection and would be regulated with many cellular and viral factors in addition to the earlier reported HCMV IE2.

#### **Sa2.70. Characterization of a Th1/Tc1 Type Immune Response to the Novel Therapeutic Hepatitis C Peptide Vaccine IC41 in Humans.**

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IC41 is a vaccine consisting of five synthetic peptides harboring relevant CD4+ and CD8+ T-cell epitopes of Hepatitis C Virus (HCV) and the synthetic Th1/Tc1 adjuvant Poly-L-Arginine. In recent multicenter studies this novel vaccine was investigated in more than 200 subjects. To monitor immunogenicity, PBMC were cryo-preserved and T-cell responses were assessed by interferon-gamma ELISpot and lympho-proliferation. In addition, a highly sensitive five-color HLA class I tetramer-binding assay was developed to quantitate and phenotype HCV-specific CD8+ cytotoxic T cells. A HLA-A\*0201-transgenic mouse reference standard was established as long-term quality control of the custom-made HCV tetramers used in these clinical longitudinal studies. Here we present the detailed analysis of a well known HCV NS3-derived HLA-A2 epitope part of IC41. This included staining for a panel of different surface markers (CD45RA, CCR7, CD27, CD28, CD38, and HLA-DR), intracellular interferon gamma and perforin. The evolution of the HCV-specific T-cell response was compared to recall responses against CMV. Moreover, CD8+ T cell responses

against the NS3 epitope seen in tetramer or ELISpot analyses were correlated with proliferative CD4+ T cell responses. Most importantly, several chronic HCV patients with T cell responses against this epitope showed a concomitant yet transient decline in HCV RNA. In summary, these data indicate that therapeutic vaccination with IC41 can induce cellular immunity against HCV, and results from these studies provide first insights in the mode of action of IC41 on human T lymphocytes.

### Sa2.71. Hepatitis B Virus X and C Protein Induce Transcription of hfgl2 Prothrombinase Gene.

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**Objectives:** Fibrinogen-like protein 2/fibroleukin (fgl2) plays a pivotal role in the pathogenesis of both experimental and human fulminant viral hepatitis. We have previously reported that the nucleocapsid protein of murine hepatitis virus 3 which causes massive hepatocellular necrosis in Balb/cJ mice induces transcription of mfgl2. The aim of this study was to investigate the key factor(s) of hepatitis B virus (HBV) that induces transcription of human fgl2 (hfgl2) and the cis-acting elements involved.

**Methods:** three eukaryotic expressing plasmids of HBV structure genes encoding C, X and S proteins respectively were constructed. Expression of these plasmids in eukaryotic cells were detected by immunohistochemistry and Western blot. A series of 5' truncated promoter of hfgl2 gene were subcloned into the luciferase report vector pGL2-basic to form the promoter-report constructs. Chinese hamster ovary cells and HepG2 cells were cotransfected with constructs expressing HBV X, C and S protein respectively and a hfgl2 promoter construct upstream of the luciferase report gene.

**Results:** HBV X protein and C protein but not S protein activates the transcription of hfgl2 gene, showing an average of 6-fold and 5.4-fold increasing in relative luciferase activity. Further more, series deletion array of hfgl2 gene promoter show that a strong regulatory domain from -816 to -468 (relative to the transcription start site) is responsible for the regulation of hfgl2 gene transcription in response to HBV proteins.

**Conclusion:** These results indicate that HBx and HBc proteins are the viral factors that involved in the hfgl2 expression, and the promoter region of -816 to -468 may contain the cis-elements in response to viral proteins C and X. This study provides new insights in understanding the interaction between virus and host gene expression and its contribution to the pathogenesis of viral fulminant hepatitis. This work was supported by the National Science Fund for Distinguished Young Investigators (No. 30225040 for Dr. Q. Ning, No. 30123019 for Dr. XP Luo).

### Sa2.72. BCG Vaccination of the Human Newborn Induces Specific Regulatory CD4+ T Cells.

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Regulatory CD4+ T cells (Tregs) are critical for control of protective immune responses to foreign organisms. We hypothesized that BCG vaccination of the newborn induces specific Tregs,

and that a dynamic equilibrium between specific regulatory and conventional immunity exists.

We incubated whole blood from 36 10-week old South African infants, routinely vaccinated with BCG at birth, with BCG and determined mRNA expression of the Treg-specific marker Foxp3 (real time RT-PCR). A median 19% (range of 0–650%) upregulation of Foxp3 strongly suggested presence of specific Tregs.

Tregs may suppress conventional immunity through either TGF- $\beta$ , or IL-10, or CTLA-4. We therefore incubated whole blood of vaccinated infants with BCG in the presence of neutralizing antibodies against these molecules, and showed a median 11% to 25% increase in soluble IFN- $\gamma$  production (ELISA), suggesting that these Treg effector molecules do indeed control conventional BCG-induced immunity.

Because these effector molecules may be made by cells other than Tregs, we correlated BCG-induced mRNA expression of these molecules with that of FoxP3. FoxP3 expression correlated with TGF- $\beta$  expression ( $r_2 = 0.9$ ,  $P < 0.001$ ), but not with IL-10 or CTLA-4 expression. This suggested that TGF- $\beta$  may be the effector molecule of mycobacteria-specific Tregs.

To determine whether IL-10-producing Tregs (Tr1 cells) were present, we measured BCG-induced CD4+ T cell-specific expression of this cytokine (flow cytometry). Low frequencies of CD4+ T cells made IL-10 (median 0.01%). However, individual cells were either IL-10+ or IFN- $\gamma$ +, but not double+, suggesting presence of both specific Tr1 and specific conventional CD4+ T cell subsets. (TGF- $\beta$  could not be measured with this assay.)

Finally, we found that BCG-induced IFN- $\gamma$  mRNA expression correlated with Foxp3 expression ( $r_2 =$ ,  $P < 0.001$ ), suggesting a dynamic equilibrium between specific regulatory and conventional immunity.

We conclude that newborn vaccination with BCG does induce specific Tregs, which may control conventional effector/memory T cell immunity.

### Sa2.73. Effect of an Anti-Inflammatory Pentapeptide Produced by *Entamoeba histolytica* upon Gene Expression Related to Inflammation/Woundhealing.

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#### INTRODUCTION

The monocyte locomotion inhibitory factor (MLIF), an anti-inflammatory pentapeptide (Met-Gln-Cys-Asn-Ser) produced by *Entamoeba histolytica*, inhibits the *in vitro* and the *in vivo* locomotion of human monocytes (PBMN), without affecting that of neutrophils or eosinophils. MLIF also virtually cancels the production of reactive oxygen and nitrogen intermediates (ROI, RNI) in PBMN and nPMN, and again spares eosinophils from this effect. MLIF also inhibits delayed hypersensitivity skin reactions to 1-chloro-2,4-dinitrobenzene in guinea pigs and gerbils. All this may contribute to the regeneration without scarring of the affected organs (i.e. liver, skin) found on recovery after treatment.

#### OBJETIVE

To determine the effect of MLIF on the profile of inflammation/woundhealing gene expression in the human cell line MRC-5, using a microarray device.

## MATERIAL AND METHODS

A 96% pure construct of MLIF and the human cell line MRC-5 were employed. The cell line MRC-5 was expanded in MEM containing 10% bovine fetal serum until reaching the adequate cell count. Cells were adjusted at  $5 \times 10^6$  cells/5 ml and were placed in 6-well plates, incubating them for 24h at 37°C with 5% CO<sub>2</sub> in: PMA-Ionomycin (10ng/10<sup>6</sup>cells/ml, 0.5µg/10<sup>6</sup>cells/ml), or PMA-Ionomycin+MLIF (10ng/10<sup>6</sup>cells/ml, 0.5µg/10<sup>6</sup>cells/ml, 50µg/10<sup>6</sup>cells/ml). Controls consisted of cells incubated without any stimulus or cells exposed to 50µg/10<sup>6</sup>cells/ml MLIF alone. Cells were treated and lysed in 1ml of TRIZOL<sup>®</sup>. Total RNA was isolated following the manufacturer's instructions and DNA was removed with DNAase I. RNA integrity was confirmed by agarose gel electrophoresis.

The synthesis of labelled cDNA with <sup>33</sup>P was started from total RNA (Human Cytokine Labelling Primers<sup>®</sup>) and purified in Sephadex G-25<sup>®</sup> columns. The cDNA was hybridized in gene microarray membranes (Panorama Human Cytokine Gene Arrays<sup>®</sup>) Membrane were exposed using BioMax MR X-ray film<sup>®</sup>. Spots were quantified by digital analysis. Average values of housekeeping genes and negative controls were used to adjust values of the genes in the membrane. We used 0.999 % confiability and a Z value of 3.090.

## RESULTS

MLIF modifies the gene expression of cultured MRC-5 cells (65 genes are over-expressed, 46 genes diminish their expression). The over-expressed genes included: MCP-1, α2 integrin and C-Kit. Under-expressed genes include: IGF-BP6, INFγ, and MIP 1β.

## DISCUSSION AND CONCLUSIONS

The MLIF effect on this panel of 375 inflammation/woundhealing genes using MRC-5 cells are multiple and complex. Possible pro, anti-inflammatory and woundhealing. Profiles may correlate with the known biological effects of MLIF (Supported by IMSS-FOFOI grant 2003-011).

### Sa2.74. Spectrum of CD1d-Restricted T Cells: Control of Anti-Viral and Anti-Tumor Responses.

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"Invariant" TCR-α+, non-invariant diverse αβ+, and γδ+ CD1d-restricted T cells are subsets of 'NKT' cells with distinct locations and functions. CD1d-restricted T cells can rapidly produce large amounts of anti- &/or pro-inflammatory cytokines and chemokines depending on microenvironment and stimulus. Systemic stimulation of invariant NKT cells leads to transient protection against numerous diseases through indirect activation of various antigen presenting cells (APC), B and T cells, all of which can express CD1d, as well as NK cells. CD1d-restricted T cell activation can rapidly stimulate APC pro-inflammatory IL-12 production. This was modeled by direct ligation of human APC CD1d in vitro. In vivo, both invariant NKT cell activation and CD1d ligation could protect against acute picornavirus infection, suggesting re-examination of results involving CD1d "blocking." CD1d-restricted T cells are also essential for optimal physiological response against picornavirus through augmentation of IL-12 production and NK cell activation. A case report further supports an antiviral role for human invariant NKT cells. However,

excessive activation of local CD1d-restricted T cells can lead (directly and indirectly) to tissue damage. High levels of IFN-γ producing Th1 resident hepatic CD1d-restricted T cells may protect against acute infection, yet contribute to pathology during chronic infections such as hepatitis C through amplified and modified Th1/Th2 responses to tissue CD1d. Analogous results were found in model viral myocarditis. Invariant NKT cells are also essential for optimal anti-tumor responses against spontaneous cancer, metastases, and tumor vaccines. Cancer progression correlates with failure of patient invariant NKT cells to activate protective anti-tumor responses. This defective response can be corrected in vitro and a phase 1 trial has been designed. Finally, the presence of NK cell markers is not essential for function. Therefore, CD1d-restricted T cells are diverse multi-functional cells at the innate-adaptive immune response interface. Therapeutic approaches exploiting distinct CD1d-restricted T cell populations are being explored, for example in graft-versus-tumor responses and graft-versus-host disease suppression.

### Sa2.75. Establishment of a Substitutive SARS Murine Model and Its Application in SARS Study.

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**Objectives:** To establish a substitutive murine model of severe acute respiratory syndrome (SARS) with murine hepatitis virus type 3 (MHV-3) and to investigate the interaction between SARS virus and host immune response, mfgl2 prothrombinase gene as a representative of host genes.

**Methods:** The Balb/cJ mice were infected with 100PFU of MHV-3 via trachea and Balb/cJ mice injected with sterilized saline were served as control. Survival rate and pathological features in organs were observed. Virus titers in different organs were determined on monolayer of L2 cells by a standard plaque assay. Virus distribution and cellular localization were studied by in situ hybridization. As a proinflammatory gene, mfgl2 expressions were measured in the lung by in situ hybridization and immunohistochemistry to investigate the role of mfgl2 in the pathogenesis of the disease.

**Results:** Mice infected with MHV-3 via trachea developed typical interstitial pneumonia with pathological characteristics of leukocyte infiltration, hyaline membranes formation, exudation of fibrin fluid within the lung, presence of vascular thrombosis in the pulmonary vessels and died within 5 days, while all mice in control group survived with no inflammation in lung. Moderate histological changes were also found in liver and spleen but mild in other organs examined. MHV-3 particles were identified in the cytoplasm of alveolar epithelia, infiltrating cells in the lung, serous gland epithelium of the trachea/bronchus, cells (lymphocytes and others) in the periphery of the germinal centers in the spleen, hepatocytes, brain neurons, epithelia of the distal renal tubules, epithelium of small intestine, myocardium cells. mfgl2 expression was evidenced in lymphocytes and mononuclear inflammatory infiltrates within stroma and in terminal and respiratory bronchioles associated with fibrin deposition and micro-vascular thrombosis.

**Conclusions:** The pathological changes in this substitutive SARS murine model mimic the characteristics of SARS in human. In addition to the physical damage of virus replication in lung and immune organs including spleen, liver, the up-regulation of novel gene mfgl2 in lung in association with fibrin deposition and micro-vascular thrombosis may play a vital role in the development of

SARS. This work was supported by national SARS 973 project of China (fund No. 2003CB514100) and National Science Fund for Distinguished Young Investigators (No. 30225040 for Dr. Q. Ning, No. 30123019 for Dr. XP Luo).

### Sa2.76. Direct Innate Immune Activation by Human Naive B Cells Via Combined TLR9 and RP105.

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Recognition of microbial infection and initiation of host defense responses is controlled by multiple mechanisms. B cells provide an important link between the innate and adaptive branches of the immune system. However, direct involvement of B cells in imparting potentially protective responses is unclear. We here focused the relevance of naive B cells via TLR9/RP105 to innate immunity by secreting essentially germline encoded polyreactive antibodies. The proliferation of CD27<sup>+</sup> memory B cells was higher than that of CD27<sup>-</sup> naive B cells by CpG DNA stimulation as reported. The proliferation of naive B cells was extremely higher than that of memory B cells by CpG DNA and anti-RP105 mAb stimulation. The expansion and cell size were dramatically increased by combined CpG DNA and RP105 signaling. The signaling through RP105 increased the expression of CD69 and CD86. The life span of naive B cells was prolonged by anti-RP105 MAb and CpG DNA plus anti-RP105 MAb. Total immunoglobulin and Staphylococcus Pneumoniae specific IgG/IgM production by adult naive and cord blood B cells were recognized in the presence of CpG DNA/anti-RP105 mAb. Interestingly, IL-21 enhanced the production of Staphylococcus Pneumoniae specific IgG/IgM. However, the differentiation of B cell to plasma cells was not affected by anti-Rp105 mAb stimulation. Both TLR9 and RP105 signalings activated NF-κB pathway. Naive B cell activation via TLR9/RP105 pathway may play a beneficial role through profound their expansion and generation of antigen specific low affinity antibodies. This innate immune system by naive B cells may detect infections and trigger antimicrobial host defense responses.

### Sa2.77. Evaluación del Número de Linfocitos T Reguladores en Pacientes Con Tuberculosis.

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#### RESUMEN

**Introducción:** Las células con función inmuno-reguladora más ampliamente caracterizadas son los Linfocitos T CD4<sup>+</sup> CD25<sup>bright</sup> cuyo papel en la regulación de enfermedades inflamatorias y autoinmunes es preponderante. Por otro lado la tuberculosis es una enfermedad causada por el bacilo *Micobacterium tuberculosis* y se caracteriza por cursar con daño tisular severo, en el pulmón, debido principalmente a la propia respuesta inmune del enfermo por lo que posiblemente los mecanismos de regulación de esta respuesta inmune quizás estén alterados. En este estudio analizamos el número de células inmuno-reguladoras en sangre periférica de pacientes con tuberculosis en comparación con sujetos sanos, así

como la expresión del gen GITR el cual se ha reportado que esta presente en estas células y que abate su función supresora.

**Objetivo:** En ratones las células T reguladoras CD4<sup>+</sup> CD25<sup>+</sup> tienen un papel preponderante en la prevención de fenómenos de autoinmunidad. Estas células reguladoras también se han descrito en humanos. En este trabajo investigamos la presencia y el fenotipo de estas células en la sangre periférica de individuos con tuberculosis pulmonar activa y en sujetos sanos.

**Métodos:** Se estudiaron doce pacientes con tuberculosis y doce sujetos sanos. Se analizó el número de las diferentes subpoblaciones de linfocitos T CD4<sup>+</sup> en sangre periférica por citometría de flujo, así como la expresión de GITR por RT-PCR en células mononucleares de sangre periférica.

**Resultados:** Los niveles de los diferentes tipos celulares tanto en pacientes como en los sujetos control en la sangre periférica tienen un rango de valores muy amplio, no observándose diferencia significativa entre ambos grupos a excepción de las células productoras de IL-10 que en los pacientes es más grande comparado con los controles. **Conclusiones:** Para las células Treg., las que producen TGFβ y las CTLA-4<sup>+</sup> nuestros resultados muestran valores similares tanto en pacientes como en sujetos sanos que nos indica que estas células no se ven afectadas con la presencia de la enfermedad solo las que son IL-10<sup>+</sup> muestran valores más elevados en el grupo de los pacientes aunque este estudio se llevo a cabo en sangre periférica en donde el número de estas células es muy pequeño con lo que pensamos que sería de gran importancia el desarrollar el mismo estudio pero directamente en el sitio de la lesión.

### Sa2.78. Direct Epitope Discovery for Yellow Fever Virus Asibi-17D.

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Major histocompatibility complex (MHC) class I molecules bind intracellular peptides for presentation to cytotoxic T lymphocytes (CTL). CTL respond to foreign peptides presented by the class I of virus-infected and cancerous cells. Identification of peptides presented by class I molecules during a yellow fever virus (YFV) infection is important for understanding *in vivo* immune responses to infection and for comparing these responses to those arising from vaccination with Yellow Fever-Asibi [17D]. YFV belongs to the family Flaviviridae and is a positive-sense, single-stranded, encapsulated RNA virus. The virus enters cells by receptor-mediated endocytosis, viral RNA synthesis occurs in the cell cytoplasm, and viral protein synthesis proceeds in the endoplasmic reticulum. Virions mature in the endoplasmic reticulum and are released through the cell's plasma membrane by exocytic fusion. YFV is spread to a human host when the saliva of an infected female mosquito mixes with capillary blood during a meal. The virus then infects vascular endothelial cells and is able to spread to the reticuloendothelial system where it replicates and secondary viremia may occur causing the immune system to be overwhelmed. To understand which peptide epitopes distinguish the class I MHC of YFV-infected cells, we selected the U937 human macrophage cell line for characterization. We transfected HLA-A\*0201 and B\*0702 into the U937 cell line and then infected the cell line with YFV strain 17D. U937 cells secreting either A\*0201 or B\*0702 are cultured in a hollow fiber bioreactor unit, the class I HLA is harvested from both infected and uninfected cells, and the peptide epitopes unique to YFV infected cells identified by comparative mass spectrometric mapping and

MSMS sequencing of peptides unique to YFV infected cells. Identification of MHC class I epitopes unique to YFV infected cells will be useful for understanding to which of the epitopes available on infected cells does the YFV vaccine elicit, or fail to elicit an immune response.

### Sa2.79. Clonal Organization of T Cell Memory: Paradigm of “Focused Diversity” Contributes to Repertoire Flexibility.

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The human memory CD8 T cell receptor (TCR) repertoire specific to the influenza A virus, HLA-A2.1-restricted M1<sub>58-66</sub> epitope was analyzed. b-TCR clonotypic sequences of tetramer-selected cells or cells from the bulk cultures revealed a clonally diverse population of responding T cells. These cells used BV17 almost exclusively and focused on a similar CDR3b structure, a paradigm we refer to as “focused diversity”. This was observed in four different HLA-A2.1 individuals. We determined a strong clonal selection based on a-TCR usage as all expressed a-TCRs originated from AJ42 and multiple AV (AV27, AV8.1, AV 8.6 and others) families. Furthermore, there was a CDR3a motif identified utilizing AGA(G<sub>n</sub>)GG or similar amino acid sequences. Some of these CDR3a only differed by the length of the run of Gly residues. Thus, the pattern of TCR repertoire in the context of “focused diversity” extends to both of the TCR chains. This complex repertoire structure was highly resistant-not only was it stable over time, but persisted under condition of highly variable antigen stimulations *in vitro*. These results suggest that the ab-TCR repertoire flexibility in the context of “focused diversity” might allow antigen-specific immune responses to accommodate extremes of antigen challenges. (NIH Grant: AI-49320).

### Sa2.80. Capture and Specific Delivery of Exogenous Lipid Antigens by Apolipoprotein E.

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T lymphocytes recognize antigens presented by antigen presenting cells (APC). Peptides are presented by the MHC system, with endogenous peptides presented by MHC class I and exogenous peptides presented by MHC class II. In contrast to the MHC system, CD1 molecules survey different intracellular compartments for lipid antigens which are presented at the APC surface to lipid-reactive T cells. Recently, saposins were shown to mediate an important step in presentation by transferring lipid antigens from lysosomal lipid bilayers to CD1 molecules. However, the mechanisms of exogenous lipid antigen delivery to CD1 antigen loading compartments are not known. Serum lipoproteins are the primary mediators of extracellular lipid transport for metabolic needs, however their role in lipid antigen

presentation has not been investigated. Here, we define the pathway mediating exogenous lipid antigen delivery by apolipoprotein E (apoE) to achieve antigen presentation by CD1. In human serum, lipid antigens sequester in the very low density lipoprotein (VLDL) fraction with antigenicity being dependent upon apoE-mediated uptake into dendritic cells. In addition, we have found that dendritic cells themselves secrete apoE, which can then efficiently capture lipid antigens. ApoE-lipid antigen complexes are internalized by receptor mediated uptake resulting in rapid and efficient antigen delivery to endosomal lipid antigen loading compartments enabling CD1 restricted T cell activation. The immune system has thus co-opted an important component of lipid metabolism for the presentation of lipid antigens.

### Sa2.81. Supertype Analysis: Definition of Overlapping Peptide-Binding Capacities for MHC Class I Molecules.

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Major histocompatibility complex (MHC) proteins are encoded by highly polymorphic genes and play a crucial role in immunity. The polymorphisms that distinguish MHC molecules are predominantly positioned to modify peptide binding. However, recent studies seem to indicate that not all genetically diverse MHC molecules are functionally different. As such, classification of MHC molecules into functional supertypes on the basis of overlapping peptide-binding specificities has become an important issue, with direct implications for the development of epitope-based vaccines with wide population coverage. Unfortunately, direct experimental validation of multiple members of these supertypes has been tremendously difficult because of lack of high quality human leukocyte antigen (HLA) molecules. In this study, we describe a direct biochemical approach for classifying HLA molecules into supertypes using recombinant soluble HLA (sHLA) proteins. A set of 53 single-specificity sHLA receptors was screened for their binding capacity towards a series of unrelated fluorescent-labeled peptide ligands. During the process, each peptide candidate was incubated with activated sHLA, and the peptide/HLA interaction was monitored over time. Data analysis showed various degrees of overlapping peptide binding capacities among the alleles tested, reflecting the ability of MHC class I alleles with genetically more or less distinct peptide binding sites to share the binding of identical peptides. As a result of this study, we were not only able to better define supertype classification but also to include additional alleles not currently characterized. Taken together, this novel HLA screening procedure represents a versatile tool for supertype-binding analysis and will have profound use in the understanding of antigenic peptide selection, degeneration and discrimination during T-cell mediated immune responses. A complete knowledge of this phenomenon finds utility in epitope design for the development of HLA based vaccines and immunotherapeutics.

### Sa2.82. Development and Validation of Fluorescence Polarization-Based Competitive Peptide Binding Assays-New Screening Tools for Epitope Discovery.

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Various approaches are currently proposed to successfully develop therapies for the prevention and treatment of infectious diseases and cancer. One of the most promising approaches is the development of vaccines that elicit cytotoxic T lymphocyte (CTL) responses. In order to facilitate CTL-specific epitope discovery and validation, we have developed a state-of-the-art biochemical HLA class I peptide-binding assay including A\*0101, A\*0201, A\*0301, A\*1102, A\*2401, A\*2902, A\*6801, A\*6901, B\*0702, B\*0801, B\*1501, B\*2705, B\*3505, B\*4001, B\*4402, and Cw\*0801. The technique is based on competition and uses FITC-labeled class I binding reference peptides in combination with highly purified, recombinant soluble HLA class I molecules to quantitatively measure the binding capacity of non-labeled peptide candidates. Fluorescence polarization allows direct, real-time measurements in solution without separation steps. The assay shows excellent reproducibility and sensitivity with dynamic ranges between 170 to 240 mP. Optimal loading of synthetic peptides into fully assembled soluble HLA-A\*0201 complexes is enabled by thermal destabilization at 53°C for 15 minutes demonstrating that efficient peptide exchange does not require the removal of endogenous peptides from the reaction environment. Multiple assay-specific parameters have been determined showing that the binding of the selected fluorescent peptides to soluble HLA is effective with many of the molecules (21–37%) binding exogenously added peptides. To further explore the specificity of binding and to demonstrate the immunologic relevance of the FP-based competition assay, a panel of HLA A\*0201-restricted and naturally processed peptides have been tested for binding to sHLA-A\*0201. In this validation approach, a panel of 15 peptides was selected to represent binders that reflect several orders of magnitude of A\*0201 binding affinity. Obtained IC<sub>50</sub> values were directly compared to the values published from direct cell-free <sup>125</sup>I-radioligand assays and cellular based fluorescence assay systems. Results proved to be in excellent agreement with each other also allowing the formulation of an FP-based category system, where peptides with an FP-based IC<sub>50</sub> value of 5 μM and lower were considered high affinity binding, 5–50 μM IC<sub>50</sub> values were considered medium-affinity binding, and 50–1,000 μM IC<sub>50</sub> values were judged as low-affinity binding. With the ability to exactly determine molecular affinity parameters, our FP-based HLA competition assays are of prime importance for the development of immunomodulating compounds. Built into a high-throughput screening platform, this new assay approach will bridge current screening gaps and considerably shorten the route to clinical development considering the myriad of possible peptide sequences, coupled with the genetic variability of MHC molecules among individuals.

### Sa2.83. Anthrax Toxins Impair Pulmonary Dendritic Cell Functions: Implications in Pathogenesis.

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Anthrax is a life-threatening disease of present and future concern, especially since it was used as a bioterrorist weapon in 2001. *Bacillus anthracis*, the agent of anthrax, secretes two critical virulence factors, the lethal toxin (LT) and the edema toxin (ET). To investigate the role of toxins in the physiopathology of inhalational anthrax, we evaluated the functions of murine lung dendritic cells (LDC) upon infection with *B. anthracis* strains secreting LT, ET, both toxins, or with a non toxinogenic strain. Briefly, isolation of LDC from BALB/c mice was performed after digestion by collagenase and DNase followed by positive selection

with CD11c microbeads. We purified three cell populations gated on CD11c/CD11b expression: (i) a CD11c<sup>high</sup>/CD11b<sup>low</sup>; (ii) a CD11c<sup>int</sup>/CD11b<sup>int</sup> and a (iii) CD11c<sup>low</sup>/CD11b<sup>high</sup> population.

Upon infection with LT expressing strains, we observed a down-regulation of CD86 and MHC class II expression on CD11c<sup>int</sup> cells. LDC infected with a non toxinogenic strain induced the secretion of TNF-alpha, IL-10, IL-6 but a low level IL-12p70. LT producing strains selectively inhibited the production of TNF-alpha, IL-10 and IL-6, whereas ET producing strains inhibited TNF-alpha, did not affect IL-10 and increased significantly IL-6 production, as compared to a non toxinogenic strain. These results suggest that anthrax toxins produced during infection impairs LDC functions and suppress the innate immune response. This may represent a new strategy developed by *B. anthracis* to escape the host response and spread through the alveolar wall.

### Sa2.84. Rheumatic Fever: Development of an Animal Model Using Streptococcal M1 Recombinant Protein.

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Rheumatic fever is an inflammatory autoimmune condition following 3% of non treated group A streptococci pharyngitis of certain M serotype strains. Heart valves are permanently damaged by inflammation leading to heart valve replacement surgery. In addition to a cellular heart valve attack, anti-basal ganglia autoantibodies arising later in the autoimmune process target caudate and subthalamic nuclei antigens, being responsible for the neurologic symptoms of Sydenham's chorea. Antibodies targeting both heart antigens and M proteins were found in blood of affected patients. M protein and cardiac valve derived proteins were shown to be recognized by T cell clones obtained from lymphocytes infiltrated in rheumatic patient cardiac valves (Guilherme et. al, 1995, Circulation 92:415-20). Therefore, antigenic mimicry between streptococcal M protein epitopes and heart components has been proposed as the triggering factor leading to the heart autoimmune attack. The understanding of the disease process and therapeutic advances has been hampered by the lack of an adequate animal model for the disease. We have injected Lewis rats with streptococcus recombinant M1 protein 500ug on day 0 followed by 500ug boost on day 7 and sacrifice on day 21, in order to reproduce a recently described animal model of rheumatic fever (Quinn, A. et al, 2001, Infect.Immun. 69(6):4072-78). Rat hearts were subjected to histopathological analyzes. Spleen and lymph node lymphocyte cells, as well as sera, were harvested and probed against ABC domains of the M1 complete extracellular protein, AB domains (N-terminus), or C domain (C-terminus) and myosin or control proteins. Rat-immunized cells were used for FACS analysis to study their phenotypic profile and cytokine production. We have obtained specific lymphoproliferative and humoral immune responses against M1 ABC, AB and C domains of the *S.pyogenes* M1 protein. The proliferation index reached above 10, and the antibody titers were significantly higher than in controls, up to the 12.800 fold dilution, but we could not detect any proliferative nor humoral response against myosin. The anathomopathological evaluation of rat hearts displayed presence of lymphomononuclear infiltration and signs of lesions suggestive of Rheumatic Fever in some of



the immunized animals. We are currently analyzing different immunization protocols in order to reproduce the disease in Lewis rats. We believe that the definition of the M protein minimal epitope(s) responsible for RF will certainly contribute to better understanding of the disease process.

### Sa2.85. Effect of the Zinc in the Viability and Differentiation in Human and Murine Cells.

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The majority of the studies done mainly in murines show the resulting alterations from Zinc deficient and the stimulus or the protection induced by the metal in some cell cultures, however, there are evidences that demonstrate that Zinc supplementation *in vitro* or *in vivo* can cause disturbs in the immune system.

Our study has as objective to establish which is the influence of Zinc in the maturity and viability of different cellular lines (U937, human monocytes and mice bone marrow macrophages), as well as the effect that and identical stimulus (proportioned by Zinc) can generate in the same cellular line but from different origin.

The monocytes were obtained from vein blood from healthy individuals through the Boyüm method. The bone marrow cells were obtained from the femur of BALB/c mice, 6 to 8 weeks old, and the U-937 cells from aliquots maintained in culture.

$5 \times 10^6$  cell/well were added in sterile plastic micro plates, then it was added the medium required for each cellular line with or without the stimulant factor required for its differentiation (supernatant of L 929 cells with the granulocyte macrophage colony stimulated factor for the bone marrow cells of mouse and PMA for the human monocyte and U-937 cells) without Zinc or with different dose of metal (0.05–1.0 mM). They were in culture for 11 days at 37°C with 5% of CO<sub>2</sub>. Microscopic observation were done at 3, 9 and 11 days, for the morphologic analysis and cellular differentiation; the viability and total recount of cells were determined on days 6 and 11.

The viability of cells of bone marrow of mouse, mononuclear phagocytes and U-937 cells incubated with 0.05 and 0.1 mM of Zinc was similar to the controls without Zinc (90%). With 1.0 mM of metal, viability decreased considerably ( $P < 0.0006$ ), 50% of the mouse bone marrow cells and human MP, 80% in U-937 cells. The number of cells increased and there was no cellular differentiation for the morphologic characteristics observed at the beginning of the culture (small and round cells) were not modified during the stated lapse of time (11 days). Deleterious effect induced with this dose of Zinc was observed in the three cellular lines.

Variations in number, size, morphology and viability of the cells observed with the different concentrations of the metal, and/or with the same doses at different times suggest that the effect exerted by Zinc depends not only of the used dose but also of the period of its interaction with cells.

### Sa2.86. IL-27/WSX-1 Signalling: An Important Role in the Pathogenesis of Experimental Septic Peritonitis.

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Sepsis represents a significant health problem worldwide. At present, there are only a few effective therapeutic approaches for the treatment of septic patients and mortality rates still remain high. In the present study we elucidated the potential role of the recently discovered heterodimeric cytokine Interleukin-27 (IL-27) in the pathogenesis of septic shock or acute polymicrobial sepsis.

In a first series of experiments, we injected mice deficient for the EB13 subunit of IL-27 or WT controls intraperitoneally with 40 mg/kg E. coli LPS. As a result, EB13 deficient mice survived significantly longer than WT mice following endotoxin administration. Furthermore, in the cecal ligation and puncture model (CLP) of polymicrobial sepsis EB13 deficient mice displayed improved long-term survival (45 %) compared to control mice, which almost all died within 72 h post CLP.

After systemic administration of recombinant IL-27 into EB13 KO mice immediately after CLP these mice died within 2 days suggesting a pathogenic role of IL-27 in the immune pathology of the disease. Mini-laparoscopic analysis demonstrated that IL-27-EB13 deficient mice had a significantly reduced inflammatory response in the peritoneal cavity compared to wildtype controls after CLP.

We next investigated cytokine levels in serum and peritoneal lavage at different time-points after induction of septic peritonitis. As a result IL-27-EB13 KO mice showed a markedly attenuated proinflammatory cytokine response as indicated by decreased expression of IL-6 in both blood and peritoneal lavage, whereas bacterial concentrations in blood were similar to those in control mice. Subsequently, we analyzed the kinetics of IL-27 expression after CLP by quantitative Realtime-PCR. The expression of both IL-27 subunits was strongly induced in liver, spleen, lung and peritoneum 4, 6 and 8 h post CLP. Interestingly, peritoneal macrophages were the major source for IL-27 expression. To determine the cells/tissues that are targets for the biological functions of IL-27 during sepsis, we analyzed the expression of the IL-27 receptor chains WSX-1 and gp130 in cells, which have been implicated in the pathogenesis of the disease. We found out, that Mast cells and neutrophil granulocytes co-expressed both receptor chains suggesting that IL-27 can induce the release of proinflammatory mediators in these cells. CONCLUSION: Our data implicate that IL-27 has a critical role in the early pathogenesis of septic peritonitis and may be a potential new therapeutic target for this disease.

### Sa2.87. Morphine Facilitates Transendothelial Migration of HIV-1 Infected Monocytes across the Blood Brain Barrier by Modulating P-Glycoprotein Expression in Brain Microvascular Endothelial Cells.

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The structural and functional integrity of the BBB is altered by various diseases of the CNS such as HIV AIDS Dementia (HAD). HIV-1 enters the CNS via the transmigration of infected monocytes across the BBB. Drug abuse in HAD patients leads to greater fluidity of cellular membranes which, increases the permeability of the BBB to immunocompetent cells. The efflux transporter P-glycoprotein (P-gp) is an important component of the BBB and an HIV-1 induced increase in P-glycoprotein expression could be one of the mechanisms for reduced intracellular accumulation and resistance to anti-retroviral agents resulting in the progression of HAD. Morphine is transported by P-gp therefore the BBB

permeability of morphine may be altered based on the level of P-gp expression in the endothelial cells of the BBB. We hypothesize that one of the mechanism by which morphine increases the transmigration of HIV-1 infected monocytes across the BBB is via the modulation of P-gp expression by the brain microvascular endothelial cells (BMVEC) that constitute the BBB. Using an in-vitro BBB model, we studied the effect of morphine ( $10^{-7}$  to  $10^{-11}$ M) on transmigration of HIV-1 infected monocytes and on P-gp gene and protein expression by BMVEC. The monocytes were infected with HIV-1 IIIB strain and were used in transmigration assays. P-gp gene expression was quantitated using real time, quantitative PCR while protein levels were measured using western blot analysis. Our results show that morphine significantly increased the transendothelial migration of uninfected monocytes by 39% ( $P < 0.01$ ) as compared to control and it further enhanced transmigration of HIV-1 infected monocytes by an additional 23%. Morphine at  $10^{-7}$  and  $10^{-9}$  M concentration alone and in combination with HIV-1 tat (10–100ng/ml) significantly increased P-gp gene and protein expression in BMVEC. Increased P-gp expression in BMVEC results in enhanced BBB permeability to HIV-1 infected monocytes contributing to the neuropathogenesis of HIV-1 in drug abusers.

**Sa2.88. Intralesional T Cell Lines Obtained from Rheumatic Heart Disease Lesions Can Be Used as a Tool for Searching for Non-Autoimmune M Protein Epitopes for Developing a Safe Vaccine Against Group A Streptococci.**

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Rheumatic fever (RF) is a post-infectious autoimmune disease prevalent in over 20 million children, mostly in developing countries. It is triggered by *Streptococcus pyogenes* and affects susceptible individuals following a non-treated throat infection. Thirty to 45% of patients develop carditis, the most serious disease manifestation leading to severe and permanent valvular lesions and to the development of chronic rheumatic heart disease (RHD). We have been studying the mechanisms leading to pathological autoimmunity in rheumatic fever and RHD for the last 15 years. Our studies have contributed to a better understanding the cellular and molecular basis of RHD, opening a gateway for the development of a vaccine for a post-infectious autoimmune disease. We studied the C-terminal region of the M protein, in which we have identified some potentially protective epitopes, recognized by both T and B lymphocytes from more than 500 healthy individuals and RF/RHD patients. In order to verify potentially autoimmune epitopes we analyzed 21 overlapping C-terminal peptides (20 mers each differing by only two amino acid residues) and 9 N-terminal peptides previously described as heart-tissue cross-reactive by proliferation and cytokines secretion assays using 09 heart-infiltrating T cell lines (HIL) obtained from rheumatic heart disease (RHD) patients. Our results showed that 5 out of 9 HIL were able to recognize 7 C-terminal peptides. Among these peptides, four {M(254-273), M(292-311) M(293-312) and M(295-314)} recognized by 02 HIL induced interferon gamma production. Two peptides M(258-277) and M(259-278) were recognized by one HIL and induced TNFa and

IL-5 production. Only one HIL simultaneously recognized the C-terminal M(269-288) and the cross reactive M5(1-20) peptides.

Our results indicate that peptides differing by only a few amino acids are capable of generating divergent immune responses, which could be the key issue in defining vaccine epitopes.

**Sa2.89. Is There Any Relation between Lymphocyte Subsets, NK Activity and Infection in Beta Thalassemia Patients.**

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In  $\beta$ -Thalassemia is multiple transfusion therapy is used to maintain nearly normal hemoglobin levels, and partially suppresses the increased, but ineffective erythropoiesis. Unfortunately, transfusion is associated with alloimmunization, risk of exposure to infectious pathogens and accumulation of iron. A major cause of morbidity and mortality in thalassemia is infections, assumed to be result of immunological changes. There is some controversy in relation between immunological status, transfusion therapy regimen and frequency of infection and to date it is not quite clear why these patients are susceptible to infection.

In this study lymphocyte immunophenotyping for CD3+ (T-cells), CD3+/CD4+ (T-helper cells), CD3+/CD8+ (T-cytolytic), CD3+,CD19+ (B-cells) and CD3-/CD16+,CD56+ (Natural killer cells) was detected in the whole blood of 30 beta thalassemic patients using a three-color flow cytometric technique, Natural Killer cell activity was also determined, with flow cytometric assay for NK cytotoxicity using PKH2-labeled K562 target cells. Serum ferritin was measured by RIA as the iron status. We recruited 30 healthy children of comparable age that had never gone under blood transfusion. Information about the first date of transfusion, history of infection and age of administering chelation therapy were obtained by interview, archive files and related physician.

Results showed an increased CD19+ lymphocytes ( $P < 0.05$ ) without alteration in T lymphocytes Or CD4+/CD8+ ratio and number of CD3-/CD16+, CD56+ cells but decreased NK activity ( $P < 0.05$ ). The results do not correlate with the tendency to infection. The study of other factors is needed to detect those who are more susceptible to infections.

Immuno-Oncology.

**Sa2.90. Dysregulation of Histone Modification and DNA Methylation in Acute Myeloid Leukemia.**

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Objectives: Like p16, p15 is a gene which inhibits CDK4 and CDK6 and negatively regulates the cell cycle. Because DNA methylation of p15 is frequently observed in patients with acute myelogenous leukemia (AML), the silencing of p15 is thought to be closely related to the onset and progression of tumors. Recently, the relationship between DNA methylation and histone modification (acetylation and methylation) has been highlighted, and treatment targeted at these changes has been devised. The present study was undertaken to examine the relationship between DNA

methylation of p15 gene and histone modification in AML. Methods: Tumor cells were isolated from the bone marrow or peripheral blood of 6 patients with AML (4 cases of primary AML and 2 cases of recurrent AML; 4 boys and 2 girls with ages ranging from 1 to 14 years). More than 90% of the isolated cells were confirmed to be leukemic cells. Bone marrow and peripheral blood mononuclear cells from healthy individuals were used as control. Leukemic blood cell line, HL60, which expressed p15 gene, and MOLT4 whose p15 was silenced by methylation were also examined. Acetylation of histones H3 and H4 and the methylation of histone H3 at lysine 9 (H3 Lys 9) were analyzed by chromatin immunoprecipitation. The methylation status of p15 gene of immunoprecipitated DNA was evaluated by PCR using methylation specific primer. To know the possible role of DNMTs in AML, we examined their expression levels by competitive PCR. Results: Bone marrow and peripheral blood mononuclear cells and HL60 cells showed acetylated histone H3 and H4, but not methylation of H3 Lys 9. On the other hand, MOLT4 cells had not only methylation of histone H3 Lys 9 but also histone H3 and H4 acetylation. All 6 AML cases showed histone H3 and H4 acetylation as well as methylation of H3 Lys 9. Interestingly the p15 promoter DNA immunoprecipitated with antibody specific for acetylated histone H3 or H4 was unmethylated in some alleles and methylated in other alleles. Coexistence of methylated alleles and unmethylated alleles were observed by methylation-specific PCR and sequencing in the case of methylated H3 Lys 9. DNMTs expression levels were not overexpressed in AML, compared with their levels in normal bone marrow and peripheral blood. Conclusion: These results suggest unbalance between histone modification and DNA methylation of p15 gene in patients with AML.

### Sa2.91. Immunological and Comparative Study of Ras Family Oncogene Product in Various Human Tissues.

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Biology in recent years has focused on identifying specific genes termed as Proto-Oncogenes, expression of which appears to underlie the process of carcinogenesis. These Proto-Oncogenes become activated as Oncogenes by a variety of mechanism: viral transduction, promoter insertion, amplification, point mutations and chromosomal translocations. The Oncogenes are classified according to sequence homology, functional similarity and site of action of their gene products. Out of which ras-gene belongs to G-protein related to 21 KD, located on the inner face of plasma membrane (P21) is playing a very important role in detecting a wide variety of normal and tumour tissues. This proteins bind guanine nucleotides with high affinity.

The present work was aimed at study of the distribution of ras-oncogene in human epithelial cancers and precancerous lesions, with confirmed histological examination. Lesions were of oral cavity, respiratory tract, gastrointestinal tract and liver, breast, kidney and urinary bladder, ovary and skin. Cryostat sections of tissues were prepared. Primary antibody Ras (Ki/Ha) P21 (lyophilized) was obtained commercially and Peroxidase-Anti-Peroxidase method was used to localize the antigens.

The staining was predominantly in the cytoplasm; appearance was fine granular/ diffused. Squamous cell carcinomas of various sites showed 95.6% positivity and 94.4% in cases of adenocarcinoma. Anaplastic carcinomas showed 71% positivity whereas

cases of verruca, transitional cell, Paget's disease showed 60% positivity. In the premalignant lesions: oral leukoplakia, submucous fibrosis showed 75% positivity. All the cases of benign lesions showed 65% positivity while normal tissues of oral cavity, respiratory tract and skin showed only 25% positivity. This study helps to compare the normal and abnormal tissues

and also provides an excellent class of tumor markers for targeting and therapy, using immunological, pharmacological or radiolabelled agents to inhibit their functioning in cancers.

### Sa2.92. Defects in TCR Associated Proteins in Relation to Immune Impairment in Oral Cancer Patients.

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Cancer of the oral cavity is easily detectable and curable in the early stages. However, recurrence and nodal metastasis are frequent often due to late diagnosis of the disease and limited understanding of the biology of the tumour. Immunosuppression is reported in cancer patients even though the mechanism remains ill-defined. Recent reports suggest T-lymphocytes to be the principal effector cells in this process and modification of signal transducing molecules to be responsible for the impairment. TCR-zeta protein is an essential component of TCR complex that binds zap 70 protein and transduces signals following TCR activation. T-cell activation also leads to translocation of NFkB components like Rel-B and c-Rel to the nucleus activating the transcription of variety of genes including IL2. This study addresses the expression status of the signal transducing proteins and its role in the immune impairment in patients with cancers of the oral cavity. 70 oral cancer patients were collected for the study, after obtaining informed consent. Healthy normal individuals were selected as controls. Percentage population of CD3 +, CD4+ and CD8+ cells were enumerated by surface phenotyping using FACS Calibur and capacity to respond to mitogens and anti-CD3 was assessed by the Thymidine incorporation assay. T-cell function was assessed by quantitation of levels of IL-2 production after stimulation with PHA/anti-CD3. The expression status of the signalling molecules TCR- $\zeta$ , CD3-  $\epsilon$ , zap-70, p<sup>56</sup>lck, PKC, rel-B and c-rel was evaluated by western blotting following stimulation of lymphocytes with anti-CD3 in presence and absence of r-IL2. Surface phenotyping of CD3 +, CD4+ and CD8+ cells showed significant decrease in CD3 and CD4 positive cells and the CD4/CD8 ratio with significant increase in the CD8+ cells. Cell proliferation assay clearly demonstrated defects in cell proliferation to T cell specific mitogen and majority of the oral cancer patients showed decreased response to PHA and anti-CD3. Production of IL-2 by the lymphocytes was also found to be reduced. Exogenous Interleukin-2 could increase the transformation response in all the controls and in 20% of the patients to different degrees varying from 10% to 90%. Low levels of the signaling molecules (TCR- $\zeta$ , CD3-  $\epsilon$ , zap-70, p<sup>56</sup>lck, PKC, rel-B and c-rel) and an impairment in the transduction of rel-B to the nucleus were observed in these lymphocytes. Decreased CD4<sup>+</sup>/CD8<sup>+</sup> ratio with an increase in suppressor cells, reduced lymphocyte proliferation and production of IL-2 suggest a defective immune regulation in cancers of the oral cavity. Impairment in the translocation of rel-B to the nucleus and the reduced levels of signal transducing proteins might be the reason for the decreased production of interleukin-2 and immune impairment in oral cancer patients.

### Sa2.93. Is the Oncogenesis Epigenetic Induced Alternative Way for Cell Survival?

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Microevolution of tumors is accompanied by all, or almost all, elements that characterize evolution in the Darwinian sense. The evolution of tumors progress on a time scale of months and years within a limited population of altered cells, involve all the phenomena observed in the long-term evolution of species. These phenomena include random changes in the genome of tumor cells, various forms of selection pressure and selection of tumor cells. Mutations of key genes endow tumor cells with a selective growth advantage and, in the presence of an unstable genome, these cells further mutate and undergo progressive 'clonal evolution'. Albeit this model of tumor survival is widely accepted, there is possibility that emergence of 'first' altered cell generation follow same axioms as mentioned phenomena of tumor growth and escape. To that effect, shifting of a normal cell to the tumor cells might be explained as activation of adaptive mechanisms for cell survival. Thanks to large genetic potential and searching for 'better solution', the 'endangered cell' or cell under abnormal 'conditions/selection pressure' may wend to malignant alteration as an alternative way for survival. For example, epigenetic factors such as 'chemical distresses' or virus-infection may activate the silenced genes that are responsible for malignant alteration. In vertebrates, evolutionary accumulation of genetic potentials is associated with phenomena of epigenetic induced adaptation and ability of species for survival in different conditions. In the same way, shifting of the cell from normal to altered might be conceived as an epigenetic induced adaptation for cell survival, as well as mechanism of tumor escape.

### Sa2.94. Anti-Interleukin-10 Strategies in Treatment of Malignant Diseases.

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Interleukin-10 (IL-10) is important cytokine for phenomena of tumor growth, escape and even carcinogenesis. This cytokine is essential for tumor cell proliferation because its neutralization decreases clonogenicity of malignant cells, whereas addition of recombinant IL-10 increases cell proliferation. IL-10 autocrine/paracrine loop plays an important role in the resistance of certain tumors to chemotherapeutic drugs. In addition, immunomodulatory/suppressive nature of IL-10 plays significant role in mechanisms of tumor escape from immune monitoring. Nevertheless, there is no clear anti-IL-10 strategy in treatment of malignant disease. In fact, there are a few hypotheses, and small number of experimental/clinical studies, that propose anti-IL-10 strategy in treatment of malignant diseases. For example, Ammonium Trichloro(dioxoethylene-o,o')tellurate (AS101) inhibits synthesis of tumor IL-10 on the transcriptional level. Therefore, AS101 treatment combined with chemotherapy may be effective in the treatment of certain malignancies. Another possibility is treatment of malignant patients by anti-IL-10 antibodies with unpredictable consequences and complications in relation to autoimmunity and immune complexes disease. The strategy that we suggest is based on extracorporeal blood filtration/purification method with a view to removing IL-10 molecules from patient's blood by anti-IL-10 antibodies attached on the filter walls. The present strategy

provides a method for enhancing an immune response in tumor sufferers to facilitate the elimination of IL-10 and/or other immunosuppressive cytokines and other molecules. The method involves the removal of immune system inhibitors from the circulation of the tumor sufferers, thus, enabling a more vigorous immune response to the tumor. Particularly useful in the strategy is an absorbent matrix composed of an inert, biocompatible substrate joined covalently to a binding partner, such as antibody, capable of specifically binding to the IL-10.

### Sa2.95. Modulation of TNF alpha Effects on Apoptosis Induction in PC Cell Line.

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TNF-alpha is a pleiotropic cytokine which can induce apoptosis in sensitive cells, but also regulated cell proliferation, cellular activation and differentiation. To be better estimated role of TNF on PC cell line, originally developed from patients with myelodysplastic syndrome at Institute of Oncology Sremska Kamenice, Novi Sad, we monitored the kinetics of changes after in vitro treatment with or without TNF-alpha in presence anti-CD45 and CD95 MoAb, IL-3, FLT3 and GM-CSF. We analyzed cell viability by cell enumeration; intracellular metabolic activity by determination of total LDH activity after cell sonication, cell proliferation, cell membrane molecule expression, apoptosis and necrosis using flow cytometry (Becton Dickinson, San Jose, USA). Analyses were performed 2, 6, 8 and 24h after treatment under some experimental conditions. Our results showed that in comparison with untreated cells, TNF-alpha induced significantly increase in apoptosis and necrosis, in PC cells, which expressed high level of CD95 and TNF alpha receptors. Immunophenotype of this cells also excluded B cell or T cell characteristics, but presence of early hematological markers was found. Pretreatment of PC cell with anti-CD45 and anti CD95 monoclonal antibodies modulated cell death induced by TNF. In addition, presence of TNF in cell culture medium induced significantly decrease in cell proliferation, stimulated by IL-3, GM-CSF or FLT-3. However, no changes in CD13 and CD33 antigen expression following cell proliferation, determined after 7 days cultures and stimulation in comparison to percentage of antigen expression before treatment. No changes in intracellular LDH activity before and after cell proliferation induced with TNF or different cytokine combinations. We conclude that sensitivity to apoptosis and evidence for their increase limited cell proliferation estimated on this cell line.

### Sa2.96. Priming of T Cells to Intracranial Tumor Antigens Does Not Confer a Survival Advantage.

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*Introduction:* The ability of T cells to recognize a tumor antigen is the most important step in immune activation. The degree to which this event occurs in the CNS remains a matter of open debate. In the present study we sought to (1) determine whether peripheral T cell priming occurred in response to intracranial tumors, (2) to evaluate whether the degree of priming offered a survival advantage, and (3) to design ways of improving the specificity and delivery of T cells to intracranial tumors.

**Methods:** The B16/F10 metastatic melanoma cell line was transduced with the H-2K<sup>b</sup> restricted SIY peptide.  $1 \times 10^3$  or  $10^6$  B16-SIY cells were implanted in the flanks or brains of H-2K<sup>b</sup> restricted C57BL/6 and 129S6 mice. ELISPOT assays were performed on spleens isolated 7 or 13 after tumor implantation to assess endogenous T lymphocyte responses. In a separate set of experiments, naïve 2C CD8<sup>+</sup> cells, which recognize the SIY peptide, were isolated from 2C/RAG2<sup>-/-</sup> mice by negative selection and stimulated on Days 0 and 4 through co-incubation with replication-incompetent, H-2L<sup>d</sup>- and B7-co-expressing P815 mastocytoma cells in a 1:5 ratio. The cells were then injected into B6/129 mice harboring B16-SIY intracranial tumors with or without local intracranial delivery of IL-2 at varying time points.

**Results:** While the B16-SIY tumor grew in the flanks of C57BL/6 mice and was associated with poor T cell priming to the SIY antigen, it did not grow in 129 mice where the T cell response was prominent. In contrast, intracranial delivery of B16-SIY was uniformly fatal in both animal models, in spite of peripheral T cell priming to the SIY antigen. Mice treated with tumor-specific T cells either before or after intracranial tumor implantation exhibited a statistically significant increase in survival, from 16 days (controls) to 21 days (T cells),  $P < 0.01$ . Co-therapy with T cells and IL-2 exhibited strong synergism: 16 days for controls, 39 days with T cell delivery prior to tumor establishment ( $P < 0.001$ ), and 58 days with T cells after tumor establishment ( $P < 0.00001$ ). Twenty-percent of mice treated with T cells and IL-2 were long term survivors of greater than 120 days. Immunohistochemical analysis of animal brains revealed local CD4<sup>+</sup> and CD8<sup>+</sup> infiltration in intracranial tumor-bearing mice that had been treated with either T cells alone or T cells and IL-2.

**Conclusions:** Our results confirm that T cell priming does indeed occur in response to CNS tumors, however, the strength of the response does not correlate with survival. While the use of tumor specific T cells offers a survival advantage, local delivery of IL-2 to the site of the tumor further augments the immunotherapeutic response. This study represents the first report in which T cells directed against intracranial antigens along with IL-2 exhibit a synergy which significantly prolongs the survival of animals with intracranial tumors.

### Sa2.97. An *In Vivo* Role for *Trypanosoma cruzi* Calreticulin in Antiangiogenesis.

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Angiogenesis leads to neovascularization from existing blood vessels. It is associated with tumor growth and metastasis and is regulated by pro and antiangiogenic molecules. Most well characterized anti-angiogenic molecules are currently under clinical trials for cancer treatment. During the last few years we have cloned, sequenced and expressed a *Trypanosoma cruzi* (*T. cruzi*) calreticulin gene (*TcCRT*). Its product, TcCRT, a 45kDa protein, is more than 50% identical to human CRT (HuCRT). Most vertebrate CRTs are known by their chaperone properties. We have recently demonstrated that TcCRT, present on the surface of trypomastigotes, binds to the collagenous portions of both C1q and mannan binding lectin and inhibits the classical activation pathway

of human complement (J. Immunology **172**: 3042-3050. 2004). Since TcCRT is highly homologous to a functional antiangiogenic fragment from HuCRT (aa 120-180), recombinant (r) and native (n) TcCRT were tested in their antiangiogenic effects, in the chick embryonic chorioallantoic membrane (CAM) assay. Both proteins mediated highly significant antiangiogenic effects in the in vivo CAM assay. This effect was further substantiated in experiments showing that the genetic plasmid construct pSecTag/*TcCRT* also displayed significant antiangiogenic properties, as compared to the empty vector, thus indicating that the parasite gene was transfected to the vertebrate host. Most likely, the fact that antiangiogenic substances act preferentially on growing neoplastic tissues but not on already established tumors, is due to their effects on emerging blood vessels. Thus, by virtue of its capacity to specifically bind to laminin, CRT may interfere with the assembly of endothelial cells and, as a consequence, in the generation of new blood vessels. In synthesis, our results indicate that TcCRT, like its human counterpart, has antiangiogenic properties. These properties may explain, at least partly, the reported antineoplastic effects of experimental *T. cruzi* infection. (Supported by: FONDECYT-Chile Grants N° 1010930 and 7010930, and MECESUP-Chile UCH0115).

### Sa2.98. Post-Transcriptional Control of MICA Expression by BCR/ABL Oncogene through PI3K/mTOR Pathway.

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MHC class I chain-related molecules (MIC) are NKG2D ligands that participate in immune surveillance of cancer. Engagement of NKG2D by cell surface MIC triggers NK cell activation and antigen-specific CTL immunity. However, the release of soluble forms of MIC (sMIC) by the tumor may impair this response by decreasing NKG2D surface expression. The molecular basis for MIC expression in tumors is unknown. We demonstrated the first direct relationship between an oncogene and MIC expression in chronic myeloid leukemia, a malignancy caused by the BCR/ABL fusion oncoprotein. At diagnosis, around one third of patients showed increased serum levels of sMICA that correlated with white blood cell count and that decreased upon therapy with imatinib mesylate (IM), a specific BCR/ABL inhibitor. In K562 cell line, IM decreased both surface MICA/B expression and NKG2D-mediated lysis by NK cells. Up-regulation of MICA/B-expression by BCR/ABL was confirmed by silencing BCR/ABL gene expression. IM did not affect MICA mRNA levels but decreased MICA protein expression and sMICA release. Likewise, MICA expression was reduced upon treatment with inhibitors of PI3K and mTOR, that are both activated by BCR/ABL. These results suggest a post-transcrip-

tional regulation of MICA by the BCR/ABL oncogene via a PI3K/mTOR mediated pathway.

### Sa2.99. Innate Immune Adherence Activity to Tumor Cells Properties of Methotrexate-Loaded Adult and Cord Blood Erythrocytes.

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**Objective:** To study the effect of MTX-loaded process on innate immune adherence activity to tumor cells of adult and cord blood erythrocytes.

**Methods:** MTX was loaded in erythrocytes using the modified hypotonic preswelling technique. We used the erythrocytes after undergoing washing, swelling and drug-loading treatment from adult and cord blood erythrocytes, and measured the rate of rosette formation.

**Results:** We observed that the innate immune adherence activity of erythrocytes from adult and cord blood after hypotonic swelling treatment significantly increased as compared with other groups ( $P < 0.05$ ), which the rate of rosette formation was up to  $(61.45 \pm 8.51)\%$ ,  $(17.00 \pm 2.16)\%$ , respectively. However, MTX-loaded treatment was no significant change than un-treated carrier erythrocytes.

**Conclusion:** MTX-loaded treatment did not affect the innate immune adherence activity to tumor cells of erythrocytes significantly.

### Sa2.100. Erythrocytes Promote the Expression of CXCR4 on Leucocyte Membrane Activated by Tumor Cell.

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**Objective:** To research the potential role of erythrocytes in leucocytes reactivity against with neoplasms.

**Methods:** 0.2ml suspension of inactivated cancer cells ( $S180, 5 \times 10^6/ml$ ) (or 0.2ml NS as control) were added into 0.2ml fresh anticoagulant whole blood (or 0.2ml white blood cells) treated by citric acid, and incubated for 1 h at  $37^\circ$ . Using monoclonal antibody of CXCR4, we compare the difference of expression level of CXCR4 in leucocytes, and compute the activation index (treated group-control group/control group).

**Results:** It was found that cancer cells could activate hematogenic immunity. In 8 normal blood donors and cancer patients, the activation index of CXCR4 in whole blood cells was significantly higher than that in white blood cells ( $t = 2.598$ ,  $P < 0.05$ ). And in the cancer groups, the modulus of CXCR4 ( $57.64 \pm 10.30$ ) in whole blood cells treated group was significantly higher than that ( $35.26 \pm 4.92$ ) in whole blood cells control group ( $t = 3.397$ ,  $P < 0.01$ ).

**Conclusion:** These results indicate that there is a road map of blood immune reaction and erythrocytes is requisite. The erythrocytes can promote the expression of CXCR4 of granulocyte. Erythrocytes plays a vital role in leucocytes reactivity against with neoplasms.

### Sa2.101. Cancer Cell Activation Study of Hemaimmune Reaction Road Map Experimental System.

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**Objective:** To establish a detection method of IL-8, IL-6 and DARC (Fy6), Which has application in the study of

cancer cell activated to hemeimmune reaction line map theory.

**Methods:** Using ELISA method of IL-8, IL-6 and Flow cytometry of Fy6. We detected Experimental system of hemaimmune reaction road map. Cancer cells ( $S180, 5 \times 10^6/ml$ ) or NS were added in fresh anticoagulant whole blood (treated by citric acid), or in red blood cell and white blood cells and plasma (or NS), and incubated for in at  $37^\circ$  to see results.

**Results:** Cancer cells (deathly cells) can activate hemaimmune reaction road map experimental system. In cancer cells added to whole blood group, level ( $531.6pg/ml$ ) IL-8 higher than that ( $<0.5pg/ml$ ) In NS added to whole blood group and in cancer cells added to whole blood group, Level (28.85) of Fy6 on red cell Lower than that (45.80) of Fy6 on NS added to whole blood group. In cancer cells added to whole blood cells group, Level (2.86 or 1) of activation index (IL-8 or IL-6) higher than that (0.36 or 0.25) in cancer cells added to white blood cells group. In cancer cells added to white blood cells group, Level ( $138-126.3pg/ml$ ) of IL-8 higher than that ( $55.99-3.90pg/ml$ ) in NS added to white blood cells group.

**Conclusion:** The results suggested that the red blood cells and complement plays a vital role in regulation (IL-8, IL-6) of hema immune reaction. And there is road map of hemaimmune reaction. Antigen (cancer cells) can activate complement, Adhere to complement (C3b), than be adhering to red blood cells, Then adhering to white blood cells to activate hemaimmune reaction. Furthermore, it can provide useful experimental system of hemaimmune reaction road map theory study.

### Sa2.102. Apoptosis-Based Therapies for Hematological Malignancies.

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Apoptosis is an intrinsic cell death program that plays critical roles in tissue homeostasis, especially in organs where high rates of daily cell production are offset by rapid cell turnover. The hemopoietic system provides numerous examples attesting to the importance of cell death mechanisms for achieving homeostatic control. Much has been learned about the mechanisms of apoptosis of lymphoid and hematopoietic cells since the seminal observation in 1980 that glucocorticoids induce DNA fragmentation and apoptosis of thymocyte and the demonstration in 1990 that depriving Colony Stimulating Factors (CSFs) from factor-dependent hematopoietic cells causes programmed cell death. From an understanding of the core components of the apoptosis machinery at the molecular and structural levels, many potential new therapies for leukemia and lymphoma are emerging. In my presentation, I will introduce some of the drug discovery targets thus far identified within the core apoptotic machinery, and describe some of the progress to date towards translating our growing knowledge about these targets into new therapies for cancer and leukemia.

### Sa2.103. The Role of CD4+CD25+ T Cells in Leukemia Relapse after BMT.

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CD4+CD25+ T cells may play an important role in mediating immune tolerance by regulating T cells which cause autoimmune disease. Evidence from murine systems suggests these cells inhibit immune responses against tumours. In order to investigate this hypothesis, we have decided to assess their role in leukemia relapse

after either bone marrow transplantation and/or chemotherapeutic treatment. Leukemias and patients were selected for this project. Immunophenotypic analysis indicates that patients with CML who relapsed after allogeneic BMT have a higher proportion of CD4+CD25+ T cells compared to those who are disease free. Our findings suggest that a proportion of CD4+CD25+ T cells in CML patients at relapse are inhibitory. Therefore, these results may reveal that a relapse after chemotherapeutic treatment may be caused by an increase in the regulatory population which may lead to the enhanced inhibition of a graft versus leukemia effect.

#### **Sa2.104. The Immunoreactivity of Serum IgA with Gliadin in Patients with Myeloma Multiplex.**

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Gluten intolerance is system immunological disorder which is characterized in part by the presence of antigliadin antibodies, which sometimes are also directed to calreticulin. The aim of this work was to determine is there any immunoreactivity of serum IgA with gliadin, or with tissue transglutaminase, in small group of patients with myeloma multiplex.

Three patients with IgA and 4 patients with IgG myeloma multiplex, 1 patient with both IgG and IgA, M components in the serum, 1 patient with non-Hodgkin lymphoma- lymphoplasmocyticum, and 7 healthy people were included in the study. For determination of the level of the immunoreactivity of antigliadin IgA antibodies a home made ELISA test was used, with 5 micrograms of crude gliadin (SIGMA) as the antigen, while 1% bovine serum albumin was used as blocker. Immunoreactivity of IgA anti-gliadin antibodies was determined in prediluted serum to 1:250, and 1:500. Blanks were wells coated with blocker 1%BSA (without gliadin), incubated with corresponding serum dilutions, treated with secondary, HRP labeled, goat anti-human IgA antibody, and additionally, wells coated with only gliadin treated with secondary, goat anti-human IgA antibody. Immunoreactivity of IgA anti-transglutaminase antibodies was determined in prediluted human serum to 1:100, using commercial. ELISA test (Binding Site), with recombinant tissue transglutaminase, as the antigen.

Results from this work showed high intensity of the interaction of serum IgA with gliadin in two patients with IgA plasmocytoma (only in patients with IgA, M component in the serum). The OD492 at 1:250 serum dilution were 0.523 and 0.206, while there was no interaction of serum IgA with gliadin in all healthy persons sera, i.e., at 1:250 serum dilution it was in the limits of the experimental error (OD492 < 0.050). The level of this reaction was of less pronounced intensity, in one patient with IgA plasmocytoma (in his serum there was no M component), OD492 was 0.107. Antigliadin IgA immunoreactivity was also found in 1out of 4 patients with IgG myeloma multiplex, OD 492 was 0.529. In one patient with non-Hodgkin lymphoma- lymphoplasmocyticum, antigliadin IgA immuno-reactivity was high, OD492 was 0.750. Surprisingly, patient with two M components, showed IgA immune reactivity with the blocker, bovine albumin, but not with gliadin.

There was no intensive reaction of serum IgA with tissue transglutaminase for all examined patients, indicating that all immune disturbances characteristic for celiac disease are not developed in these patients.

In conclusion, these preliminary results are the first showing antigliadin IgA immunoreactivity in some patients with myeloma multiplex; they set up a question on the importance of gluten intolerance, (or of some parasitic/or bacterial infection which could induce anticalreticulin/antigliadin immunity ?) in the emergence and development of myeloma multiplex.

#### **Sa2.105. Transcription Factors Abnormalities Associated with Lymphoid Malignancies.**

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Ikaros gene family encodes for a group of Kruppel-like zinc-finger proteins which act as lymphoid-specific transcription factors. Apart from Ikaros, this group includes Aiolos, Helios, Eos and Pegasus. Knock-out studies on mice have shown that Ikaros, Aiolos and Helios function as transcription regulators playing an important role in differentiation of particular subsets of lymphocytes.

All members of Ikaros gene family share the similarity in their overall structure. The four N-terminal zinc-finger motifs are responsible for sequence-specific DNA-binding, while C-terminal zinc-finger pair acts as a dimerization domain: homo- and heterodimers of Ikaros, Helios and Aiolos bind DNA with high affinity in a sequence-specific manner. These complexes can then activate or repress transcription from certain promoters if the DNA-binding domains of both members of the complex are intact. However, by the mechanism of alternative splicing different isoforms can be created that lack one or more N-terminal zinc-fingers. These non-DNA binding isoforms act as dominant-negative because complexes contain at least one such molecule cannot bind DNA efficiently. There is a possibility that up regulated production of shorter isoforms could result in phenotypes seen in knock-out mice which include complete or partial arrests in different stages of lymphocyte development, leading to development of leukemia and lymphomas. Therefore we decided to correlate the expression of long and short isoforms of these proteins in bone marrow, peripheral blood lymphocytes and lymph nodes of patients with different lymphoproliferative disorders. Using reverse-transcription-polymerase chain reaction (RT-PCR) we analyzed lymph nodes from patients with different types of leukemia. Eight human hematological cell lines were also screened for the expression of Aiolos and Helios. Preliminary results show a heterogenous pattern of expression of Aiolos and Helios among these samples. Shorter isoforms, which are probably generated in smaller amounts than fully functional molecules, will be detected by more sensitive real-time PCR and different subpopulations of cells will be analyzed using flow cytometry. The results could improve our understanding of the mechanisms of normal lymphocyte differentiation and possible pathways in development of human lymphoproliferative disorders.

#### **Sa2.106. Generation of Linear and Cyclic Peptides Revealed a Unique Fine Specificity of Rituximab and Its Possible Cross-Reactivity with an Acid Sphingomyelinase-Like Phosphodiesterase 3B Precursor.**

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The heterogeneity of effector functions displayed by Rituximab and other anti-CD20 mAb which apparently recognize the same CD20-epitope suggests that additional mechanisms, probably related to mAb fine specificity, can be responsible for B cell depletion. To improve our understanding of Rituximab function, we investigated its fine specificity. The biopanning with Rituximab of an eptapeptide cystein-constrained (c7c) phage display peptide library (c7c PDPL), of an eptapeptide linear (7-mer) PDPL, and of a dodecapeptide linear (12-mer) PDPL resulted in the isolation of 13, 9 and 10 clones respectively, which specifically reacted with Rituximab in ELISA and Western blot. The c7c PDPL-derived clone insert sequences expressed the motif A(S)NPS, which matched the CD20 amino acid stretch <sup>170</sup>ANPS<sup>173</sup>. Two cyclic peptides bearing ANPS- (Rp15-C) and SNPS- (Rp3-C) motif specifically recognized Rituximab paratope, while no reactivity was observed with Rp3-C-derived peptide mRp3-C (bearing the mouse CD20 <sup>170</sup>SNSS<sup>173</sup>). The 7- and 12-mer PDPL-derived clone insert sequences expressed the motif WPXWLE, which could be aligned only to the reverse-oriented amino acids <sup>161</sup>WPKWLE<sup>156</sup> of acid sphingomyelinase-like phosphodiesterase 3b precursor (ASMLPD3b), though linear peptide Rp1-L, Rp5-L and Rp10-L bearing the motif competed with cyclic peptide for Rituximab-paratope binding. Testing the reactivity of anti-CD20 mAb 1F5, 2H7, AT80, B1 and CAT13.6 with linear and cyclic peptides showed that only 1F5 displayed a reactivity profile similar, though not identical, to that of Rituximab. Furthermore, Rituximab reacted with ASMLPD3b-derived peptide, suggesting a possible interaction with this enzyme precursor. Our results indicate a unique fine specificity of Rituximab and suggest a possible mechanism to explain the recently reported ability of Rituximab to increase acid sphingomyelinase activity in raft microdomain.

### Sa2.107. Functional Analysis of Peripheral Blood Lymphocytes Subsets in Patients with Cancer-Associated Dermatomyositis.

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#### Objectives

Dermatomyositis is characterized by immune-mediated muscle inflammation leading to progressive weakness of the skeletal muscles and the presence of cutaneous symptoms. Patients with dermatomyositis have a higher risk of malignant disease than the normal population. The exact pathomechanism of the association is obscure and less is known about how autoimmunity meets with tumor immunity in these patients.

#### Patients and Methods

Our aim was to characterize different lymphocyte subsets in cancer-associated dermatomyositis patients compared with healthy controls and patients who had primary dermatomyositis. Fifteen patients with cancer-associated dermatomyositis and 44 primary dermatomyositis patients were included in this study. Different lymphocyte subpopulations were measured by phenotypical characterization with monoclonal antibodies. Intracellular cytokine expression of peripheral T lymphocytes was assessed after an *in vitro* incubation with an activating cocktail. The cells were labeled

with anti-CD4 or anti-CD8 antibodies and intracellular accumulation of IL-4 or IFN- $\gamma$  was detected. The frequency of different T-cell subsets was measured within the T lymphocyte population by flow cytometry.

#### Results

Concerning on the phenotypic characterization, there were no detectable differences in the percentages of CD4+, CD8+ and CD19+ cells. Compared to controls, CD3+ cells were decreased (70.9% vs. 56.6%), while CD56+ cells were elevated (8.4% vs. 15.1%) in cancer-associated dermatomyositis patients, but not in primary dermatomyositis patients. The ratio of activated CD3+/HLA-DR+ and CD3+/CD69+ cells was elevated both in cancer-associated and primary dermatomyositis cases. There was no significant difference in the ratio of Th1 cells compared to controls both in untreated and treated cancer-associated dermatomyositis patients (21.0% vs. 24.4% and 21.9%). On the other hand, significantly decreased Th1 ratio was found in active primary dermatomyositis patients (21.0% vs. 9.8%). The percentage of Th2 cells were normal in cancer-associated and in primary dermatomyositis patients as well, but it was decreased in inactive primary dermatomyositis patients compared to healthy controls.

#### Conclusion

T lymphocytes of primary dermatomyositis were less polarized towards Th1 cells, while this was not observed in cancer-associated dermatomyositis patients. Cancer-associated dermatomyositis differs from primary myositis in many aspects of clinical and immunological features. Better understanding of the precise interaction of the host immune system with the malignant disease can shed light on the association between an autoimmune disease and malignancy.

### Sa2.108. Bacteriophage HAP1: Molecular Basis of Its Antitumour Activity.

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Previously we investigated interactions of bacteriophage T4 with mammalian cells: unexpected binding of phage T4 to cancer cells and its antitumour activity. We selected *in vitro* the mutant HAP1 that was able to bind the cells much stronger (than parental T4), it was also more effective against B16 melanoma tumour and metastases *in vivo* (Dabrowska et al. Acta Virol. 48, 2004, Dabrowska et al. Anticancer Res. 24, 2004). We proposed a possible molecular basis of these interactions: reactions of beta3 integrins on target cells and T4 capsid proteins: probably gp24, which contain KGD-aminoacid motifs, i.e. RGD homologues able to bind beta3 receptors (Gorski et al., Medical Immunol. 2, 2003). Here we present further results that may highlight non-antibacterial properties of phages.

In direct sequencing of phage-head genes we found a non-sense mutation in the *hoc* gene of HAP1. Phage particle size and morphology was observed in the electron micrographs and determined by dynamic light scattering (PCS). T4 and HAP1 do not differ in their general morphology, but the head of HAP1 is smaller than the head of T4. This is in line with the well-described



morphogenesis of the T4 capsid: after incorporation of Hoc protein T4 phage head becomes visibly larger. These indicate that HAP1 lacks gp Hoc. The normal Hoc protein is balloon-shaped and it extends to about 5 nm away from the capsid surface, 160 regularly arranged units per one capsid. Because of its special localisation gp Hoc impedes access of external factors to the head surface. Without gp Hoc, there are no important spatial disturbers that can diminish the interactions of other head components with any external targets. This also applies to gp 24, which was proposed as the active protein. Differences in T4 and HAP1 interactions with platelets were also observed (Kniotek et al., *Immunology* 2004). This may suggest that Hoc could be involved. Hoc protein is not necessary for T4 viability nor for its structure, and its exact function is unknown. However, a very interesting report reveals its relatedness to eukaryotic immunoglobulin-like domains. This similarity results rather from divergence from a common ancestor, not just from convergent evolution (Bateman et al., *Virus Genes*. 14, 1997). The immunoglobulin superfamily is engaged in adhesion processes, MHC functions, T-cell receptors, and others. On the other hand, we know that higher organisms are strongly exposed to bacteriophages. They have become "an environment" for phage life cycles (Dabrowska et al., *J. Appl. Microbiol.* 98, 2005) and, one can expect, phages adapt to them. All these considerations suggest that some bacteriophage molecules are predicted to interact with eukaryotic organisms, influencing important immunological processing and/or to modulate these interactions. Hoc protein seems to be one of these molecules.

#### **Sa2.109. Thy-1<sup>+</sup> Immature Natural Killer Cells Suppress Dendritic Cell Functions during the Development of Leukemia in a Mouse Model.**

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**Objective:** Although immune response is thought to regulate the progression of various cancers, cancers often progress by escaping from hosts' immune surveillance. In the present study, we investigated the changes in the immune status during the progression of mouse leukemia.

**Materials and Methods:** We established a leukemia model by injecting in BALB/c mice with WEHI-3b cells intraperitoneally. Mononuclear cells were isolated from peripheral blood (PB) and bone marrow (BM) every 10 days, and analyzed the cell composition by flow cytometry. Cells were separated by using magnetic microbeads-conjugated antibodies.

**Results and discussion:** The numbers of peripheral white blood cells (most of them were leukemic cells) were dramatically increased after day 24 following injection of WEHI-3b cells, and reached  $1.8 \times 10^5$ /ml on day 30. During progression of leukemia, both dendritic cells (DCs, I-A<sup>d</sup> CD11c<sup>+</sup>) and DX5<sup>+</sup>CD3<sup>-</sup> cells showed marked increases in PB, although most cell types were increased. We, therefore, focused on the kinetics and functions of those two cell populations. Increased DCs expressed lower levels of I-A<sup>d</sup> than that of DCs from normal mice and had low allo T-cell stimulatory activity. In the BM of leukemic mice, only DX5<sup>+</sup>CD3<sup>-</sup> cells were continuously increased despite the progression of leukemia, and those cells were rapidly increased in PB. The increase in DX5<sup>+</sup> cells in BM was thought to be induced by soluble factors from leukemic cells, because co-culture of WEHI-3b cells with normal bone

marrow cells in trans-wells showed a selective increase in DX5<sup>+</sup> and CD25<sup>+</sup> cells. The morphology of most of the circulating DX5<sup>+</sup> cells from leukemic mice was large granular lymphocytes, and the surface markers of the cells were shown to be lineage-CD94-CD122<sup>+</sup>CD25<sup>+</sup>Ly-49-Thy-1<sup>bright</sup>c-kit<sup>dim</sup>. These data suggest that those DX5<sup>+</sup> cells were immature NK cells. Isolated circulating NK cells from leukemic mice were able to down-regulate the expression of I-A<sup>d</sup> on normal BM-derived DCs, which was possibly mediated by TGF- $\beta$  produced by those cells. Moreover, these NK cells significantly suppressed the allo T-cell stimulatory activity of DCs, which required cell-to-cell contact between NK cells and DCs, and CD25 was thought to be involved. Because direct interaction between NK cells and DCs induced IL-2 production, IL-2 might be associated with the inhibitory effect. In the '90s, Thy-1<sup>+</sup> bone marrow cells were reported to have immunoregulatory functions in bone marrow transplantation without further analyses. Immature Thy-1<sup>+</sup>NK cells with immunosuppressive activities identified in the present study might be a population of such cells.

**Conclusion:** We have identified circulating immature NK cells with immunosuppressive activities during the development of leukemia, which is important not only for understanding the development of the disease, but also for effective immunotherapy.

#### **Sa2.110. The Application of an *In Vitro* Viability Assay Followed by Subtractive Hybridization for Identification of Prognostic Markers in Acute Myeloid Leukemia.**

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A high percentage of acute leukaemia patients relapse after an initial response to treatment. Currently, risk-adapted therapy is useful for only a fraction of patients. Novel markers need to be identified to provide therapeutic direction to reduce over-treatment or under-treatment of patients. Furthermore, the mechanisms that regulate early relapse have not been identified. We studied *in vitro* viability using the MTT assay of blasts from 13 *de novo* Acute Myeloid Leukemia patients (FAB: 4 M2, 7 M4 and 2 M5a) and compared it with the duration of survival (in months) of these patients. A significant correlation ( $r = -.721$ ,  $P = 0.005$ ) between viability and duration of survival was observed. We then selected a sample with low *in vitro* viability and a longer survival period and another sample with high viability and short survival period. Subtractive hybridization was performed to enrich and amplify for differentially expressed genes in these two samples. Nucleotide sequencing of four preliminary clones showed one differentially expressed gene was a mitochondrial gene, cytochrome c oxidase. This gene was upregulated in a sample that showed high *in vitro* viability and shorter survival period. Mitochondrial genes have been indicated in leukaemia patients refractory to conventional chemotherapy and may play a role in early relapse. Thus, we conclude that *in vitro* cell viability may be useful as a prognostic marker and the study of this biological difference in acute leukemia patients may lead to a further understanding of the mechanisms that control cell survival and relapse.

#### **Sa2.111. Expression of Granzyme B and CD86 Is Associated with Persistence of Cervical Cancer.**

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**Background:** Cervical carcinoma is a human papilloma virus (HPV)-related malignancy, in which escape of the tumor from the hosts' immune response is thought to play an important role in carcinogenesis and may involve alterations in the expression of immune-regulatory molecule genes. The purpose of the present study was to evaluate the relationship between the expression levels of *CD28*, *cytotoxic T-lymphocyte-associated-4 (CTLA4)*, *inducible costimulator (ICOS)*, *ICOSL*, *CD80 (B7-1)*, *CD86 (B7-2)* and *Granzyme B (GrB)* genes and response to treatment in cervical cancer. **Materials & Methods:** The mRNA levels were determined, by quantitative real-time RT-PCR in cervical cancer specimens from 75 patients collected prior to radiotherapy or radiotherapy plus chemotherapy. After 6 months, the patients were divided into two groups, according to disease persistence ( $n = 25$ ) or not persistence ( $n = 50$ ). Target mRNA levels were normalized by the mRNA level of reference gene (*DHPS*) and expressed in relative units (RU). **Results:** *GrB* and *CD86 mRNA* levels were significantly higher in biopsies from patients with persistence than in those without persistence, with median values of 2.12 and 0.84 RU for *GrB* ( $P = 0.008$ ), 0.53 and 0.27 RU for *CD86* ( $P = 0.001$ ), respectively. No difference was observed regarding the other molecules. **Conclusion:** Besides prognostic potential concerning response to treatment, this finding also suggests association of more aggressive tumor process with a particular immune activation profile.

Supported by: FAPESP.

### Sa2.112. Potent Tumoricidal Capacity of Macrophages Activated by GcMAF and Therapeutic Use of GcMAF for a Variety of Cancers.

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Intratumor BCG administration can eradicate local as well as metastasized cancerous cells, suggesting development of immunity against the tumors. Administration of BCG into noncancerous normal tissues, however, results in no significant effect on the tumors. A potent inflammatory agent, BCG, in normal tissues activates membranous phospholipase A<sub>2</sub> to release lysophospholipids that efficiently activate macrophages. Because cancerous tissues contain alkylphospholipids, BCG-induced inflammation produces alkyl-lysophospholipids and alkylglycerols that activate macrophages with approximately 400 times more efficiently than lysophospholipids (Yamamoto et al. 1988. *Cancer Res* 48: 6044-9). These results imply that highly activated macrophages can kill cancerous cells. Inflammation-primed macrophage activation process is the principal macrophage activation cascade that requires serum vitamin D<sub>3</sub>-binding protein (known as Gc protein) and participation of B and T lymphocytes. Stepwise hydrolysis of Gc protein with  $\beta$ -galactosidase of inflammation-primed B cells and sialidase of T cells yields a potent macrophage activating factor (MAF), the protein with N-acetylgalactosamine as the remaining sugar. Stepwise treatment of highly purified Gc protein with immobilized  $\beta$ -galactosidase and sialidase generated the most potent MAF (terminated GcMAF) ever discovered which produces no side effect in human. Administrating 10 pg GcMAF per mouse

and 100 ng GcMAF per human results in the maximal activation of macrophages, which develop enormous variation of receptors. When human macrophages were treated *in vitro* with 100 pg GcMAF/ml for 3 hrs, the macrophages were highly activated. These macrophages can bind a variety of cancerous cells. Because cancer cells are too big to be phagocytized, macrophage membrane spreads over the cancer cell. These attached macrophage membrane surface is the same as phagosome membrane, which secretes superoxide and other lytic enzymes. Trypan blue begins to penetrate through the attached site and eventually stains the entire cells. Time course study of the cell death was performed with effector/target ratio of 3. In 4 hrs, 51% of prostate cancer cells LNCaP and 60% of breast cancer cells MDA-MB-231 were killed. In 18 hrs, 82% of LNCaP and 86% of MDA were killed. When prostate, breast, colon and lung cancer patients were treated with less than 25 weekly administrations of 100 ng GcMAF, the majority of cancer patients, excluding very advanced, exhibited healthy control levels of the serum prognostic index, indicating eradication of tumors. When *in vitro* cancer cell-killing study with macrophages activated by GcMAF was performed in the presence of serum or IgG fraction of GcMAF-treated prostate and breast cancer patients, greatly accelerated cell-killings (more than 80% of cells in 4 hrs) were observed. These results suggest that the macrophages activated with GcMAF kill cancerous cells via Fc-receptor mediation preferentially and that GcMAF-therapy develops IgG antibodies against the tumors.

### Sa2.113. Clonal B Cells in Blood and Bone Marrow of Patients with a Plasma Cell Dyscrasia- Light Chain Amyloidosis.

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Light chain amyloidosis (AL) is a plasma cell neoplasia, where there is a clonal expansion of terminally differentiated B cells (plasma cells) within the bone marrow (BM) and the monoclonal immunoglobulin light chain forms insoluble amyloid fibrils that deposit systemically. We have previously shown that there is a bias in light chain variable (VL) gene usage in clonal plasma cells, with 3 rarely used  $\lambda$  light chain genes (*6a*, *3r* and *2b2*) accounting for 60% of the AL light chain V gene repertoire. We now show that peripheral blood and bone marrow of these patients contain clonally related B cells, that have a mature B cell, non-plasma cell phenotype, in the absence of plasma cells. We evaluated the light chain V gene repertoire in the clonal isotype compartment ( $\kappa$  or  $\lambda$ ) of 5 AL patients ( $3\lambda + 2\kappa$  for PBL and  $4\lambda + 1\kappa$  for BM) and 2 healthy controls. The BM mononuclear cells were enriched separately for CD19+ B cells and CD138+ plasma cells. The peripheral clonal isotype repertoire had between one-third to one-half of the B cells using the same V and J genes as the clonal population in the BM. By sequence alignment of the blood B cells and BM plasma cells using the same VJ genes, there was evidence of intraclonal variations in the blood B cells compared to the BM cells. Also, there was a restriction in diversity in the VL gene repertoire with AL patients using 6–8 different genes compared to 12–16 VL genes in the healthy controls. Though there is a clonal expansion within the specific isotype compartment, there is no change in the global numbers of  $\kappa$  and  $\lambda$  B cells in blood. In the CD19+ BM population, 3 of the 5 patients tested had between 0.8-1.4% of plasma cells in the B cell population. Three of the 5 patients had between one-third to greater than two-thirds of the

CD19+ B cells in the BM using the same VJ genes as the clonal CD138+ (plasma cell) population. These patients, by sequence alignment, showed clonal homology in the CDR3 and VJ junctions. In the remaining 2 patients, one had no evidence of any clonally related CD19+ B cells while the other showed 3 populations of B cells using the same V gene but different J genes. In this latter patient, we were unable to identify a dominant clonal plasma cell population. Interestingly, the greater the number of clonal B cells in the CD19+ BM population, the more restricted the VL gene diversity. The CD138 and CD19 sorted populations in the BM from the healthy controls showed a typical polyclonal repertoire with 22–25 V $\lambda$  and 15–17 V $\kappa$  genes represented in the CD138+ plasma cells and 7–15 V $\lambda$  and 15–23 V $\kappa$  genes in the CD19+ B cells. The presence of clonal B cells in the BM and blood of AL patients is relevant for understanding the pathobiology of disease and also has significant implications for therapy. These data, while preliminary, suggests the need for a paradigm shift in AL pathogenesis.

#### **Sa2.114. Immunomodulation by Different Forms of a Chimeric Costimulatory Molecule That Selectively Binds to CD28.**

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The efficacy of several immunotherapeutics and adjuvants is limited by their specific activity and/or undesired ligand binding properties. We have used DNA shuffling to generate large libraries of chimeric adjuvant proteins and costimulatory molecules, and have selected the improved variants based on ligand binding and functional properties. This presentation describes one such chimera: CD28 binding protein (CD28BP), which is a DNA shuffled version of B7.1 that preferentially binds to CD28. Interestingly, CD28BP demonstrates adjuvanticity in a membrane-bound form yet inhibitory activity when soluble. We have combined DNA encoding CD28BP with DNA encoding an *in silico* shuffled cancer antigen that induces immune responses cross-reactive with Epithelial Cell Adhesion Molecule (EpCAM) to form the basis of a cancer vaccine candidate. In non-human primate studies we show that this approach achieves the goal of augmenting EpCAM-specific immunity with an excellent safety profile. Importantly, we demonstrate that the presence of CD28BP in the vaccine construct is required for the detection of EpCAM-specific CD8 T cell IFN-gamma production. Based on these findings, we believe this novel vaccine approach has the potential to break the immunological tolerance against EpCAM that limits current colorectal carcinoma vaccine approaches. Moreover, CD28BP has broader applications as a general DNA vaccine adjuvant. In contrast, soluble CD28BP protein has the capacity to inhibit mixed lymphocyte reactions and antigen-specific T cell responses *in vitro* making it a suitable candidate for autoimmune therapeutic applications.

#### **Sa2.115. Enhanced Melanoma Tumor Growth in CD28 Deficient Mice.**

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For the activation of naïve T cells two signals are necessary. Besides the cognate recognition of antigen in the context of MHC

molecules, co-stimulatory signals are essential for effective T cell responses and here the CD28/B7 receptor ligand system is best characterized. The role of CD28/B7 for anti-tumor immune responses is still controversial. We induced anti-Trp-2<sub>180–188</sub>/K<sup>b</sup> melanoma specific CD8<sup>+</sup> T cell responses by DC vaccination and compared their efficacy to control either subcutaneous or pulmonary metastases of B16 melanoma in CD28-deficient and wildtype mice. In both models, the tumors developed faster in CD28-deficient mice. For subcutaneous metastases, the difference was only prominent during a certain time window after tumor challenge, whereas the areal tumor burden of pulmonary metastases was significantly enhanced in CD28 k.o. mice. Furthermore, the influence of CD28 signalling on priming, homing or effector function of Trp-2<sub>180–188</sub>/K<sup>b</sup>-reactive circulating or tumor infiltrating T cells was phenotypically investigated. Trp-2<sub>180–188</sub>/K<sup>b</sup>-reactive CD8<sup>+</sup> T cells could be detected after DC vaccination in a comparable frequency in the circulation as well as among the cellular infiltrate in subcutaneous tumors in both genotypes. Clonotype mapping of tumor infiltrating lymphocytes showed that subcutaneous tumors in animals of either genotype harbored an oligoclonal infiltrate. Functional analysis of Trp-2<sub>180–188</sub>/K<sup>b</sup>-reactive cells, however, revealed that the number of IFN $\gamma$ -producing cells was substantially lower in CD28 k.o. mice. Hence, in our model priming or homing of Trp-2<sub>180–188</sub>/K<sup>b</sup>-reactive CD8<sup>+</sup> T cells does not seem to be affected in CD28 k.o. mice, but CD28 seems to be essential for effector functions of tumor specific CD8<sup>+</sup> T cells.

#### **Sa2.116. ALL and CLL Cells Express Elevated TNF-alpha upon Stimulation When Compared with Normal B Cells.**

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Rationale: We hypothesized that the malignant B cell has significant autocrine function inducing its own replication and sought to establish its role in its own survival by evaluation of its cytokine profile compared with B cells from phenotypically normal (NL) peripheral blood (PB) and bone marrow aspirates (BM). We further hypothesized that the immunologic profiles differ by the type of B cell malignancy.

Methods: Discarded BM or PB specimens from patients diagnosed morphologically and immunophenotypically as ALL ( $n = 16$ ) or CLL ( $n = 18$ ) were evaluated for induced expression of tumor necrosis factor alpha (TNF), IL2 and IL4 and compared with NL BM or PB specimens ( $n = 13$ ). Specimens were washed, resuspend at  $1-10 \times 10^6$ /ml in RPMI+10% FCS and incubated with PMA (25 ng/ml), ionomycin (1.0 mcg/ml) and brefeldin A (10mcg/ml) for 4–6 hours at 37° in 5% CO<sub>2</sub>. The cells were then washed, resuspended in PBS, incubated with monoclonal antibodies (MAb) to CD19; CD2; CD38 and CD45 (Beckman Coulter, Hialeah FL) for 15 minutes, then fixed and permeabilized with Intraprep kit (Beckman Coulter) and incubated with MAb to TNF, IL2 and IL4. (Becton Dickinson, San Jose CA) for 15 minutes. The cells were washed, resuspended in PBS and analyzed by a Coulter XL flow cytometer using standard CD45 by right angle side scatter employing logical gating strategies using PE and FITC conjugated isotypic controls antibodies to establish the histogram region negative for fluorescence. The percentage of CD19+ cells expressing TNF was recorded for each specimen. The mean for each of the ALL and CLL groups was compared with the NL group using a standard t test with unequal variance.

Results: Stimulated ALL cells showed a statistically significant increase in the percentage of cells expressing TNF (7.25%) compared with NL stimulated B cells (0.3%) ( $P = 0.012$ ). Similarly, stimulated CLL cells showed a statistically significant increased expression of TNF (4.06%) compared with NL stimulated B cells ( $P = 0.015$ ). The difference in TNF expression between stimulated ALL cells and CLL cells did not reach statistical significance ( $P = 0.17$ ). Within the CLL group, for specimens with B cell CD38 expression less than 15% ( $n = 9$ ) the mean TNF expression was 0.68% and for specimens with CD38 expression greater than 15% ( $n = 6$ ) the mean TNF expression was 10.6%. There was a significant difference in the percentage of TNF expression in those CLL cells with CD38 expression greater than 15% compared with those with CD38 expression less than 15% ( $P = 0.04$ ). There was no difference between NL cells and ALL or CLL cells and induced IL2 and IL4 expression.

Conclusion: Stimulated ALL and CLL B cells express TNF suggesting a possible role for this cytokine in the pathogenesis of these malignancies. For CLL, the degree of TNF expression may have prognostic significance given its correlation with CD38 expression. These findings support the initiation of an investigation into the possible role for available anti-TNF therapies in clinical studies of both ALL and CLL.

*This work is support by a grant from the National Leukemia Research Association.*

#### **Sa2.117. Role of the MAP Kinase Signaling Pathway in the Regulation of Antigen Expression by Human Melanomas.**

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Efforts to enhance anti-tumor immunity have led to identification of numerous tumor-associated antigens, but vaccination with such antigens have shown limited efficacy. Our studies have focused on the ability of melanomas to escape immune destruction by selective down-regulation of the very antigens that are being targeted with tumor vaccines, because even if a vaccine is able to raise highly specific cellular and humoral immunity, immune-mediated destruction requires continued expression of target antigens. With melanoma vaccines, the melanocyte-specific antigens such as Melan-A/MART-1, gp100 and tyrosinase show extensive heterogeneity with respect to their expression levels in tumor cells. During our search for agents that can retain or enhance melanoma antigen expression, we showed that the MAP kinase pathway (MEK1/2) inhibitors PD98059 and U0126 were capable of enhancing expression of both mRNA and proteins for several antigens in melanoma cell lines. While treatment of melanomas with these agents induces dramatic morphological changes, and over time induces some apoptosis (approximately 25% at 4 days), there is a dose and time-dependent enhancement of antigen expression in viable cells. In melanomas a specific B-raf V599E mutation has been detected at high frequency, which results in constitutively active B-raf and activation of the MAP kinase signaling pathway. Data obtained with the MAP kinase inhibitors therefore suggested a possible link between B-raf function and antigen expression. However, we observed no simple relationship between B-raf mutational status and antigen expression levels in 23 melanoma cell lines. Furthermore, Western blotting showed no clear correlation between phospho-ERK levels, melanoma antigen expression, and B-raf mutational status.

These data, together with MEK inhibitor enhancement of antigen expression, indicate that there are multiple influences on MAP-kinase signaling that influence levels of antigen expression. Transient over-expression of B-raf offered a direct means of modulating MAP kinase signaling in a series of melanomas. Accordingly, we found that in four antigen-positive cell lines of variable V599E B-raf mutational status (two heterozygous wild-type /mutant, one homozygous wild type, and one homozygous mutant), transient over-expression of mutant B-raf resulted in increased ERK activation and reduction of Melan-A/MART-1 and gp100 antigen levels. We conclude that inhibition of ERK activation by MAP kinase pathway inhibitors promotes antigen expression in melanoma cell lines, irrespective of original antigen expression status (high, low or absent expression of Melan-A/MART-1 and gp100). Conversely, when ERK activation is induced by transient constitutively active mutant B-raf transfection, antigen expression declines. These data indicate that while several factors may influence levels of antigen expression in melanomas, blocking MAP kinase activation enhances expression of several melanocyte-associated antigens, indicating that MAP kinase inhibition may prove helpful in targeting of melanomas in immunotherapy trials.

#### **Sa2.118. Optimization of a Manufacturing Process of Dendritic Cell Vaccine To Treat Colorectal Cancer.**

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Patients with advanced colorectal cancer have limited options for treatment. Immunotherapy, and especially dendritic cell (DC)-based vaccines, appears to be one of the most promising therapies. We identified peptides from CEA, MAGE and HER2/*neu* that stimulated CTL *in vitro* for further development. Previously, using normal healthy donors, we developed a large scale manufacturing process to produce adequate number of autologous DC loaded with the immunogenic peptides for use in a clinical trial. This process cultures mononuclear cells (MNC) in serum-free VacCell<sup>®</sup> media containing GM-CSF and IL-13. On day 7, DC enriched by elutriation were first pulsed with KLH, then matured with a bacterial membrane fraction from *Klebsiella pneumoniae* (FMKp) and IFN- $\gamma$ , and pulsed with peptides. These matured DC were finally cryopreserved. This manufacturing process is capable of generating 10–21 vaccine doses per batch from a qualification study with normal healthy donors ( $n = 5$ ). This process was further simplified by using the Gambro ELUTRA<sup>™</sup> Cell Separation System to perform the elutriation step with a single-use closed system sterile disposable set. Here, we present the qualification of this modified manufacturing procedure in three full scale Collidem<sup>™</sup> process runs. Three apheresis products from three normal healthy donors, after overnight shipping, contained  $1.2 \pm 0.1 \times 10^{10}$  MNC with monocytes ranging from 13 to 18%. MNC were cultured for 7 days in VacCell media containing GM-CSF and IL-13. Enrichment by elutriation generated  $1.1 \pm 0.3 \times 10^9$  DC, with a mean viability and purity of  $96 \pm 2\%$  and  $93 \pm 4\%$ , respectively. Upon maturation and peptide pulsing these DC showed typical patterns of phenotypic maturation, up regulation of CD25, CD80, CD86 and CD83 and down regulation of CD14 with secretion of IL-12p70 and TNF $\alpha$ . The process generated  $7.9 \pm 3.0 \times 10^8$  viable DC that is equivalent to  $23 \pm 8$  vaccine doses per process. Based on these data, the process to manufacture the Collidem vaccine was amended to use this closed system elutriation method, and a Phase I / II clinical trial is underway

to evaluate the Collidem peptide pulsed DC vaccine for treatment of colorectal cancer patients.

### Sa2.119. Hfgl2 Prothrombinase/Fibroleukin Expression in Cancer and Its Potential Clinical Significance.

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**Objectives:** Immune coagulation, microthrombus and fibrin deposition within the microvasculature are major contributors to the pathogenesis of xenograft rejection, viral induced hepatocellular injury and cytokine induced fetal loss syndrome. Here we investigated the contribution of the novel gene fibrinogen like protein 2 (fgl2) prothrombinase mediated immune coagulation in cancer and its potential clinical significance.

**Methods:** Malignant tumor tissues were obtained from 11 patients with colon cancer, 15 patients with cervical cancer, 10 patients with breast cancer, 9 patients with esophageal cancer, 12 patients with lung cancer, 16 patients with gastric carcinoma. Immunohistochemistry and in situ hybridization were used to detect the hfgl2 prothrombinase and hfgl2 mRNA in tumor tissues. Antibodies for CD8, CD57, CD68 and vWF were also used in series histological sections to determine the cellular types in which the hfgl2 was expressed.

**Results:** hfgl2 prothrombinase and hfgl2 mRNA was expressed in almost all the above cancers. Further more, it was evidenced that hfgl2 was highly expressed not just in cancer cells, but also in interstitial infiltrated cells include macrophages, NK cells, CD8+T lymphocytes and vascular endothelial cells.

**Conclusion:** The expression of hfgl2 in cancer cells and activated interstitial infiltrated cells may contribute to the characteristic statue of hypercoagulability, which in turn induces tumor angiogenesis and metastasis. The molecule of hfgl2 may serve as a potential therapeutic target for the management of tumor development. This work was supported by the National Science Fund for Distinguished Young Investigators (No. 30225040 for Dr. Q. Ning, No. 30123019 for Dr. XP Luo).

### Sa2.120. Expression of Stromal Cell-Derived Factor-1 in Brain Biopsies from Patients with Primary Central Nervous System Lymphoma.

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**Objective:** Although the pathogenesis of primary central nervous system lymphoma (PCNSL) remains unclear, it is hypothesized that specific chemokine-chemokine receptor interactions contribute to localization of malignant B lymphocytes to the brain and eye. One candidate mediator is the lymphoid chemokine, stromal cell-derived factor-1 (SDF-1, CXCL12). Although initial work focused on its critical role in hematopoiesis, more recently the participation of SDF-1 in neural development has been recognized; SDF-1 is constitutively expressed by brain neurons and endothelium, neuroglia and meningeal cells. Consequently we studied the expression of this chemokine in PCNSL.

**Methods:** Formalin-fixed, paraffin-embedded specimens from 10 patients with PCNSL were cut 3 microns in thickness and stained by standard indirect immunohistochemical methods, using

a goat polyclonal anti-human anti-SDF-1 antibody (Santa Cruz Biotechnology) at a concentration of 5 µg/mL. Following deparaffinization of the tissue, antigen retrieval was performed by boiling the sections for 10 minutes in 10 mM citrate buffer at pH 6.0. Normal tonsil, and astrocytoma and meningioma biopsies were also immunostained. Negative controls were prepared by substituting goat IgG (Sigma) for the specific antibody.

**Results:** Positive staining for SDF-1 was identified in all 10 of the PCNSL biopsy specimens. This expression was localized to neurons, endothelial cells and meningeal cells. Weaker staining was also observed in lymphoma cells that were either diffusely distributed throughout the brain tissue or present as perivascular infiltrates. Neuronal and meningeal expression of SDF-1 was noted in the astrocytoma and meningioma biopsies; tonsil stained positively for SDF-1 in the crypt and outer epithelium, and within the tonsil proper. Negative controls showed no positive staining. Interestingly, a mouse monoclonal anti-human SDF-1 antibody (R&D Systems) that recognized SDF-1 in tonsil showed no reactivity in either normal brain or PCNSL biopsy specimens.

**Conclusions:** Expression of SDF-1 occurs within PCNSL lesions in the brain, as well as normal brain tissue. Studies examining the functional relevance of this expression are indicated to assess possible involvement of SDF-1 in the pathogenesis of PCNSL.

### Sa2.121. Phase 1–2 Evaluation of Different Immunotherapy Protocols for GBM.

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Glioblastoma Multiform (GBM) cells escape immune recognition by two major mechanisms. These cells produce strong anti-inflammatory substances in the local tumor micro environment and fail to express MHC II proteins as well as co-stimulatory and adhesion molecules on their surface. Mixed Leukocyte Culture Cytoimplant (MLC) has been previously shown to function as a powerful intratumor pro-inflammatory cytokine pump, which can reverse the anti-inflammatory activity of the tumor microenvironment (Cancer, 2000, 88: 1325–1335). Tumor-B cell hybridoma vaccines (TBH) have been shown to function as antigen presenting cells, which can facilitate CD4 T cell recognition of the tumor antigen (Transfus Sci 1996, 17: 643–649). The use of each of these therapies as single agents or combined have been tested successfully in clinical trials for others tumors (for pancreatic adenocarcinoma, ASCO Proceeding 2001, 20: 264a, and for breast cancer ASCO Proceeding 2003, 22: 182.). In this phase one study we have evaluated toxicity and possible antitumoral effect of each treatment and the combination of both. As a preliminary study 11 consecutive patients with GBM according to standard selection criteria were divided in three treatment groups. Group 1: (4 patients) Debulking Surgery (DS) + Radiotherapy (Rx), second DS + MLC; Group 2: (4 patients) DS + Rx, second DS + TBH; Group 3: (3 patients) TQ + Rx, second TQ + MLC + TBH.

#### Results and Conclusions

Group I: 4 patients; 4.5 months of survival after second surgery; CR 1/4, PR 2/4, PD 1/4; 2/4 Severe Inflammation.

Group II: 4 patients; 10.75 months of survival after second surgery; CR 2/4, PR 1/4, PD 1/4; 0/4 Severe Inflammation.

Group III: 3 patients; 8.0 months of survival after second surgery; CR 2/3, PR 1/3, PD 0/3 2/3 Severe Inflammation.

50 % of the patients from group 2 are alive up to now, with non signs of relapsings. However, at the moment the Kaplan Meyer analysis does not show significant difference in the survival between the three groups. Treatment with MLC has a strong and rapid therapeutic effect, but it is limited in duration. It can generate sever encephalitis. Treatment with TBH has a slow and lasting therapeutic effect. It does not generate encephalitis. Treatment with MLC + TBH acts synergically, provoking a rapid, strong and lasting therapeutic response. In 50% of patients, it generated encephalitis. The three immunotherapy regimens seems to have strong antitumoral effect but they differ on velocity and the risk to develop secondary severe brain inflammation which is a complication that seems to be related with the MLC procedure and start around 15 days after the it was done. No other major toxic effect was observed.

### Sa2.122. Suppression of Lck Sensitizes Acute Lymphoblastic T Cells to the Antiproliferative Action of Interferon Alpha.

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We studied the effects of Lck suppression on the sensitivity of acute lymphoblastic T cells to the antiproliferative action of interferon alpha. Short interfering RNA (siRNA)-mediated Lck ablation reduced the activity of downstream signaling pathways indicating that Lck is constitutively active in lymphoblastic T cells despite the absence of T cell receptor stimulation. Lck-deficient and Lck siRNA-treated Jurkat cells were drastically more sensitive to the antiproliferative effect of interferon alpha than wildtype or untreated cells through a reduction in the rate of S-phase progression. Interestingly, Lck deficient cells were not more sensitive to the proapoptotic action of interferon alpha. Exposure of a panel of acute lymphoblastic T cells to an Lck-specific inhibitor similarly sensitized the cells to the antiproliferative effect of interferon alpha. This work provides a rational basis for the combined use of Lck-specific inhibitors and interferon alpha for the treatment of T cell acute lymphoblastic leukemia. We are currently employing reverse phase protein microarray technology to explore the mechanism by which Lck inhibition sensitizes interferon alpha activity.

### Sa2.123. Regulatory Cells and Human Malignant Gliomas.

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We have isolated and analyzed both tumor cells and tumor infiltrating lymphocytes (TILs) present in ex vivo central nervous system (CNS) tumor specimens. We used flow cytometry to examine immunologically important cell surface molecules on glioblastoma multiformae (GBM) tumor cells prior to any culturing in vitro, and screened supernatants from ex vivo GBMs for secreted cytokines. Tumor cells expressed moderate to high levels of both HLA class I and class II molecules, suggesting that tumor cells could directly interact with and influence TILs. In addition, a majority of the GBMs examined ( $n = 10$ ) secreted high amounts of the immunosuppressive cytokine IL-10. Based on these observa-

tions, we hypothesize that GBM tumor cells promote the differentiation and/or expansion of IL-10-secreting type 1 T regulatory (Tr1) cells, which in turn suppress tumor immunity in situ. Consistent with this hypothesis, additional preliminary observations suggest that a CD25+ population of CD4+ TILs, which appear to represent effector cells activated in situ, infiltrates GBMs and that these cells are notable for their secretion of IL-10. In contrast, CD4+ TILs lacking CD25 expression are notable for their secretion of IFN-g and the absence of IL-10. These data suggest a novel mechanism by which the tumor microenvironment of GBMs influences the function of TILs and hinders their ability to mount an effective tumor response.

### Sa2.124. Transfection of Breast and Lung Cancer Cell Lines with sHLA-A\*0201 and sHLA-B\*0702.

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**Introduction.** Cancer is a disease that affects more than a million individuals every year. The pathogenesis of cancer is not yet completely understood and is considered to be multifactorial. Although there is a wide range of behavior between different cancers, all cancer cells present atypical morphology, bizarre mitotic cycles and uncontrolled growth rate, ultimately leading to invasion and killing of the host. The human immune system represents a powerful mechanism for detecting and destroying cancer cells. Immune recognition is mediated by the major histocompatibility complex class I (MHC I) molecules, which scan the cell's proteome and carry small peptides of intracellular origin to the cell surface. Effector T-lymphocytes (CTL) survey MHC-peptide complexes and target cells displaying cancer-specific peptides. What epitopes differentiate the cancer cells from normal cells? Recognizing the MHC-peptide phenotype of cancer cells is a critical step in the development of CTL-activating vaccines and/or the design of new therapies. **Objective.** The process of epitope discovery from cancer cells requires large amounts of HLA to extract and identify the peptides presented by the MHC I. Therefore, our immediate goal is to *transfect and express the soluble human MHC I molecules (sHLA)-A\*0201 and -B\*0702 in two or more cancer cell lines* that will allow the production of large quantities of sHLA and facilitate the elucidation of MHC-epitopes presented on cancer cells. **Materials and Methods.** Breast cancer cell lines MCF-7 and MDA-MB-231 as well as lung cancer cell lines A549 and NCI-H292 were transfected with sHLA-A\*0201 and sHLA-B\*0702. sHLA constructs lack the intracellular and transmembrane domains of the  $\alpha$ -chain, and were modified at the 5' end by adding the last 10 carboxy-terminal amino acids of the rat VLDL receptor (VLDLr). sHLA-VLDLr were cloned into the mammalian expression vector pcDNA3.1(-) Geneticin or Zeocin (Invitrogen) using FuGene 6 Transfection Reagent (Roche). Transfected cell lines were selected with the specific antibiotic, and assayed for production of the transfected A\*0201 and B\*0702 molecules using an in-house ELISA. Several positive clones were obtained from each cell line and high producers were selected by sequential subcloning and ELISA testing for sHLA protein production. **Results.** Two breast cancer and two lung cancer cell lines were successfully transfected with sHLA-A\*0201 and sHLA-B\*0702. Stable clones producing sHLA  $\geq 50$  ng/ml are now available for the production of high quantities of sHLA and the future detection of unique peptide epitopes that distinguish cancer cells from normal cells. **Conclusions.** Stable and high sHLA-producer cancer cell lines are an

important preliminary step in the cancer epitope discovery process. Fulfillment of our immediate goal enables us with the tool to proceed in the discovery of unique epitopes that characterize the MHC-peptide phenotype of cancer cells.

### Sa2.125. Serologic Study Using a GST-Capture ELISA for Determination of Anti-HPV16 Antibodies in Tunisian Women.

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Cervical cancer is the second most tumor in Tunisian women just after breast cancer.

Human papillomavirus (HPV) infection is considered the main cause of invasive cervical cancer especially HPV16 and HPV18; Moreover, antibodies against the E6 and E7 proteins of HPV types 16 and 18 have been found to be strongly associated with cervical cancer. Many assays can be used to define presence of these antibodies but the majority of them have low sensitivity.

The present work is the first one in Tunisia in which we have used a GST-capture ELISA in order to estimate the antibody response against HPV16 E6 and HPV16 E7. These antigens were overexpressed in E.coli as GST fusion proteins as described by the research group of Pawlita.

Using this system, we have analysed 205 sera; 71 cases of cervical cancers, 64 cases of inflammation of the cervix and 70 controls. Results showed that among cancers, positivity is 37% and 42% for 16 E6 and 16 E7 respectively and it is 0% and 2% for inflammations, 3% in controls for both 16 E6 and 16 E7.

## Immunology of the Eye

### Sa2.126. The Involvement of Autoimmunity Against Retinal Antigens in Determining Disease Severity in Toxoplasmosis.

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**PURPOSE.** Ocular lesions are frequent in various individuals infected with *Toxoplasma gondii*. Disease intensity in ocular toxoplasmosis varies greatly between patients. Autoimmunity has been suggested as a possible component to retinal destruction.

**METHODS.** Immunologic parameters in the response to retina antigens were evaluated in infected persons with and without ocular lesions and in no infected controls. Subjects were divided into groups on the basis of titers of serum antibodies to *T. gondii*, presence and severity of ocular lesions, and clinical history.

**RESULTS.** Peripheral blood mononuclear cells from patients with mild disease responded to one or more retinal antigens with a significantly higher frequency than patients without disease or with severe disease. Interestingly, the cytokines produced by the proliferating mononuclear cells did not follow any specific patterns, except for the fact that IL-4 and IL-5 were seldom detected.

**CONCLUSIONS.** Our results suggest that although the presence of an immune response towards autoantigens is not protective against the development of ocular lesions by the *Toxoplasma gondii*, it may protect against the development of severe disease.

### Sa2.127. Ipsilateral Lymphadenectomy To Inhibit Corneal Allograft Rejection in Rats.

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**Objective:** To investigate use of ipsilateral lymphadenectomy therapy for inhibiting immune rejections in rat corneal transplantation. **Methods:** Corneal allogenic transplantation models were established in rats. 18 female Wistar rats were used as donors, and 36 female Sprague Dawley (SD) rats were used as recipients. After corneal penetrating transplantation, recipients were randomly divided into 3 groups: Group A was the control group; Group B, the bilateral lymphadenectomy group; Group C, the ipsilateral lymphadenectomy group. Among of 12 rats in each group, the corneas of 2 rats in each group were used for pathological study at day 14 after the transplantation, the other 10 rats were used for studying corneal immune rejection with a slit lamp. The point in time when allograft rejection occurred was recorded and mean survival time (MST) were compared. In addition, the rejection index, clarity, edema and vascularization of the grafts were examined and compared 14 days after transplantation. **Results:** MST of the grafts in group B and C were (46.30 ± 9.464)days, (44.43 ± 7.604)days, respectively, and were increased significantly ( $P < 0.01$ ) in comparison with the group A, which had MST of (10.71 ± 1.567) days. The difference in the MSTs between group B and C was not significant ( $P > 0.05$ ). Pathological study showed that at 14 days after transplantation, the corneas in group A were in a state of edema and showed disturbed collagen fibers and very significant neovascularity. However, for corneas in group B and C, no significant changes were detected compared with normal corneas except for a reduced density of endothelial cell. 14 days after corneal transplantation. The rejection index for group B and C, was much lower than that for group C. **Conclusions:** Both bilateral and ipsilateral lymphadenectomy therapies have a significant effect in preventing corneal allograft rejection. Ipsilateral lymphadenectomy is a less complex surgical procedure and is just as effective in preventing rejection.

**Key Words:** inhibit; corneal transplantation; allograft rejection

### Sa2.128. Different Circulating Levels of Endothelin-1 in Plasma from Glaucoma Patients.

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**Introduction:** The glaucomatous optic neuropathy is one of the first causes of blindness around the world. The exact cause of glaucoma is not known, however in the recent years it has been suggested that endothelin-1 (ET-1) on aqueous humor (AH) is related with the generation of glaucoma in humans and animals models.

In order to understand the participation of ET-1 in the maintenance of the different types of glaucoma, we determined the ET-1 concentration in AH and plasma from glaucoma patients.

**Materials and Methods:** After informed consent, blood and AH samples were taken from patients with glaucoma treated with trabeculectomy (Primary open-angle glaucoma-POAG-, Acute closed-angle glaucoma-ACAG-, Pseudoexfoliative glaucoma-PG-) and from patients with senile cataract treated with phacoemulsification and without antecedent of glaucoma (Control group, CG). ET-1 determination was made by a chemiluminescent immunoassay

and the results were analyzed with the Mann-Whitney Rank Sum Test considering a difference statistically significant with a  $P < 0.05$ .

Results: ET-1 concentration in AH from glaucoma patients was  $3.9 \pm 0.9$  pg/100ml, while in CG group was  $5.2 \pm 1.5$  pg/100ml; ET-1 plasma concentration from glaucoma patients was  $0.8 \pm 0.1$  pg/100ml, while in CG group was  $0.4 \pm 0.1$  pg/100ml ( $P = 0.008$ ). We do not find statistical differences in the concentration of ET-1 in AH between the different groups of glaucoma patients: POAG  $3.2 \pm 0.8$  pg/100ml, ACAG  $7.9 \pm 3.1$  pg/100ml, PG  $3.2 \pm 2$  pg/100ml, and we do not find statistical differences when we compared each group with CG group. Also, we do not find statistical differences in the concentration of ET-1 in plasma samples between the different groups of glaucoma patients: POAG  $0.9 \pm 0.2$  pg/100ml, ACAG  $0.6 \pm 0.2$  pg/100ml, PG  $0.7 \pm 0.2$  pg/100ml. However, when we compared ET-1 concentration in plasma from each group of glaucoma patients with the CG group, we found that only in the POAG patients a difference statistically significant ( $P = 0.014$ ).

Conclusions: Our results suggest that only in the groups of patients with POAG could be an association with the ET-1 plasma concentration and glaucoma. We consider that the diverse clinical presentations of glaucoma studied here are a consequence of a different molecular process, in which of them, the ET-1 could play a minimum role. At the present time, there are developing drugs against endothelin to treat glaucoma, however the differences observed in our study should be considered in order to guarantee the success of new therapies for glaucoma.

#### **Sa2.129. LPS Stimulation Induce an up Regulation of TLR-4 on Limbal Epithelial Cells without Secretion of TNF-Alpha.**

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Introduction: Little is known about the expression of natural immune receptors as toll like receptors in corneal epithelium, and their function in ocular immune response is controversial.

Objective: We sought to determine the extracellular expression of TLR-4 on human limbal epithelial cells cultivated in vitro and if so, to determine its cellular function after LPS stimulation.

Materials and Methods: From sclera-corneal rims, limbal epithelial cells were isolated and grown in the presence of supplemented hormonal epithelial medium at 37°C and 5% CO<sub>2</sub> until confluence. At passages one or two, the cells were exposed to different doses of LPS from *E. coli* for 24 h. After stimulation, the cells were recovered and stained with PE-conjugated monoclonal antibodies against human TLR-4 and analyzed by flow cytometry; mRNA was obtained and RT-PCR was performed for the identification of TLR-4, GAPDH was used as an internal control. Secretion of TNF-alpha by these cells was evaluated by ELISA on the supernatant. PBMC were used as LPS activation controls.

Results: Limbal epithelial cells expanded in vitro expressed constitutively low density TLR-4; after stimulation with LPS the expression of TLR4 was augmented taking into account the medium fluorescence intensity. A similar behavior was observed at the mRNA level, the expression was augmented after stimulus. When TNF-alpha was evaluated, interestingly, this cytokine was not detectable at any concentration of LPS and even at 48 h of

stimulus. PBMC secreted optimal concentrations of TNF-alpha after LPS stimulation.

Conclusions: Although the extra cellular expression of TLR4 on limbal epithelium stimulated in vitro up-regulates TLR4, its function seems not to be associated with the secretion of TNF-alpha on limbal epithelium.

#### **Sa2.130. CD80 and CD86 Expression on Corneal Epithelial Cells Infected with Adenovirus.**

MaCarmen Jimenez,<sup>1</sup> Herlinda Mejia,<sup>1</sup> Marisela Linares,<sup>1</sup> Alejandra Sanchez-Navarro,<sup>1</sup> Raul Suarez,<sup>1</sup> Yonathan Garfias.<sup>1</sup> <sup>1</sup>Research Unit, Institute of Ophthalmology, Fundacion Conde de Valenciana, Mexico, D.F., Mexico, D.F., Mexico.

Introduction: CD80 and CD86 belongs a family of proteins named B7. B7 molecules costimulate T cell during immune activation. Normally, the corneal epithelial cells do not have any expression of those molecules on their surface. The objective of this study is to determine if the corneal epithelial cells induce B7-molecules after a viral infection.

Materials and Methods: Corneal epithelial cells were isolated from human corneas treated with dispase-II, and grown in the presence of supplemented hormonal epithelial medium at 37°C and 5% CO<sub>2</sub> until confluence. At passages one or two, the cells were exposed to different doses of adenovirus 5 (Ad5) for 2 h. After infection the cells were washed three times and then cultured at different times. Then, the cells were recovered and stained with PE-conjugated monoclonal antibodies (mAbs) against human CD80 and CD86 and with FITC-conjugated mAbs against human cytokeratin and the results were analyzed by flow cytometry.

Results: Non infected corneal epithelial cells did not express at any time CD80 or CD86; however Ad5 infected epithelial cells were positive to CD80 since 24h (40%) raising its maximum level at 72 h (~90%), CD86 expression on epithelial corneal cells was detected also at 24h (20%) raising its maximum level at 72h (~70%). Conclusions: Our results suggest that corneal epithelial cells express CD80 and CD86 after virus infection.

#### **Sa2.131. Adamantiades-Behcet's Disease: A Trend in Time.**

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**Purpose:** To analyze differences in response to treatment of ocular Adamantiades-Behcet's disease (ABD) in the 1970's, 1980's and 1990's.

**Methods:** Medical records of thirty six patients with uveitis due to Adamantiades-Behcet's disease followed at the National Eye Institute (NEI) were reviewed.

**Results:** All the patients were divided in 3 groups according to the time of follow-up: first group was followed from 1962 until 1972, second group from 1983 until 1992 and the third group from 1992 through 2004. Visual acuities and degrees of inflammation in each group were recorded at each visit. There were 12 patients (24 affected eyes) in the 1970's group, 7 patients (14 eyes) in the 1980's group and 17 patients (34 eyes) in the most recent group. Mean follow-up in the 1970's group was 3.06 years, in the 1980's-4.68 years and in the most recent group- 4.03 years. Mean time between the diagnosis of ABD and presentation to NEI was 3.8 years in the 1970's group, 3 years in the 1980's group and 4.4 years in the most



recent group. Therapeutic agents used in the 1970's group included systemic steroids, methotrexate, 6-mercaptopurine, azathioprine, cyclophosphamide and chlorambucil. Agents used in the 1980's group included systemic steroids, cyclosporine, chlorambucil and colchicine. Medications used in the most recent group included systemic steroids, cyclosporine, azathioprine, daclizumab, mycophenolate mofetil, methotrexate and infliximab. Statistical analysis showed that average logMAR change per year was 0.6479 in the 1970's group, -0.0225 in 1980's group and -0.0272 in the most recent group.

**Conclusion:** Adamantiades-Behcet's disease is a severe blinding disorder. Though the trend for clinical improvement from 1970's to 1990's was noticed, the changes in visual outcomes did not reach statistical significance. Larger studies may be more empowered to demonstrate the trend for improvement due to introduction of newer agents and directed therapy.

### Sa2.132. Visual Outcomes in Children with Juvenile Idiopathic Arthritis-Associated Uveitis.

L. I. Kump,<sup>1,2</sup> R. A. Cervantes Castaneda,<sup>1</sup> S. N. Androudi,<sup>1</sup> G. F. Reed,<sup>2</sup> C. S. Foster.<sup>1</sup> <sup>1</sup>Ocular Immunology and Uveitis Service, Massachusetts Eye and Ear Infirmary, Boston, MA, USA; <sup>2</sup>Laboratory of Immunology, National Eye Institute, NIH, Bethesda, MD, USA.

**Purpose:** To analyse visual outcomes in children with juvenile idiopathic arthritis-associated uveitis.

**Methods:** Charts of 89 children with JIA-associated uveitis were reviewed.

**Results:** Among 269 children with uveitic syndromes referred to a tertiary eye center 89 children (33%) had juvenile idiopathic arthritis associated uveitis. The process was bilateral in 76 children. Seventy three patients were females, 84% of patients were Caucasian. Mean age of onset of uveitis was 5.7 years. Mean follow-up was 2.96 years. ANA positivity was detected in 51 patients, 42 of them females. The patients with JIA-associated uveitis developed numerous complications in the course of their disease: among 165 affected eyes 105(64%) developed cataracts, 33 (20%) developed increased intraocular pressure, 76 (46%) eyes developed band keratopathy, posterior synechiae were present in 96 (58%) eyes. Among 89 children 73% were on immunomodulators, and 40% were treated with non-steroidal anti-inflammatory agents alone or in combination with immunomodulators, and 21% were treated with topical and/or systemic steroids. Among 65 children who required immunomodulation, only one chemotherapeutic agent was used in 30 children, two agents in 21 children, and three or more in 14 children. Visual acuities of the patients were documented and compared at standard intervals. By mixed models linear regression, visual acuity improved an average of 0.0113 logMAR units at each two-month visit ( $P = 0.032$ ).

**Conclusion:** In spite of the severity of JIA-associated uveitis, much of the children's vision can be preserved if the patients are treated appropriately.

### Sa2.133. Increased Expression of I $\kappa$ B $\alpha$ Permits Tolerance Induction by TGF $\beta$ -Treated Antigen Presenting Cells.

A. P. Ghafouri,<sup>1</sup> B. Turpie,<sup>2</sup> J. W. Streilein,<sup>2</sup> S. Masli.<sup>2</sup> <sup>1</sup>HMS, Harvard Medical School, Boston, MA, USA; <sup>2</sup>Department of Ophthalmology, Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA.

Antigens introduced in the anterior chamber of the eye are processed and presented by resident antigen presenting cells (APCs) in a manner that results into antigen-specific peripheral tolerance that is characterized by suppression of Th1-mediated immune response such as delayed type hypersensitivity (DTH). Similarly, conventional APCs such as peritoneal exudates cells (PECs) or a macrophage hybridoma clone (#59) that are treated with TGF $\beta$  in vitro can induce comparable peripheral tolerance. Such TGF $\beta$ -treated APCs are known to express a unique set of genes that are essential for their tolerance inducing ability. Suppression of two genes regulated by NF $\kappa$ B, IL-12 and CD40, is critical for their tolerizing property. The I $\kappa$ B proteins that prevent nuclear translocation of NF $\kappa$ B dimers therefore, can also support suppressed expression of IL-12 and CD40. Antigen presenting cells treated with TGF $\beta$ , were found to up-regulate their expression of I $\kappa$ B $\alpha$ . In this series of experiments we have assessed contribution of I $\kappa$ B $\alpha$  to tolerance promoting properties of APCs. Macrophage hybridoma stably expressing anti-I $\kappa$ B $\alpha$  siRNA or ectopically overexpressing I $\kappa$ B $\alpha$  were examined for their ability to express I $\kappa$ B $\alpha$  protein (using western blot), suppress OVA-specific DTH and secrete IL-12 in culture supernatants. These APCs were pulsed with ovalbumin (OVA) and cultured overnight in the presence or absence of TGF $\beta$ 2 (5 ng/ml). NF $\kappa$ B activity of these APCs was assessed by measuring nuclear levels of p50 and p65 using TransAm chemiluminescence assay (Active Motif, CA). Increased levels of I $\kappa$ B $\alpha$  protein were detectable in TGF $\beta$ -treated #59 and I $\kappa$ B $\alpha$  overexpressing #59, however APCs expressing anti-I $\kappa$ B $\alpha$  siRNA failed to do so. Inhibition of IL-12 secretion by TGF $\beta$  treatment was found reversed in APCs expressing anti-I $\kappa$ B $\alpha$  siRNA. While both TGF $\beta$ -treated #59 and #59 overexpressing I $\kappa$ B $\alpha$  suppressed OVA-specific DTH, such suppression was not induced by TGF $\beta$ -treated #59 expressing anti-I $\kappa$ B $\alpha$  siRNA. Nuclear p50 levels were significantly decreased in TGF $\beta$ -treated #59 and I $\kappa$ B $\alpha$  overexpressing APCs, while such a decrease was prevented in TGF $\beta$ -treated anti-I $\kappa$ B $\alpha$  siRNA expressing #59. These results demonstrate that TGF $\beta$ -treatment of APCs increases their expression of I $\kappa$ B $\alpha$  which prevents expression of proinflammatory molecules such as IL-12 by preventing nuclear translocation of NF $\kappa$ B. Such increased expression of I $\kappa$ B $\alpha$  permits tolerance induction by these APCs.

### Sa2.134. CD36 and Thrombospondin Interaction Is Essential for the Tolerance Inducing Properties of TGF $\beta$ -Treated Antigen Presenting Cells.

S. Masli,<sup>1</sup> B. Turpie,<sup>1</sup> J. W. Streilein.<sup>1</sup> <sup>1</sup>Department of Ophthalmology, Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA.

Antigen presenting cells (APCs) in the anterior chamber of the eye are known to induce antigen-specific systemic tolerance in which Th1-mediated immune response such as delayed type hypersensitivity (DTH) is suppressed. Active TGF $\beta$ , abundantly available in the ocular environment, confers such tolerance-inducing property on these resident APCs. Similarly, in vitro treatment of conventional APCs with TGF $\beta$  converts their functional phenotype to that of tolerizing APCs. Investigation of molecular mechanisms underlying this conversion has revealed significant involvement of various molecules such as TNF $\alpha$ , MIP-2, TGF $\beta$ 2, CD40 and IL-12. Products of these genes, via various pathways contribute to systemic tolerance induced by TGF $\beta$ -treated APCs. More recently, we detected increased expression of extracellular matrix protein, thrombospondin (TSP), in TGF $\beta$ -treated APCs. In this report we have assessed molecular

interactions of TSP that lead to tolerogenic phenotype of these APCs. Conventional APCs (either hybridoma cell line #59) or thioglycollate-elicited peritoneal exudates cells (PECs) can express TSP receptors such as CD47 as well as CD36. Macrophage hybridoma #59 or peritoneal exudates cells collected from wild type(WT) or TSP-1 null or CD36 KO mice were used as APCs. These APCs, pulsed with ovalbumin (OVA) and treated with TGF $\beta$ 2 (5 ng/ml), were examined for their ability (1) to suppress OVA-specific DTH response, (2) to inhibit IL-12 secretion by ELISA and RT-PCR and (3) to enhance TSP or TNF $\alpha$  expression by RT-PCR. While WT APCs treated with TGF $\beta$  or #59 treated with TSP suppressed OVA-specific DTH, both TSP-1 and CD36 deficient APCs failed to do so in response to TGF $\beta$  treatment. In case of TSP-1 deficient APCs, exogenously added TSP restored their ability to acquire tolerogenic properties upon TGF $\beta$  exposure. Unlike WT APCs, both TSP and CD36 deficient APCs treated with TGF $\beta$  secreted increased levels of IL-12. Exogenously added TSP mimicked TGF $\beta$  in inhibiting IL-12 secretion by WT PECs and enhancing TNF $\alpha$  expression by #59. Absence of CD36 on PECs also prevented their ability to increase expression of TSP. These results support the possibility that TSP expressed by TGF $\beta$ -treated APCs contributes significantly to tolerance inducing property by facilitating inhibition of IL-12 secretion and expression of TNF $\alpha$ . Interaction of TSP with its receptor CD36 is essential for the tolerizing phenotype of TGF $\beta$ -treated APCs.

### Poster Session 1

7:30 AM–1:30 PM, 5/15/2005

### Cytokines/Chemokines

#### Su1.01. STAT-1 Mediates the Stimulatory Effect of IL-10 on CD14 Expression in Human Monocytic Cells.

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IL-10, an anti-inflammatory cytokine, has been shown to exhibit stimulatory functions including CD14 upregulation on human monocytic cells. CD14-mediated signaling following LPS stimulation of monocytic cells results in the synthesis of proinflammatory cytokines. Our flow cytometry results show that LPS-induced CD14 expression on monocytic cells may be mediated by endogenously produced IL-10. To investigate the molecular mechanism by which IL-10 enhances CD14 expression, both human monocytes and the promyelocytic HL-60 cells were used as model systems. IL-10 induced the phosphorylation of phosphatidylinositol 3-kinase (PI3K) and p42/44 extracellular signal-regulated kinase (ERK) mitogen-activated protein kinases (MAPK) as determined by western blotting with phospho-specific antibodies. By employing specific inhibitors for PI3K (LY294002) and ERK MAPKs (PD98059), we demonstrate that LY294002 either alone or in conjunction with PD98059 inhibited IL-10-induced phosphorylation of signal transducer and activator of transcription-1 (STAT-1) and consequently CD14 expression. However, IL-10-induced STAT-3 phosphorylation remained unaffected under these conditions. Furthermore, LY294002 and PD98059 inhibited the binding of the STAT-1 transcription factor to its binding site in the CD14 promoter. Finally, transfection of HL-60 cells with STAT-1 interfering RNA vectors

inhibited IL-10-induced CD14 expression. Taken together, these results suggest that IL-10-induced CD14 upregulation in human monocytic cells may be mediated by STAT-1 activation through the activation of PI3K either alone or in concert with the ERK MAPK.

#### Su1.02. Altered Cytokine Production and Reduced Membrane Signaling through the Antigen Receptor in CD4+ T Cells Overexpressing Ly-6A.2 Molecule.

Anil Bamezai, Jennifer Reed, Abraham Chacko. <sup>1</sup>Biology Department, Villanova University, Villanova, PA, USA.

Mouse Ly-6 molecules serve as excellent differentiation markers on immune cells of hematopoietic origin, but their influence on cellular differentiation is unknown. CD4+ cells over-expressing Ly-6A.2 resist polarization to either Th1 or Th2 phenotype. To understand the mechanism underlying the polarization tolerance in Ly-6A.2 over-expressing CD4+ T cells, we examined their cytokine profile in response to varied antigenic stimulation under primary culture conditions. Ly-6A.2 over-expressing cells generated higher levels of both Th1 and Th2 signature cytokines in a dose dependent manner. In addition, the CD4+ T cells over-expressing Ly-6A.2 showed reduced phosphorylation of LAT than the controls. Our results suggest that Ly-6A.2 expression on CD4+ T cells diminishes their membrane proximal signaling in response to the antigen receptor and therefore might contribute to their altered cytokine profile. These results also suggest that CD4+ T cells capable of producing high levels of Th1 and Th2 signature cytokines in response to antigen stimulation are resistant to polarization to either Th1 or Th2 phenotype.

#### Su1.03. Lymphocytes Demonstrate Different Functioning Changes Induced by Exogenous PDGF-AB and TGF-b In Vitro at Cerebral and Coronary Atherosclerosis: What Is Established?

Andrei I. Teplyakov.<sup>1</sup> <sup>1</sup>Resrarch Institute for Ecopathology and Occupational Diseases, Mogilev, Belarus.

We hypothesized that proatherogenic T cells are controlled by cytokines network balance. Among them, TGF-b has been implied in atherogenesis, but its mechanism of action remains unclear. Taken together, abrogation of TGF-b signaling in T cells able to accelerate the atherosclerosis and permit to suggest that TGF-b reduces atherosclerosis by dampening T cell activation. Inhibition of T cell activation may therefore represent a strategy for antiatherosclerotic therapy. **Goal:** to analyze the possible evidence for TGF-b action on lymphocytes functions (G<sub>0</sub> chromatin topography changes) in vitro at cerebral and coronary atherosclerosis. **Object:** 29 patients. The original research technique has been used: adding TGF-b to achievements the final concentration: 0.05, 0.5 and 5.0 ng/ml in whole blood sampled PDGF-AB for achievement final concentration in whole blood sample: 0.5, 5.0 and 10.0 ng/ml with following incubation in plastic tube for each sample. The lymphocytes chromatin nuclei behavior was estimated after 30, 60 and 360 min for each samples. The chromatin of lymphocytes nuclei was studied using Computer TV Morphodensitometry System "DiaMorph" (Russia) in the smears dyed especially for DNA. **Results** are contraindicated to habitual opinions about common atherogenic mechanisms without evaluation of organ-target influence. At first sight, it seem to be possible that mechanisms of sensitivity to PDGF have switched on (i.e. PDGF at cerebral atherosclerosis acted in other manner or in assembly to other factor, which are could be known). However, more complex manner of chromatin changes of lymphocytes, implicated in

atherogenesis, have shown, that they have undergone by most of involved components of cytokines network factors, which are reversed timely. The main detected features at cerebral in comparison to coronary atherosclerosis have consist of initial hetero- and euchromatin rebuilding. Obtained results have conflicted to becoming fixed opinion that cerebral and coronary atherosclerosis are common process, unified any atherosclerosis without locations evaluation. So it is possible, that the detected changes have resulted more disseminated vascular implication in cerebral atherosclerosis in comparison to coronary ones. **Conclusion.** Obtained results are controversial to described the dampening lymphocytes activation, induced by TGF- $\beta$ . We have no satisfied results, evidenced the TGF- $\beta$  suppressive manner on lymphocytes functioning at atherosclerosis. Received results, in our opinion, reflects the more complicated interaction between TGF- $\beta$  and lymphocytes functioning changes. It has been confirmed by the facts, that G<sub>0</sub> lymphocytes chromatin (dependent from concentration and exposition time) have changed its portrays on biphasic manner after PDGF-AB adding: initially at short period of time we detected the same nuclear activation (and gene expression activation, correspondingly) with following stable chromatin topography changes, reflected the stable lymphocytes functioning changes on pathologic manner, which is needed to more detail investigation.

#### Su1.04. Live Measurement of Chemokine Triggered Integrin Activation on a Single-Molecule Level.

R. H. Eibl.<sup>1</sup> <sup>1</sup>Institute of Pathology, Technical University of Munich, Munich, Germany.

Atomic force microscopy (AFM) based force spectroscopy can be used for direct measurement and quantification of cell adhesion forces on living cells down to single-molecule interactions. This technique is used here to study the physiologic activation of alpha(4) integrins by the chemokine CXCL12 on living cells expressing the chemokine receptor CXCR4. At low physiologic concentration of CXCL12 the rupture force of a single pair of adhesion receptors increases from 40pN to 60-80pN, whereas at higher concentrations of CXCL12 it lowers the rupture forces back to 40pN. This study confirms that arrest chemokines such as CXCL12 can rapidly modulate the affinity of an integrin receptor. To date, this is the first direct measurement of chemokine induced affinity modulation of a single (adhesion) receptor as well as of the desensitization of a chemokine receptor, both measured on a molecular level on a living cell. AFM based force spectroscopy has evolved into a completely new pharmacological test for single-molecule interactions on living cells. This technique will be used to elucidate the mechanisms involved in both physiologic leukocyte homing as well as organ-specific tumor cell metastasis.

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#### Su1.05. Sp1 Binding Site of TGF-beta1 Promoter Is the Target for Trans-Activation by HPV-16 E6 and E7 Oncoproteins.

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TGF- $\beta$ 1 is a multifunctional cytokine involved in several immunoregulatory processes and plays a critical role in the escape of some cancer from host immunity. HPV is the main etiologic agent in cervical cancer, and E6 and E7 oncoproteins have properties of transforming cell and transregulation of cellular genes. We have identified a correlation between TGF- $\beta$ 1 gene expression and the advance of the malignancy, which suggest that HPV induces TGF- $\beta$ 1 gene expression. However, the mechanisms by which HPV induce TGF- $\beta$ 1 in cervical cancer remain unclear. In this study we analyzed whether HPV-16 E6 and E7 oncoproteins are involved in the molecular mechanisms of TGF- $\beta$ 1 gene expression. For that end, we amplified the human 5'-TGF- $\beta$ 1 promoter by PCR from peripheral blood lymphocyte DNA, which was cloned in pBlue-script and sequenced. This DNA fragment was subcloned in pBLCAT3 and several constructs generated by deletion of TGF- $\beta$ 1 promoter. C33A cells were transfected with this constructs and evaluated the effect of HPV-16 E6 and E7 oncoproteins by cotransfection with pSV2E6 and pSV2E7. We observed that E6 and E7 oncoproteins induced two-fold promoter activity inside TGF- $\beta$ 1 core promoter, while had not significant effect in other TGF- $\beta$ 1 regulatory regions. We also observed several interactions DNA-protein between HPV-16 E6 and E7 oncoproteins with Sp1 regulatory elements of TGF- $\beta$ 1 core promoter by Footprinting and EMSA. In addition, we identified a super shift DNA-protein complex specific associated to Sp1 binding site of TGF- $\beta$ 1 core promoter when the anti-E6 or anti-E7 antibodies were used. The results suggest specific interactions between viral transcriptional factors inside of TGF- $\beta$ 1 core promoter. These results may explain the molecular mechanisms of TGF- $\beta$ 1 gene regulation in cervical cancer as well as tumor escape mechanisms from host anti-tumoral immune response.

#### Su1.06. Orexin-A Has Delayed Reponse to Intestinal Ischemia-Reperfusion Injury.

Ji Lin,<sup>1</sup> Guang-Tao Yan,<sup>1</sup> Xiu-Hua Hao,<sup>1</sup> Lu-Huan Wang,<sup>1</sup> Kai Zhang.<sup>1</sup> <sup>1</sup>Research Laboratory of Biochemistry, Basic Medical Institute, General Hospital of P.L.A, Beijing, Beijing, China.

**BACKGROUND:** Orexin-A, also named hypocretin-1, is a novel neuropeptide secreted by specific neurons in lateral hypothalamus. Recent findings suggest that orexin-A provides a critical link between the peripheral energy balance and central

nervous system mechanisms that coordinate sleep-wakefulness and motivated behaviors, mainly promoting food intake and suppressing energy expenditure. As orexin-A is an active mediator closely related with energy metabolism, we hypothesize that orexin-A may undergo a fluctuation during the severe metabolic impediment of acute inflammation, such as intestinal I/R injury.

**MATERIALS AND METHODS:** An intestinal ischemia-reperfusion (I/R) injury model of rats was established, and rats were divided randomly into six groups: sham-operation group, 60 min ischemia/30 min reperfusion group (I60'R30'), I60'R90', I60'R150', I60'R240' and I60'R360', 9 rats each group. A highly sensitive orexin-A radioimmunoassay was used to check the change of orexin-A concentrations in plasma and hypothalamus tissue, and RT-PCR was used to detect the change of orexin-A mRNA expression in hypothalamus tissue. Therefore, the change of orexin-A levels both in peripheral blood and central secretory tissues before and after intestinal I/R injury could be investigated.

**RESULTS:** Compared with before injury, plasma orexin-A levels of each group showed no significant difference. Compared with sham group after injury, both plasma and hypothalamus orexin-A levels of every other group showed no significant difference. Compared with sham group after injury, orexin-A mRNA expression of I60'R30' and I60'R90' decreased step by step, that of I60'R150' reached the lowest, and that of I60'R240' and I60'R360' recovered gradually to the level of sham group.

**CONCLUSION:** Orexin-A has a delayed response to acute inflammatory stimuli such as intestinal I/R injury, and it may participate in metabolic disorders in the injury as inflammatory cytokines.

**Keywords:** Ischemia-reperfusion, intestinal; Orexin-A; Radioimmunoassay; PCR; Inflammation, acute; Cytokine

#### **Su1.07. Increased Interleukin-6 Serum Levels in Subjects with Metabolic Syndrome.**

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An activation of generalized non-specific inflammation could play a role in the pathogenesis of metabolic syndrome and its components. However, the data regarding the changes of pro-inflammatory interleukins in subjects with metabolic syndrome remain controversial. Therefore, the aim of this study was to investigate the levels of interleukin-6 in the serum in subjects with metabolic syndrome compared to those without this syndrome. We studied 55 patients with metabolic syndrome (age-  $55.8 \pm 1.37$  years, BMI- $32.4 \pm 0.6$  kg/m<sup>2</sup>, data are presented as mean  $\pm$  SEM) and 50 subjects with no metabolic syndrome (age-  $52.7 \pm 1.40$  years, BMI- $27.6 \pm 0.6$  kg/m<sup>2</sup>). The diagnosis of metabolic syndrome was made based on ATP-III criteria (2001). In the group of patients with metabolic syndrome there were 18 subjects with type 2 diabetes mellitus, 16 persons with impaired glucose tolerance, arterial hypertension was diagnosed in 45 subjects. In those persons without metabolic syndrome type 2 diabetes mellitus was registered in 3 subjects, impaired glucose tolerance was found in 7 persons, arterial hypertension was diagnosed in 8 subjects. All persons studied did not receive any medications for the treatment of diabetes mellitus or arterial hypertension at the time of the examination. There were no clinical signs of any concomitant disease in patients studied. The levels of interleukin-6 were measured by specific assay. We found that the levels of interleukin-6 were significantly increased in patients with meta-

bolic syndrome compared to those without this syndrome-  $1.44 \pm 0.3$  and  $0.625 \pm 0.16$  pg/ml, respectively,  $P < 0.05$ . We can conclude that elevated serum interleukin-6 levels could reflect an activation of generalized inflammation in patients with metabolic syndrome, which could contribute to the development and progression of atherosclerosis and high risk of cardiovascular disease in these patients.

#### **Su1.08. LIGHT Regulates CD86 Expression on Dendritic Cells through NF- $\kappa$ B, but Neither p44/42 MAPK nor JNK/AP-1 Signal Transduction Pathway.**

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The members of the tumor necrosis factor (TNF) family play pivotal roles in the regulation of the immune system. LIGHT is a type II transmembrane protein belonging to the TNF family that was originally identified as a weak inducer of apoptosis. This cytokine has been extensively studied on its role in T cell regulation. Recently, we identified its role in inducing maturation of dendritic cells, such as LIGHT upregulated CD86 expression on dendritic cells in our previous report. However, the signal transduction pathway on this regulation remain unknown. In this study, we found that LIGHT activated NF- $\kappa$ B, p44/42 MAPK, but not JNK. LIGHT upregulates CD86 expression on DCs through activation of NF- $\kappa$ B, but not p44/42 signal pathway, because inhibition of NF- $\kappa$ B activity by its inhibitor could blunt the effect of LIGHT in up-regulation of CD86 expression, but neither inhibitor of p44/42 nor JNK inhibitor has this effect. Thus we demonstrate that LIGHT regulates CD86 expression through NF- $\kappa$ B signal transduction pathway but neither p44/42 MPAK nor JNK/AP-1 signaling pathway. We conclude that NF- $\kappa$ B signal plays a key role in LIGHT-mediated upregulation of CD86 expression.

#### **Su1.09. An Important Role of Inducible Histamine in P.acnes-Primed and LPS-Induced Hepatitis in Mice.**

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Histamine is a well known mediator of allergic inflammation, synthesized by one step enzymic reaction with histidine decarboxylase (HDC). The increase in vascular permeability, vasodilatation and the stimulation of nerve terminals of primary sensory neurons through the stimulation of H1-receptors contribute to the facilitation of the inflammatory response. In addition to H1-receptor-mediated effects, histamine has been demonstrated to be involved in the regulation of innate and acquired immune responses through H2-receptors. In the previous study, we demonstrated that histamine inhibited the IL-18-induced expression of ICAM-1 on monocytes and the subsequent production of IL-12 and IFN- $\gamma$  in human PBMC. Histamine also inhibited the ICAM-1 expression on monocytes in a human mixed lymphocyte reaction in the presence of IL-18. These effects of histamine were all mediated by H-2-receptors.

Fulminant hepatic failure is pathologically characterized to be diffuse intrahepatic infiltration by inflammatory cells with massive multilobular necrosis. Heat-killed *Propionibacterium acnes* (P.acnes) followed by challenge with a low dose of lipopolysaccharide (LPS) induces acute and massive liver injury, mimicking fulminant hepatic failure. In the present study, we examined a functional role of inducible histamine in the protection against hepatic injury and lethality in P.acnes-primed and LPS-induced hepatitis, using HDC knockout and H2-receptor knockout mice. Moreover, we investigated the effects of the inhibitor of histamine N-methyltransferase (HMT) on hepatitis. LPS challenge after P.acnes-priming increased HDC activity in the liver of wild-type mice, associated with a marked elevation of histamine and tele-methylhistamine levels. Western blotting showed the increase in 74 kDa HDC band in the liver. These results strongly indicated that HDC protein was induced by LPS in the liver and that the histamine produced had high turnover rate. HDC-like immunoreactivity was observed in CD68-positive Kupffer cells/macrophages. Treatment of wild-type mice with famotidine and ranitidine but not chlorpheniramine augmented hepatic injury and inhibited the survival rate. The same dose of P.acnes and LPS induced severe hepatitis and high lethality in HDC and H2-receptor knockout mice, the former were rescued by the subcutaneous injection of histamine. Histamine suppressed the expression of IL-18 and TNF- $\alpha$  in the liver, leading to the reduced plasma levels of inflammatory cytokines. HMT inhibitor had similar effects to histamine. These findings indicated that endogenously produced histamine plays a very important role in preventing excessive innate immune response in endotoxin-induced fulminant hepatitis through the stimulation of H2-receptors.

#### **Su1.10. Receptors for Vascular Endothelial Growth Factor (VEGF) on Murine Lymphocytes and Macrophages.**

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VEGF is considered to be one of the most important angiogenic factors. It is known to be chemoattractive for human monocytes and to possess some immunoregulatory influence on T-cells. The aim of the study was to evaluate the mRNA expression of VEGF receptors: VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) in murine immunocompetent cells. Isolated thymocytes, lymph node cells, whole thymus and peritoneal macrophages from intact C3HA mice were used for investigation. Peritoneal cells were allowed to adhere for 2 hours and then washed from non-adherent cells. The level of mRNA expression was studied by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated with the help of guanidine thiocyanate method. Synthesis of cDNA templates was carried out using 2  $\mu$ g of total RNA, oligo d(T)15 primers and M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Specific primers used for PCR amplifications were: VEGFR1 forward primer-5'-GAAGCGGTT-CACCTGGACTGAGACC-3'; VEGFR1 reverse primer-5'-GGC-TTTGCTGGGGGATTTCTCTAA-3'; PCR product size 432 bp; VEGFR2 forward primer-5'-ACAGACAGTGGGATGGTC-CTTGCA-3'; VEGFR2 reverse primer-5'-AAACAGGAGGT-GAGCTGCAGTGTGG-3'; PCR product size 272 bp. Primer pairs were designed from nucleotide sequences available in public database. As a control for housekeeping gene an RT-PCR

procedure for  $\beta$ -actin was also delivered. Whole thymus expressed mRNA for both VEGFR1 and VEGFR2, while isolated thymocytes expressed only VEGFR2 mRNA. Peritoneal macrophages as well as isolated lymph node cells expressed mRNA for both receptors. Incubation of peritoneal macrophages in the presence of 50 ng/ml of VEGF during 24 h resulted in the enhancement of VEGFR1 mRNA expression. The expression of VEGFR2 gene in murine thymocytes was shown for the first time. This suggests that VEGF may directly influence thymic cells and have some specific influence on the development of T-cells. As it was previously shown VEGF can modulate thymocyte mitogen-induced proliferation and spontaneous apoptosis in vitro and evokes thymic involution while administered in vivo (Ohm et al., 2003). The expression of VEGFR2 mRNA in murine peritoneal macrophages is also a novel finding. These data indicate that tissue macrophages possess both receptors unlike blood monocytes that are known to express only VEGFR1. Supported by grants: RFBR N.03-04-49186 and Scientific Schools N.540.2003.4.

#### **Su1.11. Modulation of Suppressor of Cytokine Signalling 3 (SOCS3) Expression and Stability in Human Neutrophils and in Differentiated PLB-985 Cells by G-CSF, GM-CSF and IL-4.**

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Rationale: Polymorphonuclear neutrophils (PMN) are major players in inflammation. They are the first cells to arrive at an inflammatory site and are involved in the inflammatory response. Inflammation is largely resolved when PMN undergo apoptosis and are then ingested by phagocytes such as macrophages. Many cytokines can modulate PMN functions including apoptotic rate and the ability to exert phagocytosis. This is the case with G-CSF, GM-CSF and IL-4. IL-4 is particularly known to enhance PMN phagocytosis and to delay apoptosis. After binding to their specific receptor, many cytokines activate the intracellular Jak/STAT pathway. It was recently discovered that the Jak/STAT pathway can be regulated by a family of proteins named SOCS (suppressor of cytokine signalling), that inhibit the pathway via a feedback loop mechanism. CIS, SOCS2 and SOCS3 have been reported to be expressed in PMN. The aim of the present study was to evaluate the expression of SOCS and their modulation in PMN and in the PLB-985 cell line following cytokine stimulation. PLB-985 are immature promyelocytic cells that acquire a neutrophil-like phenotype when dimethylsulfoxide (DMSO) is added to the culture medium. Methods: PMN were freshly isolated from the venous blood of healthy volunteers. Differentiated PLB-985 (PLB-985D) were obtained after incubation of PLB-985 cells with 1,25% DMSO for 6 days. Cells were incubated with either G-CSF, GM-CSF, IL-2, IL-4 or IL-6 for 1 to 8h. RT-PCR was used to detect SOCS mRNA and protein expression was studied by immunoblotting using specific antibodies. The proteasome inhibitor MG132 was used to investigate the SOCS protein pool that is rapidly degraded in the cell. Cycloheximide (CHX) was used to inhibit protein synthesis and to answer whether or not SOCS proteins are de novo synthesized. Results: G-CSF, GM-CSF and IL-4 were found to induce the mRNA expression of SOCS3 in PMN after 1h of stimulation. PMN, but not PLB-985D cells, were found to express a basal level of the SOCS3 protein. After pre-incubation with MG132, treatment of cells with G-CSF, GM-CSF or IL-4 enhanced the level of SOCS3 in PMN and induced its expression in PLB-985D

at the protein level. Stimulation with IL-6 also induced SOCS3 expression in PLB-985D but not in PMN. CHX reversed the increase of SOCS3 in PMN following stimulation with cytokines. CIS and SOCS2 mRNA expressions varied in PMN and PLB-985D, but this was not observed at the protein level. Conclusions: SOCS3 mRNA and protein expression increased in PMN and PLB-985D after stimulation with G-CSF, GM-CSF or IL-4. SOCS3 is rapidly degraded in PMN and PLB-985D, since the increased expression of protein was better observed when cells were pre-incubated with the proteasome inhibitor MG132. The elevated level of SOCS3 protein expression in PMN and PLB-985D is probably the result of de novo protein synthesis, since this was inhibited by CHX. CIS and SOCS2 mRNA levels varied in cells following stimulation with the cytokines we used, but their protein expression remained stable suggesting the existence of another mechanism governing their expression.

### Su1.12. Quantification of mRNA Expression of TNF- $\alpha$ and TGF- $\beta$ , Fas, TNFR1 and 2 Using Real-Time Quantitative RT-PCR.

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Measurement of the expression level of cytokines is important for the study of in vivo and in vitro effects of cytokines. However, sensitive and quantitative measurements of mRNA are difficult to perform because the established techniques (e.g. northern blotting) are laborious and prone to contamination. We have applied and validated a real-time quantitative PCR for cytokine mRNA quantification. This method is easy to optimize and offers sensitive, reliable and quantitative results with significantly reduced labour, allowing analysis for cytokine expression for large number of samples. We applied this method for comparing expression levels of several cytokines (TNF- $\alpha$  and TGF- $\beta$ ) and their receptors (Fas, TNFR1 and 2) between eosinophils and neutrophils isolated from peripheral blood. The results showed significance difference comparing to the volunteer samples.

### Su1.13. Role of TWEAK, a Member of the TNF $\alpha$ Family of Cytokines, in Arthritis.

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TWEAK is a macrophage-derived cytokine and member of the TNF $\alpha$  family of ligands originally identified as a weak inducer of death in certain tumor cell lines (TNF-like weak inducer of apoptosis). TWEAK exerts pleiotropic effects on a variety of cell types in vitro, including proangiogenic and proinflammatory activities. These cells express a known receptor for TWEAK, FGF-inducible molecule 14 (Fn14), which is restricted to epithelial and mesenchymal cell types, including synoviocytes and chondrocytes, and is highly upregulated in contexts of injury and inflammatory disease. It was reported that TWEAK induces human synoviocyte production of proinflammatory cytokines, chemokines and MMPs, and osteoclastogenesis of a murine macrophage cell line. Thus, TWEAK may promote joint inflammation and damage. Here we report that neutralizing TWEAK mAbs markedly reduce clinical paw severity in mouse and rat collagen-induced arthritis models. Inhibition by anti-TWEAK mAbs occurs at the challenge phase and does not appear to alter T cell priming or elicitation of

anti-collagen antibodies. Decreased clinical paw severity correlates with reduced inflammation and protection from cartilage and bone loss at the histological level. Further studies are in progress to dissect the mechanism of action whereby TWEAK promotes joint inflammation, cartilage and bone loss and to investigate the potential interplay between TWEAK and TNF in this context.

### Su1.14. The Anti-Proliferative Effect of Infliximab on T-Cells Is Prevented by CD28 Induced Co-Stimulation.

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**Introduction:** The systemic neutralization of the proinflammatory cytokine TNF $\alpha$  has been shown to be beneficial for patients with RA. Since CD28+ T-cells have been implicated in the pathogenesis of RA, we evaluated if the anti-proliferative effect of Infliximab could be prevented by anti-CD28 induced co-stimulation.

**Aim:** The aim of this study was to dissect the effect of TNF $\alpha$  neutralization on a well-defined cell population under highly standardized stimulatory conditions. The effect of anti-TNF $\alpha$  neutralization was thus examined on highly purified naïve human cord blood T-cells under different stimulatory conditions.

**Methods:** Highly purified (>90% CD3+CD45RO-) naïve human T-cells were negatively selected from cord blood. The cells were cultured in a serum free medium (AimV) with or without anti-human TNF $\alpha$  chimeric monoclonal antibody (Infliximab (100 $\mu$ g/mL)), with or without TGF- $\beta$ 1 (10ng/mL) and with or without anti-IL-10 (10 $\mu$ g/mL). The cells were stimulated for four days with anti-CD3 (10  $\mu$ g/mL) with or without anti-CD28 (1 $\mu$ g/mL). For analysis of cell division, T-cells were labeled with CFSE.

**Results:** The effect of TNF $\alpha$  neutralization resided both on the presence of CD28 induced co-stimulation and TGF- $\beta$ 1. The anti-proliferative effect was completely lost in the presence of anti-CD28 induced co-stimulation and strongly reduced in the presence of TGF- $\beta$ 1. Interestingly, the anti-proliferative potential of Infliximab exceeded that of TGF- $\beta$ 1 under suboptimal stimulatory conditions (without co-stimulation). Infliximab acted similarly upon both CD4+ and CD8+ T-cells whereas our earlier work has shown that TGF- $\beta$ 1 generally affects CD8+ T-cells more strongly than CD4+ T-cells. Finally, Anti-IL-10 did not affect proliferation except when TGF- $\beta$ 1 was present, independently of co-stimulation.

**Conclusion:** The primary T-cell responses towards anti-TNF $\alpha$  treatment is affected by CD28 induced co-stimulation and TGF- $\beta$ 1. This may affect the effectiveness of Infliximab in the RA synovium where elevated levels of CD28+ T-cells and TGF- $\beta$ 1 have been reported.

### Su1.15. Lack of Linkage between DNA Methylation and Interleukine-10 Gene Expression.

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**Objective:** Epigenetic regulation including DNA methylation profoundly influences effector cytokine gene expression such as IFN-g and IL-4 in differentiated T helper (Th) cells. However, until now the DNA methylation status of IL-10, a cinderella immunoregulator of the immune system, remains unrevealed. **Methods:** A double cytokine secretion assay allowing simultaneous isolation of cytokine-secreting cell subset of interest was used to highly purify human IL-10<sup>+</sup> IFN-g-, IL-10<sup>+</sup> IFN-g<sup>+</sup>, IL-10- IFN-g<sup>+</sup> and IL-10- IFN-g- human Th cell subsets after short-term ex vivo polyclonal stimulation. Subsequently a global quantitative DNA methylation analysis of IL-10 and other Th cell differentiation related genes was performed. **Results:** Strikingly, no methylation differences were detected between these Th-cell subsets in 15,5 kb of IL-10 genomic locus spanning 9,1 kb upstream and the complete gene encompassing 99 CpGs. This included both promoter as well as other conserved noncoding regions. In contrast, hypomethylation and hypermethylation of the IFN-g gene promoter and exon I and intron I regions were noticeable in IFN-g-producers versus nonproducers, respectively. In addition, antigen-specific IL-10<sup>+</sup> Th-cell subsets showed faded fidelity upon in vitro restimulation already after 2 weeks of in vitro expansion. **Conclusion:** Our results point out a lack of epigenetic memory for IL-10 expression in human Th cells. Thus, clinical applications of IL-10<sup>+</sup> regulatory Th cells should be evaluated carefully with respect of a stable IL-10 expression in the transferred T-cell population.

#### **Su1.16. Epigenetic Regulation of TNF alpha in Monocytes/Macrophages.**

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TNF alpha is regulated at the level of transcription, message turnover, translation and protein turnover. We have also demonstrated a strong role for epigenetic regulation of expression. This has implications for chronic inflammatory diseases where the epigenetic "print" may be altered and contribute to disease persistence. We examined histone acetylation, histone methylation, DNA methylation and nuclear localization of the TNF alpha gene in cell lines and primary cells before and after stimulation to understand the role of epigenetics in the regulation of gene expression. Histone acetylation was not altered in our model systems by acute stimulation but was increased reproducibly by increasing maturation of monocytes. Artificially increasing histone acetylation in some settings increased TNF alpha expression but not in others suggesting histone acetylation was not sufficient for transcriptional competence. In contrast, histone K4 methylation was increased in competent cells and inhibition clearly impaired production of TNF alpha suggesting that this epigenetic mark was critical for transcription. DNA methylation correlated with competence to produce TNF alpha and was also found to be developmentally regulated and critical for production of TNF alpha. Lastly, we have used FISH to define the location of the TNF alpha locus within the nucleus. A human centromere probe was used to define heterochromatin. The TNF alpha locus was generally found in euchromatin in terminally differentiated cells, however, human stem cells which are not competent to produce TNF alpha had their TNF alpha

locus in heterochromatin. From these data, we conclude there is a temporal sequence of events culminating in transcriptional competence. Early in development, the TNF alpha gene exits heterochromatin and the DNA becomes demethylated. Monocyte lineage cells further undergo modification of the H3 and H4 histones with acetylation according to their maturational state. Histone H3 lysine 4 methylation appears to be the final event leading to transcriptional competence of the TNF alpha gene. To determine whether these epigenetic marks could be altered in inflammatory disease states, we investigated patients with systemic lupus erythematosus (SLE) for evidence of altered chromatin structure. Patients with SLE had increased histone acetylation at their TNF alpha locus in monocytes compared to controls suggesting that epigenetic changes may participate in disease perpetuation. In conclusion, these data support a very strong role for TNF alpha epigenetic regulation in the control of expression of this powerful cytokine.

#### **Su1.17. A Fast and Robust Multiplexed Immunoassay Panel for Simultaneously Quantifying 24 Cytokines/Chemokines in Rat Serum or Plasma Using Luminex xMAP Technology.**

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Rat is an animal model used extensively in many areas of biomedical research, where quantification of multiple cytokines and chemokines is critical for understanding physiological and pathological processes such as inflammation. However, limited sample availability, high cost, extra time and labor associated with using multiple conventional ELISA products make it impractical to measure multiple cytokines and chemokines in rat models. We previously reported a 14-plex immunoassay panel for rat cytokines, which is currently available as a commercial product. Here we report the expansion of this multiplexed immunoassay system for simultaneous quantification of 24 different rat cytokines and chemokines (Eotaxin, G-CSF, GM-CSF, GRO/KC, IFN $\gamma$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17, IL-18, IP-10, leptin, MCP-1, MIP-1 $\alpha$ , RANTES, TNF $\alpha$  and VEGF) in a single serum/plasma sample of 5  $\mu$ L volume. The methodology includes typical sandwich immunoassays on the surface of polystyrene beads using specific immobilized capture antibodies, biotinylated detection antibodies and streptavidin-phycoerythrin as the reporter molecule. A Luminex<sup>100</sup> reader is used to quantify the fluorescent signal on the beads. Each antibody pair used for individual analyte is highly specific, with no or negligible cross-reactivities to other cytokines or chemokines within the panel. The standard curves for all analytes range from 6.4 to 20,000 pg/mL. The overall sensitivities are between <1 to 20 pg/mL in serum matrix. The assay robustness is demonstrated by acceptable precisions ( $\leq 14\%$  for inter-assay CV%,  $\leq 8.5\%$  for intra-assay CV%), an average recovery of  $93.6 \pm 23\%$  for linearity of sample dilutions, and an accuracy of  $97 \pm 18\%$  in serum matrix. Total assay time is 3.5 hour for serum-free samples or overnight for serum or plasma samples. The assay panel may also be used for samples such as cell culture supernatant, cell/tissue extract or other biological fluids. This simple, sensitive, accurate, and reproducible assay panel offers an economic and convenient tool for accurate and simultaneous quantification of multiple rat cytokines and chemokines in biological samples.

### Su1.18. Markers of Inflammation, Vitamin E and Peripheral Nervous System Function. The InCHIANTI Study.

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Aging is characterized by a decline in function of multiple physiological systems. The causes of this decline are still unclear but increased oxidative stress, disturbances in energy metabolism and a primary dysregulation of the immune system might play an important role. Aging of the peripheral nervous system is associated with several morphologic and functional changes, including a decrease of the nerve conduction velocity. These changes contribute to age-related-decline in muscle strength, sensory discrimination, and autonomic responses, thereby increasing the risk of falls and disability. The aims of this study were to investigate how nerve conduction velocity in the peripheral nervous system correlates with immunological and inflammatory markers and age-associated diseases. We measured motor nerve conduction velocity of the right superficial peroneal nerve using a standard neurophysiologic technique in a population based sample of subjects aged between 20 and 103 years old taking part in the InCHIANTI study; blood count and standard biochemistry were assayed and a sample obtained for cytokines and vitamin E measurements. The InCHIANTI study examined persons living in the municipalities of Greve in Chianti and Bagno a Ripoli, two small towns located in the surroundings of Florence (Italy), randomly selected from the local population registry. Of the 1292 subjects enrolled in the study, 573 (44.3%) were males and 719 (55.7%) females. Average NCV declined with age both in men and in women. However, in each age-group NCV was higher in women than in men. The age-associated decline in NCV was linear. A history of diabetes ( $P < 0.001$ ), stroke ( $P = 0.009$ ) and peripheral arterial disease ( $P = 0.05$ ) as well as the presence of cognitive impairment (0.004) were associated with a lower NCV, even after sex and age adjustment. Among the cytokines considered, no significant association with peripheral conduction could be found for IL-1 $\beta$ , IL-1 Ra, IL-10 and TNF- $\alpha$ . A significantly lower NCV was measured in subjects whose serum levels of the IL-6 ( $P < 0.001$ ) as well as those of its soluble receptor (s IL-6R) ( $P < 0.001$ ) were in the lower tertile. When IL-6 was adjusted for age and sex the differences among groups disappeared ( $P = 0.32$ ), while for s IL-6R ( $P = 0.03$ ), the adjustment changed only the strength of the association. At multivariate analysis, besides age and height, independent predictors of nerve conduction velocity included: diabetes (<0.001), cognitive impairment (0.001), sIL-6R (0.03);  $\alpha$ -tocopherol (0.04), uric acid (0.01), number of lymphocytes (0.01) and neutrophils (0.004). Our results support the hypothesis that inflammation and oxidative damage are involved in the aging of the peripheral nervous system, independently from age-associated diseases.

### Su1.19. Protective Effect of Galectins Against the Generalized Shwartzman Reaction of Mice.

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Galectins are animal lectins that exhibit affinity for  $\beta$ -galactosides and share certain conserved sequence elements. To date, 14 galectins have been cloned in mammals and shown to play modulatory roles in diverse biological processes such as cell adhesion and proliferation, T cell apoptosis, and immune responses.

Structurally, both galectin (Gal)-8 and Gal-9 belong to the tandem-repeat subfamily that is characterized by the presence of two distinct carbohydrate recognition domains (CRDs) joined by a linker peptide. It has shown that Gal-8 induces adhesion activity and superoxide production in neutrophils, and that Gal-9 possesses eosinophil chemoattractant activity as well as induces superoxide production and prolongs cell survival in eosinophils.

In this study, we showed that intraperitoneal administration of recombinant Gal-8 and Gal-9 strongly induced neutrophil accumulation in peritoneal cavity of mice and Gal-8 and Gal-9 exhibited neutrophil chemoattractant activity in vitro. Neutrophil play a critical role in innate immune response and complications of bacterial infection such as septic shock and septic multiple organ dysfunction syndrome. Therefore, to determine whether galectin-induced neutrophil contribute to enhance septic shock, we examined the functions of Gal-8 or Gal-9 in LPS-induced lethal shock syndrome, known as the generalized Shwartzman reaction that can be elicited by two consecutive injections of LPS. Unexpectedly, Gal-8 or Gal-9 treatment with first LPS challenge increased mouse survival and suppressed the elevation of IL-12, IFN- $\gamma$  and TNF- $\alpha$  levels. This protective effect of Gal-8 or Gal-9 against the Shwartzman reaction and suppression of these cytokine productions were diminished by depletion of neutrophils using anti-Gr-1 monoclonal antibody. Furthermore, treatment of mutant Gal-8 that lacks  $\beta$ -galactoside binding activity, with LPS could not suppress the elevation of IL-12, IFN- $\gamma$  and TNF- $\alpha$  levels, whereas neutrophil accumulation in peritoneal cavity of mice was observed.

Thus, these results indicated that galectins modified various cytokine production induced with LPS and improved mouse mortality of the Shwartzman reaction via neutrophil accumulation. Taken together,  $\beta$ -galactoside binding activity of galectins was required to modify LPS induced cytokine production.

### Su1.20. Analysis of Cytokine Network for Initiation of Immune Responses in the Hyperplastic Thymus Associated with Myasthenia Gravis.

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Myasthenia gravis (MG) is closely associated with thymic abnormality such as hyperplasia and thymoma. Unlike experimental MG, thymectomy is effective in relieving the symptom. In particular, the hyperplastic thymus contains many IL-2R expressing B-cells, including anti-AChR antibody producing B-cells, suggesting that they are developed under the influence of hyperplastic circumstances rather than simply accumulated here from the periphery as a reservoir. In the present study we aimed to analyze thymic environment for induction of B-cell responses.



MG thymi were used to examine a potential for production of T-cell cytokines regulatory for B-cell responses. For analysis of a B-cell stimulatory mechanism, nude mouse splenocytes and various cytokines detected in the MG thymus were used. In the hyperplastic thymi associated with MG, IL-2 producing cells, but not IL-4 or IFN- $\gamma$  producing cells, were exclusively detected, suggesting that conventional mature Th-1 cells that exclusively produce both IL-2 and IFN- $\gamma$  are involved at less extent in thymic IL-2 production. We found that the myoid cell conditioning medium provided a circumstance for thymic and splenic lymphocytes to produce IL-2. We also found many precursor myoid cells that produce 80-kDa and 100-kDa haemopoietic biglycan and AChR (+) cells only in their subset. These two factors and other cytokines produced by myoid cells significantly stimulated B-cell responses in a synergistic fashion with IL-2. All these results suggest that hyperplastic MG thymus contains an alternative immuno-stimulatory environment that may play an important role in breakage of anergic state against AChR.

#### **Su1.21. Age-Related Changes in Cytokine Production in Chernobyl Clean-up Workers from Latvia.**

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The statement that exposure to ionizing radiation accelerates the aging process is disputable. The latest investigations confirm aging to be associated with increased inflammatory activity reflected by increased levels of circulating proinflammatory cytokines. Chronic low-grade inflammation with following impairment of immune system functions in aging promotes the development of age-related diseases, such as cancers, degenerative and infection diseases. In the same way, increased inflammatory activities have been observed after radiation exposure. Other cytokines but proinflammatory also are known to participate in the aging process. Approximately 6000 Latvia's men were affected by ionizing radiation during they have been working in Chernobyl to clean up the after-effects of the Chernobyl power plant accident. The morbidity increases progressively among them year by year significantly exceeding that in population.

**Aim** of the present work was to evaluate the production of several cytokines by peripheral blood cells of individuals who participated in 1986 in the clean-up work of the Chernobyl nuclear power plant explosion aftereffects depending on age.

**Materials and methods.** ELISA measured plasma concentrations of sIL-1 $\beta$  and sIL-6 as well as level of IL-2 and IL-4 spontaneous and after stimulation by LPS and PHA mitogens after 24h and 96h in peripheral blood mononuclear cell culture supernatants were determined in 40 Chernobyl clean-up workers 12–17 years after their work in Chernobyl and in 40 blood-donors without a history of occupational radiation exposure. The ability of peripheral blood leukocytes (PBL) to produce IFNs was determined in 74 Chernobyl clean-up workers 12–14 years after the work in Chernobyl and in age matched 25 blood-donors. IFNs were tested in whole blood cultures by the standard virus cytopathic inhibition micromethod after their in vitro induction by Newcastle disease virus, phytohemagglutinin or double-stranded RNA. Individuals were divided in 2 age groups: the first-age 35–45 and the second-age 46–65.

**Results.** The ability of PBL to produce IFN were significantly decreased in both Chernobyl clean-up workers age groups in comparison with blood-donors (control groups) and the incidence of inability to produce IFN in the 2<sup>nd</sup> Chernobyl clean-up workers group two times exceeds that in the 1<sup>st</sup> ( $P < 0.01$ ). The concentration of sIL-6 was significantly higher in the 2<sup>nd</sup> age group. The production of IL-2 as well as IL-4 by peripheral blood mononuclear cells showed no significant differences nor between both age groups or between Chernobyl clean-up workers and donors.

**Conclusion.** The increased concentration of pro-inflammation cytokine sIL-6 together with significantly impaired anti-viral defense by decreased ability of PBL to produce IFNs in Chernobyl clean-up workers from Latvia are age dependent.

#### **Su1.22. Phosphodiesterase Regulation of TNF- $\alpha$ Expression after Spinal Cord Injury.**

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Spinal cord injury (SCI) consists of two phases, instantaneous cellular destruction and axonal damage caused by the initial mechanical trauma, followed by progressive injury termed "secondary injury," resulting from exposure of surrounding tissue to excitatory amino acids, cytokines, and oxidative metabolites from cellular debris or invading immune cells. It is known that increasing cyclic adenosine monophosphate (cAMP) has potent suppressive effects on the pro-inflammatory actions of the immune response, a critical component of secondary injury. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), a central pro-inflammatory cytokine known to be activated after SCI, is important in triggering cell death and is negatively regulated by cAMP elevation. The cAMP modulation of TNF- $\alpha$  expression is further investigated in this study.

Previous work performed in the lab has shown that the administration of rolipram, a phosphodiesterase inhibitor and a pharmacological agent capable of elevating intracellular levels of cAMP, increases the number of spared oligodendrocyte-myelinated axons after SCI. The overall goal of the current study was to elucidate putative mechanisms by which rolipram can affect TNF- $\alpha$  initiated signaling events that mediate axo-pathology and cell death following spinal cord injury. Female Fischer rats underwent a C5 moderate contusion injury and subsequent rolipram or vehicle treatment. Animals were then sacrificed at different intervals after injury according to known TNF- $\alpha$  peak expression points. ELISA testing of control untreated tissue in comparison to rolipram treated demonstrated that rolipram reduces TNF- $\alpha$  production after SCI compared to vehicle controls. The expression of *tnf- $\alpha$*  is largely controlled through an autoregulatory loop via the transcription factor, nuclear factor kappa B (NF $\kappa$ B), hetero- or homo-dimeric complexes of 5 different subunits. It has been previously demonstrated that a potential shift in the balance of NF $\kappa$ B dimers, with an increase in p50 homodimers causes transcriptional repression culminating in reduced expression of *tnf- $\alpha$*  (Bohuslav et al., *J Clin Invest.* 1998). In the current study, the role of this transcriptional control mechanism in rolipram-mediated *tnf- $\alpha$*  inhibition was explored. Western Blots of injured spinal cord from rolipram or vehicle treated animals were taken an hour after injury and probed for the p50 subunit of NF $\kappa$ B. Results showed that p50 production was greatly increased in the injured spinal cords of rolipram treated animals. Furthermore, in rolipram treated animals,

we also found increased translocation of the catalytic protein kinase A (PKA) subunit to the nucleus. We propose that elevated levels of cAMP and downstream activation of PKA after rolipram treatment, leads to an interaction of PKA at the transcriptional control level of *tnf- $\alpha$*  that alters the balance of NF $\kappa$ B subunit-mediated activation and repression of the *tnf- $\alpha$*  promoter. Further investigation of this interaction may lead to novel neuroprotective therapies for spinal cord injury repair.

**Su1.23. Analysis of IL-27 (EBI3/p28) Expression in EBV- and HTLV-1-Associated Lymphomas: Heterogeneous Expression of EBI3 Subunit by Tumoral Cells.**

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IL-27 is a novel heterodimeric cytokine of the IL-12 family composed of two subunits, Epstein-Barr virus (EBV)-induced gene 3 (EBI3) and p28. EBI3 is expressed at high levels in EBV-transformed B cell lines, and is induced in vitro by the EBV oncogene LMP1 in an NF- $\kappa$ B dependent manner. We now show that EBI3 expression is upregulated in HTLV-1-infected cell lines and IL-2 dependent leukemic cells from Adult-T cell leukemia/lymphoma (ATL) patients, compared to normal activated T cells. EBI3 expression is decreased in HTLV-1-transformed cells by treatment with BAY11-7082, an inhibitor of NF- $\kappa$ B, and is induced in Jurkat cells by expression of HTLV-1 wild-type Tax oncoprotein, but not by a Tax mutant, M22, that is defective for NF- $\kappa$ B activation. In situ analysis of EBI3 and p28 expression in Hodgkin's lymphomas (HL), in various EBV-associated lymphoproliferative disorders (LPDs) (including post-transplant LPDs and nasal-type NK/T-cell lymphomas), and in ATL showed that EBI3 is expressed by neoplastic cells in all cases of HL and of LMP1-positive EBV-associated LPD, and at variable levels in ATL cases, but rarely in control T-cell lymphomas. In contrast, in all lymphomas tested, no or few tumoral cells expressed p28. Consistent with these data, no significant p28 and IL-27 expression was detected in HL-derived cell lines and EBV- or HTLV-1-transformed cell lines. This selective overexpression of EBI3 by transformed cells suggests that EBI3 may play a role, independently from its association to p28, to regulate anti-viral or anti-tumoral immune responses.

**Su1.24. Pin1 Regulates Cytokine Expression in Human Peripheral Blood Mononuclear Cells.**

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Inappropriate cytokine production can trigger a variety of allo- or autoimmune diseases. This is particularly true in organ transplant where immuno-suppressors like FK506 or cyclosporin A have revolutionized the field. As part of their action, these 2 drugs function as peptidyl-propyl isomerase (PPIase) inhibitors. A third member of the PPIase family, Pin1 has been implicated in cell-cycle progression but its function on cytokine expression has not been investigated. In this study, we analyzed the role of

Pin1 on cytokine expression in human peripheral blood mononuclear cells (PBMC). PBMC from normal donors were activated with PHA plus PMA or anti-CD3 plus anti-CD28. Pin1 was inhibited with varying concentrations of juglone, an irreversible active site inhibitor of Pin1. At 1  $\mu$ M, juglone strongly inhibited (60 to 80 % inhibition) GM-CSF, TNF- $\alpha$ , IL-8 and IL-4 mRNA levels by activated PBMC. At concentrations as low as 0.1  $\mu$ M, juglone still reduced GM-CSF, TNF- $\alpha$  and IL-8 mRNAs by 65, 35 and 34 %, respectively but had little effect on IL-4. In order to further demonstrate Pin1's role in cytokine mRNA regulation, we transduced the WW domain of Pin1 (TAT-WWPin1) into PBMC. The WW domain affects Pin1-protein interactions and prevents Pin1 functions as a dominant negative. At 20nM, TAT-WWPin1 decreased GM-CSF mRNA accumulation by 50% but IL-4 mRNA level remained unchanged. These data suggest Pin1 is critical for the mitogen-induced elaboration of cytokines and its inhibition maybe a powerful immuno-suppressant.

**Su1.25. Cytokine and Enzyme Spectrum in Tracheobronchial Aspirate of Newborns with Pneumonia Treated with Recombinant IL2.**

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In tracheobronchial aspirate (TBA) of newborns, suffering from intrauterine pneumonia high levels of IL-1 $\beta$ , IL-8 and TNF $\alpha$  ( $P < 0,001$ ) were determined. The high levels of the proinflammatory cytokines accompanied by the elevation up to 23 times of neutrophil elastase (NE) and in 21 times of neutrophil myeloperoxidase (MPO). The levels of studied enzymes in control were  $70 \pm 21$  ng/ml and  $250 \pm 75$  mg/ml, respectively. Group A of patients were treated with including into traditional therapy endotracheal and intravenous infusions of recombinant IL2 ("Roncoleukin"-Biotech, Russia). The duration of lung mechanical ventilation period of newborns of group A decreased in 2.5 times ( $P < 0,001$ ), in comparison to traditionally treated patients (group B); the duration of antibacterial therapy reduced in 1.5 times ( $P < 0,05$ ); hospitalization period-in 1.4 times and death rate-in 5 times. On the 7<sup>th</sup> day of therapy a significant decrease in IL-8 and TNF $\alpha$  levels have been noticed in patients of group A. The concentration of IL1 $\beta$  was 3 times higher than in traditionally treated group. NE levels have been decreased more than to  $347 \pm 109$  ng/ml ( $1041 \pm 303$  ng/ml-in group B). MPO also decreased more than in control group ( $2978 \pm 1032$  mg/l and  $6686 \pm 1371$  mg/l, accordingly). On the 21 day of monitoring the cytokine's and enzyme's spectrum in TBA of rIL2 treated newborns dose not differ from healthy babies, while the levels in the control group were remain high.

**Su1.26. Cytokines and Anticytokines Therapy in Severe Acute Pancreatitis.**

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Recent studies show an important role of cytokines in the pathophysiology of acute pancreatitis. The increased levels of pro-inflammatory cytokines determine progression of disease and development of its systemic complications. Imbalance between pro- and anti-inflammatory cytokines may play the pivotal role in the development of the septic complications in severe course of acute pancreatitis.

The serum levels of interleukins (IL) 1-beta, 6, 8, 10, IL-1-receptor antagonist (IL-1Ra), tumor necrosis factor (TNF-alpha), CD8, and CD4 lymphocytes were studied in 83 patients with acute pancreatitis.

The increased levels of all pro-inflammatory cytokines at the time of admission were noted in all patients. By that, the highest levels of these cytokines were in patients with severe course of the disease. The initial levels of anti-inflammatory cytokines were higher in patients with mild pancreatitis in compared with severe once. Development of septic complications accompanies by the rapid overexpression of IL-10. Such changes in IL-10 expression in patients with septic complications may be explained by the inhibition of IL-10 the bactericidal function of neutrophils. At development of purulent-septic complications increase others anti-inflammatory cytokines was observed. It was combined with is persistent imbalance of immunoregulatory T-lymphocytes with suppression predominance. Good results are received at application anticytokines therapies (pentoxifylline, dexamethasone) in initial stage of disease. In remote period there are indications to application immunoregulatory cytokines, such as interleukin 2.

Thus, the therapy directed on correction of cytokines status is perspective in acute pancreatitis.

#### **Su1.27. Cytokine Production by Dendritic Cells Stimulated with Microbial Products Modulated by Epigallocatechin Gallate (EGCG).**

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Dendritic cells (DCs) are important monocytic phagocytes essential for innate immunity, especially to opportunistic intracellular microorganisms like *Legionella pneumophila*. In this study DCs were stimulated *in vitro* by either infection with *Legionella* or the microbial stimulant LPS from *E. coli* or MDP from Gram positive bacteria. Production of the Th1 helper cell activating cytokine IL-12 and the proinflammatory cytokine TNF $\alpha$  was rapidly induced. These cytokines are important for antimicrobial immunity and were assessed in the DC cultures by ELISA assay. Treatment of the stimulated DCs with EGCG, the primary polyphenol catechin in plant products, inhibited in a dose dependent manner production of IL-12 important for activating immune cells against intracellular bacteria like *Legionella*. In contrast, treatment of the DCs with EGCG enhanced production of TNF $\alpha$  in a dose dependent manner. TNF $\alpha$  is important in inflammation and considered crucial for the inflammatory response. Thus the results of this study show that EGCG, the major catechin widely present in plant products such as green tea, has marked effects on production of immunoregulatory cytokines by stimulated DCs known to be involved in antimicrobial immunity, especially innate immunity. Further studies are warranted to determine the mechanisms of the effects of this and other catechins on cytokine production by immune cells, such as DCs.

#### **Su1.28. TSG-6 Protein Up-Regulates Cyclooxygenase-2 Expression and Prostaglandin Biosynthesis in Macrophage Cell Line.**

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TSG-6 protein, the secreted product of TNF-stimulated gene 6 (TSG-6), is an important endogenous mediator of inflammation

and female fertility. Previous studies have shown that TSG-6 has a protective effect in murine models of experimentally induced arthritis, but the mechanism responsible for this effect has remained elusive. To gain insights into the role of TSG-6 we investigated whether TSG-6 protein affects the expression of inducible cyclooxygenase-2 (COX-2), a key enzyme in inflammation and immune responses. We found that TSG-6 protein up-regulates the COX-2 protein expression in the RAW 264.7 murine macrophage cell line. The effect of TSG-6 was abolished by heat denaturation, trypsin digestion, or anti-TSG-6 specific antibodies. TSG-6 treatment of cells also resulted in a rapid increase in COX-2 mRNA levels, suggesting that TSG-6 up-regulates COX-2 gene expression. Up-regulation of COX-2 was accompanied by an increase in the biosynthesis of prostaglandins, with a predominance of PGD<sub>2</sub>. The PGD<sub>2</sub> metabolite, 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub>, can act as negative regulator of inflammation through the activation of the nuclear peroxisome proliferator activator receptor- $\gamma$ , and inhibition of multiple steps in the nuclear factor- $\kappa$ B signaling pathway. Thus, induction of COX-2 expression in macrophages and preferential PGD<sub>2</sub> synthesis may underlie the protective effect of TSG-6 observed in experimental models of arthritis. Hyaluronan, a molecule with which TSG-6 interacts, modulates the COX-2-inducing activity of TSG-6 protein. This work provides the first demonstration that TSG-6 can influence the expression of a gene regulating inflammation in a cell culture system, which may help to gain a better understanding of the physiological and pathophysiological functions of TSG-6.

#### **Su1.29. The Experimental Study on the Antitumor Effect of 4-1BBL *In Vivo*.**

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**Objectives:** To observe the oncogenicity *in vivo* of mouse gastric carcinoma cell lines MFC, transfected with 4-1BBL gene and its effect on mouse immune function.

**Methods:** 1. Mouse gastric tumor cell lines (MFCs) was transfected with pMKITneo/4-1BBL, pMKITneo through Lipofectamine<sup>TM</sup>2000 mediation. 2. The MFC cells high-expressing 4-1BBL were chosen by slot-blotting. 3. The MFC/4-1BBL cells, MFC/pMKITneo cells and wild-type MFC cells were injected subcutaneously into 615 mice to observe their oncogenicity. 4. The numeral changes of NK cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells in peripheral blood of bearing-tumor mice were measured by FACS. 5. The apoptosis ratio and the cycle distribution of tumor node cells in tumor-bearing mice was detected by FACS.

**Results:** The 4-1BBL gene had been introduced successfully into mouse gastric carcinoma cell lines MFCs. The oncogenicity of MFC/4-1BBL cells was obviously lower than that of MFC/pMKITneo cells and wild-type MFC cells. The peripheral blood NK cells and CD4<sup>+</sup> T cells in mice inoculated with MFC/4-1BBL cells were notably more than that in mice inoculated with MFC/pMKITneo cells and MFC cells, but to CD8<sup>+</sup> T cells had no difference as compared with before injection of MFC/4-1BBL cells.

**Conclusions:** The oncogenicity of MFC/4-1BBL cell distinctly declines, and it can induce the proliferation of NK cells and CD4<sup>+</sup> T cells in tumor-bearing mice. This study provides experimental basis for the further developing of the gene-modified tumor vaccine. **[Key words]** 4-1BBL gene, gene transfection, tumor immune.

### Su1.30. Enhanced Immunol Efficacy of mIL-23 Gene Modified Tumor Vaccine.

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**Objectives:** To evaluate the effects of mIL-23 gene modified murine colon carcinoma cell (Colon26/IL-23) vaccine on macrophages and T cells.

**Materials and Methods:** Colon26/IL-23, Colon26/LXSN(transfected with vector) and Colon26 cells were treated with mitomycin C to prepare tumor cell vaccines. BALB/c mice were immunized with those vaccines by i.p. injection. Griess assay was used to detect the level of NO in peritoneal macrophages. The proliferation of macrophages and splenic cells was determined using (<sup>3</sup>H]-TdR) incorporation method. Macrophage mediated tumor cytotoxicity and CTL activity were assessed by radioactive (<sup>3</sup>H]-TdR) release assay and LDH assay respectively. The survival of the mice i.p. immunized with the vaccines were also observed.

**Results:** Cytotoxicity of macrophage and CTL activity to tumor were enhanced in the mice i.p. immunized with Colon26/IL-23 vaccine and the level of NO secreted by the macrophages from the mice injected with Colon26/IL-23 vaccine was higher than those in controls. The Colon26/IL-23 vaccine also promoted the proliferation of macrophages and splenic cells and increased survival time of mice which were challenged with wild Colon26 cells.

**Conclusions:** mIL-23 gene modified tumor vaccine can activated macrophages and T cells and also has anti-tumor effect.

### Su1.31. Expressions of mIL-23 Gene in Murine Colon Carcinoma Cells Induces Production of NO and TNF- $\alpha$ in Murine Macrophages.

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**Objectives:** To examine whether expression of mIL-23 in murine colon carcinoma cells (Colon26/IL-23) could induce murine macrophage to secrete NO and TNF- $\alpha$ .

**Materials and Methods:** Normal BALB/c mice were injected by i.p. with Colon26/IL-23, Colon26/LXSN (transfected with vectors), and Colon26 cells respectively. On days 5 and 10 after the cell implantation, peritoneal macrophages were prepared by lavage with HBSS and collecting the adherent cells. NMA (N<sup>G</sup>-monomethyl-L-arginine) and LPS were inhibitor and inducer of NO production respectively. The production of NO was determined by Griess assay. The levels of TNF- $\alpha$  and IL-12 were detected by ELISA. Some mice were used for observation of survival.

**Results:** The levels of NO and TNF- $\alpha$  produced by the macrophages from the mice injected Colon26/IL-23 cells were higher than those from control mice at both time-points. NWA inhibited the production of NO in all groups. The production of NO and TNF- $\alpha$  of the LPS stimulated macrophages from the mice injected Colon26/IL-23 cells was more than those in control groups. The level of IL-12 of the macrophages from the mice injected Colon26/IL-23 cells is similar to controls. The average survival time of the mice injected Colon26/IL-23 cells is longer than that of control groups.

**Conclusions:** Expressions of mIL-23 in murine colon carcinoma cells can induce murine macrophages to secrete higher levels of NO and TNF- $\alpha$  and has anti-tumor effect.

### Su1.32. Control of Apoptosis of Primary Human Peripheral CD4+ T Cells.

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Objective of the study: Peripheral T cell homeostasis is accomplished by balancing between proliferation and apoptosis. This is essential in establishing prompt immune responses but at the same time to avoid hypersensitivity reactions, immune-mediated diseases, and lymphoproliferative disorders. Apoptosis is controlled via two different pathways. Antigen stimulation can induce Fas-dependent cell death, called activation-induced cell death (AICD), whereas absence of survival signals leads to activated T cell autonomous death (ACAD). Human adenotonsillar CD4+ T cells are critically located at the point of entry of foreign inhaled and digested antigens in the pharynx. This population includes cells that show expression of activation antigens. We used these in vivo activated adenotonsillar CD4+ T cells as a model to study homeostasis of peripheral CD4+ T cells. Materials and methods: Adenotonsillar tissue samples were obtained from children aged 1 to 4 years who underwent surgery because of adenotonsillar hyperplasia or infections. Naive phenotype CD4+ CD45RA+ and memory phenotype CD4+ CD45RO+ T cells were purified from homogenized tissues after ficoll centrifugation using antibodies conjugated with magnetic beads. To stimulate cells through TCR, cells were incubated with different concentrations of CD3 antibody. The involvement of the Fas-FasL interactions was determined using FasL antibody and recombinant Fas protein. To inhibit apoptosis, cells were treated with cytokines. Apoptosis was detected by analyzing phosphatidyl-serine translocation, DNA degradation, and caspase-3 activity. Results: Some adenotonsillar naive phenotype CD4+ CD45RA+ and most memory phenotype CD4+ CD45RO+ T cells expressed high levels of activation antigens, such as CD69. CD4+ CD45RA+ T cells, but not CD4+ CD45RO+ T cells, were sensitive to Fas-dependent apoptosis upon stimulation with a high concentration of anti-CD3 antibody. CD4+ CD45RO+ T cells were susceptible to rapid and spontaneous apoptosis in vitro that could be partially inhibited by cytokines IL-2, IL-15, and IL-7 that bind to the cytokine receptor common gamma chain, the chemokine CXCL12, and by IL-6, but not by interfering Fas-FasL engagement or TCR signaling, or by elimination of reactive oxygen species by a synthetic superoxide dismutase mimetic. Conclusions: Apoptosis of adenoidal naive phenotype CD4+ CD45RA+ T cells could be induced with a high concentration on anti-TCR antibody and was Fas-dependent, thus resembling AICD-type cell death. Apoptosis of CD4+ CD45RO+ T cells was reminiscent to ACAD-type cell death as it could be inhibited by various cytokines and it was not Fas or TCR dependent. Thereby, homeostasis of peripheral human CD4+ T cells is first achieved by deletion of naive CD45RA+ T cells by high concentrations of antigens, such as auto-antigens or nutrients. The magnitude of the immune response is then fine-tuned by various cytokines that can inhibit the death of activated memory phenotype CD45RO+ T cells.

### Su1.33. Regulation of Inducible Heparanase Gene Expression in Human T-Cells by Soluble and Immobilized TNF $\alpha$ .

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In the course of an inflammatory response immune cells need to acquire directional migrating phenotype. This is done by means of degrading extracellular mesh with enzymes, and by promoting adhesion and migration of the cells by active molecules, such as cytokines. One of the enzymes is heparanase, a heparan sulfate specific endoglycosidase, secreted by immune cells, which combines cytokine-like properties with conventional enzymatic activity. Upon acidification of cellular environment heparanase degrades sugar chains, whereas at basic pH it increases adhesion of the cells to extracellular matrix and promotes their movement along chemokine gradients.

Given the versatile functions of this molecule, finding the prerequisites for its gene expression regulation is important. Our major goal was to investigate inducible heparanase gene transcription in primary human T-cells in response to exogenous signals.

In our work we utilized 95% pure CD3+ T-cells, derived from healthy human donors. As an activator we used TNF $\alpha$ , a major pro-inflammatory cytokine, which delivers a stress signal to cells, bearing TNF receptors, and influences their transcriptional programs. T-cells were exposed to soluble TNF $\alpha$ , or the cytokine was first pre-complexed with fibronectin, and then the cells were placed on this immobilized TNF $\alpha$ .

We measured the heparanase mRNA levels and protein content in cell lysates using real-time quantitative PCR, and heparanase enzymatic assay, respectively.

The cytokine in its soluble and fibronectin-immobilized form promotes the accumulation of heparanase mRNA and protein in a strictly defined time- and concentration-dependent manner. To assess the intracellular signaling pathways, involved in TNF $\alpha$ -induced signal transduction, necessary for the heparanase gene transcription, we used chemical components, known to inhibit key players of signal transduction cascades, initiated by TNF receptor ligation. We show, that the heparanase mRNA accumulation depends on PKC, p38, JNK, PI3k signaling cascades, whereas it is not influenced by NF $\kappa$ B and ERK. To further strengthen the notion of specificity of TNF $\alpha$  signaling, we also demonstrate, for the first time, a strong correlation between the effects of active TNF $\alpha$  concentrations on the upregulation of relevant transcription factors, such as GATA-3, Ets-1, Egr-1, but not Ets-2, measured by immunoblotting, and heparanase mRNA accumulation. Finally, we show, that these effects are reproduced, although to a limited degree, by other members of TNF superfamily, FasL and TRAIL, suggesting the involvement of intracellular signaling components, shared by these ligands.

In conclusion, we claim that heparanase, an important agent in the autoregulatory loops of inflammatory lesion, is responsive to the signals, delivered by the common stress signals, such as TNF $\alpha$ .

### Su1.34. Serum Free PBMC Freezing and Testing Conditions Afford Enhanced Detection of Antigen-Specific T Cells and Standardization of Immune Monitoring.

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Serum has been essential for both freezing and testing of human PBMC. Serum contains a plethora of bioactive molecules whose variable concentrations makes every serum batch unique, with unique effects on T cell performance in vitro. We developed a serum free protocol for freezing PBMC. The thawed PBMC displayed > 80% viability regardless whether they were frozen with serum, or serum free. Moreover, the thawed PBMC maintained full functionality compared to the fresh cells when tested in ELISPOT assays against a panel of 23 individual peptides and 5 protein recall antigens recognized by CD8 and CD4 cells, respectively. Strikingly, the PBMC performed frequently better under serum free conditions: increased numbers of cytokine producing cells were elicited by the recall antigens without an increase of activity in the medium control. The magnitude of signal enhancement under serum free conditions was considerably larger than with inclusion of costimulatory antibodies, such as anti-CD28. Apparently, serum contains suppressive factors (such as IL-10 and TGF-beta) that can interfere with T cell activation. Thus, serum free freezing and testing media are not only a convenient alternative to bypass the need for screening for "optimal" serum batches, but also such media enhance and standardize T cell monitoring.

### Su1.35. Serum High Mobility Group B1 [HMGB1] Is a Damage Associated Molecular Pattern [DAMP] Cytokine Elevated in Chronic Hepatitis C Patients and Diminishing Following Orthotopic Liver Transplantation.

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**Introduction.** Hepatitis C virus (HCV) infection is the leading cause of chronic liver disease in the U.S., affecting approximately 4 million people. The impact in healthcare-related costs is enormous, with over \$600 million being spent annually in loss of work and medical costs. The immune mechanisms that lead to chronic liver damage and cirrhosis in hepatitis C have not been elucidated but are increasingly being attributed to nonapoptotic, unscheduled cell death and release of a variety of DAMPs including uric acid, S100 proteins, heat shock proteins, and HMGB1, each acting in a cytokine like role with specialized immunoglobulin type receptors including RAGE and TLR2 and TLR4. HMGB1 is a highly conserved nuclear protein that has a surprising extracellular role. It binds DNA, increasing access to transcription factors, and also recruits cells across endothelial barriers, promoting local production of TNF, IL-6, and IFN $\gamma$ . It is released from necrotic cells, activated macrophages, NK cells and mature DCs, but not neutrophils. Following DNA damage as a result of apoptotic death, ultraviolet irradiation, or platination, it is sequestered in the nucleus. Detectable in serum and tissue it serves as a leaderless cytokine, prototypic of the DAMPs that drive repair and a previously underappreciated role in inflammation. **Results.** HMGB1 was measured in a highly sensitive sandwich ELISA developed in our laboratory and did not

correlate with other serum analytes including ALT, AST, total bilirubin, or albumin. Pretransplant HMGB1 was detectable in the serum of all 17 patients evaluated, ranging from 9.9–487.1 ng/ml including three pts with hepatoma (62.6, 74.1, and 485.9). We evaluated whether HMGB1 correlated with disease activity at baseline or at 3, 6, 9 or 12 months of follow-up with the presence of hepatoma, histologic activity index [HAI], rejection activity index or stage (degree of fibrosis). The highest 12-month HMGB1 (161), was associated with the largest HAI (6). This was the only significant ( $P = 0.02$ , Pearson correlation) association. Following orthotopic liver transplantation, HMGB1 levels diminished at 3mo. [ $n = 16$ ] by a median ratio of .41 compared to baseline [ $P < .05$ ] and at 12mo. [ $n = 14$ ] by a median ratio of .13 [ $P < .001$ ]. One pt with a baseline level of 74.1ng/ml with hepatoma increased post transplant to 581.3ng/ml and is being reevaluated for recurrence. **Conclusion.** We conclude that HMGB1 represents an independent measure of tissue damage and injury, as well as cancer that may be useful in the assessment of patients with HCV infection. Controlling HMGB1 activity and release is an approach being developed as an experimental therapy for patients with sepsis, arthritis, cancer, and other inflammatory disorders.

### **Su1.36. Ex Vivo Pro-Inflammatory Cytokines Expression in Patients with Aggressive Periodontitis.**

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**RATIONALE:** Periodontitis includes a broad spectrum of immuno-inflammatory responses to periodontal pathogens from oral biofilm that destroy the supporting tissue of the teeth. In this pathology the balance between pro and anti-inflammation is tipped to pro-inflammatory activity mediated by cytokines that include TNF $\alpha$  and IL-1 $\beta$ . **OBJECTIVE:** We explored the relationship of TNF $\alpha$  and IL-1 $\beta$  expression and the aggressive periodontitis (AP) condition in Chilean patients. **METHODS:** Until now we recruited 42 individuals with AP and 40 control subjects. For cytokine expression blood culture were stimulated with LPS from *E. coli* or *P. gingivalis* extracts. TNF $\alpha$  and IL-1 $\beta$  were measured by an ultra-sensitive ELISA kit.

**RESULTS:** The LPS-induced TNF $\alpha$  levels in AP patients were higher than controls ( $P = 0.0414$ ) while means of serum TNF $\alpha$  concentrations in patient group were 3 times higher than those found in controls ( $P = 0.000$ ). Although no significant the highest mean for LPS-induced IL-1 $\beta$  level was obtained by the AP group.

**CONCLUSION:** The mononuclear cells from AP patients express higher induced TNF $\alpha$  and IL-1 $\beta$  levels than healthy individuals in an *ex vivo* culture system.

Financed by Fondecyt-Chile 1040860, Fondef-Chile D0311055 and University of Chile Clinical Hospital 016/03.

### **Su1.37. IL-22 Increases the Innate Immunity of the Skin.**

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Interleukin(IL)-22 was discovered in 2000. It belongs to the IL-10 family of cytokines whose members (additionally IL-10, IL-19, IL-20, IL-24, and IL-26) are structurally related molecules. We have previously shown that IL-22 is mainly produced by activated T cells, particularly the Th1 subset. The data presented here surprisingly show that IL-22, unlike IL-10, does not act on immune cells. This conclusion is based on a systematic study at all possible levels of analysis: on receptor expression, signal transduction, effects *in vitro*, and effects *in vivo*. In contrast, the quantitative analyses of a wide range of tissues and corresponding primary cells and cell lines showed that many non-immune tissue cells are target of IL-22 as they express both chains of the IL-22 receptor complex. Very high levels of IL-22 receptor chains were found in skin and keratinocytes. In primary human keratinocytes these levels were further upregulated by IFN-g suggesting an increased sensitivity of these cells towards the IL-22 action under T1 conditions. The receptor complex on these cells was functional and induced STAT3 tyrosine phosphorylation. For the first time, this study also identified effects of IL-22 on keratinocytes, namely the upregulation of the antimicrobial agents b-defensin 2 and b-defensin 3. This effect was transcriptionally regulated, and independent on protein *de novo* synthesis and alternative protein secretion indicating a direct effect of IL-22. Additionally, this induction was time- and dose-dependent, and enhanced upon cellular differentiation. The extent of induction was comparable to that by other known inducers of b-defensins. In skin from patients with psoriasis, high levels of IL-22 were highly significantly associated with strongly upregulated expression of b-defensin-2 and b-defensin-3 suggesting a protective effect of IL-22 in this disorder. Taken together, IL-22 does not serve the communication between immune cells but is a T cell mediator that directly promotes the innate, non-specific immunity of the skin. The observation that activated T1 cells directly regulate the non-specific immune defense in tissues demonstrates a so far unknown but very important side of the immune system.

### **Su1.38. IL-7 in Human B Cell Development.**

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Human B cell production has been thought to differ from that in mouse with respect to the requirement for IL7. In mice, IL7 increases the *in vitro* production of B cells from **adult** murine bone marrow by 20–30 fold. In contrast, human studies have focused on B lymphopoiesis from **fetal** precursors and show little increase in B cell production with IL7 (~2 fold). A re-examination of IL7 knockout mice has shown that IL7 is required to generate **adult-derived** B2 cells (CD5 $^{-}$ ), but not for the production of **fetally-derived** CD5 $^{+}$  B1 cells. Here we examine the role of IL7 in human B cell development from hematopoietic stem cells (HSCs) isolated from human umbilical cord blood (CB), a source likely to contain both HSCs that give rise to B1 cells, as well as those capable of generating B2 cells. FACs analysis of human CB shows mutually

exclusive expression of IL7 $\alpha$  and CD5 on CD34+CD19+ pro-B cells. When FACS-sorted Lin- CD34+ CB HSCs were placed in stromal cell co-cultures, the addition of IL7 increased the *in vitro* production of human B lineage cells up to 60-fold as compared to cultures with anti-IL7 neutralizing antibody. IL7-induced increases observed on human stroma were ~10-fold more than those with the murine S17, MS-5, or OP9 stromal cell lines. A titration of IL7 into co-cultures showed that the threshold for IL7-induced increases in human B cell production was 10 to 100 fold lower in co-cultures with human stroma as compared to those with murine stroma. Surprisingly, murine stromal cells provided better support for human B cell production in the absence of murine or human IL7 activity. Our data show that IL7, in the presence of human stroma, can dramatically impact B cell production from at least a subset of progenitors in human CB. On the other hand, murine stroma support IL7-independent B cell production from human CB progenitors. Using Ki-67 and Annex V/7AAD staining we are examining the extent to which proliferation and/or anti-apoptotic effects, at the pro-B and pre-B stages, contribute to IL7 effects seen *in vitro*. Our studies suggest that the roles of IL7 in human and murine B cell development may be more similar than previously believed.

### Su1.39. An Important Role for CX<sub>3</sub>CR1 in Vascular Remodeling.

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**Objectives:** A functional polymorphism in the chemokine receptor CX<sub>3</sub>CR1 has recently been associated with protection from coronary artery disease (CAD) and internal carotid artery (ICA) occlusive disease in man. CX<sub>3</sub>CR1 is functionally expressed on monocytes and vascular smooth muscle cells (VSMC), both of which cell types are critical for the vascular remodeling found in atherosclerosis, angioplastic restenosis, and ICA occlusive disease. Our objectives in this study were to investigate whether CX<sub>3</sub>CR1 was involved in the inflammatory process of vascular remodeling, and the mechanisms by which it may play a role. **Methods:** Femoral arteries of CX<sub>3</sub>CR1-deficient and wild type mice were subjected to injury by the passage of an angioplasty guidewire or sham operation. After 5, 14, and 28 days, mice were euthanized, perfused with 4% paraformaldehyde, and tissues harvested for immunohistochemistry. In some cases, mice were injected with bromodeoxyuridine (BrdU) for 24 hours prior to tissue harvest for cell proliferation studies. **Results:** CX<sub>3</sub>CR1 deficiency resulted in protection from vascular remodeling following guidewire-induced injury. The incidence of vascular inflammation or remodeling was decreased in CX<sub>3</sub>CR1<sup>-/-</sup> mice (38.1% vs. 86.4% for WT,  $P = 0.001$ ). At d5, the injured arteries of WT mice had a marked monocyte infiltration into the intima compared to non-injured arteries on the control side of each animal ( $13.6 \pm 11.5$  vs.  $0.0 \pm 0.0$ ,  $P = 0.006$ ). At d14, WT injured arteries had neointimal hyperplasia with a mixture of monocytes and VSMC. By d28, the intimal hyperplasia comprised primarily of VSMC with an average intima to media ratio (I/M) of  $0.78 \pm 1.04$  compared to 0.0 in non-injured arteries ( $P = 0.02$ ). In CX<sub>3</sub>CR1<sup>-/-</sup> mice, there was no detectable monocyte invasion to the intima at d5 (100% decrease compared to WT,  $P = 0.006$ ), and there was an 86.8% decrease in the numbers of

monocytes in the intima at d14. At d28, the intima area was decreased in CX<sub>3</sub>CR1<sup>-/-</sup> mice by 62% compared to WT mice ( $P = 0.11$ ), with a concomitant decrease in the numbers of VSMC in the intima (87.1%,  $P = 0.055$ ). As expected, the number of VSMC in the media declined during the injury response in WT animals (33.4% decline,  $P = 0.025$ ) while it remained unchanged in the media of CX<sub>3</sub>CR1<sup>-/-</sup> mice (9.9% decline,  $P = 0.22$ ). The percentage of actively proliferating cells in injured vessels was also decreased in CX<sub>3</sub>CR1<sup>-/-</sup> mice (12% vs. 27% in WT,  $P = 0.027$ ). **Conclusions:** CX<sub>3</sub>CR1 plays a critical role in vascular remodeling following femoral artery injury. One of the primary mechanisms involves monocyte recruitment to and/or retention within the injured vessel intima. Although the VSMC defects seen at d28 were downstream of the monocyte defects observed at d5, we can not rule out a possible contribution of CX<sub>3</sub>CR1 to the proliferation and media-to-intima migration of VSMC in the course of vascular remodeling.

### Su1.40. Chromatin Regulation of Interleukin 10 Gene Expression at the Allele Level.

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It is generally assumed that most mammalian genes are transcribed from both alleles in most situations. But it is now clear that several genes do not have this transcriptional behavior. In general these genes are grouped in 3 different classes, a) imprinted genes, b) most genes in the X chromosome of female cells and c) autosomal genes that are stochastically monoallelic transcribed. Interleukin 10 (IL-10) is a multifunctional cytokine, its activities array from the classical inhibition of effector functions of T lymphocytes, monocytes and macrophages, to regulation of growth and differentiation of B lymphocytes and dendritic cells, and its recent key role in differentiation and function of the T regulatory cell. In the 15 years since IL-10 was first described, most studies aimed the biological function but few addressed its genetic regulation. Making use of IL-10eYFP<sup>kl</sup> mice, in which transcription from different alleles is easily distinguishable, we observed that IL-10 shows a pseudo-monoallelic transcription pattern in CD4<sup>+</sup> T cells. Most CD4<sup>+</sup> T cells express stochastically only from one allele while only few transcribe it from both. Allelic exclusion in this case does not seem to take place and a bias towards bi-allelic expression is obtained with increased TCR signaling. We propose that this pseudo-monoallelic expression is the reflection of a tightly controlled low frequency expression of the IL-10 gene. We also show that chromatin appears to play a major role regulating this process. Thus at least two levels of regulation seem to be implicated in the bona fide regulation of IL-10 expression. TCR signaling through activation of transcription factors and regulation of chromatin opening seem to act together to provide efficient control of IL-10 expression. We think that this two-layer regulation might reveal an extremely important mechanism in controlling genetic expression in biological situations of low frequency expression of genes and as such might apply to many more genes than the few ones studied so far.

### Su1.41. Local Interleukin-1 Immunotherapy Effectiveness Depends on Cytokine Genetic Background.

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Interleukin-1 (IL-1) is one of the main mediators involved in the control of inflammation and acute phase response. IL-1 family molecules regulate tissue repair, inflammatory and immune reactions. Important question of IL-1 biology today is how to use this pleiotropic highly biologically active molecule in the therapy of various human diseases. Systemic IL-1 administration induces adverse effects limiting IL-1 therapeutic use. Very promising is IL-1 local application just to the lesion or inflammatory site. Human recombinant IL-1 beta has been used for local therapy in patients with chronic bacterial purulent rhinosinusitis after failure of routine antibiotic therapy. IL-1 beta solution application (10 ng/ml) directly to the inflammatory site daily for 3–5 days led to a clinical improvement in most of treated patients. Endogenous IL-1 beta and IL-1 receptor antagonist (IL-1RA) ex vivo production and gene polymorphism studies for the distribution of IL-1b and IL-1RA alleles were performed in all patients. Patients with more rare IL-1b (+3953)<sup>+</sup> allele had elevated IL-1 beta production and patients with IL-1RA 2\*(VNTR)<sup>+</sup> allele had higher antagonist production compared with patients bearing normal alleles. These abnormal alleles were found in 30% and 90% of patients respectively that was much more frequently compared to healthy subjects suggesting for genetic predisposition to chronic rhinosinusitis probably due to the dysregulation in the IL-1 family cytokines production. We have found that recombinant IL-1 therapy was highly effective in patients carrying IL-1RA 2\*(VNTR)<sup>+</sup> allele with increased endogenous IL-1RA levels. In these patients IL-1 beta local application significantly increased functional activity of leukocytes isolated from the inflammatory sites tested in the assays of neutrophil migration to fMLP, superoxide production, adhesion and phagocytosis. Cytological analysis showed that the reduction in inflammatory manifestations was accompanied with the decrease in the proportion of neutrophils and increase in the numbers of monocytes/macrophages. Negative results of IL-1 beta therapy was obtained in patient who had very rare combination of homozygous IL-1b (+3953)<sup>+</sup> and normal IL-1RA 2\*(VNTR)<sup>-</sup> allelic variant that led to elevated IL-1 beta production and normal IL-1RA levels. According to obtained results IL-1 family cytokines gene polymorphisms are linked to chronic rhinosinusitis and may influence results of cytokine immunotherapy.

#### **Su1.42. Local T-Lymphocytes from Subacute and Chronic Hypersensitivity Pneumonitis Show Phenotypic and Functional Differences.**

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##### **Background:**

Hypersensitivity pneumonitis (HP) is a complex lung syndrome of varying intensity and clinical presentation that result of an immunologically-induced inflammation in response to a large variety of inhaled antigens. HP may present as acute, subacute, or chronic form. HP is characterized by a remarkable T-cell alveolitis. However, the evaluation of lymphocyte phenotypes, primarily CD4<sup>+</sup>/CD8<sup>+</sup> T cell subsets has given inconsistent results. Likewise, studies of other T-cell phenotypes in human HP like T-helper 1 (Th1) versus Th2 are scanty. We hypothesized that some divergence in T-cell subsets may play a

role. We analyzed a variety of T-lymphocyte phenotypes in cells obtained by bronchoalveolar lavage of subacute and chronic patients.

##### **Methods:**

Forty one patients with HP induced by avian antigens and classified as subacute (21 patients), or chronic (20 patients), 5 healthy subjects. HP BAL cells were stained with mAbs to CD3, CD4, CD8, CXCR3, CCR4 for surface staining and intracellular IFN- $\gamma$ , IL-2, IL-4, IL-10. The antigen specific secretion of IFN- $\gamma$ , IL-2, IL-4, IL-10 following pigeon serum stimulation was determined by CBA, and this was used to identify specific CD4 Th1 and Th2 subpopulations. In order to precise the best cut point of CD4<sup>+</sup>/CD8<sup>+</sup> ratio, an analysis of their sensitivity versus 1-specificity (ROC curves) was used. The test with higher sensitivity and the lower 1-specificity was considered to be the most useful.

##### **Results:**

BAL CD4<sup>+</sup>/CD8<sup>+</sup> ratio was highly heterogeneous, and although higher levels were revealed in patients exhibiting chronic HP, results did not reach statistical significance ( $3.5 \pm 3.7$  versus  $1.7 \pm 1.5$  and  $1.2 \pm 0.4$  from subacute patients and controls respectively). However, 14 chronic patients showed values over 2.0 compared with 7 subacute patients. ROC curve showed that a CD4<sup>+</sup>/CD8<sup>+</sup> ratio 1.5 was the best cut point to separate chronic from subacute cases displaying sensitivity (S) of 67%, a specificity of 80%, a positive predictive value of 78%, and a negative predictive value of 70%. A significant difference in the receptor chemokine expression was noticed. Thus, subacute patients exhibited significantly higher numbers of T-cells expressing CXCR3 ( $40.6 \pm 10.7$  % versus  $25.6 \pm 7.3$ % in chronic cases). By contrast, CCR4 was higher expressed in chronic patients  $6.0 \pm 1.3$ % versus subacute  $2.1 \pm 1.3$ %.

Intracellular IFN- $\gamma$ /IL-4 ratio was significantly higher in subacute patients  $1.6 \pm 0.9$ % versus chronic  $0.7 \pm 0.45$ % after antigen specific stimulation. Also IFN- $\gamma$  /IL-10 ratio measured in supernatant cultures was significantly higher in subacute patients  $152 \pm 24.2$  versus chronic  $0.6 \pm 0.7$ .

##### **Conclusions:**

Our results revealed several differences in chronic patients when compared with subacute patients, including an increase in CD4<sup>+</sup>/CD8<sup>+</sup> ratio and an increase in Th2-like response.

#### **Su1.43. Phenotypic and Functional Analysis of Memory CD4 and CD8 T Lymphocytes in Subacute and Chronic Hypersensitivity Pneumonitis.**

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**Background:** The chemokine receptor CCR7 has been used in previous studies in combination with the CD45RA antigen as a tool to define different populations of memory CD4 and CD8 T lymphocytes with different homing and function capacities and at different stages of differentiation. Using this approach four populations of memory CD4 and CD8 T lymphocytes have been defined in patients with subacute and chronic hypersensitivity pneumonitis (HP) in bronchoalveolar lavage and blood. The following pattern of differentiation of CD4 and CD8 T cells has been demonstrated: CD45RA<sup>+</sup>CCR7<sup>+</sup> → CD45RA<sup>-</sup>CCR7<sup>+</sup> → CD45RA<sup>A-C</sup>CCR7<sup>-</sup> → CD45RA<sup>+</sup>CCR7<sup>-</sup>.

**Methods:** Peripheral blood mononuclear cells (PBMC) and bronchoalveolar lavage cells (BALc) obtained from 5 subacute and



5 chronic HP patients were stained with mAbs to CD4, CD8, CCR7, CD45RA, CD62L. The secretion of IFN- $\gamma$  following specific stimulation was determined on antigen-specific CD4 and CD8 lymphocytes and this was used to identify specific CD4 and CD8 effector cells.

**Results:** The CD8 memory blood T lymphocytes was equally composed pre-terminally differentiated CD45RA<sup>-</sup>CCR7<sup>-</sup> (CD8 subacute: 30  $\pm$  12%; CD8 chronic 30  $\pm$  2.1 vs controls 10.5  $\pm$  2.3%  $P$  < 0.05) and terminally differentiated CD45RA<sup>+</sup>CCR7<sup>-</sup> (CD8 subacute: 20  $\pm$  7%; CD8 chronic: 30  $\pm$  14% vs controls 15.2  $\pm$  3.7) in subacute and chronic patients. In contrast CD4 blood T cells was composed pre-terminally differentiated (CD4 subacute: 34  $\pm$  15%; CD4 chronic: 38  $\pm$  27% vs controls 10.3  $\pm$  3.8  $P$  < 0.05) and a higher proportion of terminally differentiated memory CD4 T lymphocytes (CD4 subacute: 66  $\pm$  16%; CD4 chronic: 62  $\pm$  28% vs controls 16.1  $\pm$  3.5  $p$  < 0.01). In BAL the CD4 and CD8 memory T cells was predominantly composed pre-terminally differentiated T memory cells in both CD4 and CD8 cells (CD4 subacute: 83  $\pm$  8.2%; CD4 chronic: 94  $\pm$  4% vs controls 85.6  $\pm$  9.3; CD8 subacute: 88  $\pm$  5.4%; CD8 chronic 95  $\pm$  0.7% vs controls 36.4  $\pm$  4.7  $P$  < 0.05).

**Conclusions:** A number of studies suggest that effector and memory cells with differing functional and migratory capabilities arise as an inevitable consequence of antigen encounter. The present results demonstrate that selective impairment of the differentiation of antigen specific subacute and chronic HP memory cells in blood and BAL indicate a different distribution of CD4 and CD8 memory lymphocytes.

#### Su1.44. Pro-Inflammatory and Anti-Inflammatory Cytokines Track with KIR Haplotypes A and B in Octo/Nonagenarian Subjects.

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**Background:** Natural Killer cells (NK) play a pivotal role in the innate immune response, controlling tumour and infection-related responses. The cytolytic activity of NK cells is controlled by an array of activating and inhibitory cell surface receptors, including the killer immunoglobulin-like receptors (KIRs), which can be grouped into the most common haplotype groups called A and B. Cytokine changes occur in ageing and we wondered whether changes in serum cytokines were related to group A and B KIR haplotypes.

**Methods:** KIR haplotyping available from 300 subjects from the Belfast Elderly Longitudinal Free-living Aging Study (BELFAST) was matched with a subset of corresponding serum cytokines.

**Results:** Subjects categorized into the KIR haplotype associated with activation, showed increased serum cytokine values for both pro-inflammatory cytokines IL-2, IL-6, sIL-6, TNF $\alpha$  and IL-12 as well as increased values for the anti-inflammatory cytokines IL-10 and TNF $\beta$ . There was no apparent sex-related difference.

**Conclusions:** KIR haplotypes A and B in octo/nonagenarians do track with their activation status in relation to cytokines and could be effectors of tumour and infection-related responses and the immune-related changes in ageing.

#### Su1.45. Evaluation of T<sub>H</sub>1/T<sub>H</sub>2 Cytokine Modulations in Chronically HIV-Infected Adults Who Received Therapeutic Vaccination and Intermittent HAART Following an Initial HAART Intensification (CTN-140 Pilot Trial).

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Evaluation of T<sub>H</sub>1/T<sub>H</sub>2 cytokine modulations is important to determine the immune competence and/or restoration in chronic HIV patients undergoing initial highly active antiretroviral therapy (HAART) intensification (GM-CSF, Hydroxyurea, and ddI), therapeutic vaccination (Remune<sup>TM</sup>) and structured treatment interruption (STI). We evaluated serum levels of interferon (IFN)-gamma, interleukin (IL)-2, tumor necrosis factor (TNF)-alpha, IL-15, IL-4, and IL-10 in 10 patients enrolled in the Canadian HIV Trials-140 pilot study. All patients were in viremic control, i.e., virus load (VL) was < 50 copies mL<sup>-1</sup> at the time of baseline samplings. Nine sampling points were at baseline, HAART-intensification, therapeutic vaccination, 1<sup>st</sup> peak VL (rebound during STI), 1<sup>st</sup> < 50 VL, 2<sup>nd</sup> peak VL, 2<sup>nd</sup> < 50 VL, 3<sup>rd</sup> peak VL, and 3<sup>rd</sup> < 50 VL, respectively. All cytokines were measured by competitive enzyme-linked immunosorbent assays (ELISA). The difference between group means at a given time-point and at baseline was determined by t-test. We found that IFN-gamma levels were comparable to baseline value of 58.88 pg mL<sup>-1</sup> at all time points except at 2<sup>nd</sup> peak VL and at 2<sup>nd</sup> < 50 VL samplings when the levels were significantly reduced. IL-2 levels were elevated significantly ( $P$  < 0.05) at 1<sup>st</sup> time as VL was below 50 copies mL<sup>-1</sup> compared with baseline value of 58.43 pg mL<sup>-1</sup> and high levels (higher than in age-matched HIV-seronegative controls) were sustained thereafter. Conversely, IL-10 levels were reduced significantly at 1<sup>st</sup> time as VL was < 50 copies mL<sup>-1</sup> than baseline concentration of 109.90 pg mL<sup>-1</sup> and low levels were maintained at all subsequent time points. Regarding both TNF-alpha and IL-4, all modulations were found to be statistically non-significant ( $p$  > 0.05) as compared with baseline mean concentrations of 124.0 pg mL<sup>-1</sup> and 75.35 pg mL<sup>-1</sup> respectively. However, IL-15 levels were comparable to baseline concentration of 241.10 pg mL<sup>-1</sup> (which was higher than those in controls) until 1<sup>st</sup> time as VL was < 50 copies mL<sup>-1</sup> and the levels were significantly reduced thereafter. Therefore, it was concluded that these immunomodulatory therapeutic interventions were able to induce/maintain a T<sub>H</sub>1-dominant cytokine profile in these patients whereas suppressed IL-15 levels at 2<sup>nd</sup> peak VL and thereafter might be due to a deregulated innate immune response.

#### Su1.46. Cytokine Expression in CD4+ Cells Exposed to the Monocyte Locomotion Inhibitory Factor Produced by *E. histolytica*.

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The Monocyte Locomotion Inhibitory Factor (MLIF), could so contribute to the sparse delayed inflammation observed in amebic

abscess of the liver, either directly through its effects upon MP (i.e. depression of chemotaxis and respiratory burst), or indirectly through the modulation of the production and/or secretion of cytokines involved in the recruitment of MP in the late stages of inflammation. We evaluated the effect of MLIF on the production of pro/anti-inflammatory cytokines in CD4+ cells.

#### Materials and Methods

T CD4+ lymphocytes were purified by negative selection using a commercial kit (MACS- Reagen's isolation of human CD4+ T cells. One  $\times 10^7$  PBMC cells were placed in propylene tubes with 80ml PBS-albumin-EDTA and 20  $\mu$ l antibody cocktail to eliminate cells other than CD4+ (10 min at 4 °C). The CD4+ lymphocyte purified fraction was found to be 95% pure. MLIF (96% pure) was commercially obtained (American Peptide Co, Sunnyvale, CA, USA). Five  $\times 10^5$  T CD4+ cells were placed in RPMI-1640 with 10% fetal calf serum (FCS) and were stimulated for 24 h in the presence of PMA (50 ng/ml), MLIF(50  $\mu$ g/ml) or PMA+MLIF. The supernatant fluids were analyzed by ELISA.

#### Results

**Pro-inflammatory cytokines.** Cells activated with MLIF expressed an increased production of *constitutive* pro-inflammatory cytokines IL-1 $\beta$ , IL-2 and IFN- $\gamma$  (262, 245, 46 pg/ml respectively) when compared with the production of *constitutive basal* RPMI cell controls (26, 18, 10 pg/ml respectively). PMA enhanced the *induced* production of the same pro-inflammatory cytokines (192, 384, 84 pg/ml respectively). The expression of IL-1 $\beta$  and IFN- $\gamma$  *induced* by PMA was significantly inhibited by MLIF (25% and 20% respectively), which did not occur with IL- 2.

**Anti-inflammatory cytokines.** MLIF, increases the production of *constitutive* anti-inflammatory cytokines when compared to *basal* IL-5, IL-6 and IL-10 (188, 114, 134 pg/ml respectively) when compared to the *basal* production in control cells RPMI (15, 16 and 13 pg/ml respectively). With PMA, an increase of production of *induced* IL-5, IL-6 and IL-10 (575, 250, 326 pg/ml respectively), while the mixture of PMA+MLIF was found to decrease the production of *constitutive* PMA of IL-5 and IL-6, but not of IL-10, that significantly increased its expression.

#### Conclusions

Pro/anti-inflammatory cytokines were produced by MLIF, in amounts similar to those induced by PMA. With the PMA+MLIF mixture the IL-1 $\beta$ , IFN- $\gamma$ , IL5 and IL-6 (all with pro-inflammatory potential) production was inhibited, but not that of IL-10 (the prototype of an anti-inflammatory cytokine), which disclosed a significant increase of its expression. Thus MLIF can orchestrate an anti-inflammation pattern of cytokines that contribute to the exiguous inflammation found in advanced lesions of invasive amebiasis (CONACYT GRANT 38104-M).

#### Su1.47. Interleukin 21 Modulates Cytokine Pathways That Contribute to the Pathology of Arthritis.

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Interleukin 21 (IL-21) is secreted by activated T cells and modulates immune cell functions with both pro- and anti-inflammatory effects. Interleukin 21 receptor, (IL-21R) homologous to IL-2R beta and IL-4R alpha, associates with gamma common chain upon ligand binding. IL-21R is constitutively expressed on many cell types in the immune system, and is upregulated by IL-21. **Rationale:** Since blockade of the IL-21

pathway with soluble IL-21RFc resulted in a reduction of clinical signs of arthritis in rodent models, we examined the effect of this pathway on rat and murine responses *in vitro* to understand the potential mechanisms of IL-21 regulation in arthritis. **Methods:** Arthritis was induced in DBA/1 male mice with bovine type II collagen, and in the Lewis Rat with Freund's Complete Adjuvant. Animals were treated with soluble mIL-21RFc, which neutralizes both murine and rat IL-21 bioactivity. Draining lymph node cells from collagen-immunized mice were cultured *in vitro* with collagen and either IL-21 or IL-21RFc and assayed for proliferation and cytokine secretion. Cytokines and anti-collagen specific IgG levels were also measured in the serum by ELISA analysis. In the rat, Con A stimulated spleen cells or anti-CD3 activated splenic T cells were assayed for proliferation and cytokine secretion in response to IL-21 and IL-21RFc. **Results:** When compared with control animals, 3x weekly treatment with IL-21RFc resulted in less severe signs of disease in both rat and murine prophylactic and therapeutic models of CIA. Addition of murine IL-21 to collagen restimulated LN cells resulted in a dose dependent inhibition of proliferation, and a decrease in IFN-gamma, GMCSF, and IL-6 and increase in IL-10 in the culture supernatant. Conversely, addition of IL-21RFc enhanced the collagen proliferation response and resulted in increased IFN-gamma, GMCSF and IL-6 and decreased IL-10 production suggesting that IL-21RFc modulated endogenous IL-21, as well other antigen-induced cytokines. **Conclusion:** These results suggest that Interleukin 21 plays a role in modulation of antigen-specific T cell responses and this may contribute to the pathology of arthritis.

#### Su1.48. Critical Role of STAT1 on Self-Renewal Capacity of CNS Stem Cells by an Inducible Interferon- $\gamma$ Genetic Program.

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**Objective:** To investigate the molecular mechanisms of neural stem cells (NSCs) responses to the proinflammatory cytokine interferon- $\gamma$ .

**Background:** Self-renewal capacity is a fundamental property of NSCs, in which stem cells proliferate forming multipotent neurospheres in the presence of FGF-2. NSCs respond to inflammation, however it is not known what molecules account for their response during disease. Our hypothesis is that NSCs express functional immune related genes that interact with stemness genes, and control NSC intrinsic properties.

**Materials and Methods:** We analyzed multiple microarray datasets for inflammatory genes expressed by NSCs then performed gene clustering by Gene Ontology, and data mining using several bioinformatics databases. For testing self-renewal capacity, NSCs were isolated from different regions of mouse brain, and cultured in DMEM/F12 with N2 supplement and bFGF (20 ng/ml). For gene expression analysis mRNA from duplicate NSC cultures exposed to interferon- $\gamma$  for 24 h, were processed on Affimetrix microarrays. Genes showing a change in expression of >2-fold with a p-value of  $P < 0.001$ , were confirmed using a different microarray platform, confocal microscopy and western blot.

**Results:** We found that NSCs express several interferon related genes, we confirmed their expression *in vivo* on E14.5 germinal zone by confocal microscopy. IFN- $\gamma$  at doses of 50 to 500 U/ml added to NSC cultures from E14.5, newborn, or adult brain, decreases the frequency of FGF-2 induced neurosphere

formation ( $39.6 \pm 9.2\%$  vs.  $4.6 \pm 3.5\%$ ,  $P < 0.002$ ) and neurosphere diameter ( $131 \pm 33.2$  vs.  $42.2 \pm 14$ ,  $P < 0.0001$ ). To determine the genes affected by IFN- $\gamma$ , primary NSCs were treated with IFN- $\gamma$  and subjected to microarrays. We identify >100 affected genes, clustered in 3 groups by a Terrain Gene Map: a) IFN- $\gamma$  highly inducible genes (absent in untreated NSCs with 20–2000 fold increase) b) MHC genes and proteasome genes increased 2–10 fold c) Decreased genes, 50% of which are related to stemness including cell cycle, histone and lifespan genes. We confirmed the expression of selected genes by confocal microscopy and western blot. To elucidate the molecular pathways affected by IFN- $\gamma$ , we analyzed downstream pathways of IFN- $\gamma$ , and we found a 20-fold increase in stat-1 gene expression. Interferon- $\gamma$  induced rapid phosphorylation of STAT1 Tyr-701 and nuclear translocation of STAT 1 in NSCs 15 min after treatment. Using NSCs isolated from stat-1 $^{-/-}$  mice, we found that the reduction of self-renewal capacity by IFN- $\gamma$  in wild type mice was absent in stat-1 $^{-/-}$  NSCs. Instead, stat1 $^{-/-}$  brains contain more NSCs and IFN- $\gamma$  in vitro increased their neurosphere formation by 3-fold (self-renewal capacity). Experiments of differential gene expression and behavior in vivo of stat1 $^{-/-}$  vs wildtype mice after IFN- $\gamma$  treatment are under way to further evaluate the importance of stat1 in NSCs.

**Conclusion:** NSCs have a functional interferon genetic program. Interferon- $\gamma$  induces this program and leads to the activation of multiple genes including stat-1, which appear to form a central checkpoint for NSCs self-renewal capacity.

#### **Su1.49. Role of CXCL1/CXCR2 in Promotion of Glial Survival during Experimental Demyelination.**

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Inadequate repair is thought to underlie chronic demyelination in Multiple Sclerosis (MS). Oligodendrocyte precursor cells (OPCs), which are present in the adult central nervous system (CNS), have the ability to migrate into lesions and differentiate into mature oligodendrocytes. OPC proliferation and differentiation is driven by growth factors such as the chemokine CXCL1. Administration of CXCL1 may enhance OPC function and remyelination. We have examined the role of CXCL1 and its receptor (CXCR2) in the corpus callosum of female C57BL/6 mice during cuprizone-induced demyelination. Response to the demyelination causes astrocytes, microglia and OPCs to accumulate in the corpus callosum. Removal of the toxin cuprizone allows remyelination to occur. We found a transient increase in CXCL1 mRNA in the forebrain prior to OPC accumulation, as early as 1 week after the initiation of cuprizone treatment. The level of CXCL1 mRNA peaked at two weeks and then declined to normal levels. CXCR2 positive cells also increased in the corpus callosum during demyelination. PDGF receptor alpha positive OPCs were shown to express CXCR2, suggesting a possible mechanism for migration of these cells into the corpus callosum. To test this, we injected a replication-defective adenovirus encoding CXCL1 via the cisterna magna which introduced the virus into the cerebrospinal fluid without inflammation. Virus was injected at 0 weeks or 3 weeks after initiation of cuprizone treatment and mice were sacrificed at either 3 weeks or 6 weeks. Virally encoded CXCL1 mRNA was detected in the cerebellum up to 6 weeks after virus injection. Immunohistochemical detection of NG2 positive OPCs showed no effect of the CXCL1 encoding adenovirus on the number of OPCs in the corpus callosum. These data suggest that

endogenous CXCL1 is sufficient for OPC mobilization and remyelination. Deletion strategies are currently underway.

#### **Su1.50. Standardizing Multiparameter Flow Cytometry for Evaluation of Cytokine-Secreting Activity in T Cells Via Automation of Sample Preparation and Analysis.**

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The utility of evaluating T cell functional assays is well recognized in the context of determining an efficacious vaccine strategy for infectious diseases/cancer, a tolerance profile in autoimmunity and transplantation, as well as for understanding the basic mechanisms of T cell immune responses in disease pathogenesis. However, the variability associated with T cell functional assays continues to be problematic, especially in studies involving multi-center clinical trials as well as in longitudinal studies. The variable natures of these assays are associated with a variety of factors ranging from source of the T cells, the processing methodology (isolation, freezing, thawing, and culturing), the sample preparation for staining and finally, data analysis, and data reduction. With a view to reducing variability and standardizing targeted steps of the assays involving T cell function, an automated methodology for staining and analysis of intracellular cytokines via flow cytometry was developed.

A modification to available sample preparation instruments was performed that enabled the automated pipetting, incubation, and staining of intracellular and surface molecules of human stimulated whole blood or PBMC for flow cytometric analysis. A 5-color flow cytometry assay (2–3 surface markers; 2–3 intracellular cytokines) was developed to characterize the “restricted polyclonal” Th1 versus Th2 cytokine profile response in T cells stimulated by SEB/CD28. The automated sample staining and analysis greatly reduced variability between specimens while reducing the hands-on sample preparation and analysis time. The complex nature of the cytokine profile and inter- and intra-subject variability was revealed on evaluation of several cytokines in the context of multiparametric evaluation of the T cell responses. The use of automation thus provides a greater degree of standardization in these functional assays, allowing for a correlation of variability and complex immune response profiles, to vaccine efficacy or disease progression without as significant an interference due to the variable nature of the manual assay.

#### **Su1.51. LT $\beta$ R Is Expressed, Functional, and Regulated on Endothelial Cells during Contact Sensitization.**

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High endothelial venules (HEVs) are specialized lymph node blood vessels that allow entrance of lymphocytes into lymph nodes. Through our analysis of genetically deficient and transgenic mice, we have previously determined that the LT $\alpha\beta$  complex is critical for expression of several HEV genes during development and that the LT $\beta$ R is expressed on HEV. The specific objectives of this study were to determine: 1) whether LT $\beta$ R signaling is crucial for HEV gene expression in adult wild type mice and 2) whether this is a direct effect on endothelial cells in induction of HEV gene expression. These questions were investigated *in vivo* in C57BL/6 mouse peripheral lymph node (PLN) and *in vitro* in the bEnd.3 cell line. The regulation of HEV genes (GlyCAM-1, MAdCAM-1, SLC, an HEV specific GlcNAc-

6-sulfotransferase [HEC-6ST], and LT $\beta$ R itself) was evaluated by immunohistochemistry and real time PCR, using an LT $\beta$ R activator, an LT $\beta$ R inhibitor and contact sensitization. LT $\beta$ R was expressed constitutively on HEV in PLNs and was inhibited by one injection of an LT $\beta$ R-Fc fusion protein. HEV genes were regulated by contact sensitization with oxazolone in C57BL/6 mice. LT $\beta$ R was regulated in parallel with HEC-6ST after contact sensitization with oxazolone. An interesting kinetic pattern was detected for these and several other HEV genes: there was an initial decrease in gene expression and a rebound by 7 days in most of the genes with the exception of MADCAM-1, where there was an exactly opposite effect. The initial phase of gene regulation after contact sensitization was independent of TNFRI, though the optimal rebound of gene expression was partially dependent on that receptor. LT $\beta$ R signaling was essential for the optimal rebound of gene expression after sensitization. bEnd.3, a mouse endothelial cell line, prominently expressed the LT $\beta$ R and upon stimulation with a hamster anti-LT $\beta$ R antibody showed a dramatic up-regulation of MADCAM-1. These *in vitro* and *in vivo* data taken together indicate that the LT $\beta$ R is expressed on endothelial cells, can signal directly, is crucial for constitutive HEV gene expression and is regulated during an immune response. These data also provide information regarding plasticity of HEV gene expression during an immune response, suggesting an initial reversion to an immature phenotype and recovery through a developmental program to an adult phenotype.

Supported by NIH CA 16885 to NHR and the Anna Fuller Fund (SL).

#### **Su1.52. Interferon- $\alpha$ Inducing TLR9 Agonists.**

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Bacterial and synthetic DNA containing CpG dinucleotides (CpG DNA) have been shown to be the natural ligands for TLR9, a molecular pattern recognition receptor. CpG DNA activation of TLR9 has been shown to produce Th1 type immune responses, including IFN- $\alpha$  induction. Through extensive DNA structure-activity relationship studies, we have identified novel synthetic dinucleotides and DNA structures (immunomers) that are recognized by TLR9. The combination of novel DNA structures with synthetic stimulatory dinucleotides provided us a portfolio of immunomodulatory oligonucleotides (IMOs). In general, IMOs produce distinct TLR9-mediated immune responses with high IL-12 and low IL-6 cytokine secretion profiles compared with natural CpG dinucleotide motif. Based on human cell culture assays and *in vivo* non-human primate studies, we have identified novel IMOs consisting of synthetic stimulatory motifs, which induce high levels of IFN- $\alpha$ . The induction of IFN- $\alpha$  by IMOs is dependent on the presence of specific nucleotide sequences and the activation of plasmacytoid dendritic cells. These novel IMOs also induced human B cell proliferation and activated B cells to express surface markers and secrete cytokines. IMOs induced expression of a number of cytokine and chemokine genes, including IFN- $\alpha$  gene and a number of interferon inducible genes such as 2'5'-adenylate synthetase in human PBMCs. In non-human primates, IMOs administered s.c. at 1 mg/kg dose induced high levels of IFN- $\alpha$  secretion up to 72 hr. These results suggest that IMOs that induce high levels of IFN- $\alpha$  may be suitable candidates for the treatment of hepatitis C and cancer, where recombinant IFN- $\alpha$  therapy is commonly used.

#### **Su1.53. TGF-Beta in Foxp3 Expression and Development of Peripheral CD4+CD25+ Treg.**

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Although CD4+CD25+ regulatory T cells (Treg) are instrumental in the maintenance of immunological tolerance, one critical question is whether CD4+CD25+ regulatory T cells (Treg) can only be generated in the thymus or can differentiate from peripheral CD4+CD25- naïve T cells. We have shown that conversion of naïve peripheral CD4+CD25- T cells into CD4+CD25+ regulatory cells can be achieved through co-stimulation with TCR and TGF-beta. These converted regulatory cells are not only unresponsive to TCR stimulation, but also inhibit normal T cell proliferation *in vitro*. Importantly, TGF-beta-converted transgenic CD4+CD25+ suppressor cells proliferate in response to immunization, but inhibit antigen-specific naïve CD4+ T cell expansion *in vivo* in an OVA peptide TCR transgenic adoptive transfer model. In a murine asthma model, co-administration of TGF-beta-induced suppressor T cells prevents house dust mite (HDM)-induced allergic pathogenesis in lungs. Significantly, TGF-beta induces Foxp3 gene expression in TCR-challenged CD4+CD25- T cells, which mediates their transition toward a regulatory T cell phenotype. The TGF-beta induction of Foxp3 is attributed to the CD4+CD25- naïve T cells, but not selective expansion of a small number of pre-existing regulatory T cells. Accordingly, TGF-beta induced-Foxp3 expression in naïve CD4+CD25- T cells can be detected as early as 16 hours after stimulation. TGF-beta induces Foxp3 expression in CD4+CD25- T cells in the 5C.C7 TCR transgenic mouse on a Rag2-/- and IL-2-/- background. TGF-beta 1-/- mice exhibit reduced number of peripheral CD4+CD25+CD62L+ T cells. Finally, CD28 signaling is involved in TGF-beta induction of Foxp3 in CD4+CD25- T cells. The data provide new evidence in understanding the mechanisms for generation and development of CD4+CD25+ regulatory T cells, and open a unique opportunity to intentionally manipulate this pivotal population of cells for immune tolerance and T cell-based immunotherapy.

#### **Su1.54. Osteoprotegerin Synthesis by Circulating T Cells Is Related to Osteoporosis in HIV-Infected Patients.**

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Osteoprotegerin (OPG), a cytokine of the TNFR superfamily, is the decoy receptor for Receptor Activator of NF- $\kappa$ B ligand (RANKL). OPG counteracts bone resorption induced by interaction of the receptor-ligand pair RANK-RANKL. OPG, present in blood, is also implicated in diverse aspects of vascular function and in immune regulation. The aims of this study are to better understand the different functions of circulating OPG through an elucidation of the role of immune cells in its regulation and to explore new aspects of interactions between the immune and skeletal systems. Our objectives, formulated in this broad framework, would be:

1. To delineate the subpopulation(s) of peripheral blood mononuclear cells responsible for the synthesis of osteoprotegerin and to identify the factors of regulation.
2. To study the functional impact of OPG regulation by immune cells in an appropriate physiopathological context i.e in HIV infected individuals.

To address these issues we used enzyme immunometric assays combined with immunoblots to examine the synthesis and the regulation of OPG by subpopulations of normal human peripheral blood mononuclear leucocytes (PBMCs), enriched by immunomagnetic separation, under different conditions of incubation. RNA synthesis profiles were studied using RT-PCR and Northern blot analyses. We provide evidence that, amongst PBMCs, OPG is constitutively secreted only by CD4+ T lymphocytes, and that T cell production of OPG is up-regulated by an anti-CD3 antibody, IL-1 $\beta$ , TNF- $\alpha$ , GM-CSF, vitamin D<sub>3</sub>, but not TGF- $\beta$ . The T cell synthesis of OPG is strongly increased by IL-4, unlike IL-10 which inhibits the constitutive and IL-4-induced production of OPG. To verify the pathophysiological importance of T cell-derived OPG, we chose to study circulating concentrations of OPG of patients infected with HIV-1 knowing that disease induced by HIV-1 is specifically associated with both compromised CD4+T cell number & function, and an increased incidence of osteoporosis. Given that OPG is presently considered one of the final components that decides outcome of bone resorption, we compared circulating concentrations of OPG in HIV-infected individuals to age-matched normal subjects and remarked a significant decrease of OPG concentrations in plasma from HIV-infected patients. Additionally, normal T lymphocytes in the presence of recombinant HIV-1 gp120 had an unequivocal reduction of their constitutive OPG production. Moreover, nelfinavir, a protease inhibitor used in highly active antiretroviral therapy, significantly inhibited the constitutive OPG synthesis by normal T cells. These results are the first to suggest a causal link between HIV-induced T cell malfunction, i.e a dysregulation of its capacity to produce OPG, and secondary osteoporosis. We highlight a new role for T lymphocytes in the regulation of circulating OPG, and suggest the therapeutic potential of OPG or OPG mimetics in the decreased bone mineral density associated with HIV infections.

#### **Su1.55. Detection of Cytokines in Patients with Hypersensitivity to Metals.**

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**Introduction:** In the dental praxis, dentist are often confronted with suspect delayed hypersensitivity reaction to various metals used in prosthetic and restorative dentistry. Cytokines have been involved in most disease processes including hypersensitivity. In this study a new method of detection of cytokines so called "Human Cytokine Array" was used in ten patients with suspect delayed hypersensitivity to metal components of dental alloys.

**Methods:** The clinical part of study involves examination of the oral cavity with respect to the presence of dental alloys including dental radiograph. Special questionnaire focused in general health status was also used. In the immunological examination mononuclear cells separated from peripheral blood were used to study the production of cytokines. By using Ray Bio™ Human Inflammation Antibody Array III it was possible to identify the expression profiles of 40 cytokines. The detection of cytokines was performed after 3 days cultivation with mercury and nickel chlorides.

**Results:** Nickel chloride stimulated production of IL-6, IL-10, IFN-gamma, IL-1 alfa, TNF alfa and TNF beta. Mercury chloride stimulated production IL-4, IL-6, IL-10, IL-1 alfa and sTNF RII.

**Conclusion:** Mercury chloride activated rather clones of TH2 lymphocytes, while nickel chloride TH1 lymphocytes. Both metal salts activated regulatory clones Tr.

**Acknowledgment:** This project was supported by grant of Czech Ministry of Health No. NK 7437-3.

#### **Su1.56. SCYA4L Polymorphism Genotyping by Fluorescent Resonance Emission (FRET) Probes in Real-Time PCR.**

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Human CCL4/MIP-1beta and CCL3/MIP-1alpha are two highly related molecules that belong to a cluster of inflammatory CC chemokines located in chromosome 17. CCL4 and CCL3 were formed by duplication of a common ancestral gene, generating the SCYA4 and SCYA3 genes, which, in turn, present a variable number of additional non-allelic copies (SCYA4L and SCYA3L1). Moreover, our group found that SCYA4L locus is polymorphic and displays a second allelic variant (SCYA4L2) highly represented in HIV+ patients compared to the one described originally (SCYA4L1).

Recent evidences have pointed out that variation in SCYA3L1 gene copy number play a key role in susceptibility to HIV infection. Probably SCYA4L will be also related with this phenomenon. Similarly this gene dosage variation could be involved in other diseases where these chemokines play important roles.

We developed a FRET probes Real-time PCR based method to determine easily, fast and reliably the genotype for the polymorphic SCYA4L locus. This polymorphism consists in a single nucleotide change situated in the second intron acceptor splice site (+590A>G). Our approach uses specific primers for SCYA4L locus and a pair of FRET hybridization probes. We have tested the use of the sensor probe complementary to each one of the allelic variants, obtaining a better melting resolution with the SCYA4L2 specific probe, while SCYA4L1 sensor probe is more useful for total SCYA4L copy number determination. Based on this approach we are setting up a protocol to determine the copy number of the SCYA4L gene whereas it informs about copy number belonging to SCYA4L1 or to SCYA4L2 allelic variants.

#### **Su1.57. Correlation of Interleukin-4 and Chronic Periodontitis.**

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**Aim:** Regarding to the presence of numerous B cells in chronic periodontitis and since TH2 cells have important role in inducing the humoral responses, so the aim of this study was to determine the correlation between IL-4 (as a most important cytokine for differentiation of TH2 cells from TH0 cells and IL-12 (as a most important cytokine for differentiation of TH1 cells from TH0 cells) and chronic periodontitis.

**Materials and Methods:** For this purpose, 20 gingival samples from healthy patients and 20 gingival samples from patients with chronic periodontitis were collected during periodontal surgery. Tissue samples were cultured for 72 hours, then ELISA was used for determining the concentration of IL-4 and IL-12 in supernatant fluids.

**Results:** IL-4 and IL-12 were found in all of samples. There was no significant difference between case and control groups

regarding IL-4 or IL-12 concentration. But there was significant difference between two groups regarding the ratio of IL-4 to IL-12 (IL-4/IL-12). Also there was correlation between IL-4/IL-12 and attachment loss ( $P \sim 0.028$ ).

**Conclusion:** It is concluded that in chronic periodontitis, probably the number of TH2 cells is more than TH1 cells but in active phases of inflammation and tissue damage, the number of TH2 cells could be decreased by the reduction in IL-4 production.

### **Su1.58. Interleukin-21 Maintains T Cells in a Naive Phenotype.**

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Of the large number of cytokines released during the development of the immune response, T cell growth factors (TCGFs) such as interleukin-2 (IL-2), IL-15 and IL-21 play major and distinct parts in T-cell activation. These TCGFs determine whether activated T cells undergo apoptosis or persist as regulatory/memory cells after robust immune activation, the key to peripheral T-cell homeostasis.

IL-21 is a major TCGF and part of the innate and adaptive immune response. IL-21 co-stimulates naive, but not memory T cells, contrary to IL-15 that is a major stimulus of memory CD4<sup>+</sup> and particularly CD8<sup>+</sup> T cells. Unlike IL-2 and IL-15, IL-21 has no significant effect on the proliferation of T cells in the absence of anti-CD3 or other stimuli. Here we demonstrate that IL-21 plays a potent role in maintaining the naive phenotype of human CD4<sup>+</sup> T lymphocytes, with the persistence of specific cell-surface markers. PBMC were isolated from healthy young donors, CD45RO<sup>-</sup> (naive) and CD45RO<sup>+</sup> (memory) CD4<sup>+</sup> T cells were subsequently isolated on a FACSvantage, sorter. The selected CD4<sup>+</sup> cells were cultured for up to 3 weeks with IL-2 or IL-21. The phenotype of naive and memory CD4<sup>+</sup> T cells was assessed by immunofluorescence. Markers such as CD45RA, CD27, CD62L and particularly CCR7 were clearly upregulated in CD4<sup>+</sup> T cells cultured with IL-21 as compared to IL-2. Less than 1% of CD4<sup>+</sup> T cells cultured with IL-2, but 43% of CD4<sup>+</sup> T cells cultured with IL-21 express CD45RA at day 21. This means that the majority of naive CD4<sup>+</sup> T cells cultured with IL-2 switched to a CD45RO<sup>+</sup> memory cell phenotype by day 21. The expression of CCR7 on CD4<sup>+</sup> T lymphocytes cultured with IL-21 induces the migration of these cells through the chemoattraction of CCL21. Moreover, IL-21 reduces the frequency of proliferating naive T cells compared to IL-2, but proliferation markers, such as Ki-67 (B56) and proliferating cell nuclear antigen (PCNA) were expressed equally in both cell-culture conditions.

Therefore, IL-21 is a unique TCGF able to maintain CD4<sup>+</sup> T lymphocytes under a naive phenotype, and to increase their cell-surface expression of CCR7 and their migration through chemoattraction to CCL21. These findings suggest that IL-21 may play a unique role in innate and adaptive immune response due to the persistence of naïve T lymphocytes.

### **Su1.59. Osteopontin, a Th1 Cytokine, Promotes Cell Survival and Inhibits Programmed Cell Death of Activated T Cells.**

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Osteopontin (OPN), previously regarded as an adhesive bone matrix protein, has recently been shown to be a pleiotropic cytokine regulating early cell-mediated immunity. Our previous studies have shown OPN to play a critical role in autoimmune demyelinating diseases. In multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) OPN expression is significantly increased in the affected tissues. Here we show that OPN inhibits TCR-mediated apoptosis in vitro without affecting cell-cycle progression as measured by BrdU incorporation and CFSE labeling. To investigate the effect of OPN on activation-induced T cell death in vivo, we activated V $\beta$ 8(+) T cells by injecting Staphylococcal enterotoxin B (SEB) into OPN<sup>-/-</sup> mice and monitored the deletion of V $\beta$ 8(+) T cells. We found that the death of these stimulated T cells in vivo is accelerated in OPN<sup>-/-</sup> mice. Adoptive transfer of T cells from OPN<sup>-/-</sup> mice to Rag-1<sup>-/-</sup> recipients revealed that OPN secreted from T cells prolongs survival of activated T cells in vivo. We also found in OPN<sup>-/-</sup> mice that expression of the pro-apoptotic Bcl-2 family protein, Bim, was increased in both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Furthermore, compared to wild type mice, OPN<sup>-/-</sup> mice display a remarkably reduced number of certain T cell subsets. In OPN<sup>-/-</sup> mice, in comparison to the T cell subpopulations CD62L(lo)CD44(hi) and CD62L(lo)CD44(int), CD62L(hi)CD44(hi) express higher levels of pro-survival protein Bcl-2. Despite of increased cell death in OPN<sup>-/-</sup>, after T cell activation the level of active caspase-3 was lower in OPN<sup>-/-</sup> cells than wild type cells. Furthermore, treatment of OPN<sup>-/-</sup> and wild type lymphocytes with a cell-permeable caspase inhibitor reduced the death of activated wild type but not OPN<sup>-/-</sup> T cells, which suggests that activation-induced cell death in OPN<sup>-/-</sup> T cells is mediated by an alternative, caspase-independent pathway. In this study, we suggest that OPN plays a role in regulating programmed cell death of activated T cells and that OPN signaling promotes T cell survival.

### **Su1.60. Comparison of RNA Preparation Methods and Their Effect on Cytokine/Chemokine Gene Expression.**

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**Objective:** Commercially available blood collection tubes for gene expression studies lyse red blood cells and stabilize RNA to ensure detected transcript profiles reflect the physiological state of the subject. We assessed two such systems for their ability to detect and quantify differential expression of cytokine and chemokine genes. **Method:** Peripheral blood samples from 8 healthy volunteers were collected in both Tempus Tube™ blood collection tubes (Applied Biosystems (ABI), Foster City, CA) and PAXgene™ Blood RNA Tubes (PreAnalytiX, Valencia, CA). All samples were isolated according to the manufacturers' instructions. RNA purity and yield were assessed prior to analysis. Samples were preprocessed as follows: 1) Blood was drawn directly into Tempus™ and PAXgene™ tubes or 2) Samples were drawn in LI Heparin tubes, stimulated with PHA, and then transferred into the two RNA collection tube types. We analyzed both: 1) Transcript detection at single time-points and 2) Differential expression between baseline samples and samples stimulated with 25  $\mu$ g/ml PHA after 3 hrs. Affymetrix HG-U133 2.0 GeneChip© with 47K transcripts and quantitative RT-PCR for 184 cytokine and chemokine transcripts (probe primers from ABI)

were run. **Results:** Initial analysis of 2 out of 8 participants generated a GeneChip<sup>®</sup> analysis set of 1,245 reliably detected transcripts. Clustering using standard correlation generated the following differential expression profiles: Set A (370 transcripts) showing up regulation using both tubes; Set B (336 transcripts) showing down regulation using both tubes; Set C (180 transcripts) showing no change, but having elevated baseline values in Tempus<sup>™</sup> but not PAXgene<sup>™</sup>; Set D (249 transcripts) showing no change, but having elevated baseline values in PAXgene<sup>™</sup> but not in Tempus<sup>™</sup>. Transcripts in Set C and D were not cytokine/chemokine related. Transcripts in Set A with greater than 10 log scale expression include chemokine (c motif) ligand 1, class 1 MHC-restricted T cell associated molecule, chemokine (C-X-C motif). Quantifying differential expression by RT-PCR showed high similarity between the tubes for all cytokine/chemokine transcripts. Some allergy relevant cytokines, such as IL-5, were not detectable at high levels in the GeneChip<sup>®</sup> assay, but were detected at high levels using RT-PCR. **Conclusion:** Different kinetics for each tube during the lysing process may account for detection level differences at single time-points for non-cytokine/chemokine related transcripts found using GeneChips<sup>®</sup>. These time-point differences could not be confirmed using RT-PCR since cytokine/chemokine RT-PCR probes were used. This demonstrates that the selection of a particular tube for RNA studies may depend heavily upon the transcripts of interest. For cytokine/chemokine transcripts, both tube types provide essentially equivalent expression profiles.

#### **Su1.61. Regulation of Interferon Gamma Expression by Tumor Necrosis Factor Receptor Type 1 Signaling.**

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Cytokine networks play a crucial role in the disease progression of both multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE). The study of these networks is necessary for understanding the disease and for developing treatments. However, cytokines are multifaceted and may fulfill contradictory roles depending upon a variety of factors, e.g. stage of disease. Tumor necrosis factor (TNF) is an important cytokine in both EAE and MS. Mice that lack TNF receptor type 1 (TNFR1 KO) develop mild EAE but express high levels of interferon gamma (IFN-gamma) in the spinal cord. IFN-gamma is a pro-inflammatory cytokine traditionally considered detrimental in MS, but which has been shown to be protective in EAE. The present study examines the mechanism by which lack of TNFR1 results in increased IFN-gamma production using an in vitro co-culture approach. Wild type (WT) or TNFR1 KO mice were immunized with MOG35-55 peptide in complete Freund's adjuvant. Seven to ten days later, lymph node T cells were isolated by MACS using negative selection. Splenic antigen presenting cells (APCs) from unimmunized mice were also obtained by MACS using negative selection. WT or TNFR1 KO T cells were co-cultured with WT or TNFR1 KO APCs in the presence of MOG35-55 peptide, and cytokine expression was analyzed at various time points by ELISA or real-time PCR. Both WT and TNFR1 KO T cells produced significantly more IFN-gamma when co-cultured with TNFR1 KO APCs compared with WT APCs. This observation was not altered by the addition of neutralizing anti-TNF antibody, demonstrating that the enhanced

IFN-gamma secretion was not due to TNF actions through TNFR2. Furthermore, these data demonstrated that TNFR1 signaling on the APC is critical in regulating IFN-gamma production. APCs can regulate T cell IFN-gamma production by secretion of IFN-gamma-inducing factors, such as IL-12. Preliminary results show that mRNA expression of the p40 subunit of IL-12 was higher in co-cultures containing TNFR1 KO APCs compared to WT APCs. This suggests that the absence of TNFR1 on APCs leads to higher levels of IL-12, which results in higher IFN-gamma secretion by T cells. The p40 subunit is a component of IL-23 as well as IL-12. IL-12 promotes IFN-gamma-secreting T cells, whereas IL-23 promotes IL-17-secreting T cells that have recently been shown to be critical for EAE pathology. Protein array analysis revealed high levels of IL-17 in our co-cultures, and future work will further investigate the role of IL-17 in our co-culture system. In conclusion, this study shows that lack of TNFR1 on APCs leads to greater IFN-gamma production by T cells, potentially due to a change in p40 expression. These data further extend our understanding of the cytokine networks underlying CNS inflammation. This study was funded by the Canadian Institutes of Health Research and the Multiple Sclerosis Society of Canada.

### **General Autoimmunity**

#### **Su1.62. Bacterial HSP70 Immunization Inhibits Proteoglycan-Induced Arthritis.**

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Immune responses to heat-shock protein 70 (HSP70) are seen in inflammatory diseases, such as rheumatoid arthritis (RA), diabetes and atherosclerosis. Although HSPs have well described roles as chaperones for intracellular proteins, the significance of immune responses to HSP is only now becoming clear and indicate that HSPs are targets for disease suppressive immune regulation. In experimental disease models HSP can be used as therapeutic agents to prevent or arrest inflammatory damage and first clinical trials using HSP peptides have shown a shift in pro-inflammatory cytokine profiles of peptide specific T cells to production of anti-inflammatory cytokines. In order to analyze the mechanisms leading to HSP induced immune regulation we set out to test the possible protective effects of HSP70 in a novel mouse (BALB/c) arthritis model. This proteoglycan-induced arthritis (PGIA) model can be induced by immunization with cartilage PG emulsified with the adjuvant dimethyl dioctadecyl ammonium bromide (DDA).

A single i.p. injection of 100 µg mycobacterial HSP70 given 10 days prior to the first PG injection (both given in DDA) resulted in a two-week delay of the arthritis onset. Furthermore, HSP70 preimmunization induced a significantly milder arthritis, as assessed by clinical score (mean maximum arthritis score  $0.7 \pm \text{SEM } 0.4$ ; compared with  $3.5 \pm \text{SEM } 1.3$  in the control mice;  $P < 0.001$ ). The protective effect of HSP70 was accompanied with HSP70-specific T cell proliferation, IFN- $\gamma$  and IL-10 production, in mice pretreated with HSP70. Interestingly, in

these mice we could also detect IL-10 production against the arthritis inducing proteoglycan, which was not present in control mice. In addition, joint sections of HSP70-pretreated mice showed less leukocyte infiltration, reactive synovial cell proliferation and cartilage destruction compared to the control group.

In conclusion, HSP70-initiated modulation, could dramatically suppress the development of inflammation and subsequent tissue damage in PGIA. In addition, we demonstrated that HSP70 preimmunization, indeed, activated T cells, altered the cytokine profile and modulated the immune regulatory response.

### Su1.63. Characterization of Functionally Important Regions of CD200:CD200R for Immunoregulation Using Blocking Synthetic Peptides and/or mAbs.

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**Objective:** We have previously defined the immunoregulation which occurs, allowing prolongation of allograft survival and suppressing collagen-induced arthritis in mice, following interaction of the cell surface molecules, CD200 and CD200R. The CD200R1 isoform is most important in delivering direct immunosuppressive signals. Other groups have used a biophysical approach to investigate the structurally important regions for these interactions, and have suggested the N-terminal (extracellular domains) of the molecules are crucial. We report on our investigations of the important interactions using an approach investigating perturbation of various biological functions which follow CD200:CD200R interactions.

**Materials and Methods:** A series of 15-mer peptides were synthesized which defined continuous sequences of the extracellular region of the murine and human CD200 molecule. In addition, peptides mapping to the presumptive CDR1, CDR2 and CDR3 of the human and mouse CD200R1 molecules were synthesized. We assessed the ability of these different molecules to block the interaction of CD200 with CD200R1 in a competitive ELISA using plate-bound CD200R1Fc and biotinylated CD200Fc, as well as by FACS using FITC-conjugated CD200Fc binding to 24hr-LPS-activated adherent cells. Peptides were also used to modulate the suppression of MLC reactivity in vitro which follows from inclusion of CD200Fc in MLC cultures, measuring development of CTL after 5 days of culture.

In addition, using a newly prepared panel of mAbs to mouse and human CD200Fc, we compared the rank activities of antibodies for binding (FACS or ELISA) to CD200 with their abilities to augment immune reactivity in MLCs.

**Results:** Peptides defining discrete regions in the N-terminal regions of CD200 and CD200R1 were functionally active in the different assays, including the assay which investigated blockade of CD200Fc-mediated suppression. Moreover, infused in vivo, the same mouse-specific peptides suppressed protection from graft rejection afforded by injection of soluble immunosuppressive CD200Fc.

Using the panel of mAbs, we observed again that only mAbs defining epitopes in the N-terminal domain could augment MLC reactivity (or block immunosuppression by soluble CD200Fc), while mAbs targeting C-domain epitopes, although reactive in ELISA or FACS (targeting cell surface CD200) were inactive in MLCs. In a final assay we compared by rank these different anti-

CD200s for FACS staining of CD200 expressed on various cell types, including fresh dendritic cells and lymphoid tumor cells. These data suggested there exists a significant heterogeneity in expressed CD200.

**Summary:** Our data confirm that the N-terminal regions of CD200 and CD200R1 are crucial to mediate immunoregulation, and suggest that it will prove possible to generate smaller molecular weight antagonists of this interaction which may have clinical utility.

### Su1.64. Gene Identification of Single Chain Format Variable(scFv) Anti- $\beta_2$ -Glycoprotein-I ( $\alpha\beta_2$ GP-I) and Anti-Prothrombin (aPt) Antibodies Obtained from a Primary Anti-Phospholipid Syndrome (PAPS) Patient by Phage Display.

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**Introduction:** Anti-phospholipid syndrome (APS) is an autoimmune disease characterized by thrombotic and hemocytopenic manifestations in patients with high titers of autoantibodies directed against phospholipids and phospholipids cofactor proteins. Molecular studies of anti-phospholipids (aPL) had been performed on monoclonal autoantibodies obtained from EBV transformed B cells, which do not represent the repertoire of the autoimmune aPLs. Using phage display technology is possible the study of a wide repertoire of  $V_H(D)J_H$  and  $V_LJ_L$  rearrangements that recognize specific antigens. **Objective:** To study by phage display the  $V_H(D)J_H$  and  $V_LJ_L$  rearrangements of anti- $\beta_2$ GP-I and anti-Pt from a patient with PAPS with high titers of these autoantibodies.

**Methods:** cDNA was synthesized from RNA obtained from peripheral blood mononuclear cells. The  $V_HDJ_H$  and  $V_LJ_L$  ( $\kappa$  and  $\lambda$ ) rearrangements were amplified. Heavy and light chains fragments were linked to obtain scFv fragments. The scFv were ligated to a pSyn2 vector. *E. coli* XL1-Blue were transformed with the products of the ligation. Cultures were co-infected with the M13/KO7 phage to rescue the antibodies in the format phage-scFv antibody. Four rounds of selection were done against  $\beta_2$ GP-I and Pt. Positive clones were sequenced and analyzed.

**Results:** Two clones that recognize  $\beta_2$ GP-I (beta 1 and 2) and one that recognize Pt (Prot-1) were obtained.  $V_H$  genes from the Beta1 and 2 belong to the  $V_H4$  family. Beta1 rearrangement shows 96.7 % of homology with the VHSP/VH4.22 gene, no D gene was identified and the  $J_H$  gene used was the product of the  $J_H5$  gene with 98.03 % of homology. Light chain is composed by the product of the 1b.366F5/DPL5 gene that belongs to the  $V\lambda 1$  family, it shows 99.4 % of homology, no  $J_L$  gene was identified. Beta2 clone shows 98.97 % of homology with the VIV-4/4.35+ gene, no D gene was identified and it was rearranged with the product of the  $J_H4b$  gene with 81.25 % homology, light chain used the product of the gene V2-14+ which belong to the  $V\lambda 3$  family with 88.88 % of homology and it is rearranged with the product of the  $J_L3b$  gene. The third clone, Prot1 carries the product of the DP-47/V3-23 gene that belongs to the  $V_H3$  family with 98.6 % of homology, no D gene was identified and the  $J_H$  gene identified



was the product of the J<sub>H</sub>4b with 87.5 % of homology, light chain is the product of the 1b.366F5/DPL5 gene, which belongs to the V<sub>λ</sub>1 family with 93.6 % of homology, it was re-arranged with the product of the J<sub>L</sub>3b gene, and it shows 89.47 % of homology.

**Conclusions:** We confirm the bias through the use of V<sub>H</sub>3 and V<sub>H</sub>4 gene families. The number of somatic mutations suggests that the rearrangements are the product of antigen driven clones. The antibodies in format scFv represent well those autoantibodies found in patients with PAPS.

### **Su1.65. A Novel Network of Human B Cell Effector Cytokines Is Implicated in Autoimmunity: Dysregulation in Patients with Multiple Sclerosis and Potential as Therapeutic Target.**

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**Background:** Beyond their roles as potential antibody producing cells, there is growing interest in the fundamental roles that B cells may play in regulating immune responses. Emerging animal studies point to an important contribution of B cell effector cytokines to autoimmunity. We set out to study the potential involvement of human B cells and their cytokines in immune regulation and autoimmunity.

**Results:** We report that the profile of human B cell cytokine production is context dependent, being critically influenced by the balance of signals through the B cell receptor and CD40. B cells appropriately stimulated by sequential B cell receptor and CD40 stimulation proliferate and secrete tumour necrosis factor (TNF)- $\alpha$  and lymphotoxin (LT) which contribute to germinal center reaction and thereby amplify the ongoing immune response. In contrast, CD40 stimulation alone—a mimic of a B cell receiving bystander T cell help in the absence of specific antigen recognition—induces significantly less pro-inflammatory cytokines ( $n = 24$ ;  $P < 0.0002$ ) but significantly enhanced production of IL-10 ( $P < 0.004$ ) that serves to suppress inappropriate immune responses. We further observed that this novel network of reciprocal regulation of B cell effector cytokines, is abnormal in a subgroup of patients with relapsing remitting multiple sclerosis where B cells maintain their capacity to proliferate and produce the proinflammatory cytokines TNF- $\alpha$  and LT ( $n = 18$ ;  $P = 0.46$  compared to normals), but have a significantly diminished capacity to produce IL-10 ( $n = 18$ ;  $P < 0.02$ ). This B cell cytokine network can be targeted therapeutically, as evidenced in patients treated with mitoxantrone (Novantrone<sup>®</sup>), a recently approved immune suppressant in MS. Compared to B cells from untreated patients, B cells from mitoxantrone treated MS patients produce significantly less of the pro-inflammatory cytokines TNF- $\alpha$  ( $n = 8$ ;  $P < 0.02$ ) and LT ( $P < 0.05$ ), but significantly more of the anti-inflammatory cytokine IL-10 ( $P < 0.05$ ). We further observed that treatment was associated with a decrease in the proportion of circulating memory B cells, suggesting that the apparent reciprocal regulation of B cell cytokines mediated by mitoxantrone, related to differential effects on distinct B cell subsets. This guided further studies of the fundamental B cell cytokine network and enabled us to ascribe unique effector cytokine profiles to normal memory and naive human B cell subsets.

**Summary:** We ascribe active roles for memory and naive human B cell subsets in appropriately promoting or suppressing

local immune responses in the normal state through production of distinct effector cytokines. We further suggest that dysregulation of this B cell cytokine network occurs in some patients with the human autoimmune disease multiple sclerosis, and that this network represents a potential therapeutic target.

### **Su1.66. Class Switch Recombination in B-Lymphopoiesis Is a Novel Pathway for Development of Autoimmune B Cells.**

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Activation of autoreactive B cells is pivotal for the initiation and propagation of many autoimmune diseases. It is generally thought that this activation reflects a breakdown in peripheral tolerance, although the nature of the development of these cells is not completely understood. Many studies in B lymphopoiesis have shown that IgM receptors drive development and construction of naive B cell repertoire, whereas IgG receptors promote formation of the memory B cell compartment. This isotypic change results from a gene rearrangement process called class switch recombination (CSR), and is thought to occur in mature peripheral B cells upon activation and providing of appropriate T cell help. In the absence of T cell help, activated B cells undergo Fas-mediated apoptosis, a process thought as a peripheral mechanism for self-tolerance. We have recently shown that CSR spontaneously occur during normal B lymphopoiesis in vivo and in vitro, and that these cells acquire a memory phenotype. We found that CSR in B lymphopoiesis is T cell-independent, but further expansion of the cells is regulated by T cells. To further study these cells we have used the Igm-deficient mouse model (mMT), where B cell development is limited by the ability of the cells to undergo CSR. We found that isotype switched B cell precursors can mature to the periphery and differentiate to antibody-producing plasma cells. These B cells generate a gH-driven repertoire that is oligo-monoclonal and bearing self-tissue reactivity. Further studies revealed that isotype-switched B cell precursors are negatively selected by Fas signaling, as blocking the Fas/FasL interaction rescues the development of isotype-switched B cells in vivo and in vitro. Our studies propose a novel developmental pathway driven by gH-isotypic receptor that effectively circumvents peripheral tolerance requirements. This pathway, however, is strictly controlled by the Fas signaling to prevent B cell autoimmunity.

### **Su1.67. The Role of VCAM-1 in Self-Reactive T Cell Fate.**

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Vascular Cell Adhesion Molecule 1 (VCAM-1) plays a role in leukocyte migration and adhesion, and is found on a variety of cells including endothelial cells, thymic epithelium, and dendritic cells. VCAM-1 binds to its principal ligand, Very Late Antigen-4 (VLA-4) found on B cells and T cells. Disruption of VCAM-1/VLA-4 signaling is associated with prolonged allograft acceptance and impaired T cell responses. Therefore a role has been established for VCAM-1 as a positive factor for T cell activation and proliferation. It is our hypothesis that in the absence of VCAM-1 expression on dendritic cells, antigen specific, self-reactive CD4<sup>+</sup> T cells will fail to proliferate as effectively as when VCAM-1 is present. It is also hypothesized that some self-reactive T cells activated in the absence of VCAM-1 will survive and acquire a regulatory T cell

phenotype. To test this hypothesis we will use 3A9 antigen specific T cells that recognize Hen Egg Lysozyme (HEL) protein presented as peptide by antigen presenting cells. Transgenic mice expressing membrane-bound or soluble HEL have been crossed onto conditional VCAM-1 knockout mice lacking functional expression of VCAM-1 on all subsets of splenic dendritic cells. By adoptive transfer of labeled 3A9 T cells into either HEL or VCAM<sup>-/-</sup> HEL recipient mice, the fate of these antigen specific T cells can be followed over time.

### **Su1.68. The Autoimmune Response to Modified Human Low-Density Lipoprotein.**

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Human LDL modified in vitro has been used to develop antibodies that allow the detection of spontaneously modified LDL in circulation. Most modified lipoproteins circulate as antigen-antibody complexes (immune complexes, IC) that can be characterized after precipitation with 4% PEG. Chemical and immunological analysis of IgG and LDL isolated from IC demonstrated that the human immune system recognizes malondialdehyde lysine, carboxymethyl lysine and other yet uncharacterized epitopes. The IgG fraction isolated from precipitated IC contains antibodies reactive with copper-oxidized LDL (oxLDL) and AGE-modified LDL (AGE-LDL) predominantly of the pro-inflammatory subclasses 1 and 3. Comparison of the IgG subclass and dissociation constants of antibodies contained in precipitated IC and remaining in the supernatant after PEG precipitation showed that antibodies included in IC have statistically significant higher avidity and relatively higher IgG1 concentrations. Affinity chromatography isolation of circulating antibodies to oxLDL and AGE LDL showed that IgG antibodies predominated over IgM antibodies, and that IgG subclasses 1 and 3 predominated over subclasses 2 and 4, for both antigens. Because IgG1 and IgG3 antibodies can activate complement and deliver activating signals to phagocytic cells as a consequence of their interaction with Fcγ receptors, the pathogenic potential of modified LDL-IC is unquestionable. Furthermore, clinical studies carried out in patients with type 1 diabetes have clearly demonstrated that high levels of modified LDL-IC predict cardiovascular events. Our observations have also additional clinical implications. On one hand, we have demonstrated that the assay of modified LDL and corresponding antibodies in serum is extremely inaccurate, due to the interference of IC. On the other hand, our studies suggest that measurements of modified LDL in IC, rather than serum, are required for the evaluation of the pathogenic potential of modified LDL. In conclusion, the autoimmune response triggered by spontaneously modified LDL seems to be a significant factor in the pathogenesis of atherosclerosis.

### **Su1.69. Studies on Signal Transduction Pathways Downstream of CD28/IL-2 That Regulate the E3 Ligase, GRAIL.**

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Anergy can be induced in CD4<sup>+</sup> T cell clones by a strong TCR signal in the absence of costimulatory signals. CD28/B7 costimu-

lation has been shown to play a critical role in preventing anergy induction. Two models have been proposed to explain these results: one predicts that CD28 costimulation has a direct inhibitory effort on factors involved in anergy induction. The other suggests that CD28 costimulation results in the production of growth factors, such as IL-2, which in turn prevents anergy.

We have identified GRAIL (gene related to anergy in lymphocytes) using differential display. GRAIL contains a highly conserved zinc binding ring domain and exhibits E3 ligase activity in vitro. Our studies have shown that constitutive expression of GRAIL, but not the enzymatically inactive form, H2N2 GRAIL, is sufficient to induce anergy in naïve CD4<sup>+</sup> T cells, suggesting that GRAIL is an anergy factor. Furthermore, we have shown that pharmacological inhibition of downstream targets of CD28 signaling such as PI3K and mTOR, as well as blocking IL-2 signaling using an antagonist antibody, all result in a significant increase in GRAIL expression, indicating that GRAIL is regulated by CD28. Our results suggest that GRAIL functions to maintain anergy in T cells through the ubiquitination pathway to degrade essential signaling molecules that are involved in T cell immunity. Our studies also shed light on the signal transduction pathways downstream of CD28/IL-2 that are involved in GRAIL expression, thereby elucidating the basis of costimulation blockade. A better mechanistic understanding of GRAIL and its regulation by costimulatory molecules could lead to novel treatments for patients suffering from autoimmune disease or from transplantation complications.

### **Su1.70. Immune Monitoring the Effects of hOKT3γ1 Ala-Ala in a Patient with New Onset Type 1 Diabetes Mellitus.**

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**Background:** Type 1 diabetes (T1D) is an autoimmune disease caused by the pathogenic action of T lymphocytes which destroy insulin producing beta cells. An effective immunomodulatory anti-CD3 antibody, hOKT3γ1(Ala-Ala), used to treat new onset T1DM had direct effects on pathogenic T cells and induced regulatory cells. **Methods:** In a Phase II trial sponsored by the Immune Tolerance Network, we studied the effects of the hOKT3γ1(Ala-Ala) on immune responses in patients with new onset T1D. Six patients were treated with hOKT3γ1(Ala-Ala) I.V. daily for 12 days. Immunophenotyping; T cell proliferation by CFSE-based flow cytometry; and cytokine secretion were performed. **Results:** Transient depletion of T cells in patients followed by recovery of the cells after the 12 day antibody course was observed in all but one patient who demonstrated a prolonged reduction in the number of circulating CD4<sup>+</sup> T cells. Nine months after therapy, the patient's CD4 count was 102 × 10<sup>6</sup>/mL and did not return to normal levels till 29 months post therapy (512 × 10<sup>6</sup>/ml). In contrast, the CD8<sup>+</sup> T cell count recovered rapidly and reached 86% of baseline by 30 days. No obvious difference in either coating or modulation of CD3 on T cells was observed on the CD4<sup>+</sup> T cells as compared to the other treated individuals. Trough levels of drug were higher but not exceptionally different than observed in the majority of

treated individuals. However, there was selective recovery of CD45RO cells and CD4+CD62L+CD25hi Treg population increased during the treatment. In spite of the reduced CD4+ T cell count, the T cells functioned normally on a per cell basis as demonstrated by CFSE proliferation and IL-2 secretion. Clinically, the patient had an uneventful clinical course with only a transient viral syndrome shortly after treatment, with all cultures and serological studies negative. The patient was maintained on antimicrobial prophylaxis until her CD4+ count recovered. The patient's c-peptide levels remain high at 2 years (77% of baseline) while maintaining a normal Hemoglobin A1c at 18 months comparable to that noted in other responders. *Discussion:* In the present clinical study of hOKT3g1 (Ala-Ala) one patient experienced a prolonged decrease in CD4+ T cells. The therapy did not lead to impaired CD4 function *ex vivo*, or to opportunistic infections. The unremarkable clinical course may relate to lack of depletion centrally, and/or failure to cause more generalized immunosuppression. Since the higher circulating levels did not result in a better clinical response, the data support dosing at the previous lower phase I/II levels helping define the window for safety and efficacy with this agent. [Author's note: one of the authors of this study holds the patent on the antibody under investigation.]

#### Su1.71. Essential Role of the Co-Receptor CD72 in B Cell Peripheral Tolerance.

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CD72, a type II transmembrane protein expressed predominantly on B cells, acts as a negative regulator of B cell activation through the B-cell receptor (BCR). However, whether CD72 plays a role in autoimmunity is unknown. We found that aged CD72-deficient mice develop lupus-like autoimmune features such as anti-nuclear Ab, anti-DNA Ab, and glomerulonephritis. To study the mechanism of this spontaneous autoimmune disease, we employed a transgenic mouse model of autoimmunity to elucidate how CD72-deficient B cells break down tolerance *in vitro*. B cells from double-transgenic (dTg) mice expressing an anti-HEL (hen egg lysozyme)-specific BCR and soluble HEL in the circulation are anergic (unresponsive) to HEL antigen (Ag) stimulation. We found that B cells from CD72 HEL dTg mice are hyperproliferative in response to HEL Ag stimulation, as compared to the anergic response of B cells from wild-type (WT) HEL dTg mice. To further understand the signal transduction pathways employed by CD72, the downstream signaling events such as NF- $\kappa$ B, NF-AT, ERK, p38, and JNK pathways were examined. The results show that B cells from CD72 HEL dTg mice have (1) greater NF- $\kappa$ B p65 DNA binding activity, (2) greater NF-ATc1 activation, (3) greater ERK activity, (4) greater JNK activity, and (5) greater p38 activity, as compared to those of B cells from WT HEL dTg mice. We next examined the early events of signaling. We found that the calcium flux in response to HEL Ag stimulation is greatly enhanced in CD72 HEL dTg B cell when compared to the WT HEL dTg B cells. To identify the targets of CD72 signaling immediately following stimulation, we examined the phosphorylation status of many early signaling molecules such as Lyn, Syk, Ig- $\alpha$ /b, PLC $\gamma$ -2, Blnk, Vav, and Cbl etc. Our results show that Cbl-b is a direct

target of CD72 signaling. To determine whether these effects of CD72 on B cell tolerance have clinical relevance to the development of autoimmune disease, we induced EAE (experimental autoimmune encephalomyelitis) in both WT and CD72KO mice. We found that MOG (35-55) peptide induced an earlier onset and more severe EAE in CD72KO mice as compared to WT mice. Taken together, these findings indicate that CD72 plays an essential role in modulating B cell peripheral tolerance.

#### Su1.72. Identification of the Target Self-Antigens in Ischemia/Reperfusion Injury.

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Ischemia/reperfusion injury (I/R), a potential life-threatening disorder, represents an acute inflammatory response following periods of ischemia resulting from myocardial infarction, stroke, surgery or trauma. The recent identification of a monoclonal natural IgM that initiates I/R led to the identification of conserved self-antigens as the target. Utilizing proteomics and phage-display-peptide library, we have identified the antigenic epitope of I/R specific antigen and developed peptide inhibitors for injury in three different models (intestine, skeletal muscle, and heart). These results identify a novel pathway in which the innate response to a highly conserved self-antigen results in tissue destruction through a generalized autoimmune mechanism.

#### Su1.73. Shared Epitopes among HLA Class II Molecules in Common Autoimmune Diseases.

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Our previous studies revealed common HLA class II haplotypes that confer predisposition or protection to IDDM, MS, JCA, pemphigus vulgaris (PV) and SLE in the Bulgarian population. In order to evaluate further the role of HLA antigens in those autoimmune diseases we looked for common sequence motifs in the hypervariable regions of molecules encoded by protective and predisposing DRB1 and DQB1 alleles. We found that the b-chains coded by the common predisposing DRB1\*0301, 0401, 0402, 0404 alleles have similarities in HVR<sub>III</sub>: a motif 57D58A in pocket 9 and an electric charge of pocket 4, generated by residues 70-74. Similar characteristics are observed for molecules coded by other predisposing DRB1 alleles in our population: 1401 and 1404 (PV); 1501 and 1001 (MS); 0701 (SLE). The protective allele DRB1\*1104 codes a molecule with a neutral charge of pocket 4 and absence of 57D58A in pocket 9. Predisposing DQB1\*0302 and 02 alleles encode a common motif 13G, 26L, 57A. Part of this motif (13G, 26L) is shared by other predisposing for our population alleles: DQB1\*0602 (MS), 0604 and 0303 (SLE). The b-chain, encoded by the common protective DQB1\*0301 is characterized by different, hydrophobic amino acids-13A, 26Y, and has D at position 57 similarly to DQB1\*0503-another protective for IDDM allele in Bul-

garians. In conclusion, based on these results we suggest that more than one amino acid regions in b-chains of DR and DQ molecules, predicted to be involved in processed antigen binding and helical regions, are important for disease predisposition and protection.

#### **Su1.74. *In Vivo* Blockade of Human IL-2 Receptor (IL-2R) Induces Expansion of CD56<sup>bright</sup> Regulatory NK Cells in Patients with Active Uveitis.**

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*In vivo* blockade of the human IL-2 receptor (IL-2R) by antibody has been used for immunosuppression in transplantation, therapy for leukemia and autoimmune diseases. In this study, we report that administration of a humanized IL-2R blocking antibody induced a 4- to 20-fold expansion of CD56<sup>bright</sup> regulatory NK cells in uveitis patients over time. The induced CD56<sup>bright</sup> regulatory NK cells from the treated patients exhibited the same phenotype as those from normal donors except that they had a lower level expression of CD161. In addition, patients with active uveitis had a significantly lower level of CD56<sup>bright</sup> regulatory NK cells in the periphery as compared to normal donors ( $P < 0.01$ ). The induction of the CD56<sup>bright</sup> regulatory NK subset occurred prior to the onset of a clinically therapeutic effect. Our observation may have implications for the potential role of CD56<sup>bright</sup> regulatory NK cells in autoimmune diseases and to IL-2R blockade therapy.

#### **Su1.75. Premature Senescence of the Immune System in Rheumatoid Arthritis and Multiple Sclerosis Patients.**

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The decline in immunocompetence with age is accompanied by a steadily increasing incidence of autoimmune diseases. A reduced thymic output, which is an important aspect of immunosenescence, will induce compensatory auto-proliferation. This process can lead to a premature senescence of T cells and contribute to the immune abnormalities associated with autoimmunity and aging. In this study we tested whether the immune system of patients with rheumatoid arthritis (RA), multiple sclerosis (MS) and ankylosing spondylitis (AS) shows signs of an age-independent aging. Therefore, we measured two indicators of aging, the number of T cell receptor excision circles (TRECs) and the percentage of CD4+CD28<sup>null</sup> T cells, in peripheral blood mononuclear cells (PBMC). In addition, characteristics of senescent CD4+CD28<sup>null</sup> T cells were analyzed. PBMC were isolated from blood of 60 RA, 30 MS and 20 AS patients as well as 40 healthy controls (HC). The percentage of CD4+CD28<sup>null</sup> T cells was measured by flow cytometry. The number of TRECs in 100 ng of gDNA was determined by real-time PCR analysis. Intracellular FACS analysis and CDR3 fragment length analysis were used to determine the cytokine profile and the clonal origin of the CD4+CD28<sup>null</sup> T cells, respectively. In RA and MS patients, TREC numbers were significantly decreased compared to age-matched HC. TREC numbers were comparable between AS patients and HC. Furthermore, an increased percentage of CD4+CD28<sup>null</sup> T cells was detected in 40% of RA patients, 32% of MS patients and 12% of AS patients versus 10% of HC. In HC and RA patients, the presence of the HLA-DR4 haplotype, a genetic risk factor for

developing RA, was found to be linked with the percentage of CD4+CD28<sup>null</sup> T cells. CD4+CD28<sup>null</sup> T cells were also detected in the synovial tissue of RA patients. Analysis of the CD4+CD28<sup>null</sup> T cells revealed clonal expansions and pro-inflammatory properties. This study provides indications for a premature senescence of the immune system in both RA and MS patients. Premature aging, associated thymic involution and compensatory auto-proliferation might play an important role in the pathogenesis of autoimmunity. CD4+CD28<sup>null</sup> T cells have tissue damaging properties, are prematurely increased in both RA and MS patients and might therefore play an active role in the pathogenesis of these autoimmune diseases. Further study will be necessary to reveal the exact role of an aged immune system and more particular the role of CD4+CD28<sup>null</sup> T cells in the pathogenesis of autoimmunity.

#### **Su1.76. Histamine Release and Autoantibodies in Chronic Idiopathic Urticaria and Cough.**

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It has been shown the presence of autoantibodies in patients with thyroid immunity disease and IgG, IgA and IgE antibodies in patients with Helicobacter pylori infection.

Chronic "idiopathic" urticaria and cough are present in almost 50% of patients with either diseases. In a subset of chronic "idiopathic" urticaria patients, the disease is caused by the presence of IgG autoantibodies against the high affinity IgE receptor FcεRIα and anti-IgE autoantibodies. These functional autoantibodies stimulate normal activation of mast cells and basophils via FcεRIα causing whealing, angioedema and cough.

The aim of the study is:

to evaluate in patients with chronic idiopathic urticaria and/or cough:

the presence of Helicobacter pylori antibodies and/or thyroid autoimmunity the histamine release and the prevalence of IgG autoantibodies against FcεRIα and anti-IgE autoantibodies in patients positive for thyroid autoimmunity or Helicobacter pylori infection before and after levothyroxine sodium or HP eradication therapy

Methods

All patients had chronic urticaria and /or cough, for all of them the following investigations were performed:

skin prick test for aeroallergens and food allergens, blood test for antithyroid antibodies (AAT, ATPO), FT3, FT4, TSH., Helicobacter pylori antibodies (IgG, IgA), total IgE, autologous serum skin test (ASPT) in patients with positive antithyroid and HP antibodies, histamine- release activity, serum IgG anti-Fcε RI α and anti-IgE in all patients' sera

Results

Our preliminary data show:

100% of patients were with normal thyrotropin levels

100% of patients with thyroid autoimmunity had cough and 33% of them also had urticaria

100% of patients with HP positive had only urticaria and 41% of them also had cough

85% of the patients had ASPT and antibodies positive, 80% had ASPT and histamine release positive

70 to 100 % remission rate (either partial or complete) of urticaria and cough in patients treated with levothyroxine.

All patients treated with HP eradication therapy had negative breath test after treatment; 80% of them had remission of urticaria.

85% of the patients had ASPT and antibodies positive, 80% had ASPT and histamine release positive

#### Conclusions

These results suggest that histamine releasing autoantibodies are important in the pathogenesis of chronic urticaria and cough by stimulating or facilitating degranulation of basophils and cutaneous mast cells through cross-linking cell surface IgE receptors.

### **Su1.77. A Polymorphism of the Inhibitory Receptor, FcγRIIb, Prevents Its Access to Lipid Rafts and Alters Macrophage Responses to Immune Complexes and Opsonized Bacteria.**

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Receptors for the constant region of IgG (FcγRs) link the humoral and cellular arms of the immune system. Their dysregulation has been implicated in the development of autoimmune conditions particularly systemic lupus erythematosus (SLE). A polymorphism at position 232 in the transmembrane domain of the inhibitory receptor, FcγRIIb, results in an isoleucine to threonine substitution is prevalent in SLE patients. Using the FcγRIIb-negative monocytic cell line, U937, we assessed the consequences of expression of either wild type (wt) or mutant (232T) FcγRIIb on activatory Fcγ receptor function. Expression of wt but not 232T prevents lysosomal trafficking of immune complexes and superoxide generation, inhibits phagocytosis of opsonised pneumococci and reduces pro-inflammatory cytokine release. The 232T receptor is unable to partition into lipid rafts, excluding it from the pro-inflammatory signalling complex. Primary macrophages from 232T homozygotes show similar defects in FcγR control, correctable by lentiviral introduction of wt FcγRIIb. Thus, this receptor polymorphism radically alters the cellular response to opsonised particles and may predispose to the development of SLE. This mutation is the first described which could contribute to disease by excluding a surface receptor from lipid rafts, potentially representing a novel mechanism underlying human genetic disease.

### **Su1.78. On the Role of Th1 Cytokines (gIFN, IL18) and Growth Factor TGFβ<sub>1</sub> in Autoimmune Thyroid Disease.**

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#### INTRODUCTION

In a number of previous investigations it has been demonstrated the crucial role of different immune cells and subsets in the pathogenesis of autoimmune thyroid disease (AITD). The role of different T cell cytokines and the growth factors is now appreciated in development and progression of AITD (Hunt et al, 2000; Paschke et al., 1994; Ajjan et al., 1997; Hrda et al., 2003 etc.).

#### THE AIM OF INVESTIGATION

The aim of the investigation was the study and compare of in vitro synthesis and serum concentrations of gIFN, IL18 and TGFβ<sub>1</sub> in patients with AITG.

#### THE MATERIALS AND METHODS

25 patients with AITD were studied. The control group consisted of 12 healthy volunteers with similar to the investigated patients in sex and age. The serum concentrations of gIFN, IL18 and TGFβ<sub>1</sub> and MNC supernatant levels (24–48 h culture) of cytokines were determined using ELISA method.

#### RESULTS

An increase in the serum concentrations of both cytokines (gIFN, IL18) and growth factor TGFβ<sub>1</sub> were noted in the studied group when compared to the control group. The increase in serum concentrations of gIFN, IL18 accompanied by their increased synthesis by peripheral MNC more than 2.5–3.2 times in comparison to control data. The average serum concentration of TGFβ<sub>1</sub> in AITD was 274 pg/ml and in control group-47.4 pg/ml ( $P = 0,02$ ). The serum concentrations of gIFN and IL18 were from 3.8 to 4.5 times higher than in control group.

#### CONCLUSION

The increase of the serum concentrations of gIFN, IL18 and TGFβ<sub>1</sub> and increased synthesis by peripheral MNC of gIFN and IL18 suggest that Th1 cytokines as well as growth factor TGFβ<sub>1</sub> plays an important role in AITD.

### **Su1.79. Autoimmunity: Common Origin for Diverse Diseases.**

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**Objective:** The current study investigated the presence of autoimmune diseases (AID) among first degree relatives (FDR) of primary Sjögren's syndrome (pSS) and type 1 diabetes mellitus (T1DM) patients. **Materials and Methods:** To this purpose, a case-control study was undertaken in which individuals were interviewed personally using a questionnaire that sought information about demographic and medical characteristics including AID family history. **Results:** In pSS, there were 101 women defined according to the Revised European Criteria and 124 matched controls without AID. We found that 38 (38%) families had at least one FDR having an AID, while in controls there were 27 (22%) (OR: 2.2, 95%CI = 1.2-3.9,  $P = 0.01$ ). An AID was registered in 56 (6%) of 980 FDR of patients as compared with 33 (2%) of 1433 FDR in controls (OR: 2.6, 95%CI = 1.66-3.98,  $P < 0.0001$ ). Sixty-four AID were observed in the 56 FDR of patients, of whom five had more than one AID. No FDR among controls had more than one AID (OR: 2.96, 95%CI = 1.9 –4.5,  $P < 0.0001$ ). In patients with T1DM, defined according to the Expert Committee Criteria, there were 107 patients and 113 matched controls without AID. In patients, there were 25 (26%) FDR with at least one AID while in controls there were 9 (8%) with the same characteristics (OR: 3.96, 95%CI = 1.74-9,  $P = 0.0006$ ). Among the T1DM patients, an AID was registered for 26 (8.3%) of the 312 FDR as compared with 9 (2.4%) of the 362 FDR among controls (OR: 3.56, 95%CI = 1.64-7.32,  $P = 0.0008$ ). The most frequents AID registered in FDR of pSS patients were autoimmune hypothyroidism and rheumatoid arthritis, and in FDR of T1DM patients were autoimmune hypothyroidism and T1DM. **Conclusion:** These results indicate

that familial autoimmunity is a major trait in AID, thus sustaining the common variants/multiple disease hypothesis which emphasizes that similar immunogenetic mechanisms underlie AID.

**Su1.80. Selective Unresponsiveness of TNFR1<sup>-/-</sup> Macrophages to IFN- $\gamma$  Stimulation Is Critical in Resistance to Experimental Autoimmune Uveoretinitis.**

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In experimental autoimmune uveoretinitis (EAU), an animal model of autoimmune inflammatory eye disease, activated macrophages play a critical role in tissue destruction in the retina, since depletion of macrophages reduces retinal damage without concomitant reduction in numbers of other infiltrating cells. Macrophage activation is modulated by signals received in the local micro-environment from soluble cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , as well as signals received from cell surface receptor interactions, for example of CD200 with CD200R. Using NOS2 mediated production of nitrite (NO) as a measure of activation in vitro and EAU to study the course of disease in vivo, we have analysed the activation of wild type or TNFR1<sup>-/-</sup> macrophages.

As in the model of multiple sclerosis, experimental autoimmune encephalomyelitis, TNFR1<sup>-/-</sup> mice are resistant to the induction of EAU. They have a lower incidence of disease and lower disease scores. Priming of T cells is only modestly impaired, but by the time the animals reach peak disease (day18–21), antigen specific proliferation in the spleen is significantly reduced in the TNFR1<sup>-/-</sup> mice. To investigate this further macrophage function was assessed in vitro by activation with various stimulators. In response to stimulation with IFN- $\gamma$ , wild type mice produce substantial amounts of NO. On the other hand TNFR1<sup>-/-</sup> mice cannot be stimulated to produce NO by IFN- $\gamma$  activation. To test whether this was due to autocrine stimulation by low levels of TNF- $\alpha$ , we stimulated wild type macrophages with IFN- $\gamma$  in the presence of sTNFR-Ig fusion protein, which binds free TNF- $\alpha$ , and showed that this also blocked NO production. In contrast with this impaired activation of NO synthesis, MHC class II upregulation was indistinguishable in wild type and TNFR1<sup>-/-</sup> mice stimulated with IFN- $\gamma$ . We then addressed whether TNFR1 signalling was essential for NO production, by stimulating macrophages with LPS. Under these conditions both wild type and TNFR1<sup>-/-</sup> mice produced equivalent amounts of NO.

Together these results show that selected aspects of IFN- $\gamma$  mediated activation of macrophages are controlled by autocrine secretion of TNF- $\alpha$ , but that this control is bypassed in the presence of signals generated by pathogen-associated molecular pattern recognising receptors. Macrophage activation in a pro-inflammatory milieu may therefore be modified by the presence of pathogen derived signals, and may be different in autoimmune disease where inflammation occurs in the absence of foreign immune system activators. This implies that in this model, activation sufficient for priming occurs in the presence of mycobacterial derived products in draining lymph nodes, but not when macrophages are exposed to IFN- $\gamma$  alone in the retina.

**Su1.81. Suppressor Potency among Regulatory T Cells Is Discriminated by Functionally Active CD44.**

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CD4CD25<sup>+</sup> regulatory T cells (Tregs), are fundamental to the maintenance of peripheral tolerance and have great therapeutic potential. However, efforts have been hampered by limiting cell numbers in vivo, an anergic phenotype in vitro, and only a rudimentary understanding of the molecular basis for the functional state of CD4CD25<sup>+</sup> Tregs. We have previously shown that T cell activation through the TCR results in the activation of CD44 to bind its ligand hyaluronan (HA), and that the CD44/HA interaction is used for T cell extravasation at sites of chronic inflammation. Here we show that CD4CD25<sup>+</sup> cells more rapidly and extensively activate the functionally active, hyaluronan-binding form of CD44 (CD44<sup>act</sup>) after TCR triggering, with ~85% of cells expressing this marker compared to ~25% of CD4<sup>+</sup>CD25<sup>-</sup> cells after 40 hrs of activation. Moreover, CD44<sup>act</sup> expression is strikingly correlated with suppressor activity in CD4CD25<sup>+</sup> T cells, in contrast to other surface markers or Foxp3 expression. Within 16 hr after in vitro activation, CD44<sup>act</sup> can discriminate enhanced suppressive function in in vitro proliferation assays and in vivo models. After separation of CD4CD25<sup>+</sup> Treg into HA-binding and non-HA-binding fractions, multiple cytokines, including IL-4, IL-10, and TGF are preferentially expressed by the HA-binding cells. These cells also show the preponderance of suppressive function in vitro and in two in vivo models of allogeneic GVHD. CD44<sup>act</sup> is induced on resting CD4CD25<sup>+</sup> cells in vivo with antigenic stimulation, with similar functional consequences. These results reveal a cell surface marker that delineates functional activity among CD4CD25<sup>+</sup> regulatory T cells, thereby providing a novel tool to aid in identifying regulatory activity and enriching for maximal suppressor potency.

**Su1.82. Characterization of Immune Response in  $\beta$ -Tubulin Induced Murine Autoimmune Hearing Loss.**

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The main feature of autoimmune disease such as autoimmune inner ear disease including Meniere's disease is the development and persistence of inflammatory processes in the apparent absence of pathogens, leading to destruction of the target tissues. Western blot analysis has shown that 59% of Meniere's disease patients produce antibodies to a 55 kD inner ear membranous and neural protein identified to be  $\beta$ -tubulin. Hearing loss in mice can be induced by immunization with  $\beta$ -tubulin using ABR and DPOAE recorder and spiral ganglion as well as hair cells damages can be observed by morphological study of temporal bones. But the precise immunological mechanism of inner ear disease remains obscure.

In the current study, balb/C mice were subcutaneously injected with  $\beta$ -tubulin in dosage of 100, 200 and 300  $\mu$ g with CFA per mouse, immunizations were boosted in IFA with varying doses of tubulin twice at one-week intervals. Control mice underwent subcutaneous injection of PBS and CFA/IFA as well. After 2 weeks of last boosting, we have found that the antibodies activity to  $\beta$ -tubulin increased in dose dependent compared with controls, all control subjects were relatively unresponsive. Moreover, IFN- $\gamma$  level was markedly increased in both serum and supernatant of lymphocytes, protein levels of TGF- $\beta$ , IL-4, IL-5 IL-10 and IL-13 were significantly reduced following  $\beta$ -tubulin immunization. Interestingly, flow cytometric analysis of spleen cells from  $\beta$ -tubulin induced mice and control mice have been showed that 2.72% of total splenocytes in control mice were CD25<sup>+</sup>CD4<sup>+</sup> regulatory T cells (Treg cells), the population of Treg cells was

reduced in  $\beta$ -tubulin induced mice and followed dose dependent (such as 1.68% in 300 $\mu$ g, 2.16% in 200  $\mu$ g and 2.19% in 100  $\mu$ g), the Treg cells in naïve mice is 2.6%. Moreover, IFN- $\gamma$  and IL-2 level was markedly increased in supernatant of Treg cells culture, protein levels of TGF- $\beta$ , IL-4, IL-5, IL-10, IL-12p40 and IL-13 were significantly reduced following  $\beta$ -tubulin immunization.

Thus, these data indicate an immune reactivity against  $\beta$ -tubulin, which might be responsible for the autoimmune inner ear hearing loss. The further study is required to elucidate the role of CD25<sup>+</sup>CD4<sup>+</sup> T cells in the pathogenesis of this disease, which would eventually result in better therapy.

### Su1.83. Endogenous Expression of IRBP Is Dispensable for Generation of CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cells That Protect Against IRBP-Induced Retinal Autoimmunity.

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“Natural” CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T-reg) develop in the thymus, apparently as a result of interaction with their cognate antigen. We previously showed that susceptibility to experimental autoimmune uveitis (EAU), that is elicited in mice with the retinal autoantigen IRBP and serves as a model for human uveitis, is controlled by thymus-derived T-reg. In this study we examine whether endogenous expression of IRBP is necessary to generate EAU-relevant T-reg, by comparing IRBP knockout (KO) and wild type (WT) mice. We hypothesized that, if endogenous IRBP is needed to generate EAU-relevant T-regs, depletion of CD25<sup>+</sup> cells from IRBP KO mice will not alter their responses to IRBP. Mice were depleted of CD25<sup>+</sup> cells with the monoclonal antibody PC61. To induce EAU, mice were immunized with IRBP in complete Freund’s adjuvant (CFA), or were infused with T cells from IRBP-immunized donors. Some mice were immunized with IRBP in incomplete Freund’s adjuvant (IFA), a non-uveitogenic regimen. EAU scores and associated immunological responses (delayed hypersensitivity and proliferation to IRBP) were examined. When immunized with IRBP/CFA, CD25 depleted WT and KO both had enhanced immune responses to IRBP. Furthermore, IRBP primed T cells of CD25 depleted KO donors elicited more severe disease in WT recipients than did cells from non-depleted KO donors, suggesting that an EAU-relevant T-reg had been removed. However, when immunized with IRBP/IFA, only the depleted WT, but not the depleted KO mice, had enhanced IRBP-specific immune responses, suggesting that IRBP specific T-regs were present only in WT. We conclude that, since endogenous expression of IRBP appears to be needed to generate IRBP-specific T-regs, the EAU-relevant T-regs in IRBP KO are not IRBP-specific. We propose that these are T-regs activated by microbial components present in CFA, that inhibit development of IRBP-specific effector T cells in a bystander fashion. Thus, the innate immune stimulation that is needed to elicit EAU also activates regulatory T cells that help control the disease.

### Su1.84. Prevention and Treatment of Experimental Autoimmune Myocarditis with Altered Peptide Ligand (APL).

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**Background:** Current treatment options for autoimmune conditions are unable to specifically prevent or stop the autoimmune process. Therefore, there is a need for new antigen specific therapies for autoimmune diseases. We evaluated the efficiency of a new APL technology referred to as Ligand Epitope Antigen Presentation System (L.E.A.P.S.™) to prevent or treat experimental autoimmune myocarditis (EAM). J-My-1 is a conjugate of My-1 peptide from the  $\alpha$  chain of murine cardiac myosin, which is used to induce EAM, and J peptide that is a part of the human  $\beta$ -2 microglobulin molecule.

**Methods:** To evaluate prophylactic or therapeutic vaccination, 6–8 weeks old female A/J mice were injected with J-My-1 emulsified in ISA-51 adjuvant on days-14 and-7 prior to EAM induction and sacrificed on day 28 (prophylactic), or injected on days 0, 7, 14, 21 following EAM induction and sacrificed on day 28 (therapeutic). EAM was induced by immunization with My-1, emulsified in CFA on days 0 and 7 and injection of pertussis toxin on day 0. Control groups were vaccinated with PBS emulsified in ISA-51, using the same schedule. Experiments were repeated at least twice with 8–10 mice per group. Myocarditis severity was evaluated by histology and by the heart / body weight ratio. My-1 specific antibodies (total, IgG1 and IgG2a) were analyzed by ELISA. Th1 and Th2 cytokines were evaluated by Linco-Multiplex (for 22 cytokines) from sera as well as from heart and spleen homogenates by ELISA. To evaluate whether the mechanism of J-My-1 action was due to general anergy or specific response towards My-1 peptide, we examined serum antibodies against mycobacterial antigens (PPD) in the CFA by ELISA and antigen (My-1) specific lympholiferation *in vitro*.

**Results:** J-My-1 administration reduced the incidence and severity of EAM when given before or after the EAM induction. In both cases, the J-My-1 treatment significantly lowered the heart/body weight ratio (prophylactic,  $P = 0.047$ ; therapeutic,  $P = 0.006$ ), severity of myocarditis assessed by histology (prophylactic,  $P = 0.0187$ ; therapeutic,  $P = 0.002$ ) and lower levels of anti-My-1 antibodies. The decreased immune response was antigen specific, as J-My-1 had no effect on antibody responses to mycobacterial antigens. Spleen lymphocytes from J-My-1 treated mice had reduced proliferative responses to My-1. The severity of EAM was not reduced with My-1 and ISA51 in contrast to J-My-1. J-My-1 resulted in an increased level of IL-13 and decreased levels of IFN- $\gamma$  in heart homogenate compared to controls.

**Discussion and Conclusion:** Using the L.E.A.P.S. altered peptide ligand as a preventive vaccine as well as a treatment, we were able to significantly reduce incidence and severity of experimental autoimmune myocarditis. The protective effect of the vaccine was antigen specific. As a result of such treatment, we observed increased levels of IL-13 (which we found to be protective) and levels of proinflammatory IL-1 $\beta$  in sera and IFN- $\gamma$  in heart decreased. Supported by NIH grants 1R43HL71352-01A1 and R01 HL67290.

### Su1.85. T Cell Responses Induced by Myelin Oligodendrocyte Glycoprotein Are Suppressed by CD4<sup>+</sup>CD25<sup>+</sup> FOXP3<sup>+</sup> Regulatory T Cells from Thymus and Cord Blood.

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Activation of self-reactive T cells in healthy adults is prevented by the presence of autoantigen-specific CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (CD25<sup>+</sup> Treg). To better understand where human autoantigen-reactive CD25<sup>+</sup> Treg are selected and expanded we investigated if thymic CD25<sup>+</sup> Treg from children and CD25<sup>+</sup> Treg from cord blood are able to suppress proliferation and cytokine production induced by specific antigens. CD4<sup>+</sup>CD25<sup>-</sup> and CD4<sup>+</sup>CD25<sup>+</sup> cell fractions were purified by magnetic cell separation and proliferation and cytokine production were analysed after stimulation with the neural antigen myelin oligodendrocyte glycoprotein (MOG) and the polyconal stimuli staphylococcal enterotoxin B (SEB). CD4<sup>+</sup>CD25<sup>-</sup> thymocytes proliferated in response to MOG but the addition of an equal number of thymic CD25<sup>+</sup> T cells did not significantly suppress this proliferation. However thymic CD25<sup>+</sup> T cells inhibited IFN- $\gamma$  production induced by MOG, which indicates the presence of MOG-responsive CD25<sup>+</sup> Treg in the thymus. In contrast, cord blood CD25<sup>+</sup> Treg suppressed both proliferation and IFN- $\gamma$  production induced by MOG. Interestingly, thymic but not cord blood CD25<sup>+</sup> T cells suppressed proliferation and cytokine production induced by SEB. Both cord blood and thymic CD25<sup>+</sup> Treg expressed *FOXP3* mRNA, but *FOXP3* expression was lower in cord blood than in thymic CD25<sup>+</sup> T cells. This is probably due to the presence of CD45RA<sup>-</sup> *FOXP3*<sup>low</sup> cells in addition to CD45RA<sup>+</sup> *FOXP3*<sup>high</sup> cells in cord blood. In summary, suppression of immune responses to MOG were more easily detected in cord blood than in thymus which, suggests that MOG-reactive CD25<sup>+</sup> Treg are further expanded in the periphery. However, cord blood derived CD25<sup>+</sup> T cells did not suppress SEB induced responses and expressed lower levels of *FOXP3*, which indicates that all CD25<sup>+</sup> T cells in cord blood are not regulatory.

#### **Su1.86. Pinocytosis by Human Dendritic Cells Targets Exogenous Material Selectively to Intracellular Cathepsin S.**

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Antigen processing in the MHC II compartment of human dendritic cells (DC) is poorly understood, however, strategies that might interfere with the processing of e.g. autoantigens are warranted. In particular, information about the nature and the sequence of proteases, especially cathepsins (cat), attacking a given exogenous antigen after uptake by live human DC is lacking. Monitoring the uptake of bead-coupled chemical affinity probes for cathepsin (cat) activity it has recently been demonstrated that murine DC selectively route such probes to catS-containing compartments. Using a similar approach, we have here followed the uptake and cathepsin-binding of such affinity probes by live human DC generated from monocytes *in vitro*. We found that, in contrast to murine DC, human DC only very poorly internalize exogenous material by phagozytosis, but are much more efficient in fluid phase-endocytosis-mediated uptake of the probe, compared to murine cells. While in cell lysates active catS, catB, catH, catZ and catL were decorated by the probe, internal-

ization of the probe by live human DC revealed only a selective labelling of catS and catB, but no decoration of catH and catZ by endocytosis. In a pulse chase analysis, the signal for active catS selectively increased over a chase period of 60 min, indicating that exogenously added material was selectively routed to catS-containing compartments after fluid phase endocytosis in human DC. Treatment of DC with chloroquine reduced cellular uptake and cathepsin labelling, as expected, while the H<sup>+</sup>-ATPase-inhibitor Concanamycin B selectively abrogated labelling of CatB, most likely due to the pH-dependent activity of the enzyme. Treatment of DC with LPS not only reduced the total amount of catS reached by the probe due to decreased internalization, but also abrogated the increase in catS labelling that was seen in untreated DC without a maturation stimulus, while the total amount of active catS remained unchanged. This suggested that not only internalization, but also the intracellular transport to catS-containing compartments is affected by DC maturation. In summary, catS is likely to present the functionally dominant cysteine protease for antigen processing in the MHC class II compartment of live human DC, because exogenous pan-cathepin-reactive probes selectively bind to catS after internalization by fluid-phase endocytosis.

#### **Su1.87. Induction of CD4<sup>+</sup>CD25<sup>+</sup> Treg Suppressive Activity and IL-10 Production by IL-2.**

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*Objective of the study:* CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Treg) suppress T cell activation *in vitro* and regulate multiple immune reactions *in vivo*. IL-2 has been shown to be required for Treg homeostasis. Here, we show that IL-2 is in addition an important activator of Treg suppressive activity. Moreover, since Treg do not produce IL-2 by themselves but depend on IL-2 produced by activated T cells we also provide a new concept for the regulation of Treg activity by their own target cells via IL-2.

*Materials and methods:* We used *in vitro* cultures systems of purified T cell populations to analyse the specific effect of IL-2 on Treg. In addition we studied the role of IL-2 for Treg activity in an *in vivo* T cell transfer system.

*Results:* We and others have previously shown, that *in vitro* blockade of the IL-2 receptor on Tregs during co-culture with responder T cells completely blocks their suppressive activity. Here we demonstrate, that the uptake of IL-2 during priming of Treg is required to induce the capacity to produce IL-10 upon restimulation. IL-4 which induces significant production of IL-10 in naive T cells is not sufficient to induce IL-10 production from Treg but acts synergistically with IL-2. Furthermore, *in vivo* application of IL-2 by gene-gun immunization in normal mice selectively activates Treg and induces the complete suppression of proliferation of a transferred population of Ova-specific T cells in response to vaccination with ovalbumin.

*Conclusion:* Our results show that IL-2 is a potent inducer of Treg suppressive activity including the production of IL-10. The regulation of Treg activity by IL-2 produced from their own target cells allows to adapt Treg effector function to the strength of an immune response. Furthermore, the specific activation of Treg suppressive activity *in vivo* may represent a new strategy for the therapeutic intervention in autoimmunity and chronic inflammation.



**Su1.88. Comparison of Anti- DNA Antibodies for Their Ability To Restore Mesenteric Ischemia/Reperfusion-Induced Injury and Remote Tissue Damage in *Rag 1* Deficient Mice.**

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Natural antibodies as well as antibodies from an autoimmune strain of mice (B6.MRL/*lpr*) have been shown to mediate mesenteric ischemia/reperfusion (I/R)-induced tissue injury. Intestinal injury is initiated when neoantigens revealed on ischemic or apoptotic cells are bound by antibody and complement is activated. In addition to the local (intestinal damage), remote tissue damage in the lung occurs. In the autoimmune mice it has been reported that injury in older (five month) animals is accelerated with increased severity. Mice deficient in *Rag1* or deficient in complement components, such as *Cr2*, are resistant to I/R-induced tissue damage. Passive antibody transfer, of either purified natural antibody or IgG serum from older B6.MRL/*lpr* mice, into *Rag1* deficient mice restores I/R- induced tissue damage. These previous studies indicate that auto-antibodies contribute to tissue damage through complement activation after a tissue or organ suffers from another damaging event such as ischemia. However it is not clear if a particular neoantigen(s) is dominant in initiating the I/R-induced tissue damage. Nor is the relationship between local (intestinal) and remote (lung) tissue damage clear. Subsequently, we have expanded upon the autoimmune studies to examine if auto-antibodies with specificities for either dsDNA or ssDNA at varying concentrations would cause equivalent local and remote I/R-induced tissue damage. As expected, at high concentrations, the passive transfer of all anti-DNA antibodies into *Rag1* -/- mice resulted in local intestinal and remote damage. However at lower concentration the anti-dsDNA antibody was more efficient in facilitating mesenteric I/R-induced tissue damage. When remote lung damage was evaluated the transfer of the anti-dsDNA antibody resulted in increase septal thicken and cellular infiltrate in the lung compared to the transfer of anti-ssDNA antibodies or from wild type control mice. Further studies addressing the recruitment of cellular infiltrate, levels of complement deposition, and the signals controlling polymorphonuclear neutrophil infiltration into intestinal or lung tissue are ongoing. These studies may help clarify the role of circulating auto antibodies in organ damage in patients and mice with systemic lupus erythematosus.

**Su1.89. The Functional Role of CD48-Related Pathways in Autoimmunity.**

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CD48 is a member of the CD2 superfamily of costimulatory molecules and has an important role in regulating the immune response. CD48 is broadly expressed on B cells, T cells, dendritic cells, macrophages and NK cells, and may indicate that this molecule participates in immune regulation via various cell types. The importance of the interaction between CD48 and its receptors,

CD2 and CD244 (2B4), in autoimmunity has not been clearly addressed. Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by production of autoantibodies, in particular anti-nuclear antibodies, B cell hyperactivity and activation of self-reactive T cells. Genetic studies indicated that three regions on different mouse chromosomes (SLE 1,2 and 3) are linked with susceptibility to the SLE at different severities. A sub-region of the SLE 1 locus on mouse chromosome 1 (SLE1b) includes members of the CD2 superfamily. The present study investigated the role of CD48 in tolerance and autoimmunity by studying the spontaneous autoimmune disease that occurs in aging CD48-/- mice. Our data indicates that C57BL/6 CD48-/- mice have elevated levels of dsDNA antibodies as detected by ELISA. In CD48-/- mice, anti dsDNA IgG antibodies levels were higher than IgM but levels of both were at least 2-fold higher in 6-month old compared to 3-month old mice. Similarly, these antibodies could be clearly detected in kidney tissue sections of CD48-/- mice but not in WT mice. Phenotypic analysis of T cells in 6-month old mice showed that levels of activation markers including CD62L, but also CD69 and CD25 were increased at least 2-fold on CD4 and CD8 T cells of CD48-/- mice compared to their WT counterparts. By T cell cloning, we have demonstrated the presence of anti-nucleosomal T cell responses in CD48-/- mice. The breakdown in tolerance is not a result of a defect in activation-induced cell death therefore further studies are required in order to delineate the mechanism underlining the regulatory effect of the CD48 pathway. Overall, the present study presents evidence suggesting that CD48 plays a key role in the control of autoimmunity.

**Su1.90. The Lifecycle of In Vitro Generated CD4+CD25+FoxP3+ Human T<sub>R</sub>: Generation, Expansion, and Persistence.**

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A small population of CD4<sup>+</sup> T cells present in both humans and mice has the ability to regulate immune responses. These cells, known as regulatory T cells (T<sub>R</sub>) are characterized by the expression of CD25 and FoxP3. Originally, CD4+CD25+ T<sub>R</sub> were thought to be derived from the thymus. Recent data suggests that adaptive CD4+CD25+ T<sub>R</sub> can be generated in the periphery. In a previous report, we have shown that activation of human CD4+CD25- results in expression of FoxP3 and CD25, as well as, acquisition of a cell contact dependent, cytokine independent regulatory activity by a portion of the original CD4+CD25- T cells. Recently, we have focused on determining the factors involved in generating this type of T<sub>R</sub> and their lifecycle. Results from our lab show that the addition of IL-2 to generation cultures results in an increased number of resulting regulatory T cells, whereas, blockade of the cytokines TGF-β and IL-10 by the addition of blocking antibodies to the generation culture has no effect on the number or function of the resulting T<sub>R</sub>. This demonstrates that the generation of these T<sub>R</sub> is independent of TGF-β or IL-10 and is enhanced in the presence of IL-2. Furthermore, results show that the CD4+CD25+FoxP3+ T<sub>R</sub> resulting from the generation cultures will retain their suppressive ability upon restimulation and when reactivated in the presence of high dose IL-2 can be expanded 50-fold. Together these results suggest that generated T<sub>R</sub> may be a

good candidate for immunotherapy due to their ability to retain suppressive activity and their capacity to be expanded.

### Su1.91. siRNA Targeted Reduction of IL-23 Production by Human Dendritic Cells Increases IL-10 Production and Decreases Antigen Presentation Capacity.

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**Background:** IL-23 is a heterodimeric cytokine that comprises a p19 subunit that associates with the IL-12/23p40 subunit. Like IL-12, IL-23 is expressed predominantly by activated dendritic cells (DCs) and phagocytic cells and both cytokines induce IFN-secretion by T cells. Unlike IL-12, IL-23 also functions by stimulating secretion of the pro-inflammatory cytokine IL-17 from T cells. A central role for IL-23 in autoimmune inflammation is supported by the observation that induction of experimental autoimmune encephalitis (EAE) occurred in mice lacking IL-12, but not in mice with targeted disruption of IL-23 or both IL-12 and IL-23. Thus IL-23 expression in dendritic cells (DC) may play an important role in the pathogenesis of human autoimmune diseases. One of the ways to explore the role of IL-23 in DC function is by down regulating DC gene expression. The aim of this study was to investigate the role of IL-23 in DC function and to evaluate the use of IL-23 specific antisense oligonucleotides (AS-oligos) and small interfering RNAs (siRNA) as a tool to silence IL-23 expression.

**Methods:** We transfected human monocyte derived- DCs (mo-DCs) with specific AS-oligos to IL-23p19, IL-12 /23p40 and IL-12p35 and small interfering RNAs (siRNA) to IL-23p19. We compared the surface markers by FACS analysis and the APC function of mo-DCs that secrete lower amount of IL-23 and /or IL-12 to mo-DCS transfected with control AS-oligos and control siRNA.

**Results:** We found that mo-DCS are efficiently transfected with AS-oligos and siRNA (90–60%, respectively). Transfection of DCs with the AS-oligos and siRNA specific for the IL-23 p19 and IL-12 p35 genes resulted in potent suppression of gene expression and blockade of bioactive IL-23 and /or IL-12 p70 production without affecting unrelated genes or cellular viability. Inhibition of IL-23 and IL-12 was associated with increased IL-10 production and decreased TNF alpha production. Furthermore, transfected mo-DCs were poor allostimulators in a mixed lymphocyte reaction.

**Conclusions:** We demonstrate that AS-oligos and siRNA can be used for immune modulation by targeting human dendritic cell (DC) gene expression. Targeted reduction in the expression of either IL-23 and /or IL-12 resulted in DCs that are less potent APCs and that secrete more IL-10. Knocking down the expression of IL-23 by AS-oligos or siRNA may be used as an avenue to develop therapy that target DCs in chronic inflammatory autoimmune diseases.

### Su1.92. Opposite Effect of IL-4 and IL-13 in Experimental Autoimmune Myocarditis (EAM).

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**Background:** In a previous study, we found that the severe form of EAM in A/J mice exhibits a Th2-like phenotype. Blocking

IL-4 with anti-IL-4 monoclonal antibodies (mAb) reduced the severity of EAM in A/J mice, showing that IL-4 plays a pathogenic role in EAM. We therefore speculated that another important Th2 cytokine; IL-13; could have an additive effect to IL-4. **Methods:** 6–8 weeks old IL-13 knockout mice (KO); IL-4 KO; IL-4/IL-13 double knockout (DKO) on BALB/c background and BALB/c wild type (WT) were immunized with myosin BALB/c peptide emulsified in CFA on days 0 and 7, injected with pertussis toxin on day 0. To confirm our previous results in A/J mice, we also blocked IL-4 with anti-IL-4 mAb in BALB/c WT. Myocarditis severity was evaluated by histology and by the heart/body weight ratio (HW/BW). Myosin peptide specific antibodies were analyzed by ELISA. Th1 and Th2 cytokines were evaluated from heart and spleen homogenates by ELISA. **Results:** Absence of IL-13 in IL-13 KO mice as well as in IL-4/IL-13 DKO resulted in a significant increase in myocarditis incidence and severity assessed by histology and the HW/BW ratio. IL-13 KO mice had a higher histology score ( $P = 0.0000001$ ) as well as HW/BW ratio ( $P = 0.00008$ ) compared to BALB/c WT. IL-13/IL-4 DKO mice had still greater EAM severity than BALB/c WT mice; however, their phenotype was intermediate between IL-13 KO and BALB/c WT. It was challenging to show clearly that IL-4 is a pathogenic factor in BALB/c EAM as we observed in A/J EAM. BALB/c are moderate responders to the EAM induction; therefore we could not show a statistically significant lowering of myocarditis severity by histology in absence of IL-4 on BALB/c background. However, the HW/BW ratio was significantly lower in IL-4 KO mice than in BALB/c WT ( $P = 0.025$ ), suggesting that IL-4 promotes disease in BALB/c mice. Next to increased EAM severity, IL-13 KO mice showed significantly higher levels of anti-myosin peptide IgG, IgG1, IgG2a and IgG2b compared to BALB/c WT. IL-13 deficiency resulted in increased levels of IL-1 $\beta$  and IFN-g in heart homogenates compared to BALB/c WT. **Discussion and Conclusions:** By inducing EAM in IL-13 KO mice, we were able to significantly increase the incidence and severity of myocarditis, showing a protective effect of IL-13 in EAM in BALB/c mice. The protective effect of IL-13 was also observed in IL-4/IL-13 DKO mice, in which myocarditis severity was intermediate between IL-13 KO and BALB/c WT mice. This intermediate phenotype, together with the reduced disease in IL-4 KO and BALB/c with IL-4 blocked by anti IL-4 mAb, suggests a pathogenic role of IL-4 in EAM in BALB/c mice. The protective effect of the IL-13 is associated with increases of two key regulatory cytokines (IL-1  $\beta$  and IFN-g) in a heart homogenate from IL-13 KO mice. The two main Th2 cytokines, IL-4 and IL-13, thus have opposite effects on EAM, IL-13 being a major protective factor and IL-4 being a pathogenic factor. Supported by NIH grant R01 HL67290.

### Su1.93. CTLA4-Ig Inhibits IL-2 Production and In-Vivo Expansion of Antigen-Stimulated Memory CD4 T Cells.

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Costimulation via the CD28/B7 pathway is required for activation of naive T cells; however, the precise role of this costimulatory pathway in memory T cell function has not been elucidated. In this study, we directly tested the role of the CD28/B7 costimulation pathway on CD4 memory function, by assessing the ability of CTLA4-Ig to block memory CD4 T cell function and expansion *in vitro* and *in vivo*. We used antigen specific CD4 T cells derived from transgenic mice expressing a transgene encoded T cell receptor specific for Influenza Hemagglutinin (HA) peptide

(110-119) and I-E<sup>d</sup> and generated HA-specific memory cells by *in vitro* priming with HA and antigen presenting cells followed by adoptive transfer of HA-activated cells into syngeneic RAG2<sup>-/-</sup> hosts. HA-specific memory CD4 T cells were harvested from these adoptive hosts 8–10 weeks post transfer. *In vitro* recall of HA-specific memory CD4 T cells with HA in the presence of CTLA4-Ig led to a fifty percent reduction in the number of IL-2 producing cells, while IFN- $\gamma$  production was comparable between CTLA4-Ig and isotype control (IgG2a) treated cells. To test the effect of CTLA4-Ig on antigen stimulated HA-specific CD4 memory T cells *in vivo*, we transferred CFSE labeled HA-specific memory CD4 T cells into secondary BALB/c hosts and treated the recipient mice with CTLA4-Ig or IgG2a as an isotype control, then boosted these mice with HA peptide or PBS. We observed a reduced expansion of HA-specific CD4 memory T cells in HA-boosted recipient mice treated with CTLA4-Ig compared to IgG2a treated mice. The total antigen-specific memory CD4 T cell recovery of HA-boosted mice that received CTLA4-Ig versus control IgG2a was 20% of control in the spleen and 44% of control in the lymph nodes. These results indicate that the CD28/B7 pathway plays a key role in IL-2 production and *in vivo* expansion of re-stimulated memory CD4 T cells. These findings suggest a key role for CD28/B7 in promoting optimal memory T cell responses.

#### **Su1.94. Both Fc $\gamma$ R and C5a Are Indispensable in Antibody Dependent Autoimmune Disease.**

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**Introduction:** Complement C5a is a candidate target molecule for the treatment of inflammatory diseases such as respiratory distress syndrome and sepsis. The participation of C5a and C5aR in the development of autoimmune disease has not been investigated. The phagocytic responses by the two activating Fc receptors, Fc $\gamma$ RI and Fc $\gamma$ RIII, have been directly implicated in the pathogenesis of antibody-dependent autoimmune diseases, such as autoimmune vitiligo, immune thrombocytopenic purpura and autoimmune hemolytic anemia. In contrast, however, C5aR has not been thought to play a significant role in these diseases. Using an experimental model of autoimmune hemolytic anemia (AIHA) in C5aR-deficient mice, we show here that C5aR, although a nonphagocytic receptor, promotes cellular immune destruction in antibody-mediated autoimmune disease through a mechanism of bidirectional interaction between C5a and activating Fc $\gamma$ R.

**Methods:** Lethal AIHA was induced by a single intraperitoneal (IP) injection of  $\alpha$ MRBC autoantibodies 34-3C. Kupffer cells were isolated by percoll gradient of the liver homogenate followed from control and anemic mice by sorting from the interphase by Mac-1-coated magnetic beads. Surface protein was analysed by FACS and mRNA expression by Taqman RT-PCR. The liver supernatant was assayed for C5a-dependent chemotactic activity. **Results and**

**Conclusions:** In the present study, we identified C5a as a key mediator of AIHA and showed that mice lacking C5aR are resistant to this IgG autoantibody-induced disease model. Upon administration of anti-erythrocyte antibodies, C5aR deficiency resulted in impaired Fc $\gamma$ R-mediated *in vivo*-erythrophagocytosis. The upregulation of the activating Fc $\gamma$ R on Kupffer cells induced

by the early C5a produced is also absent, in contrast to that observed in wild-type mice. Surprisingly, in mice deficient for Fc $\gamma$ RI and Fc $\gamma$ RIII, anti-erythrocyte antibody induced C5a production is abolished identifying activating Fc $\gamma$ R as dominant mediators of autoantibody-induced release of C5a. Thus our data are consistent with a pathogenic mechanism of antibody-dependent hemolytic anemia in which IgG-opsonized erythrocytes trigger Fc $\gamma$ R-mediated C5a release that in turn activates a positive cellular feedback signal upon engagement of C5aR, which results in increased Fc $\gamma$ RI and Fc $\gamma$ RIII expression required for effective erythrophagocytosis. This shows that development of a full blown antibody-dependent autoimmune disease requires co-operation of C5a and the activating Fc $\gamma$ R in the same activation pathway and suggest therapeutic benefits of C5a-C5aR blockade in AIHA and other disease closely related to type II autoimmune injury.

Work supported by the Deutsche Forschungsgemeinschaft (Ge 892/8-1).

#### **Su1.95. Regulatory T Cells: Differential Requirement for Innate and Adaptive Immune Cells Inhibition *In Vitro*.**

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CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells (T<sub>R</sub>) have been implicated in the control of autoimmune diseases, allergy and inflammation. Development of *in vitro* model systems has facilitated the analysis of the functional properties of these cells. In this regard, inhibition of conventional T cell proliferation by T<sub>R</sub> is currently the most used assay. However, T<sub>R</sub> suppressor mechanism in these *in vitro* assays remains unknown and more importantly, appears to differ from the *in vivo* scenario. Whereas in the latter case, inhibitory cytokines, such as TGF- $\beta$  and IL-10, have been shown to play a role, this is not the case *in vitro*. In addition, while competition for IL-2 has been proposed to be the main suppressor mechanism *in vitro*, the presence of saturating amount of IL-2 in culture media was shown not to affect T<sub>R</sub> ability to suppress IL-2 production by the target cells. In this work, we first present evidences that Foxp3<sup>+</sup> T<sub>R</sub> consume significant amounts of IL-2 in standard suppression assays and this consumption positively correlates with their suppressor efficiency. As similar results were obtained when testing Foxp3-negative suppressor cells, we conclude that IL-2 consumption by CD25-expressing T cells participates in a non-specific manner to the overall suppression of proliferation observed in these cultures. We next describe a suppression assay that does not rely on IL-2 consumption, as it monitors the inhibitory function of T<sub>R</sub> on components of the innate immune system. We show that Foxp3<sup>+</sup> T<sub>R</sub> specifically suppress TNF- $\alpha$  production by LPS activated peritoneal cells. This control requires expression of MHC class II molecules, is independent of the inhibitory cytokines IL-10 or secreted TGF- $\beta$  and appears as cell contact dependent. Moreover, in this assay, regulatory activity is not abrogated when saturating amounts of IL-2 are added to the cultures. The physiological relevance of these *in vitro* assays is currently being tested *in vivo*.

#### **Su1.96. CCR6 Expression Defines Regulatory Effector/Memory-Like Cells within the CD25+CD4+ T Cell Subset.**

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**Objective:** The identification of naive and memory subsets of T cells has been fundamental for the current understanding of the immune system. It is now generally accepted that regulatory CD25+CD4+ T cells are a central element of peripheral tolerance. Little is known, however, about phenotypic and functional characteristics of these cells with regard to memory. Aim of this study was therefore to identify regulatory memory cell subsets and to determine their markers and specific functions. **Methods:** T cells were purified by FACS/MACS-sorting. Phenotypic characterization (naive vs. memory, effector vs. suppressor) was carried out by flow cytometry, expression-levels of transcription factor foxp3 was determined by real time RT-PCR. Functional studies included *in vitro* proliferation and cell migration assays. As experimental *in vivo* systems congenic (SJL) and TCR-tg EAE models (TG4) were used. Induction of memory phenotype was demonstrated *in vivo* by adoptive transfer experiments of CFDA labelled cells. **Results:** In this study we show that the chemokine receptor CCR6 is expressed on a distinct subset of mouse Treg cells. Similar to their CD25-counterparts, CCR6+ Treg cells exhibit markers of activation, memory and expansion that are indicative for an effector-memory function. They are memory-like cells, generated *in vivo* from CCR6-CD25+ T cells after the encounter of antigen. As conventional CD25- effector-memory T cells, they have a high turn-over rate and, in contrast to CCR6- Treg cells, they respond rapidly to restimulation *in vitro* with up-regulation of IL-10. CCR6+ Treg cells are enriched in the peripheral blood and accumulate in the CNS after induction of EAE. Importantly, these cells are also present in humans. Here the expression of CCR6 fully co-segregates with CD45RO, an established marker of human memory T cells. **Conclusions:** The subset of CCR6+ Treg cells seems to represent a population of 'regulatory effector-memory' T cells (T<sub>REM</sub>), destined to control potentially destructive immune responses directly in inflamed tissues. T<sub>REM</sub> cells therefore seem to be the natural counter-players of effector-memory T cells (T<sub>EM</sub>), involved primarily in front-line suppression.

#### **Su1.97. Small Molecules Trigger Peptide Loading of HLA-DR by Allele-Specific Induction of a Peptide-Receptive State.**

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**Objectives:** Class II MHC molecules, upon losing their peptide ligand, rapidly acquire a non-receptive state that prevents rebinding of new peptides. Here we show that certain small molecules are able to reverse this process. We had recently identified a number of organic compounds that accelerate ligand exchange reactions of HLA-DR molecules in a similar fashion as the natural catalyst HLA-DM. In contrast to HLA-DM, however, these compounds are effective even at neutral pH and can therefore

mediate ligand-exchange directly on the surface of living cells. Aim of this study was to identify the molecular mechanism by which small molecules mediate ligand exchange and whether these compounds can exhibit allele specificity. **Methods:** The experiments were carried out with soluble HLA-DR molecules expressed in insect cells. The kinetics of binding and release of peptide ligands was determined by ELISA experiments. The effect was validated in a cellular *in vitro* T cell system by using HLA-DR transfected fibroblasts as APC and by a flow cytometry based peptide binding assay. **Results:** The mechanism was found to be based on the induction/stabilization of the peptide-receptive state and facilitates loading of class II MHC molecules with both peptide antigens or with full-length proteins. Importantly, some of these compounds were active only on a subset of HLA DR molecules. While they catalyse efficiently the loading of peptide ligands onto allelic variants such as HLA DR1, they completely failed to accelerate peptide binding to HLA DR2. **Conclusions:** Small molecular compounds can influence the ligand composition on the surface of antigen-presenting cells in an allele-specific manner. While they represent a novel class of molecular tools which can be used in therapeutic settings to facilitate antigen loading they might also represent environmental risk factors for allergy and autoimmune diseases. In this respect the strict allele-specificity of these compounds could even have an influence on the apparent linkage between incidence of certain diseases and HLA DR allele.

#### **Su1.98. B Cell Receptor Editing Begins in the Bone Marrow.**

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**Objective:** It has been postulated that the bulk of B cell receptor (BCR) editing detected in peripheral B cell populations in both human and murine systems results from a tolerogenic process that occurs earlier in ontogeny, during B cell maturation. This possibility is substantiated by studies demonstrating that bone marrow B cells cultured *ex vivo* are capable of editing their BCRs when faced with self antigen. The purpose of the current study is to determine whether immature B cells isolated directly from murine bone marrow display evidence of BCR light chain editing.

**Methods:** Hybridoma studies of IgH56R mice (with the anti-DNA 56R V<sub>H</sub>DJ<sub>H</sub> transgene inserted in the IgH locus upstream of C<sub>μ</sub>) have suggested that IgK editing is reflected in two ways: by preferential rearrangements to the Jk4 and Jk5 gene segments and also by skewed distributions of Vk gene usage. The hybridoma approach, however, is unable to distinguish between events early and late in B cell ontogeny because only mature splenocytes are captured via cell fusion. We have looked at IgK gene usage in a strictly defined immature B cell population from the bone marrow of IgH56R mice. B220<sup>+</sup> CD43<sup>-</sup> IgD<sup>-</sup> cells further phenotyped as IgMa<sup>+</sup> IgMb<sup>-</sup> (expressing the IgH transgene) or IgMa<sup>-</sup> IgMb<sup>+</sup> (expressing an endogenous IgH) were single cell sorted into 96-well plates. RT-PCR amplification and sequencing of expressed IgK genes were performed and identities of Jk segments and Vk gene families were assigned using the NCBI IgBlast website ([www.ncbi.nlm.nih.gov/igblast](http://www.ncbi.nlm.nih.gov/igblast)).

**Results:** Evidence of *in vivo* receptor editing in IgH56R bone marrow is revealed by comparing IgK gene expression in IgMa B cells (*n* = 143) to the IgMb control population (*n* = 184). In the

potentially autoreactive IgMa population, J segment rearrangements are skewed towards the downstream Jk4 and Jk5 elements ( $P = 0.02$ ). Furthermore, the two subsets of immature B cells have significantly different predilections for Vk family gene usage ( $P = 0.0007$ ). In particular, expression of anti-DNA generating Vk1 and Vk4 genes is substantially reduced in the IgH56R expressing cells versus controls ( $P = 0.017$  and  $0.006$ , respectively).

**Summary:** This description of IgK gene expression in primary B cells from the bone marrow of IgH56R transgenic mice demonstrates that receptor editing begins as early as the immature B cell stage of development. Nonetheless, comparison of our observations to other sequence analyses of 56R B cells from a variety of sources clearly indicates that selective forces continue shaping the maturing B cell repertoire beyond this stage of development and probably well after their migration from the murine bone marrow to peripheral lymphoid tissues.

### **Su1.99. The Macrophage C5a:C5aR-Ga<sub>12</sub>-FcγRIIB/FcγRIII-Axis Defines the Inflammatory Response in the Arthus Reaction.**

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**Introduction:** IgG immune complex (IC) induced inflammatory reactions can be triggered by two pathways; the complement system (especially C5a) or the cellular receptors for IgG, FcγR. In mice, IC can interact with either of three cellular receptors, two of which FcγRI and FcγRIII lead to cellular activation, while FcγRIIB is an inhibitory receptor. A hallmark of the immune response to IC is its ability to modulate the balance between these receptors. Recent reports identified C5a dependent inverse regulation of FcγRIII versus FcγRIIB on lung macrophages in the mouse model of acute alveolitis. **Objectives:** This study was carried out to analyze the underlying cellular and molecular mechanism of their cooperation in immune-regulation of IC lung pathology. **Methods:** The methods used in the study include adaptive cell transfer experiments in which alveolar macrophages (AM) depleted C5aR and FcγR deficient mice were reconstituted with ex- vivo modified AM from CD45.1 congenic mice. C5a:C5aR-dependent modulation of FcγR expression and its functional consequence was analyzed in presence of specific pathway inhibitors and quantified by IC injury, chemotaxis, TaqMan RT-PCR and FACS analysis. **Results and Conclusion:** Here we analyzed a novel regulatory C5aR-FcγR cross-talk on AM as the dominant event in the lung Arthus reaction, the classical animal model of immune complex (IC) disease. Strikingly, initial contact between IC and AM results in cellular regulation leading to plasma complement-independent C5a production; selective Gα<sub>12</sub>-dependent C5aR signaling; and C5aR-G<sub>7</sub>-mediated FcγR alterations towards FcγRIII, the main inducer of TNFα and CXCR2 ligand production. Distinct inhibitors of this refined inflammatory cascade are each effective in disease prevention, thus indicating cellular components of the C5aR-FcγR-axis, like Gα<sub>12</sub>, as

potential new therapeutic targets in the treatment of inflammation and autoimmune diseases. In summary, our results imply a revised multimolecular model of the inflammatory cascade. Remarkably, our findings showed that macrophages function as an alternative source of C5a. These data also highlight the importance of local C5a-induced cellular mechanisms in disease pathogenesis.

Supported by MD PhD Program, MHH and Deutsche Forschungsgemeinschaft (GE892/8-1).

### **Su1.100. T Cells and Autoimmunity: Immunoregulation by CD40 Expressed on CD4<sup>+</sup> T Lymphocytes.**

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CD40 is known to play a significant role in the pathogenesis of inflammation in autoimmunity. CD40 can stimulate autoreactive B cells directly, resulting in autoantibody production. However, recently published data indicate that autoaggressive CD4<sup>+</sup> T lymphocytes also express CD40, including T cells from mice with collagen induced arthritis (CIA). Our lab has extensively studied signaling by CD40 in B cells and these reports prompted us to produce mouse (2B4.11) and human (Jurkat) T cell lines expressing transfected human (h)CD40. The objective of this study was to compare CD40 signaling in B vs. T cells, as well as characterize CD40 as a possible costimulatory molecule on T lymphocytes. As in B cells, CD40 in T cells binds to cytoplasmic adaptor proteins called TNF-R associated factors (TRAFs), including TRAF2 and TRAF3. However, CD40 mediated TRAF degradation is less efficient in T cells compared to their B cell counterparts. CD40 stimulation in T cells leads to JNK and NFκB activation and TNF-α production. Of particular interest is the ability of CD40 to act as a costimulatory molecule for T cell receptor signals, just as it does for the B cell receptor. Stimulating hCD40 transfected 2B4.11 T cells, or T cells expressing CD40 from mice with CIA, with anti-CD3, anti-CD28, or anti-CD40 alone leads to minimal secretion of IL-2 or IFN-γ. However, stimulating via anti-CD3 in conjunction with anti-CD28 and/or anti-CD40 results in significantly increased secretion of these cytokines. This increase in cytokine production is paralleled by increased NFκB activation via both classical (NFκB1) and alternate (NFκB2) pathways, as well as increased activation of JNK. These findings are particularly exciting and suggest that T cell specific inhibition of CD40 signaling could be a means for blocking the autoimmune response without altering general immune function.

### **Su1.101. Induction of Tolerance by Gene Therapy with B-Cells Expressing Ig Fusion Proteins: Mechanisms and Pre-Clinical Success in Naïve and Immune Hemophilia A Mice.**

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Our lab has demonstrated that B-cell blasts, activated by LPS, anti-Ig or CD40L and transfected with a retrovirus encoding an IgG-peptide fusion protein are tolerogenic in both normal and primed recipients. Success has been achieved with multiple antigens in different mouse strains and in rats, and in preclinical models for MS, uveitis and diabetes. We also have demonstrated that class II MHC on the presenting B cells is necessary for tolerance and that the Ig carrier enhanced the degree and duration of tolerance. *We have now adapted this system to a murine model for hemophilia A.* Approximately 25% of hemophilia A patients

make inhibitory antibodies that block clotting and therefore reverse the potential therapeutic benefit of factor VIII delivery. We know that most of these inhibitors are directed at epitopes in the C2 and A2 domains of factor VIII. Thus, for clinical application in hemophilia, we inserted residues S2173-Y2332 of the factor VIII C2 domain and S373-R740 of the fVIII A2 domain onto the IgG heavy chain backbone, respectively, to induce tolerance in hemophilia A mice. Specific tolerance to each domain was induced by this protocol. Importantly, a combination of A2-IgG and C2-IgG expressing B cells induced tolerance to the full length fVIII molecule, a result which supports the dominance of these domains in the immune response to fVIII. Tolerance was manifested in terms of ELISA titers, T-cell proliferation and especially Bethesda Unit titers (95% reduction). Importantly, similar results were obtained even when treatment was initiated after mice had preformed anti-fVIII titers, indicating its potential for the treatment of patients with "inhibitor" titers. Recent data suggest that CD25+ T regulatory cells are needed for *in vivo* tolerance induction with transfected B cells. These studies hold promise for a future clinical trial in hemophilia A patients. (Supported by NIH grants HL061883, AI035622 and a Lab Grant from the National Hemophilia Foundation.)

#### **Su1.102. Increase of Pancreatic NK Cells at Early Onset of Autoimmune Diabetes in IFN $\beta$ Transgenic NOD Mice.**

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Genetic and environmental factors are decisive in the etiology of type 1 diabetes (T1D). Viruses have been proposed as a triggering environmental event and some evidences have been reported, i.e. type I IFNs exist in the pancreata of diabetic patients and transgenic mice expressing these molecules in beta cells develop diabetes. Our group has generated the first NOD mice expressing a type I IFN (RIP-IFN $\beta$ ) in the islets, a new model of autoimmune diabetes. These mice develop accelerated diabetes at 3 weeks of age (equivalent to childhood in humans), with a cumulative incidence reaching 60% at 30 weeks age. This early-onset diabetes is characterized by selective destruction of beta cells, MHC class I hyperexpression in the islets, severe insulinitis, and a high number of Natural Killer cells in the pancreas. NK cells are innate immune cells that control certain virus infections and tumors, and have been recently associated to destructive forms of pancreatic islet autoimmunity in NOD mice. A significant increase of NK cells and NKT cells has been observed in the insulinitis of the NOD RIP-HuIFN $\beta$  and NOR RIP-HuIFN $\beta$  transgenic mice at the early onset of diabetes when compared to healthy subjects. This high amount of pancreatic NK cells has not been found in transgenic mice or NOD wild type developing diabetes after 12 weeks of age. The percentage of NK cells in spleen and pancreatic regional lymph nodes is not altered in diabetic animals (early or late) when compared to healthy animals. This subset of NK cells is not maintained during the disease, decreasing quickly after 24 hours of the clinical onset. An imbalance NK / NKT subsets exists at the early onset of T1D. Transgenic mice NOD-Scid RIP-IFN $\beta$ , unable to produce mature T and B lymphocytes although they have unaffected NK subset, do not develop diabetes thus suggesting the need of interaction between NK cells and other

lymphocyte subsets (T, B) for autoimmunity. Microarray experiments demonstrate a correlation of the NK cell subset in early onset diabetes to the islet expression of adhesion molecules, costimulatory molecules (CD86), cytokines (IFN $\gamma$ , IL6) and NK attractant chemokines: CCL2 (MCP-1), CCL3 (MIP-1 $\alpha$ ), CCL5 (RANTES), CXCL10 (IP10) and XCL1 (lymphotactin). Since type I IFNs production takes place in cells infected mainly by viruses, our transgenic model could be an interesting tool for studying how the production of IFN $\beta$  causes cellular stress, generates danger signals, inflammation and eventually NK cell-mediated autoimmunity. The characterization of the role of NK cells at early onset of T1D may help us to understand and to prevent this autoimmune disease.

#### **Su1.103. Rapid Protocol To Generate Tolerogenic Dendritic Cells Using Microbial Lipopeptide (BLP).**

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Dendritic cells (DCs) are potent antigen presenting cells capable of antigen uptake and presentation. In the immature developmental stage DCs are thought to induce T-cell anergy. Immature DCs have a high rate of endocytosis and low levels of MHC class II (MHC-II), and CD80 and CD86 co-stimulatory molecules expression. Upon maturation, DCs down-regulate mechanism of antigen capture and increase co-stimulatory molecules expression. In the steady state, meaning in the absence of acute infection and inflammation, DCs deliver the antigen to T cells without of essential co-stimulatory molecules inducing T cell tolerance. The key role of DCs in the induction of immunity to infectious agents, malignancy and transplanted allografts, and in the maintenance of T cell tolerance in the periphery has prompted the interest in their use as immunotherapeutic agents.

**OBJECTIVE:** In this study we examine whether bacterial lipopeptide (BLP) may induce phenotypic and functional changes associated with DCs maturation through Toll-Like Receptor 2 (TLR2). **METHODS:** DCs were generated from bone marrow precursors (BMDCs) by using granulocyte-macrophage colony stimulating factor (GM-CSF). On the days 3 and 5 appropriated cytokines were added. We assessed DCs maturation using LPS, TNF and BLP as stimulators. CD11c, MHC-II, CD80 and CD86 surface molecules expression was determined using murine monoclonal antibodies conjugated to phycoerythrin (PE) and isothiocyanate (FITC) by flow cytometry. The capacity of BMDCs to endocytose antigen was determined by flow cytometry using FITC-conjugated dextran. **RESULTS:** We demonstrated that BMDCs generated *in vitro* by using GM-CSF and BLP for two days show a high MHC-II expression and an increased yield of differentiated DCs. The expression of CD80 and CD86 molecules was not affected by the presence of BLP. These phenotypic features of DCs correspond to a semi-mature (sm-DCs) developmental stage. In contrast, bone-marrow precursor cultured for five days under BLP stimulus or alternative standard protocols using TNF or LPS for over eight days yield the same number of differentiated DCs and a mature phenotype demonstrated by functional assays. **CONCLUSION:** This protocol has shown to be more rapid and efficient to generate sm-DCs with tolerogenic characteristic and mature DCs that will be use in immunological therapy.

Financed by Fondecyt-Chile 1040860, Fondef-Chile D03I1055 and Meceseup UCH0115.

### Su1.104. Mucosal Administration of Anti-CD3 Antibody Suppresses EAE, Collagen Arthritis, Diabetes and Prolongs Cardiac Allograft Survival.

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**Background and Objectives.** Mucosal administration of antigen is an established method to induce immunologic tolerance and mucosal administration of auto and alloantigens is effective in treatment of animal models of autoimmunity, inflammation, and transplantation. Parenteral administration of anti-CD3 is efficacious in animal models of autoimmunity and in humans anti-CD3 is an approved therapy for transplant rejection and positive results have been reported in patients with new onset type 1 diabetes treated with parenteral anti-CD3. We investigated the effect of mucosally administered anti-CD3 in animal models of autoimmunity and transplantation. **Methods.** We orally or nasally administered anti-CD3 or Fab'2 fragments of anti-CD3 (or appropriate isotype control antibody) in doses ranging from 0.5ug to 500ug. **Results.** In the SJL model of PLP induced EAE anti-CD3 suppressed EAE when given prior to or at the peak of disease. The PLP specific immune response of anti-CD3 fed animals immunized with PLP demonstrated a decreased proliferative response, reduced IL-2 secretion and increased secretion of IL-10, IL-4 and TGF- $\beta$ . Oral anti-CD3 was associated with an increase in the numbers of CD4+ TGF- $\beta$  latency associated peptide (CD4+LAP+) in the mesenteric lymph node. CD4+LAP+ cells suppressed proliferation of CD4+CD25-LAP- T cells in vitro and adoptive transfer of CD3+LAP+ cells suppressed EAE in a TGF- $\beta$  dependent fashion. No modulation of CD3 on the surface of CD4+ T cells occurred after oral anti-CD3. Similar results were obtained in MOG induced chronic EAE in the NOD mouse. Pathologically there were less CD4+ cells and macrophages in the spinal cord. Suppression also occurred with Fab'2 fragments of anti-CD3. In the NOD model of diabetes, oral anti-CD3 given in the neonatal period suppressed the incidence of diabetes. In streptozocin induced diabetes oral anti-CD3 Fab'2 suppressed diabetes in association with CD4+LAP+ cells whose suppressive function increased after oral anti-CD3. In vivo neutralization of TGF- $\beta$  reversed the suppressive effect. In the DBA2 model of collagen induced arthritis, nasal anti-CD3 Fab'2 suppressed the incidence of arthritis, was associated with decreased levels of TNF in the joints, and was more effective than mucosally administered collagen. In allogeneic cardiac transplantation (Balb/c into C57BL/6), oral anti-CD3 was given on day -5 and continued until day +10 post transplantation. Cardiac transplants in mice receiving oral anti-CD3 survived an average of 16.2 days vs. 8.4 days for controls ( $P = 0.0004$ ). **Conclusions.** Anti-CD3 given orally or nasally is immunologically active at mucosal surfaces. The Fc portion of the Ig molecule is not required for the immunologic effect. Oral anti-CD3 induces regulatory T cells characterized by surface LAP that function in a TGF- $\beta$  dependent fashion. These results identify a novel and physiologic mechanism to induce regulatory T cells that is clinically applicable to a variety of immune mediated disorders.

### Su1.105. The Role of Ultralarge Complexes (ULC) in Heparin Induced Thrombocytopenia (HIT).

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HIT is a serious complication of heparin therapy caused by antibodies (Ab) to complexes between high molecular weight heparin and an endogenous protein, Platelet Factor 4 (PF4), leading to limb and/or life-threatening thrombosis in ~50% of affected patients. We are interested in understanding the basis of an important clinical observation: HIT auto-Ab form in most heparinized patients, but only a small percentage of these develop thrombocytopenia and/or thrombosis. In the present work we show by gel filtration that PF4 and heparin form antigenic ULC over a very narrow molar ratio (~1:1) of reactants. ULC are stable and visible by electron microscopy, but can be dissociated into smaller complexes (SC) upon addition of heparin. Formation of ULC is inefficient when PF4 is incubated with low molecular weight heparin, and none form with the pentasaccharide fondaparinux. Mutation studies show that formation of ULC depends on the capacity of PF4 to form tetramers, which then assemble into multimolecular lattices on a heparin scaffold. ULC are capable of binding >1 HIT-like monoclonal Ab/complex, are more antigenic than SC and are more capable of causing platelet activation in an Ab- and Fc $\gamma$ RIIA-dependent. Additional of PF4 to human or mouse platelets and other vascular cells leads to the self-assembly of antigenic complexes presumably nucleated by membrane glycosaminoglycans (GAG). PF4 evokes binding of HIT-like monoclonal Ab to cells in a dose-dependent manner over a narrow range of concentrations and induces Fc $\gamma$ RIIA-dependent platelet activation analogous to ULC. We conclude that the capacity of PF4 to form ULC composed of multiple PF4 tetramers arrayed in a lattice with several molecules of heparin and/or GAG may play a fundamental role in autoAb formation. The capacity of ULC to bind multiple Ab and cross-link/trigger Fc $\gamma$ RIIA promotes platelet activation and explains the propensity for thrombosis. We are currently testing the hypothesis that the patients with high steady-state level of surface PF4 based on genotype and acquired through platelet activation are those most likely to generate autoAb, form ULC and develop HIT.

### Su1.106. Effect of Freezing/Thawing Conditions and Their Optimization for Quality Control of PBMC Viability and Functional Assays.

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**Objective:** To compare and optimize freezing protocols for PBMCs taken from whole blood for detection of autoreactive T cells.

**Background:** To detect the effects of potentially immunomodulatory drugs on autoreactive T cells, clinical trials performed by the Immune Tolerance Network employ assays that quantify T cell responses to autoantigens. Centralized assay facilities are used to reduce the problem of inter-site variability. However, some inter-site variability can occur during the preparation of PBMC samples at various geographic locations. These steps require freezing of PBMCs prior to shipping, as well as thawing of samples prior to assay. We have therefore examined the effects of our freezing and thawing procedures to minimize inter-site variability of the results of T cell assays.

**Methods:** PBMCs from healthy donors and MS patients were isolated by ficoll separation. Parts of the samples were exposed to different doses of tetanus toxoid, myelin antigens, PHA or no

antigen, while the other part was frozen for 3 weeks using various freezing media and temperatures prior to stimulation in T cell assays. Proliferation ( $^3\text{H}$ -Thymidine incorporation), cytokine production (ELISA, flow cytometry (FACS) and elispot) and cell death/apoptosis (FACS) were assayed in parallel in both fresh and frozen samples. Subpopulations of PBMCs were phenotyped by FACS analysis.

**Results:** Cell viability and recovery decreased after a cycle of freezing/thawing (2 to 10% and 20 to 60%, respectively). The viability as well as PBMC response to antigens were significantly improved when human AB serum (+10% DMSO) was used to freeze the cells. The viability of PBMCs using fetal bovine serum (+10% DMSO) resulted in a fair viability (91%) but high background for proliferation and cytokine production. Using cold (4°C) freezing media decreased both the viability (by 4%) and the response of T cells to antigens (by 20%). PBMCs were better preserved (numerically and functionally) and retained a greater response to antigens when the temperature of freezing medium used was kept at 25°C than 4°. Using these optimal conditions we did not find significant differences in the cell population percentage between the fresh and frozen regarding CD3, CD4, CD8, CD14, CD19, activated or memory T cells.

**Conclusions:** We have found as expected that freezing and thawing of PBMCs decrease cell viability (2 to 10%) and T cell response (50%). This decrease in count and viability did not disproportionately affect a specific cell population among those examined. The best combination of freezing conditions was obtained using human AB serum+10% DMSO at 25°C. Thus, we have identified procedures optimal for PBMC viability and T cell responses to PHA and a panel of nominal and self-antigens. This protocol has been adopted as standard operating procedure for all studies conducted by the ITN and therefore expands the ITN's capability to evaluate mechanisms of disease and treatment response in multicenter trials.

#### **Su1.107. MBLA/C Deficient Mice Display Defective Apoptotic Cell Clearance but No Autoimmune Phenotype.**

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MBL is a member of the collectin family with structural similarities to the lung collectins, SPA and SPD. In addition, MBL has functional similarities to C1q as they both activate complement; C1q activates the classical pathway and MBL the lectin pathway. Like C1q, MBL is a circulating serum protein that is sequestered to sites of inflammation and infection. Limited reports of patients deficient in MBL have raised the possibility that lack of MBL, in a manner similar to C1q deficiency, might be associated with autoimmunity. Cells dying by apoptosis are an important target for the autoantibodies that develop in systemic lupus and failure of removal of dying cells has been implicated in development of autoimmunity in C1q deficiency. Here we show that MBL, like the other collectins and C1q, is able to bind apoptotic cells *in vitro*. Using mice deficient in both mouse MBL genes, MBLA and MBLC, we demonstrate that MBL null animals show a 50% defect in their ability to clear apoptotic cells, confirming a role for MBL in clearance of apoptotic cells *in vivo*.

Analysis of MBL null animals demonstrated expanded B1 cells but no spontaneous activation of antigen presenting cells. Importantly, despite demonstrating a defect in apoptotic cell clearance comparable to that seen in the C1qA<sup>-/-</sup> animals, MBL null animals did not develop spontaneous autoimmunity, lymphoproliferation or germinal centre expansion. These data demonstrate an important *in vivo* role for MBL in clearance of dying cells and add the MBL null animals to the short list of animals with demonstrable *in vivo* apoptotic cell clearance defects. Furthermore, it demonstrates that failure of apoptotic cell clearance can be dissociated from autoimmunity indicating that other factors must be required for autoantibody generation and end organ damage.

#### **Su1.108. Study of the Thymic Function in Patients with Autoimmune Thyroiditis.**

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Autoimmune thyroid disease (AITD) is a term that includes various clinical entities, among them Hashimoto's thyroiditis (HT) and Graves' disease (GD). Lymphocytic infiltration by T-, B-lymphocytes and DC and FDC that is often organized as functional lymphoid follicles with germinal centers is an almost constant feature. In order to investigate specific homing of recent thymic emigrants (RTE) to the thyroid and the distribution of recent RTEs in the different subsets of PBLs in patients with AITD, TCR excision circles (TRECs) have been measured in CD3<sup>+</sup> intrathyroidal lymphocytes (ITL) and in peripheral blood (PBMC) of the same patients. TRECS were measured by real-time PCR in CD3<sup>+</sup> lymphocytes obtained by cell sorting from dispersed cell preparations of thyroid glands ( $n = 10$ ) and from peripheral blood from ( $n = 15$ ) patients and ( $n = 12$ ) healthy controls. The comparison of TREC levels ITLs and PBMCs in each individual showed two patterns: in one half of patients TRECs in ITL were higher than in PBMCs whereas in the other half was higher in PBMCs. On the other hand TREC content in PBMC from AITD patients was not significantly different from controls ( $1.799 \pm 2.62/10^4$  cells vs.  $3.212 \pm 3.06/10^4$  cells,  $P = 0.902$ ,  $t$  test). To assess the influence of T cell proliferation on TREC levels, we measured telomere length in B- and T-lymphocytes in groups of 10 patients with AITDs (ITL and PBMC) and controls (PBMC). In AITD patients, the relative telomere length (RTL) in ITL was significantly lower than in PBMC ( $11.21 \pm 1.22$  vs.  $13.73 \pm 2.21$ ,  $P = 0.0266$ , paired  $t$  test) but in PBMC there was no difference with healthy donors ( $13.73 \pm 2.21$  vs.  $13.02 \pm 1.06$ ,  $P = 0.6165$ ,  $t$  test). RTLs were also measured in thyroid infiltrating and peripheral B cells. ITL CD19<sup>+</sup> B infiltrating lymphocytes had longer RTLs than peripheral B cells ( $17.69 \pm 2.70$  vs.  $15.06 \pm 1.53$ ;  $P = 0.0260$ , paired  $t$  test) in AITD while in PMBCs RTLs from AITD were similar to those in healthy donors ( $15.06 \pm 1.53$  vs.  $15.13 \pm 2.13$ ;  $P = 0.6669$ ,  $t$  test). No correlation was found between the RTL in T cells and the levels of TRECS. Taken together, these results suggest the existence of proliferation in ITL CD3<sup>+</sup> cells within the thyroid or regional lymph nodes although selective homing of memory T cells to the thyroid could not be excluded.



### Su1.109. A Novel Low-Calcemic Vitamin D Analog as a Potential Therapeutic Treatment for Autoimmune Diseases.

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Several autoimmune diseases such as psoriasis, multiple sclerosis and Crohn's disease, are associated with an imbalance between Th1/Th2 towards Th1 cells. This creates a state of chronic inflammation with predominance of Th1 cytokines such as IFN- $\gamma$ , TNF- $\alpha$  and IL-2. In contrast, the Th2 cytokines like IL-4 and IL-10 have shown beneficial effects and manipulation of Th1/Th2 balance is part of the current strategy developed against Th1-mediated immune diseases. 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (calcitriol) has been shown to exert several immunomodulatory functions on T cells and antigen-presenting cells (APC) both *in vitro* and *in vivo*. Our goal was to evaluate the immunomodulatory effects of a new vitamin D analog, QW1624F2-25SO<sub>2</sub>-1, for the therapeutic treatment of autoimmune diseases. **Methods:** QW1624F2-25SO<sub>2</sub>-1 was assessed for its ability to activate the VDR-responsive gene CYP24 by real-time PCR and to bind to VDR. We also examined, using ELISA, the effect of our compound on Th1/Th2 cytokines in human peripheral blood mononuclear cells (PBMC) and on cytokines secreted by macrophages. The use of vitamin D analogs is currently limited by the induction of hypercalcemia; therefore, the effect of QW1624F2-25SO<sub>2</sub>-1 on serum calcium level was evaluated in mice. **Results:** Transcriptional analysis in human PBMC revealed that calcitriol is a strong inducer of the VDR-responsive gene CYP24 which indicates these cells are responsive to VDR-mediated gene transcription. In a macrophage cell line, very low concentrations of QW1624F2-25SO<sub>2</sub>-1 significantly induced CYP24 expression. Interestingly, the ability of this analog to bind to VDR was weaker than calcitriol. Calcitriol has been shown to repress IFN- $\gamma$ , GM-CSF, IL-2 in T cells and IL-12 in APC, an effect that is dependent on the presence of VDR. Our results revealed that QW1624F2-25SO<sub>2</sub>-1 significantly decreased pro-inflammatory Th1 cytokine production (IFN- $\gamma$ , TNF- $\alpha$  and IL-2) and increased the production of the Th2 cytokine IL-4 in human PBMC. This analog also affected macrophage functions by inducing the secretion of IL-10 in LPS-stimulated U937 cells. *In vivo*, this analog did not induce hypercalcemia even at the highest dose of 40  $\mu$ g/kg body weight. **Conclusion:** These results demonstrate that QW1624F2-25SO<sub>2</sub>-1 is a low-calcemic analog and a strong inducer of vitamin D-dependent transcription. This novel compound modulates Th1/Th2 balance in favor of the Th2 and macrophages function suggesting its potential for the treatment of Th1-mediated autoimmune diseases.

### Su1.110. SP-A May Be a Candidate of "Qi" Molecule Triggers Some Autoimmune Diseases.

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**INTRODUCTION:** The IR-SP-A was found in alveolar, parenchyma, pleura of lung; myelin sheath of brain; epithelia of Bowman's capsule, glomerulus and renal tubules of kidney; epithelia of colon, stomach, duct of salivary gland, pharynx; and blood vessel wall and connective tissue of extracellular matrix. The positive signal was blocked by pre-absorbed SP-A antigen from recombinant or BAL. The regularity of distribution is likely

compatible with the primary injury sites of some autoimmune diseases. SP-A or like molecules is a candidate of "Qi" (meaning air) molecule and it may trigger some autoimmune diseases.

**IR-SP-A in Lung:** In lung, strong signal of IR-SP-A was detected in alveolar type II cells, the surface of alveoli and bronchiole, and pulmonary parenchyma. Our results of IR-SP-A in the sites of pulmonary parenchyma and blood vessel indicate the primary involvement in the pulmonary autoimmune diseases. Many clinicians recognized that certain autoimmune diseases are often associated with the initial pulmonary abnormalities such as alveolitis, hemorrhage.

**IR-SP-A in Extrapulmonary Sites:** In Kidney, the strong positive signals were located in some glomerulus, renal tubules and vasculature. In glomerulus, positive signal was detectable on some epithelia of Bowman's capsule, and glomerular basement membrane. In brain it showed strong IR-SP-A positive signals were located in myelin sheaths of cerebrum, cerebellum and walls of blood vessels. In digestive system, IR-SP-A positive signal in epithelia of stomach and large intestine, and very faint positive signal in blood vessels of connective tissue. In blood vessel and connective tissues, IR-SP-A was also detectable in blood vessel and connective tissue of lung and some extrapulmonary tissues. The positive signal in the blood vessel of brain was very strong, and the signal in other tissues is very faint and barely detectable. The SP-A or likely molecules is likely compatible with primary injury sites of some autoimmune diseases. The etiology of those diseases is still unknown, but the epidemiological investigation showed that non-specific environment exposure is a high risk factor. **Conclusion:** Thus far the mechanisms of "Qi" communication in Traditional Chinese Medicine have not been well established. In fact, current technology remains insufficient for the understanding of the relationship between the local stimulation and whole body response by acupuncture, Qigong and other Chinese medicine. Herein, we analyze the molecular structure and regularity of SP-A or SP-A like proteins sharing common antigenic determinants. Those molecules may act in dual manner, either enhancing or suppressing inflammatory response. The distribution of SP-A molecule is compatible with the primary autoimmune injury site, thus is likely a candidate of "Qi" communication in Traditional Chinese Medicine.

### Su1.111. The Inhibition of Autoreactive T-Cell Functions by a Peptide Based on the Complementarity Determining Region-1 of an Anti-DNA Autoantibody Is Via TGF $\beta$ Mediated Suppression of LFA-1 and CD44 Expression and Function.

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We have previously shown that systemic lupus erythematosus (SLE), which is characterized by the increased production of autoantibodies and by defective T-cell responses, can be induced in mice by immunization with a human anti-DNA monoclonal antibody (mAb) which expresses a major idotype, designated 16/6Id. We also showed that a peptide based on the sequence of the complementarity determining region (CDR) 1 of the 16/6Id (hCDR1) ameliorated the clinical manifestations of SLE, and down-regulated *ex-vivo*, the 16/6Id-induced T-cell proliferation. Herein, we examined the mechanism responsible for the hCDR1-induced modification of T-cell functions related to the pathogenesis

of SLE. We found that hCDR1 treatment of BALB/c mice resulted in a marked elevation of expression of TGF $\beta$  in vivo, and in TGF $\beta$ -induced suppression of 16/61d-stimulated T-cell proliferation ex vivo. In addition, we provide evidence that one possible mechanism underlying the hCDR1 and TGF $\beta$ -induced inhibition of T-cell proliferation is by down-regulating the expression, and therefore, the functions, of a pair of key cell adhesion receptors, LFA-1 ( $\alpha$ L $\beta$ 2) and CD44, which operate as accessory molecules in mediating antigen presenting cell (APC)-T-cell interactions. Indeed, T cells of mice treated with hCDR1 showed a TGF $\beta$ -induced suppression of adhesion to the LFA-1 and CD44 ligands, hyaluronic acid and ICAM-1, respectively, induced by SDF-1 $\alpha$  (CXCL12) and PMA. The latter suppression is through the inhibition of ERK phosphorylation. Thus, the down-regulation of SLE by hCDR1 treatment may result from the influence of the up-regulated TGF $\beta$  on the expression and function of T-cell adhesion receptors, and consequently, T-cell stimulation, adhesion, and proliferation.

hCDR1 (Edratide) is under a clinical development for the treatment of SLE by Teva Pharmaceutical Ind.

#### **Su1.112. Fas Ligand (CD95L)-Transduced Monocyte-Derived Killer-DC Are Protected from CTL-Induced Cytotoxicity and Delete Antigen-Specific CD8+ T Cells.**

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**Rationale:** Numerous studies have been performed in vitro and in various animal models to modulate the interaction of DC and T cells by Fas (CD95/Apo-1) signaling to delete activated T cells via induction of activation-induced cell death (AICD). However, similar studies with primary human cells have not been performed. Recently, we could demonstrate that Fas Ligand (FasL/CD95L)-expressing "Killer-DC" can be generated from human monocyte-derived mature DC using adenoviral gene transfer. To evaluate, whether these FasL-expressing DC (DC-FasL) could eliminate human CD8+ T in vitro, coculture experiments were performed.

**Methods:** Human CD8+ T cells and a CTL clone specific to the HLA-A2 binding Melan-A26-35 peptide were activated in a first mixed lymphocyte reaction (MLR) with mature DC pulsed with Melan-A peptide. Activated T cells were rescued and a second MLR was established with either DC-FasL, EGFP-transduced control DC (DC-EGFP) or untreated DC loaded with Melan-A- or control peptide at different ratios. As a read-out system proliferation (thymidine incorporation) and apoptosis (Annexin V/PI staining) of T cells were determined.

**Results:** FACS-analysis of PKH26 labeled DC revealed that FasL-transduced DC but not DC or DC-EGFP loaded with Melan-A peptide were protected from cytotoxicity mediated by Melan-A specific CD8+ T cells. In addition, no proliferation could be observed in Melan-A specific CD8+ T cells cocultured with DC, DC-EGFP or DC-FasL loaded with control peptide. In contrast, proliferation of activated Melan-A specific CD8+ T cells was markedly reduced in cocultures with Melan-A peptide-loaded DC-FasL, whereas a strong secondary proliferative T cell response could be observed in cocultures with DC-EGFP or DC. Inhibition of T cell proliferation was directly related to the numbers of DC-FasL present in cocultures, and at a ratio of 1:1, T cell proliferation was completely inhibited by DC-FasL, which was due to induction of

apoptosis in the majority of Melan-A specific CD8+ T cells (approximately 70%). Spontaneous apoptosis detected in CD8+ T cells cocultured with Melan-A peptide-loaded DC or DC-EGFP was low (approximately 25%).

**Conclusion:** The present results demonstrate for the first time that human DC-FasL were protected from CTL-mediated cytotoxicity (counter attack). In addition, Melan-A specific CD8+ T cells were efficiently eliminated by Melan-A peptide-loaded DC-FasL, supporting the concept to apply FasL-expressing "Killer-DC" as a novel strategy for the treatment of T cell dependent autoimmune disease.

### **Inflammatory Bowel Diseases**

#### **Su1.113. The Peripheral Cannabinoid Receptor CB2 Is Required for the Normal Formation of B and T Cell Subsets.**

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G $\alpha$ i2  $-/-$  mice are deficient in the formation of certain B and T cell subsets and are susceptible to immune dysregulation, notably developing inflammatory bowel disease (IBD). A key issue is the identity of the Gi-coupled receptors mediating this G $\alpha$ i2 requirement for lymphocyte development. Here, we test the prediction that CB2, the G $\alpha$ i2-coupled peripheral endocannabinoid receptor, is one such receptor. B and T cell subsets, isolated from tissues of the peripheral, mucosal, and serosal lymphoid compartments of CB2 null and sufficient mice were quantified by flow cytometry. Mice bearing the CB2 null phenotype had profound deficiencies in both B and T cell subsets, particularly splenic marginal zone (MZ) and peritoneal B1a cells, as well as splenic memory T cells and intestinal NK and NKT cells. CB2 is required for the formation of many immunoregulatory B and T cell subsets. These findings phenocopy and extend the developmental disorder associated with G $\alpha$ i2  $-/-$  and suggest that the endocannabinoid system is required for the formation of T and B cell subsets involved in immune homeostasis.

#### **Su1.114. The Effect of Bifidobacterium on Murine Experimental Colitis.**

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**BACKGROUND AND AIM:** The etiology of inflammatory bowel disease (IBD) is still unknown, but more and more reports show gut microflora plays some role in pathogenesis of IBD. Microflora in gut of IBD patients becomes aberrant, with normal microflora decreased, harmful and potential harmful bacteria increased. As have been reported, there has close relationship between gut aberrant microflora and mucosal immune function disorder, and modification of intestinal microflora may have therapeutic effect on IBD. In the experiment, we used dextran sulfate sodium (DSS)-induced colitis in mice which has same

pathological property as human IBD, and fed mice with Bifidobacterium before inducing colitis, to study the therapeutic effect of supplement with bifidobacterium on pathogenesis of colitis, and explore the possible mechanism.

**METHODS:** Mice were randomly divided into four groups: Control, DSS, SASP, and Bifidobacterium (Bif in short). Mice of groups DSS, SASP, and Bif were fed with 5% DSS (w/v) solution for 7 days to induce colitis, mice of Control just drink distilled solution, and disease activity index (DAI) was calculated every day. Mice of SASP were fed with SASP every day during inducing colitis, and mice of Bif were given Bifidobacterium by oral gavage from 7 days before the experiment to the end of experiment. The expression of TNF- $\alpha$ , NF- $\kappa$ B P65, Fas and MPO in inflamed colon of each group mice was measured at the end of experiment.

**RESULTS:** Mice of group SASP, Bif showed lower DAI than those of group DSS since the fourth day of experiment. There were lower expression of TNF- $\alpha$  and MPO in murine inflammatory colon, lower NF- $\kappa$ B P65 appearance in nuclei of inflammatory cells, lower Fas expression in colonic epithelia of group SASP, Bif compared with group DSS at the end of experiment.

**CONCLUSION:** Treatment with bifidobacterium has beneficial effect on murine experimental colitis, the mechanism may be involved in such respect: Bifidobacterium inhibits proinflammatory cytokine secretion and NF- $\kappa$ B activation in inflammatory cells, limits colonic inflammation, downregulates Fas expression in colonic epithelia of murine inflamed colon, alleviates inflammatory damage of colonic epithelia and protects the integrity of intestinal mucosal barrier.

**Key words:** probiotic experimental colitis  
Dextran sulfate sodium inflammation

### **Su1.115. Anti-Murine TNF-alpha Reverses TNBS Colitis in Mice but Not Oxazolone Colitis: Potential Role of Apoptosis Induction.**

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**Background:** Differences and similarities of cytokine patterns in Crohn's disease (CD) and Ulcerative colitis (UC) have been reported. Proinflammatory cytokines like TNF- $\alpha$ , are up-regulated in the mucosa of CD as well as UC patients. However, administration of infliximab (a chimeric anti-TNF mAb) has shown beneficial effect in clinical trials in CD but is less effective in UC. **Aim:** 1) to investigate the effect of anti-TNF on trinitrobenzenesulphonate (TNBS) and oxazolone (Oxa) colitis in mice as models for CD and UC respectively; 2) to study the apoptosis-inducing effect of anti-murine TNF both in vitro and in vivo in mice. **Method:** 1) Colitis was induced by rectal administration of 1mg TNBS or Oxa in 50% ethanol after 2 pre-sensitizations via the skin. Anti-murine TNF- $\alpha$  was given intraperitoneally (i.p.) daily. 2) Thioglycollate-elicited peritoneal macrophages were treated in vitro with LPS and anti-murine TNF- $\alpha$  or non-specific control antibody. Annexin V & propidium iodide and 7AAD were used to study apoptosis on the cell membrane and DNA level respectively. 3) For in vivo analysis of anti-TNF effects on macrophages, anti-murine TNF- $\alpha$  or control antibody was administered to SCID

mice. Peritoneal macrophages were recovered and apoptosis was analyzed as described above. **Results:** 1) In the TNBS colitis model, mice treated with anti-TNF recovered more rapidly compared to the control treated mice. Histological analysis revealed less severe signs of colitis; on the contrary, no beneficial effect of anti-TNF was found in the Oxa colitis mice. 2) Apoptosis was induced by anti-murine-TNF in peritoneal macrophages. In vitro, apoptotic cells in the presence of anti-TNF amount to ~40% compared to ~20% in the control cultures. In vivo, ~30% less macrophages could be harvested from the anti-TNF treated group. A higher percentage of peritoneal macrophages were apoptotic (~50%) compared to the control group (~25%). **Conclusion:** Anti-TNF treatment rescued TNBS colitis mice but had no effect on Oxa colitis. These results correlate with difference in efficacy of anti-TNF treatment in UC and CD respectively. The apoptosis-inducing-effect of anti-TNF could be demonstrated in peritoneal macrophages. The data suggest a different involvement of TNF-expressing cells in the pathogenesis of both disease models.

### **Su1.116. Evaluation of 5 vs 10 Granulocyteapheresis Treatments in Patients with Moderate Active Steroid Dependent Ulcerative Colitis: A Prospective Multicenter Randomized Trial.**

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**Introduction:** Increasing evidence indicates that granulocyteapheresis (GCAP) is effective and safe therapeutic strategy in treatment of the ulcerative colitis (UC). However the ideal treatment scheme is not established yet.

**Objective:** This study was carried out to evaluate the efficacy and safety of 5 as compared to 10 GCAP treatments in patients with moderate active steroid dependent UC.

**Materials and methods:** In this prospective multicenter randomized clinical trial 20 patients were randomized to 5 or 10 GCAP treatments (1 weekly). Each treatment consisted in a 1-hour apheresis session with Adacolumn® at 30 ml/h with 1,8 l of blood being processed. The principle efficacy variable was the remission rate defined by the Rachmilewitz index (CAI) at week 17. Secondary variables included CAI at all weeks, quality of life questionnaires (IBDQ and EuroQoL), endoscopic activity index (EAI) at week 17, as well as steroid consumption and analytical parameters.

**Inclusion criteria** were: active UC (CAI between 6 and 12), EAI >4, total colon affected length >25 cm and steroid dependency defined as at least one unsuccessful attempt to taper down steroids plus use of >400 mg of prednisone within 4 weeks prior to the study start. All patients gave written informed consent. Clinical remission was defined as CAI  $\leq$ 4 and clinical response as a drop in CAI  $\geq$ 3.

Since some patients are still in the follow-up, efficacy for each treatment regimen, as well as other secondary variables analysis will be reported on completing the study.

**Results:** 12 males and 8 females were included, being the mean age 41, 7  $\pm$  15, 2 years and mean disease duration 80, 7 months (6-528). Two patients had chronic active UC whereas mean number of flare ups during the year prior to the study entry was 2, 2  $\pm$  1 for remaining patients. All patients were previously treated with 5-ASA

and steroids, 55% with immunosuppressants and 15% with cyclosporine. 9 patients were randomized to 5 GCAP sessions and 11 patients to 10 GCAP sessions. Global mean CAI was  $8 \pm 1$ , 2 at study entry ( $n = 19$ );  $4, 2 \pm 2, 5$  at week 6 ( $n = 14$ );  $4, 7 \pm 3, 5$  at week 11 ( $n = 11$ ). Global mean prednisone dose was  $39, 7 \pm 15, 8$  at baseline,  $23, 3 \pm 11, 9$  at week 6 and  $5, 5 \pm 6, 1$  at week 11. Five out of 11 patients were steroid free at week 11. Mean IBDQ score was  $133, 4 \pm 55, 1$  at baseline, going up to  $175, 5 \pm 40.5$  at week 6 and at  $188.1 \pm 47.4$  at week 11. Two not serious adverse events, and one community pneumonia were reported.

**Conclusions:** Granulocyteapheresis is a safe and effective treatment for moderate active steroid dependent ulcerative colitis. In addition, it shows an important steroid sparing effect in a severely steroid dependant population, even allowing complete steroid withdrawal.

### Su1.117. Cytokine and Chemokine Transcript Profiles in Acute Pouchitis.

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**Background:** After ileo-anal pouch anastomosis (IAP) 10-40% of patients with ulcerative colitis (UC) but only 5% of patients with polyposis coli (FAP) develop a pouchitis. Immunoregulatory abnormalities might be of importance in the pathogenesis of the disease. Therefore we characterized cytokine and chemokine transcripts in inflamed and non-inflamed pouches in patients with UC and FAP.

**Methods:** Mucosal biopsies were taken from 42 patients with IAP (UC ( $n = 37$ ) or FAP ( $n = 5$ )). Patients with active ileal Crohn's disease (CD;  $n = 14$ ), active UC ( $n = 8$ ), specific colitis (infectious colitis, ischemic colitis;  $n = 15$ ) and patients with non-inflammatory conditions ( $n = 13$ ) served as controls. Expression of 30 pro-inflammatory gene transcripts were quantified using real-time PCR.

**Results:** Compared to normal ileal mucosa from controls, biopsies from non-inflamed mucosa from IAP (UC and FAP) patients expressed elevated transcript levels for MRP-14, IL-8, IL-1 $\beta$ , IFN- $\gamma$  and MIP-2 $\alpha$ . In addition in UC patients expression of MMP-1 and IL-23 was increased. In UC-IAP patients MRP-14 (2,9-fold), IL-8 (2,6-fold) and IL-1 $\beta$  (3,8-fold) transcripts were elevated in their inflamed mucosa in comparison to their non-inflamed mucosal biopsies. No differences were found concerning TNF- $\alpha$ , IP-10, IL-18 and ELC. Levels of TNF- $\alpha$ , IFN- $\gamma$  and IL-23 were elevated in inflamed CU pouch mucosa in comparison to specific colitis, suggesting a predominantly Th1 mediated inflammation. Transcripts of IL-2, IL-4, IL-6 and IL-12p35 could not be detected. A good correlation between pouchitis activity (PDAI) and MMP-1 and MIP-2 $\alpha$  transcripts was observed.

**Discussion:** In acute pouchitis in UC patients after IAP anastomosis transcript levels of pro-inflammatory cytokines and chemokines of predominantly Th1 origin were found elevated, even if these data cannot completely explain the immunological etiology. Quantification of transcript levels allows to estimate the extent of mucosal inflammation.

### Su1.118. Inflammatory Transcript Profiles Reflect Onset of Clinical Remission in Patients with Steroid Refractory Crohn's Disease after Treatment with Cyclophosphamide or Infliximab.

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**Background:** Concentrations of proinflammatory cytokines are increased in the intestinal mucosa of patients with active CD. We investigated in a prospective study whether cytokine profiles reflect clinical activity and predict onset of remission in patients with refractory CD.

**Methods:** Cytokine transcripts were quantified using Real-time PCR in mucosal biopsies from 13 patients with active, steroid refractory CD. Patients were treated with infliximab ( $n = 7$ ; one single infusion (5 mg/kg body weight)) or cyclophosphamide ( $n = 6$ , 3 cycles of monthly treatment of IV cyclophosphamide (750 mg)) and were followed for up to 8 weeks. Based on a group of 48 patients with active CD and 16 patients without gastrointestinal disease normal values and pathologic ranges of cytokines/chemokines transcripts were calculated and transferred into a simple scoring system (Inflammatory Bowel Disease Mucosal Inflammation Transcript Index (IBD-MITI)) ranging from 0 to 12 points.

**Results:** 5 of 7 infliximab and 5 of 6 cyclophosphamide treated patients enter into remission. The IBD-MITI based on MRP-14-, MIP2 $\alpha$ -, IL-8- and MMP-1-transcript levels was strongly elevated in 12 of 13 patients (median: 9 points). Normalization of increased values was predictive for onset of remission in these patients.

**Discussion:** Real-time PCR quantification represents a simple and objective method for grading inflammation of intestinal mucosa and has considerable potential in the analysis of clinical disease activity in CD patients, especially in patients with immunosuppressive therapies.

### Su1.119. Long Term Follow-Up of Ulcerative Colitis (UC) Treated with a Probiotic Containing the Pingel/BM Non-Pathogenic Strain of *E. coli*.

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Patients with UC have different *E. coli* compared to controls, including both higher concentrations of specific serotypes, specific cytotoxic antibodies, higher amounts of adhesive *E. coli*, and cross-reactive epitopes on colon mucosa. These putative pathogenic *E. coli*, may be normal flora in controls who are not genetically susceptible to UC. If they could be replaced by antigenically dissimilar, non-adhesive *E. coli*, then remissions should occur. The following cases support this hypothesis in a subgroup of UC.

Beginning in 1972 patients with severe UC were selected for treatment. The first was a 17 year old boy scheduled for colectomy. He was anemic from blood loss and growth retarded. Following temporary sterilization of his bowel with a 2 day course of non-absorbable antibiotics, a benign *E. coli* incubated in buttermilk was administered by mouth. The patient made an unexpected complete recovery. He remained well for 10 years after which he relapsed. Based on this case the procedure was refined and additional 27 patients were so treated.

The non-pathogenic *E. coli* was selected from a donor who had life-long normal bowel function. It was extensively studied for cytotoxicity including all known pathogenic serotypes. The non-absorbable antibiotics vancomycin, cefamandol, gentamycin and nystatin were selected which regularly rendered the feces temporarily sterile after 36 to 48 hours given P.O. q 4 to 8 hrs with a clear liquid diet. On day three 60 ml of Pingel/BM *E. coli*, containing 7-9 billion organisms/ml were administered q 4 h with sterile food. On day four *Lactobacillus acidophilus*, DDS-1 strain was added and

then *B. bifidum* in similar doses. All probiotic treatment was discontinued when remissions occurred, (although we discovered later that probiotics needed to be continued long-term.)

Results: In patients so treated, with rare exceptions, remissions regularly occurred, lasting days to months. However most eventually relapsed. The exceptions were four patients who sustained complete remissions for over 10 years. In three of these, by contrast to those who relapsed, the Pingel/BM *E. coli* replaced the resident coliform and was consistently cultured from the stool as the predominant coliform. They also elected to continue to take prophylactic probiotics. Follow up colonoscopies and biopsies were normal. In one case, AP, the donor strain disappeared from feces following prophylactic ceftin therapy for a knee replacement. He gradually relapsed, the treatment was repeated successfully, and the Pingel/BM strain was reestablished. He remains well 12 years after he declined colectomy. A 9 year old girl was treated in 1990. She remains well and continues to take both *E. coli* (Mutaflor, Ardeypharm Co, Germany) and *L. acidophilus*. Her 20 year old brother was also treated 10 years later but he did not colonize. He is improved but unlike his sister, he has not sustained a complete remission. Other cases with intermediate results will be presented which suggest that this approach should be pursued with controlled studies and especially using continued prophylactic probiotics containing a non-pathogenic *E. coli*.

**Su1.120. Blockade of Interleukin 21 with Soluble IL21RFc Reduces Inflammatory Cytokines and Correlates with Suppression of Disease in Mice Adoptively Transplanted with CD45RB<sup>hi</sup> CD4<sup>+</sup> T Cells.**  
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Interleukin 21 (IL-21), a member of the common gamma-chain family of cytokines, is secreted by activated T cells and can have a diverse range of immunomodulatory effects dependent upon the particular context of the immune response. Interleukin 21 Receptor (IL-21R) is expressed on many immune system cell types including activated T cells. *Rationale:* In situ hybridization studies have shown that IL-21R is highly expressed in the lymphoid compartment in the gut, and the human IL-21R gene has been mapped to chromosome 16 within the Crohn's Disease susceptibility region, suggesting that the IL-21 pathway may be involved in regulation of gut homeostasis. We examined the role of IL-21 in a T cell mediated model of intestinal and skin inflammation. *Methods:* CD45RB<sup>hi</sup> CD4<sup>+</sup> naive T cells were transferred into severe combined immunodeficient (SCID) mice and housed under different caging conditions so that the mice developed colitis or colitis with skin lesions resembling psoriasis. Mice were treated with soluble murine IL-21RFc or an IgG2a control Antibody and assessed for development of disease. Purified CD45RB<sup>hi</sup> cells were also cultured with anti-CD3 and IL-21 or IL-21RFc to determine the effect on proliferation and cytokine secretion. *Results:* In culture, anti-CD3 stimulated CD45RB<sup>hi</sup> (naive) but not CD45RB<sup>lo</sup> (memory) CD4<sup>+</sup> T cells proliferated in response to IL-21 and secreted increased levels of IL-2, IL-4, IL-10, IL-17, IL-18, IFN- $\gamma$  and TNF- $\alpha$ . Blockade of endogenous IL-21 with neutralizing soluble IL-21RFc resulted in decreased levels of cytokines in these cultures. In mice that developed skin inflammation, treatment with thrice-weekly IL-21RFc seven weeks after CD45RB<sup>hi</sup> cell transfer, resulted in reduced erythema, scaling and hair loss when compared to IgG2a-treated controls. Treatment of CD45RB<sup>hi</sup> recipient mice

with 200 ug IL-21RFc, 3 times per week at the time of cell transfer, resulted in a significant reduction of clinical signs of colitis as measured by body weight loss and stool score when compared with control -treated mice. Macroscopic evaluation of colons from control treated CD45RB<sup>hi</sup> recipients showed severe thickening and swelling which was almost completely suppressed in mice treated with IL-21RFc. Microscopically, control- treated mice also exhibited a greater degree of epithelial hyperplasia and leukocyte infiltration in the lamina propria/submucosa when compared with IL21RFc-treated mice. *Conclusions:* Taken together these results suggest that IL-21 is a potent potential player in the inflammatory responses in this model and blockade of this pathway may be of therapeutic benefit in Th1 mediated diseases such as Crohn's and psoriasis.

**Su1.121. Role of ICOS in Regulating Mucosal Tolerance.**  
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Inducible costimulator (ICOS) is a CD28 homologue that is induced upon T cell activation. ICOS binds to its ligand ICOSL which is expressed on fibroblasts, endothelial cells, some epithelial cells and constitutively at low levels on resting B cells, on some macrophages and dendritic cells. ICOS ligation enhances T cell proliferation and the production of several cytokines such as IFN- $\gamma$ , IL-4 and has a key role in IL-10 production. IL-10 plays an important role in the induction of regulatory T cells and suppression of autoimmunity. We have used ICOS deficient (-/-) mice to investigate the role of ICOS on 1) the induction and function of regulatory T cells, and 2) the function of pathogenic effector T cells using the colitis model developed by Powrie and colleagues. In this colitis model, the transfer of CD4 + CD25-CD45RB<sup>high</sup> T cells (Teff) from normal mice to C.B-17 SCID recipients leads to the development of a Th1-mediated inflammatory bowel disease similar to IBD in humans. Intestinal inflammation is the result of the development of a Th1 response driven by enteric bacteria. Colitis induced by transfer of Teff cells can be prevented by cotransferring cells contained within the CD4 + CD25 + CD45RB<sup>low</sup> population (Treg).

Surprisingly, we found that ICOS-/- regulatory T cells protected mice from colitis, indicating that ICOS is not required for the induction of a functional regulatory T cell population. However, we found that wild type regulatory T cell could not protect from IBD induced by T effector cells lacking ICOS. Without ICOS the balance appears to be shifted in the favour of autopathogenic Th1 cells, such that these effector cells cannot be controlled by regulatory T cells, thereby resulting in more severe clinical disease. Thus, these studies suggest that ICOS is a critical regulator of the balance between regulatory T cells and effector T cells.

**Su1.122. A Critical Regulatory Role of Th2-Like Transcription Factor c-Maf in Th1-Mediated Experimental Colitis.**

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In this study, we investigated the role of c-Maf, a transcription factor known to induce IL-4 production, in inflammatory bowel

diseases. Although Crohn's disease (CD) is associated with low IL-4 production by T-bet-expressing Th1 cells in the lamina propria, surprisingly a higher expression of c-Maf in these cells was found as compared with control patients. The relevance of this finding was further evaluated in an animal model of CD induced by adoptive transfer of CD4 + CD62L+ T cells in RAG-deficient mice. In this Th1-mediated model, an increase of c-Maf-expressing T lymphocytes in the lamina propria over time was observed. Interestingly, adoptive transfer of c-Maf transgenic CD4 + CD62L + T cells in RAG-1-deficient mice resulted in an IL-4-dependent inability to induce colitis and suppressed colitis activity induced by wild-type CD4+CD62L+ T cells.

In contrast, transfer of CD4+CD62L- T cells from c-Maf transgenic, but not wild-type mice, induced colitis and augmented a colitis induced by CD4 + CD62L+ T cells from wild-type mice in an IL-4-independent pathway, as determined by macroscopic, histologic, and endoscopic criteria. This was associated with an accumulation of CD4+ T-bet + CD25 + effector Th1 cells in the lamina propria of colitic mice. Our results reveal a novel regulatory role of c-Maf in colitis. Although overexpression of c-Maf in naive T cells prevents Th1-mediated colitis, overexpression of c-Maf in memory T-bet+ Th1 cells regulates CD25 expression and augments such colitis. Targeting of c-Maf in memory T cells in CD appears to be an attractive target for therapeutic interventions.

#### **Su1.123. Dual Immune Suppressive Activity of 4AZA1378 Alleviates TNBS-Induced Colitis in Mice.**

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**Introduction:** Elevated production of TNF-alpha and activated T cells play a central role in the pathogenesis of Crohn's disease (CD). Recently, 4AZA1378 was identified as a phosphodiesterase-4 (PDE4) inhibitor (IC50; 31 nM), which explains its inhibitory effect on LPS-induced TNF-alpha production in vitro (IC50; 245 nM) as well as in vivo. PDE4 inhibition can however not account for the strong inhibitory effect in the Mixed Lymphocyte Reaction (MLR) assay (IC50; 4 nM) which suggests another target in this assay. The latter was confirmed by the inability of Rolipram (a specific PDE4 inhibitor) up to 50.000 nM to inhibit the MLR. **Aim:** To investigate the efficacy of 4AZA1378 in trinitrobenzenesulphonate (TNBS) induced colitis in mice, a model of Crohn's disease. **Methods:** Colitis was induced by rectal administration of 1mg TNBS in 50% ethanol after 2 pre-sensitizations via the skin. 4AZA1378 (20 mg/kg) was given intraperitoneally daily. **Results:** Mice treated with 4AZA1378 had less severe signs of colitis and recovered more rapidly, as evidenced by more rapid weight recovery, and histologically by a reduction of inflammatory lesions, less edema, a reduction of goblet cells loss and reduced wall thickness. Cell infiltration, especially infiltration of neutrophils, as shown by myeloperoxidase activity, was reduced in 4AZA1378 treated animals. **Conclusion:** Our findings show that 4AZA1378 has a strong remission-inducing effect in TNBS colitis.

This study supports further pre-clinical and clinical development of this novel molecule for treatment of Crohn's disease.

#### **Su1.124. Immunohistochemical Localization of Interleukin-6 in Pancreatitis and Normal Pancreas.**

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##### **INTRODUCTION:**

Experimental and clinical studies have indicated that cytokines play an essential role as mediators of inflammatory process associated with pancreatitis. One of the most important mediators of inflammation is interleukin-6 (IL-6). Interleukin-6 is a 22–30-kDa glycoprotein produced by many cell types and has a wide variety of biologic, differentiation, and growth-promoting effects in a variety of target cell types.

The importance of IL-6 in the acute phase has been confirmed by the observation that it stimulates the synthesis of acute phase proteins, including C reactive protein (CRP), from hepatocytes *in vitro* and *in vivo*. IL-6 levels are raised in patients with acute pancreatitis (AP) and correlate with disease severity. Serum concentrations IL-6 of patients during the first 48 hours of hospitalization is a valuable marker permitting the differentiation of various types of pancreatitis (AP, CP-chronic pancreatitis, CEP-chronic exacerbated pancreatitis).

The immunohistochemical detection of IL-6 in pancreas during pancreatitis, to our knowledge, has not been previously described. In previous studies, the presence of IL-6 in human pancreatic cells had only been shown on biopsy material from diabetic patients or normal gland.

##### **AIMS & METHODS:**

The aim of the study was to identify immunohistochemically the localization of IL-6 and to determine IL-6 expression in CP and CEP. Samples of tissues of normal pancreas ( $n = 5$ ) (obtained at autopsy) and CP ( $n = 14$ ), CEP ( $n = 2$ ) were verified histopathologically and then IL-6 was localized by immunohistochemical staining using the monoclonal anti-human IL-6 antibody (R&D Systems USA) and test LSAB2-HRP (DAKO,USA) to visualize IL-6/Ab complexes.

##### **RESULTS:**

We found only scarce acinar cells staining positively for IL-6 in the normal human pancreas (–/+); islets cells did not show IL-6 immunoreactivity. In slices of the pancreas, derived from patients with CP and CEP, a much stronger immunohistochemical reaction (+++; ++++; diffused and focal) was noticed as compared to controls. IL-6 was localized in exocrine and islet cells of the pancreas. The immunohistochemical reaction of ducts cells was also strong. Interestingly, this cytokine was detected in cytoplasm and very close to nucleus. Moreover, in cases of CP and CEP with inflammatory infiltration, there were a markedly stronger IL-6 expression (++++), than that observed in specimens without infiltrate.

##### **CONCLUSION:**

In conclusion, the results presented herein clearly demonstrated a moderate and strong expression of IL-6 in exocrine and endocrine cells of patients with CP and CEP. This suggests that elevated IL-6 levels of patients with pancreatitis are probably

owing to leakage of this cytokine in the circulation following massive pancreatic cells destruction.

**Su1.125. Tumor Derived TGF-beta Suppresses Inflammation Dependent Colon Cancer Development by Inducing FoxP3 in Tumor Infiltrating CD4+ T Cells.**

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Recent data suggest that thymus derived CD4+CD25+ regulatory T cells play an important role in the tolerance of the immune system towards tumors. These regulatory cells specifically express the transcription factor FoxP3 which is believed to be a master regulator of regulatory T cell development. In contrast to this thymic development, recent data have demonstrated that CD4+CD25+ regulatory T cells can also be induced from naïve cells in the periphery. We and others have further provided evidence that TGF-beta is critical for this peripheral induction of regulatory cells by inducing the expression of FoxP3. Here we now demonstrate that the induction of FoxP3+ regulatory T cells by TGF-beta is a physiological event in the colon with a potential role in the pathogenesis of colon cancer.

Accordingly mice were injected with a single dose of the mutagenic agent azoxymethane followed by three weekly periods of Dextran sulphate (DSS) in drinking water, interrupted by each 14 days of recovery. Treated animals developed numerous tumors in the colon. In a recent study we have shown that tumor growth in this colitis associated colon cancer model was driven by inflammatory CD4+ T cells infiltrating the tumor. As described for human colon cancer, we now demonstrate that dysplastic epithelial cells produced very high amounts of TGF-beta. Interestingly CD4+ cells isolated from the same tumors expressed high levels of FoxP3 mRNA while CD4+ cells isolated from surrounding non-dysplastic colon tissue did not. Our findings were further confirmed by immunohistochemical staining. Accordingly, FoxP3 expressing cells were found in high numbers in the lamina propria of the tumor closely associated to dysplastic epithelial cells. In contrast only few FoxP3 positive cells were detectable in the lamina propria of surrounding normal colon tissue. Based on these data, we speculated that tumor derived TGF-beta may induce FoxP3 in tumor infiltrating T cells. In order to functionally analyze whether tumor derived TGF-beta may play a role in the induction of FoxP3+ regulatory T cells in vivo we induced colon tumors in mice overexpressing a dominant negative TGF-beta receptor specifically in T cells. Tumors collected from these mice showed a similar infiltration with CD4+ T cells as compared to wildtype mice. However, CD4+ T cells isolated from tumor tissue of such transgenic mice showed strongly diminished expression of FoxP3 mRNA as compared to tumors of wildtype animals. Strikingly, tumors in transgenic mice were larger than wildtype tumors implicating that in this model of inflammation dependent colon cancer, tumor induced regulatory T cells control tumor growth.

Based on our findings we propose a model in which tumor derived TGF-beta induces FoxP3 expression in infiltrating CD4+ T cells giving them a regulatory phenotype. Such tumor induced regulatory T cells in the case of inflammation dependent cancer can control tumor growth. However, in spontaneous cancer development tumor induced regulatory T cells may mediate tolerance towards the tumor by inhibiting anti-tumor immunity.

**Su1.126. Cross-Linking of Lipid Rafts on CD4+ T Cells by an Epithelial Lectin, Galectin-4, Contributes to the Exacerbation of Intestinal Inflammation.**

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Accumulating studies has addressed inflammatory bowel disease as an autoimmune disease. However, it is not known whether the intestinal epithelial cell-derived antigens are involved in generation of mucosal immune responses or are the target of the pathogenic process. By using a modified Serological Analysis of Recombinant cDNA Expression Libraries (SEREX) which is an antigen-screening approach utilizing humoral and cellular immune responses, we herein identify an epithelial cell-derived endogenous lectin, galectin-4 (G4), as a pathogenic mediator to exacerbate intestinal inflammation. G4 specifically stimulated the production of IL-6 by CD4+ T cells but not other cell types present in the diseased colon of CD45RB<sup>high</sup>-transfer and DSS-induced colitis models and TCR $\alpha$  knockout (KO) mice. In contrast, G4 was unable to stimulate IL-6 production by CD4+ T cells present in the normal colon of these colitis models as well as wild type mice. Confocal microscopic analysis showed that G4 interacts with the immunological synapse on colonic CD4+ T cells as indicated by specific binding of G4 to the lipid raft-accumulating regions. Interestingly, the binding intensity of G4 on CD4+ T cells and the G4-mediated IL-6 production were increased under intestinal inflammatory conditions. G4 has been recently shown to specifically bind to sulfated (non-sialylated) core1 O-glycan structure. Indeed, expressions of a member of sialyltransferases (ST6GalNAc-2, 3, 6 and ST8Sia-1,3) that are specifically utilized for the modification of O-glycans were markedly downregulated in the CD4+ T cells only under inflammatory conditions. In addition, the exposure of core1 O-glycan without sialylation on the CD4+ T cells was confirmed by intensified binding of PNA that specifically binds to this oligosaccharide structure. Mechanistically, the G4-mediated IL-6 production by CD4+ T cells is mediated by protein kinase C (PKC)  $\theta$ -associated cascade as indicated by a fact that colonic CD4+ T cells from PKC $\theta$  KO mice treated with DSS were unable to respond to G4 to produce IL-6. Functionally, administration of anti-G4 mAb not only led to the attenuation of chronic colitis in B cell-deficient TCR $\alpha$  double knockout mice but also effectively enhanced the recovery from 3.5% DSS-induced acute colitis. These studies not only indicate the presence of an immunogenic epithelial lectin that contributes to the exacerbation of intestinal inflammations but also provide a novel insight into the biological role of lectin/CD4+ T cell interactions under inflammatory conditions.

**Su1.127. Immunohistochemical Localization of Copper/Zinc-Containing Superoxide Dismutase in Normal Pancreas and in Pancreatitis.**

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Medicine, Wrocław, Poland; <sup>3</sup>Department and Clinic of Gastrointestinal and General Surgery, Wrocław University of Medicine, Wrocław, Poland.

#### INTRODUCTION:

The role of oxidative stress in the pathogenesis of pancreatitis and benefits of antioxidants have been suggested in various studies. Superoxide dismutase (SOD), a primary antioxidant enzyme, scavenges reactive free radicals by catalyzing the dismutation of superoxide anion into molecular oxygen and peroxide. Copper and zinc-containing SOD (Cu/Zn SOD) is the intracellular enzyme, which activity depends on metals. It has been shown that metallothionein (MT), also antioxidant protein, in high concentration in the pancreas serves a function of exceptionally sensitive indicator of Zn status. Our recent studies confirm that MT is present in exocrine and endocrine cells of patients with chronic pancreatitis, particularly in acinar cells of pancreas (1).

In the last years, evidence was provided that there appears to be a decrease in Cu/Zn SOD expression in pancreatic cells from normal pancreas to chronic pancreatitis (2).

#### AIMS & METHODS:

The aim of the study was to identify immunohistochemically the distribution pattern of the Cu/Zn SOD in chronic pancreatitis (CP) and chronic exacerbated pancreatitis (CEP). Samples of tissues of normal pancreas ( $n = 5$ ) (obtained at autopsy) and CP ( $n = 14$ ), CEP ( $n = 2$ ) were verified histopathologically and then Cu/Zn SOD was localized by immunohistochemical staining using the primary polyclonal anti-human Cu/Zn SOD antibody (Calbiochem, UK) and second anti-sheep peroxidase conjugated antibody (Sigma, Germany) to visualize Cu/Zn SOD- Ab complexes.

#### RESULTS:

We found only scarce acinar cells staining positively for Cu/Zn SOD in the normal human pancreas (the body and tail of pancreas) (-/+; +); islets cells did not show Cu/Zn SOD immunoreactivity. In slices of the pancreas, derived from patients with CP and CEP, a much stronger immunohistochemical reaction (+ +; + + +) was noticed as compared to controls. Cu/Zn SOD was localized in both acinar and islet cells of the pancreas. Interestingly, immunohistochemical reaction of ducts cells was considerably stronger (+ + + +) than that of islet and acinar cells (+; ++). We also compared expression of Cu/Zn SOD and metallothionein (MT), at the same histological specimens and experimental conditions. Whereas, Cu/Zn SOD was markedly manifested in ducts cells of pancreas, MT did not appear in it.

#### CONCLUSION:

In conclusion, these studies clearly demonstrate a moderate and strong expression of Cu/Zn SOD in acinar, islets and duct cells of patients with CP and CEP. This suggests that pancreatitis induce expression Cu/Zn SOD in pancreas. The overexpression of this enzyme in ducts cells may function as an intracellular antioxidant and can compensate for the lack of MT in the cells of pancreas.

1. Milnerowicz H., Chmerek M., Rabczynski J., et al.: *Pancreas* 2004, 29: 28-32.

2. Cullen J.J., Mitros F.A., Oberley L.W.: *Pancreas* 2003, 26: 23-27.

#### **Su1.128. The Response of Blood Monocytes of Coeliac Patients to Gliadin.**

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Celiac disease (CoD), an intestinal damage, is induced by gliadin, an alcohol soluble fraction of gluten. Peptic fragments of gliadin were shown to activate cells of innate immunity including macrophages/monocytes to cytokine and chemokine production. The aim of this study was to examine whether: (i) peripheral blood monocytes (PBMoC) respond to peptic digest of gliadin by IL-8 and or TNF- $\alpha$  production, (ii) this activity depends on the presence of IFN- $\gamma$ , (iii) there exist a difference in response of PBMoC isolated from blood donors, active and treated (GFD) coeliac patients (including analysis of the causal factors) (iiii) the signaling pathway is mediated via NF- $\kappa$ B molecule activation.

Methods: PBMoC were incubated with various doses of peptic digest of gliadin alone or together with IFN- $\gamma$ , or pre-incubated with IFN- $\gamma$  before adding the gliadin. HLA genes were typed using PCR with sequence-specific primers (SSP-PCR). NF- $\kappa$ B DNA binding activity was detected by TransAM NF- $\kappa$ B transcription factor assay kit.

Results: The capacity of monocytes isolated from active CoD patients and patients on GFD to produce IL-8 was significantly higher than that of healthy donors. The simultaneous addition of IFN- $\gamma$  had no enhancing effect on IL-8 production and the prestimulation of cells with IFN- $\gamma$  for 24 hours resulted in a significant increase of IL-8 production mainly in cells from healthy controls. The enhanced TNF- $\alpha$  secretion was detected mainly in gliadin stimulated monocytes from CoD patients and was markedly increased by simultaneous addition of IFN- $\gamma$ . Interestingly, prestimulation of cells with IFN- $\gamma$  for 24 hours increased gliadin-induced TNF- $\alpha$  production in the group of healthy donors and patients on a GFD, and slightly in the group of active CoD patients. This effect reduced the differences in TNF- $\alpha$  production among tested groups. The signaling pathway triggered by gliadin was mediated via NF- $\kappa$ B subunits p50 and p65. Specific inhibitors suppressed DNA binding activity of NF- $\kappa$ B as well as gliadin induced IL-8 and TNF- $\alpha$  secretion. The impact of HLA-DQ2/DR3 antigen expression in healthy donors and the keeping of GFD in treated patients on cell response were evaluated.

Conclusions: IL-8 and TNF- $\alpha$  produced by the cells of innate immunity could enhance the effect of gliadin specific lymphocytes and participate in the cascade leading to the damage of intestinal mucosa in celiac patients.

#### **Su1.129. Eosinophilic Esophagitis (EE): Improved Clinical Responses without a Concomitant Reduction in Esophageal Eosinophilic Infiltration.**

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**Objective:** EE is an allergic inflammatory disorder of the esophagus with a significant recent increase in the number of reported pediatric cases. The initiation of the esophageal inflammatory injury has been theorized to be due to food allergies and possibly aeroallergen sensitivities. We reviewed the cases of 32 pediatric patients (pts) with EE in order to discern clinical,



histological, and treatment correlates and to acquire a better understanding of disease pathogenesis.

**Methods:** Allergy (food and aeroallergen) testing was completed on 26/32 pts. Serial Esophageal Biopsies were performed on 23/32 pts and serial clinical scores were determined on 25/32 pts. Clinical scores (0 to 5) were based upon the presence/absence of Abd pain, Vomiting, Dysphagia, Wt loss/gain, and Chest pain.

**Results:** 22/25 pts (88%) demonstrated clinical improvement based on a comparison of clinical scores from baseline until after 3 to 45 months of treatment. Treatment: 11/22 pts-Elimination Diet (ED) + Steroids (swallowed Flovent or oral steroid); 8/22 pts- ED only; 2/22 pts-steroids only; 4/22 pts-noncompliant or PPI only.

While the majority of pts (65%) demonstrated diminished esophageal eosinophilic infiltration (15/23 pts who underwent serial biopsies), 8/23 demonstrated clinical improvement with no evidence of diminished esophageal eosinophilic infiltration. No differences in treatment regimens existed between these groups. Further, 2/8 pts had been treated longer than average lengths of time, respectively for 30 and 34 months.

**Conclusions:** EE is an allergic inflammatory disorder that in most cases clinically responds to a treatment regimen of ED and steroids. A subset of patients demonstrated an improved clinical outcome without diminished esophageal eosinophilic infiltration. These results may reflect an incomplete therapeutic response which requires close follow-up in order to detect an end of disease remission. Alternatively, these findings may be characteristic of a unique patient subset that merits further investigation.

## Poster Session 2

3:30 PM–5:30 PM, 5/15/2005

### Immunodeficiency: Primary or Acquired

#### Su2.01. Rheumatic Manifestations in a South Italy Population HIV Positive: Correlation with CD4 Count.

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Infection by human immunodeficiency virus can involve several clinical manifestations affecting almost every organ or system in the body. Rheumatic manifestations may develop at any time of the clinical spectrum, but usually are seen in late stages, although sometimes they constitute the initial presentation of the viral illness. 46 patients (43 males, 3 females), with infection by HIV, from South Italy, were recruited for the study. HIV positivity was detected by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western Blot. Patients were asked to complete a questionnaire containing 10 questions pertaining to rheumatic diseases. The following data were collected: age, sex, duration of HIV infection, CD4 count. Rheumatologic manifestations were found on clinical evaluation by questionnaire administration and physical examination in 11 of 43 patients (23.9%). RESULTS: Arthralgias were the commonest manifestations, occurring in 9 patients (82%). Pain was usually intermittent and of moderate intensity and involved 2 or more joints. One patient had Reiter's syndrome (9%) with a mild oligoarthritis of the knees and spondylitis responding promptly to NSAIDs. Another patient had a mild oligoarthritis (9%) involving knees and ankles. CD4 count was  $481 \pm 264$  (mean  $\pm$  standard deviation) in the group of patients with HIV without arthralgias and  $385.5 \pm 170.3$  in the group of patients with arthralgia,

without significant correlation between CD4 count or viral load and arthralgias.

#### Su2.02. Quality Control of siRNA, Optimizing It's Transfection Efficiency and Monitoring CD4 Gene Silencing Effect with a Microfluidic Chip Device.

T. Preckel,<sup>1</sup> C. Buhlmann,<sup>1</sup> M. Valer.<sup>1</sup> <sup>1</sup>Liquid Phase Analysis, Agilent Technologies, Waldbronn, BW, Germany.

Gene silencing with RNA interference (RNAi) is a new breakthrough technology with high potential for development of therapeutics. Here, the delivery of small interfering RNA (siRNA) into cells is of key importance in elucidating gene and protein function. Differing types of interfering RNAs and several methods of delivery into cell types exist. All require transfection optimization, as the efficiency of transfection can be affected by many factors. Furthermore, the selection of the best silencing sequence at optimized siRNA transfection conditions is based on the integrity and purity of siRNA, siRNA uptake and cell viability. Given the complexity of monitoring and optimizing these types of experiments a new tool is required that would allow for minimal sample and reagent consumption in a fast and easy to use format.

We describe the use of a microfluidic chip-based system to quickly verify siRNA quality and to determine the optimal conditions for gene silencing experiments. *First*, RNA integrity and purity is assessed. *Second*, fluorescently labeled siRNA is used to optimize transfection parameters in mammalian cells. Life cell staining is performed on-chip reducing the overall analysis time for 6 samples to less than 50 minutes from harvesting the cells to final results with actual hands-on-time of less than 10 minutes. Transfection efficiency is measured as the percentage of cells with a strong siRNA uptake within the live cell population. *Third*, gene knockdown is measured with the same system. Here, staining the protein of interest, e.g. CD4, with fluorescently labeled antibodies demonstrates the downregulation of proteins after siRNA transfection. The gene silencing mechanism can also be verified in a given cell line by using a GFP-tag and co-transfecting the tagged protein and a Cy5 labeled siRNA against that protein. Successful silencing can be measured by reduced GFP expression within Cy5 positive cell population at different timepoints after transfection.

#### Su2.03. Cardiac Thrombus in Omenn Syndrome.

S. S. Kilic.<sup>1</sup> <sup>1</sup>Pediatric Immunology, Uludag University School of Medicine, Bursa, Turkey.

Omenn syndrome is characterized by generalized erythematous skin rash, lymph node enlargement, hepatosplenomegaly, increased serum Ig E levels, eosinophilia, and evidence of severe combined immune deficiency. Patients develop fungal, bacterial, and viral infections. A-three-month-old girl with Omenn syndrome developed right ventricular thrombosis. Echocardiographic investigation revealed a rounded structure filling the apex and corpus of the the right ventricle. We investigated for hypercoagulation state and discussed ventricular thrombosis which is uncommon in Omenn syndrome.

#### Su2.04. The Frequency and Effects of Vitamin A Deficiency in Common Variable Immunodeficiency Patients.

S. S. Kilic.<sup>1</sup> <sup>1</sup>Pediatric Immunology, Uludag University School of Medicine, Bursa, Turkey.

Common Variable Immunodeficiency (CVID) is a heterogeneous group of B-cell deficiency syndromes characterized by hypogammaglobulinemia, impaired antibody production and recurrent bacterial infections. Vitamin A (Vit A), a naturally occurring antioxidant micronutrient, has immunomodulating effect in patients with immunodeficiency, including an influence on cytokine production and lymphocyte growth and functions. Vit A deficiency is associated with a shift from type 2 cytokines to predominantly type 1 cytokines. The aim of this study was to investigate vitamin A levels in CVID patients and the effects of Vit A deficiency on cytokine production.

Nineteen CVID patients and 13 healthy controls who attended to the Department of Pediatric Immunology in Uludag University School of Medicine, Turkey, were involved in this study. Serum Vit A, serum neopterin (indicator of chronic inflammatory state), serum type 1 (TNF- $\alpha$ , IL-2) and type 2 cytokine levels (IL-4, IL-10) were determined. 9-cis retinal which is Vit A derivative was added to lymphocyte cultures of CVID patients and controls and the effects on cytokine production were investigated.

While serum Vit A levels of CVID patients were obviously low in CVID patients (in 75%), serum neopterin levels were higher than control group (in 31%). Elevated serum neopterin levels and low Vit A levels in CVID patients suggests that serum Vit A levels were lower secondary to recurrent infections seen in these patients. Serum IL-4 level was found lower in CVID patients than controls. The results of lymphocyte culture in CVID patients showed that IL-4 was higher in 9-cis retinal stimulated group than unstimulated group. However the expected change on other cytokines was not seen.

As a result, our study shows that CVID patients have low serum Vit A levels and this finding correlates with their chronic inflammatory condition and supplementation with Vit A may have role in downregulation of inflammatory responses.

#### **Su2.05. Pulmonary Abscess Due to *Aspergillus* spp. in Patients with Chronic Granulomatous Disease.**

*S. S. Kilic.*<sup>1</sup> <sup>1</sup>*Pediatric Immunology, Uludag University School of Medicine, Bursa, Turkey.*

Chronic granulomatous disease (CGD) is the most common phagocytic disorder. Invasive fungal infections are an important cause of morbidity and mortality in CGD patients, with *Aspergillus* spp. being the most frequent fungal pathogens.

A fifteen-month-old boy with a cavitation in the right upper lobe presented with persistent weight loss, fever, cough and roentgenographic evidence of right upper lobe abscess resistant to antibiotic therapy. A lung biopsy led to the diagnosis of pulmonary aspergillosis. A respiratory burst assay revealed the diagnosis of CGD.

He was treated with high doses of liposomal amphotericin B (7/ mg/kg) for an invasive pulmonary *Aspergillus nidulans* infection. The infection regressed during 12 weeks of treatment to the addition of interferon-gamma.

#### **Su2.06. Hodgkin's Lymphoma Developing in a 4.5-Year-Old Girl with Hyper-IgE Syndrome.**

*Mohammad Amin Kashef,*<sup>1</sup> *Sara Kashef,*<sup>2</sup> *Farhad Hanjani,*<sup>3</sup> *Mehran Karimi.*<sup>4</sup> <sup>1</sup>*Allergy Research Center, Shiraz University of Medical Sciences, Shiraz, Fars, Islamic Republic of Iran;* <sup>2</sup>*Immunology and Allergy, Shiraz University of Medical Sciences, Shiraz, Fars, Islamic Republic of Iran;* <sup>3</sup>*Dermatology, Shiraz University of Medical Sciences, Shiraz, Fars, Islamic Republic of Iran;* <sup>4</sup>*Pediatric Oncology, Shiraz University of Medical Sciences, Shiraz, Fars, Islamic Republic of Iran.*

We report a case of Hodgkin's lymphoma developing in a 4.5-year-old child with hyper-IgE syndrome. This is one of few cases of malignancy reported in this syndrome. A white girl with asthma, recurrent pneumonia, bronchiectasis, atopic dermatitis, recurrent skin infections and growth retardation. Immunologic evaluation revealed high level of immunoglobulin E (7000 IU/dl) and peripheral eosinophilia.

She was found to have normal values for serum IgG, IgM, IgA, sweat chloride test, WBC chemotaxis and serum complement function. The occurrence in this patient of Hodgkin's lymphoma suggests that individuals with hyper-IgE syndrome may also be at increased risk for developing premature malignancies, although the precise immunologic defect in this syndrome is still unknown.

#### **Su2.07. The Prognostic Importance of Variants of Allele Genes HLA DRB1 and IL-4 in HIV-Infection.**

*Loudmila P. Sizyakina, Julia V. Sokolova.* <sup>1</sup>*Center of Clinical Immunology, State Medical University, Rostov-on-Don, Russian Federation;* <sup>2</sup>*Russian Federation.*

The purpose of our study is developing of distribution of variants of allele genes HLA DRB1 and IL-4 (on SNP in a promotor site-C-590T) in HIV-infected patients for an establishment of immunological and genetic markers of determination and/or resistance to HIV-infection.

HLA-detecting of HLA DRB1 genes and IL-4 in 30 HIV-infected patients was made using the method of multiprimer amplifications sequence-specific primers basis on PCR at a level of allele groups with the set of reagents: ("DNA-technology", Moscow). Variants of allele gene IL-4 were defined with the help of PCR with the subsequent restriction of products of amplification.

Immunological and genetic analysis revealed, that the most significant protective effect have the specificities DR B1\*01 (RR = 0,46; PF = 0,15), DR B1\*04 (Rr = 0,52; PF = 0,175) and DR B1\*13 (Rr = 0,6; PF = 0,108). The strongest positive associative correlation has been revealed in specificities DR B1\*16 (Rr = 4,3; EF = 0,124) and, probably, DR B1\*14 (Rr = 4,76; PF = 0,051).

Distinction in distribution of variants of allele promotor genes IL-4 in HIV-infected and healthy European people has been revealed. Quantity of HIV-infected people with homozygous variant C/C was lower, than in healthy European people (28,6 % and 83,3 %, accordingly), while with variants T/T (21,4% and 0%) and C/T (50,0% and 16,7 %) are essentially higher. Besides distinctions in formation of the immune response and cytokine status in HIV-infected patients have been revealed various variants of promotor allele gene IL-4.

The received results testify that immunological and genetic factors have essential influence on processes of infecting and development of disease and can be used for defying of probability of case of infection, and the individual predicting of disease, and for the development of the individualized approach for monitoring and diving adequate immunocorrection therapy of the HIV-infection.

#### **Su2.08. Recurrent Infections and Cytokine Imbalance in Hyper-IgE Syndrome Are Not Due to a Defect in Toll-Like Receptor Pathways.**

*E. D. Renner,*<sup>1</sup> *F. Hoffmann,*<sup>1</sup> *I. Pawlita,*<sup>1</sup> *V. Hornung,*<sup>2</sup> *D. Hartl,*<sup>1</sup> *M. Albert,*<sup>1</sup> *A. Jansson,*<sup>1</sup> *S. Endres,*<sup>2</sup> *G. Hartmann,*<sup>2</sup> *B. H. Belohradsky,*<sup>1</sup> *S. Rothenfusser.*<sup>2</sup> <sup>1</sup>*University Children's Hospital,*

*Dr. von Haunersches Kinderspital, Ludwig-Maximilians-University, Munich, Germany;* <sup>2</sup>*Department of Internal Medicine, Division of Clinical Pharmacology, Ludwig-Maximilians-University, Munich, Germany.*

Hyper-IgE syndrome is a rare primary immunodeficiency of unknown etiology characterized chronic eczema, elevated total serum IgE, recurrent infections of skin and respiratory tract, and variable associated skeletal symptoms. Recent reports about pyogenic bacterial infections in patients and animals with defects in Toll-like receptor (TLR) pathways lead us to search for related defects in patients with hyper-IgE syndrome.

Cytokine profiles in six patients with hyper-IgE syndrome were analyzed in serum samples, in T cells after stimulation with PMA/Ionomycin and in peripheral blood mononuclear cells stimulated by TLR ligands and bacterial products including LPS (TLR4), peptidoglycan (TLR2), PolyIC (TLR3), R848 (TLR7/8), CpG-A and CpG-B (TLR9), zymosan and heat killed listeriae monocytes. Results were compared to healthy controls.

Reduced percentage of IFN-gamma, IL-2, and TNF-alpha producing T cells after PMA stimulation in patients with hyper-IgE syndrome was found implying an impaired inflammatory T cell response. Augmented serum levels of IL-5 point to an associated Th-2 shift. However, normal production of cytokines (TNF-alpha, IL-6, IL-10, IFN-gamma, IP-10) and upregulation of CD86 on B cells and monocytes after TLR stimulation ruled out a defect in TLR signaling pathways.

In conclusion, a dysbalance in T cell responses of patients with hyper-IgE syndrome was detected as described before, but no indication for an underlying defect in Toll-like receptor pathways was observed.

### **Su2.09. Defective Functioning of Dendritic Cells in Common Variable Immuno Deficiency (CVID): A Common Denominator for the Multiple Symptoms Displayed by CVID Patients.**

*Jagadeesh Bayry, Olivier Hermine, Eric Oksenhendler, Michel D. Kazatchkine, Srinivasa Kaveri.* <sup>1</sup>*Memory Group, The Edward Jenner Institute for Vaccine Research; INSERM U681, Compton, Nr Newbury, Berkshire, United Kingdom;* <sup>2</sup>*CNRS-UMR 8147, Necker Hospital, Paris, France;* <sup>3</sup>*Department of Clinical Immunopathology, Saint Louis Hospital, Paris, France;* <sup>4</sup>*INSERM U681, Institut des Cordeliers, Paris, France;* <sup>5</sup>*INSERM U681, Institut des Cordeliers, Paris, France.*

#### **Objective:**

Common variable immunodeficiency (CVID) is a heterogeneous immunodeficiency with characteristic recurrent bacterial infections due to hypogammaglobulinemia and incapacity to generate memory B cells. In addition to dysfunctional immunoglobulin production, patient's T-cell activation is impaired, resulting in low production of cytokines and decreased T-cell proliferation. Patients further suffer from gastrointestinal infections, autoimmune disease, and various B-cell neoplasm. We addressed whether CVID is associated with impairment in the dendritic cell (DC) compartment, as DCs play a central role in the development of adaptive immunity.

#### **Materials and Methods:**

Heparinized blood samples were collected from CVID patients at least 21 days following the last infusion of intravenous immunoglobulin (IVIg) or from newly diagnosed naive CVID patients prior to IVIg therapy. As control, blood samples were obtained from patients with selective antibody deficiencies who

received IVIg similar to CVID patients, and healthy controls. Monocyte-derived DC from patients' blood and from control groups were generated after differentiation for six days in the presence of GM-CSF, IL-4 and 10% autologous plasma.

For allogeneic mixed lymphocyte reaction, DCs from CVID patients and healthy donors were exposed to CD4<sup>+</sup> T-cells of third-party healthy donors. The CD4<sup>+</sup> T cells were isolated by magnetic cell sorter (MACS) cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Cytokines were quantified by Quantikine ELISA kit (ImmunoTech, Marseilles, France).

#### **Results:**

Dendritic cells (DC) from CVID patients have severely perturbed differentiation and maturation. Although the total number of DC appears normal as determined by CD11c expression, they express only nominal levels of CD1a, a hallmark of Langerhan's cells, and completely fail to up-regulate CD83, typically expressed on fully mature DC. Moreover, patients' DC express markedly reduced levels of the costimulatory molecules CD80 and CD86 that are critical for T-cell stimulation; and HLA class II, the antigen presenting molecule. In turn, patients' DC induced weak proliferation of allogeneic T cells and produced significantly low amounts of interleukin-12 (IL-12) upon CD40 signaling.

#### **Conclusions:**

Since DC play a central role in T-cell activation, and in immunoglobulin synthesis, a failure of DC to mature into fully stimulatory cells and to present antigens may provide a more general explanation for the various symptoms of CVID patients. Multiple defects in the immune system, including malfunctioning of DC, appear to be prominent features of CVID patients.

### **Su2.10. Clinical Profile of Primary Immunodeficiencies Diseases Patients Followed up at Hospital das Clínicas da Universidade Federal de Minas Gerais.**

*L. A. O. Cunha,<sup>1</sup> D. B. Greco,<sup>2</sup> J. A. Pinto.<sup>1</sup>* <sup>1</sup>*Paediatrics, Faculdade de Medicina da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil;* <sup>2</sup>*Infectious and Contagious Diseases, Faculdade de Medicina da Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.*

**Objective:** To describe the clinical and social demographic profile of patients with Primary Immunodeficiencies Diseases followed up at HC-UFGM. **Methods:** The patients followed up at HC-UFGM, diagnosed as Primary Immunodeficiency Disease according international consensus criteria, were studied. In these patients, a questionnaire was applied after informed consent, obtained from the parents and patients. It was performed nutritional evaluation by body mass index for age. The statistical analysis was performed using Epiinfo 6.0 package. **Results:** We studied 46 patients, 61% male sex. The followed diseases were found: common variable immunodeficiency, X-linked agammaglobulinemia, C1-esterase deficiency, IgA-deficiency, IgG subclass deficiency, specific antibody deficiency, chronic granulomatous disease and cyclic neutropenia. The median age was 14 years old and the median age at onset of symptoms was 1 year old. The diagnosis was made at median age of 9,5 years old. Only familial history was significant factor in early diagnosis ( $P = 0,03$ ). The patients showed high frequency of infectious diseases, hospitalization and need of intensive care. They present also allergic, autoimmune and chronic inflammatory disease. **Discussion:** The study shows the need of adequate investigation of patients with increased susceptibility to infection. The careful investigation

including familial history and immunological assessment may contribute to reduce morbidity and mortality.

### Su2.11. New Single Nucleotide Polymorphisms in the Gene Encoding the Phagocyte Oxidase 67 kDa Protein.

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The NADPH oxidase of phagocytes produces reactive oxygen species, necessary for the innate immune response against a variety of bacteria and fungi. One of its essential components is p67-phox, a 67-kDa cytosolic protein is encoded by the NCF2 gene located in the long arm of chromosome 1. Varied mutations involving the NCF2 gene result in an autosomal recessive form of chronic granulomatous disease (CGD), a primary immunodeficiency characterized by severe and recurrent infections. We have described nucleotide changes in the NCF2 gene of normal subjects and some p67-phox-deficient patients that apparently were not responsible for the CGD phenotype, but they potentially modify the expression of NCF2 gene. In order to determine if one of these changes corresponds to a non-pathogenic polymorphism, we analyzed its occurrence in the general population. This nucleotide change corresponds to an A→G transition in position -21 of the 3' end of intron 10 (IVS10-21A→G), within the branch acceptor sequence necessary for RNA splicing. This substitution was analyzed in 114 subjects: 36% were homozygous for A, 43% were heterozygous and 21% were homozygous for G. The region between exons 9 and 13 containing of NCF2 cDNA was analysed by RT-PCR amplification and sequencing. Complete (452 bp) and alternative (284 bp, without exon 11) fragments were observed in G genotypes. Functional analysis included respiratory burst activity and NCF2 gene expression. The DHR test as assayed by flow cytometry was 99.6% for G/G individuals, 99.45% for A/G and 99% for the A/A individuals ( $P = 0.81$ , Kruskal-Wallis Test). The NCF2 gene expression was assayed by real time PCR. The A/A genotype was used as a reference for comparing the A/G and G/G genotypes. The NCF2 gene expression was similar among subjects carrying the 3 genotypes ( $P = 0.937$ , Kruskal-Wallis test). We conclude that the IVS10-21A→G substitution corresponds to a polymorphism in the NCF2 gene, and its location within a branch acceptor sequence gives rise to alternative splicing. However it does not alter NCF2 gene expression or the respiratory burst activity under certain experimental conditions.

### Su2.12. Chronic Granulomatous Disease in Latin American Patients: Clinical Spectrum and Molecular Genetics.

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**Background:** Chronic granulomatous disease (CGD) is a primary immunodeficiency characterized by early onset of recurrent and severe infections. The molecular defects causing CGD are heterogeneous and lead to absence, low expression, or malfunctioning of one of the phagocyte NADPH oxidase components. The aim of this work was to analyze the clinical features and to investigate the molecular genetic defects of Latin American patients with CGD.

**Procedures:** The study included 14 patients. The diagnosis was based on a history of recurrent severe infections, impaired respiratory burst, and the demonstration of an underlying mutation by single strand conformation polymorphism (SSCP) or RT-PCR analysis, followed by genomic DNA or cDNA sequencing.

**Results:** Seven unrelated patients were found to have the X-linked form of CGD. Heterogeneous mutations affected the *CYBB* gene: 2 insertions, 1 substitution, and 4 splice site defects; two of them are novel. Seven patients presented with one of the autosomal recessive forms of CGD (A47-CGD); all had the most common mutation, a  $\Delta$ GT deletion in exon 2 of the *NCF1* gene. Pneumonia was the most frequent clinical feature, followed by pyodermatitis, sinusitis, otitis, and liver abscess. Patients with X-CGD were more likely to have initial infections before age 2 years and to have inflammatory obstructive granulomas later. None of the patients had severe adverse reactions to BCG immunization.

**Conclusions:** X-CGD patients from Latin America showed a high degree of molecular heterogeneity, including two novel mutations. Their clinical characteristics included early onset of infections and eventual obstructive granulomas. A47-CGD represented 50% of the reported cases, a higher prevalence than reported in other series.

### Su2.13. Hereditary Angioedema in Childhood-A

#### Practice Guideline.

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Hereditary angioedema is an uncommon condition which usually first presents in childhood. In the absence of appropriate treatment hereditary angioedema has a high mortality. There are a number of reports of successful short and long-term treatment with individual agents in those who suffer from hereditary angioedema, and the availability of these agents varies internationally. A recently published international consensus statement provides guidance for the management of such patients in adulthood, but there remains little guidance in the literature for the management of children and adolescents with hereditary angioedema. The needs of this age group are different to those of adults-in particular their symptomatology differs from adults, and the adverse effects of androgen treatment are different in children and adolescents. Here we review the treatment options available for children and adolescents with hereditary angioedema, and propose a simple evidence based guideline for the management of such patients.

### Su2.14. Abnormal Neutrophil's Chemotactic Activity in Children with Congenital Insensitivity to Pain with Anhidrosis (CIPA): The Role of Nerve Growth Factor in Chemotactic Activity.

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Congenital insensitivity to pain with anhidrosis (CIPA) is a rare and severe genetic disorder. It comprises absence of sensation to noxious stimuli and inability to sweat. Loss of pain sensation leads to skin lacerations followed by deep tissue infections. CIPA has a relatively high prevalence in the consanguineous Israeli-Bedouin population, who carry a common mutation in the TrkA gene encoding the tyrosine kinase receptor for nerve growth factor (NGF). This defect in the NGF receptor results in complete absence of nonmyelinated and small myelinated fibers in the dorsal root ganglia. NGF has been shown to have chemotactic activity on neutrophils in-vitro and in animal models. We analyzed neutrophil functions in children diagnosed with CIPA and hypothesized that neutrophil chemotaxis is impaired as a result of the abnormal activity of the TrkA receptor.

Twelve children genetically diagnosed with CIPA and carrying a 1926 ins-T mutation in the gene encoding for TrkA were recruited for the study. Two independent analyses of neutrophil functions including: chemotaxis, superoxide generation and phagocytosis, were performed in each child. Chemotaxis was assessed toward fMLP on agarose plates. Superoxide production was measured by reduction of acetyl- ferricytochrome c after stimulation of PMA, OZ and fMLP. Phagocytosis was assayed by ingestion of zymosan particles opsonized by pooled human serum.

Chemotactic migration of CIPA patients' neutrophils was significantly suppressed compared to controls ( $68.9 \pm 4.7\%$ ), while superoxide production and phagocytosis were normal. NGF alone did not act as a chemoattractant to neutrophils obtained from healthy donors. However, in the presence of NGF, neutrophils migration toward fMLP was elevated.

Our results demonstrate an impaired chemotactic activity of neutrophils in CIPA patients. The chemotactic defect can account for the high prevalence of severe staphylococcal skin and bone infections of these children. Decreased chemotactic migration can be attributed to the molecular defect in the TrkA receptor as it was revealed that NGF has a strong adjuvant effect on fMLP-directed migration of neutrophils.

### **Su2.15. The IgG 2F5-Like Antibody in Serum of Mexican Patients with AIDS Progression Prior to and after the Highly Active Anti-Retroviral Therapy.**

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The mAb 2F5 is one of the few cloned human antibodies with strong neutralizing activity against a broad range of HIV-1 laboratory and primary isolates. The evaluation of its anti-viral role in the infection and in the disease development usually meets difficulties because of its low amount or even absence in HIV-1-infected individuals. Using synthetic peptides containing the ELDKWA sequence from the gp41 membrane-proximal region recognized by the mAb 2F5 and mAb 2F5 as a positive

control, we assayed in ELISA the reactivity with ELDKWA of IgG purified from sera of 30 Mexican patients with disease progression prepared prior to and after the initiation of Highly Active Antiretroviral Therapy (HAART). We found all sera to contain different but substantial levels of antibody reacting with ELDKWA. In addition, initiation of HAART led to elevation of the seroreactivity, which decreased gradually to the initial level after 24-hours of therapy. Serum of a patient with highest reactivity was investigated in a detail and shown the reactivity level to correlate with the decrease in viral load. The detection of 2F5-like antibody in all 30 patients, not reported for other countries, may either be explained by differences in the infection phases investigated or assigned to the local genetic variations of HIV-1 found in recent epidemiological survey. Since the epitope eliciting the antibody is conserved in clade B population, and the epitope is linear and available in form of synthetic peptide as antigen, we explore the possibility that the seroreactivity with ELDKWA peptides may be a diagnostic marker of the infection and disease progression in this country. Further studies are in progress to verify the hypothesis that the observed elevation of ELDKWA-seroreactivity by the therapy is due to the improved immunological status resulting from a suppression of the viral load in the critical phase of the disease progression.

### **Su2.16. Genetically Determined Deficiency of the Innate Immune Defence Protein, MBL-Associated Serine Protease-2 (MASP-2).**

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The innate immune defence is important for preventing infections or for eliminating microorganisms, which have penetrated the mechanical barriers of the body. Cell-bound as well as humoral molecules participate in recognizing the microorganisms by their pathogen-associated molecular patterns (PAMPs). Among the first group (termed pathogen recognizing receptors, PRRs) are the Toll-like proteins, while collectins and ficolins represent the latter group, and thus belong to the broader group of pathogen recognizing molecules, or PRMs. One of the collectins, mannan-binding lectin (MBL) and two ficolins, H- and L-ficolin are capable of activating the complement system. MBL, H-ficolin and L-ficolin each, thanks to their oligomeric structure provides for 12 or more clustered recognition domains, which allows for high avidity binding to suitably spaced PAMPs. MBL, and the mentioned ficolins are all three found in complex with proenzymes, named MBL-associated serine proteases (MASPs). Three MASPs, MASP-1, MASP-2 and MASP-3 exist. After recognition of PAMPs the binding allows for the activation of the MBL-associated serine proteases. MASP-2 in turn activates C4 and C2 to generate the C3 convertase, C4bC2b. The outcome is the direct killing of the invader by the membrane attack complex or opsonization and killing via phagocytosis.

When examining about 100 patients with suspect immunodeficiency we identified one patient who had normal levels of MBL but did not have the capacity to activate the complement system when probed on a carbohydrate surface. The patient was found to

be homozygous for a SNP in the first domain of MASP-2 (the CUB1 domain) (Steengard-Petersen et al, *New Eng J Med*, 2003). This mutation, changing an aspartic acid to a glycine, prevents the association of MASP-2 to MBL and the ficolins. We have since observed homozygosity in two more patients. The SNP occurs in Caucasians with a gene frequency of about 1%, giving rise to deficiency in about 1/10,000. The gene frequency of 5.5 % stated in the above mentioned paper was based on 100 blood donors and was independently verified; however it appears to be a statistical aberration. We did not observe this MASP-2 SNP when analyzing samples from 200 healthy Asians from Hong Kong.

As innate immune system factors may be involved in poor outcome in cystic fibrosis 112 of such patients (aged 4–45 years), were screened for MBL and MASP-2 levels and for MBL genotypes and for the MASP-2 SNP. We observed a higher frequency of the MASP-2 SNP in this patient population (no homozygous individuals) but no correlation to lung function was seen with MASP-2 SNP or with MASP-2 levels.

Theoretically MASP-2 deficiency is a more serious condition than MBL deficiency, since MASP-2 is also required for complement activation by the H- and L-ficolin.

#### **Su2.17. Phenotypic Analysis of Peripheral Blood and Tissue Memory B Cells in Common Variable Immunodeficiency (CVID).**

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CVID is a heterogeneous primary immunodeficiency disorder characterised by hypogammaglobulinaemia and repeated bacterial infections. Whilst many cellular abnormalities have been described in such patient, recent interest has focused on subtyping such patients based upon the presence or absence of peripheral B cell memory subsets. We have developed a whole blood assay for the detection of naïve and memory B cells in peripheral blood and compared this to the previously described PBMC separation techniques for subtyping peripheral B cells. Additionally, we have analysed a cohort of CVID patients using this assay and compared their peripheral blood memory B cell subset distributions to that seen within their peripheral secondary lymphoid organs.

Four colour flow cytometry was undertaken upon lysed whole blood and density centrifugation separated peripheral blood mononuclear cells. A series of 10 healthy controls was used to compare the 2 methods and a further 30 controls analysed to set up a reference range using the whole blood assay. 11 CVID patients were subtyped by this method into MBO (no memory cells), MB1 (no class switched memory cells) and MB2 (normal memory cells) classifications. Patients within the MB0, MB1 and MB2 classifications that had secondary lymphoid tissue archived, were analysed by immunohistochemistry for the presence of memory B cells.

The whole blood assay performed well and correlated with the density centrifugation separation technique previously described. For the IgM memory cells the correlation coefficient ( $R^2$ ) between the two assays was 0.958 and for class switched cells it was 0.984. A normal reference range was established using healthy controls. As a percentage of peripheral B cells, naïve B cells =  $64\% \pm 24\%$  (2SD), total memory cells =  $32\% \pm 24\%$ , IgM memory cells =  $17\% \pm 16\%$  and class switched memory cells =  $15\% \pm 12\%$ .

11 CVID patients were analysed and 5 subtyped as MBO, 4 as MB1 and 2 as MB2. These subtypes were stable over a 12 month period. Tissue sections were analysed for a representative patient of each of the 3 groups. A lymph node from a MBO patient showed no evidence of germinal centre CD27 B cells compared to a normal tonsil. Similarly a lymph node biopsy of a MB1 patient showed no evidence memory B cells in a lymph node. Finally, a duodenal biopsy of a MB2 patient did show evidence of CD27 positive memory B cells within this tissue.

The whole blood assay appears to be as informative as the previously described assay for assessing B cell memory status. This assay is more cost effective and convenient to undertake within a routine laboratory. The reference ranges establish a minimum level of 12% for total memory B cells (mean  $\pm$  2SD) with no individual having less than 14% total memory cells, 4% IgM memory cells or 9% class switched memory cells. Such B cell memory subsets are constant over time in adult patients with CVID and correlate with the B cells identified in their peripheral lymphoid tissues.

#### **Su2.18. Plasma Interleukin-7 Levels before Highly Active Antiretroviral Therapy May Predict CD4+ T-Cells Recovery in HIV-Children.**

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**Objective:** The recovery of CD4+ T cells in children after HAART supports the existence of some homeostatic mechanism that detects low levels of CD4+ T-cells and activates the necessary mechanisms to achieve a T-cell repopulation. In our experience, the thymus plays a key role in this repopulation, being thus these homeostatic mechanisms likely to lead to an increase in the thymic production of new T cells. In order to establish the role of plasma IL-7 levels before HAART as prognostic marker of CD4+ T-cells recovery in HIV-infected children, a retrospective longitudinal study was performed in HIV-children on first-line of highly active antiretroviral therapy (HAART).

**Patients and methods:** The inclusion criteria were: a) to begin HAART with protease inhibitor; b) CD4+ T-cells  $\leq 20\%$  at entry to the study; c) at least 6 months on follow-up; d) more than one year of age. From initial 67 HIV-infected children on first-line HAART, only 27 HIV-children with CD4+ T-cells  $\leq 20\%$  had biological samples and they could be selected to be studied. Those HIV-infected children were divided into two groups according to their percentile 75 (P75) of plasma IL-7: a) **Low IL-7:** 21 HIV-children on HAART with **low IL-7** (IL-7  $\leq P75$  (11.97 pg/ml)) at baseline; b) **High IL-7:** 6 HIV-children on HAART with **high IL-7** (IL-7  $> P75$  (11.97 pg/ml)) at baseline.

**Results:** HIV-infected children with plasma IL-7 levels  $> 11.97$  pg/ml achieved CD4+ T-cells  $\geq 25\%$  faster than HIV-children with plasma IL-7 levels  $\leq 11.97$  pg/ml ( $P = 0.017$ ). This way, HIV-children with plasma IL-7 levels  $> 11.97$  pg/ml achieved CD4+ T-cells  $\geq 25\%$  to 10.6 (Confidence interval of 95% (CI95%): 0; 23.1) months and HIV-children with plasma IL-7 levels  $\leq 11.97$  pg/ml achieved CD4+ T-cells  $\geq 25\%$  to 37.1 (CI95%: 4.7; 69.4) months. The RP of immunological response to HAART were also calculated, and high IL-7 group had a RP to achieve CD4+ T-cells  $\geq 25\%$  of 3.24 (CI95%: 1.16; 9.0).

**Conclusion:** Our data indicate that IL-7 was a good marker of CD4+ T-cells recovery in HIV-infected children.

### Su2.19. Different Profiles of Immune Reconstitution in Children and Adults with HIV-Infection after HAART.

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**Objective:** To assess the different profile of immune reconstitution after HAART in HIV-children and HIV-adults who recovered normal CD4<sup>+</sup> T-cell counts on highly active antiretroviral therapy (HAART).

**Design:** Cross-sectional study.

**Patients and methods:** We carried out a study in 9 HIV-Rec-adults and 10 HIV-Rec-children on HAART with viral load (VL)  $\leq 400$  copies/ml at least during 6 months before beginning the study, and with CD4<sup>+</sup>  $\geq 500$  cells/ $\mu$ L at the start of immunologic studies and CD4<sup>+</sup>  $\leq 300$  cells/ $\mu$ L anytime before. These patients were compared with 9 HIV-A-adults and 8 HIV-A-children who never had had CD4<sup>+</sup>  $< 500$  cells/ $\mu$ L; and 15 Healthy-adults and 20 Healthy-control (HIV seronegative subjects).

**Results:** Children had higher values of TRECs and lower IL-7 levels than adults. When we compared Z-score values of HIV-adults and HIV-children, we observed that HIV-Rec-adults had lower TRECs levels ( $P = 0.036$ ) and similar IL-7 levels than HIV-Rec-children. The HIV-children had higher values of CD4<sup>+</sup>CD45RA<sup>hi</sup>CD27<sup>+</sup> counts than HIV-adults. However, HIV-children had similar percentage of CD8<sup>+</sup>CD45RA<sup>hi</sup>CD27<sup>+</sup> than HIV-adults, and higher values of CD8<sup>+</sup>CD45RA<sup>hi</sup>CD27<sup>+</sup>/ $\mu$ L than HIV-adults. HIV-children had similar values of memory CD4<sup>+</sup> T-cell counts and CD8<sup>+</sup>CD45RO<sup>+</sup> percentages than HIV-adults, but lower values of CD8<sup>+</sup>CD45RO<sup>+</sup>/ $\mu$ L than HIV-adults. Only, HIV-Rec-children had lower values of CD8<sup>+</sup>CD45RA<sup>hi</sup>CD27<sup>+</sup> Z-score than HIV-Rec-adults ( $P = 0.055$ ). Also, HIV-A-children had lower values of CD8<sup>+</sup>CD45RA<sup>hi</sup>CD27<sup>+</sup> ( $P = 0.046$ ) and CD8<sup>+</sup>CD45RO<sup>+</sup> ( $P = 0.015$ ) Z-scores than HIV-A-adults.

**Conclusion:** The restoration of CD4<sup>+</sup> and CD8<sup>+</sup> T cells after HAART in HIV-infected adults seems to be mainly the consequence of antigen-independent peripheral expansion of T-cells, although a role of the thymus in immune reconstitution in adults cannot be discarded.

### Su2.20. Understanding HIV Infection through Dynamical Modeling: Vaccines Won't Work, What Will?

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**Mathematical** modeling the transmission and dynamics of HIV infection can lead to a deeper understanding of the disparate experimental data and identify therapies most likely to be effective in controlling the disease. **Dynamically** modeling the time-dependent HIV prevalence in the Nairobi Sex Worker cohort lead to the prediction, *subsequently observed*, that the observed resistance or immunity to HIV infection exhibited by some members of the cohort is not absolute but transient. Thus, whatever cellular or other type of immunity that is responsible for this observed resistance to HIV infection is not strong enough to reliably prevent infection. **The** dynamics of HIV infection below the viral density threshold can only be inferred from a dynamical model extrapolated into this region. A model analysis of viral load growth during the *primary* HIV infection leads to the conclusion that the viral load doubling time immediately following infection

varies between 0.16 and 0.46 days. Analyzing published modeling results on the *steady-state* HIV infection in *chronically* infected patients leads to the conclusion that the viral load doubling times immediately following HAART interruption are virtually identical to the above results for primary infection patients. Moreover, the doubling times at threshold for infected patients treated with vaccine and/or IL-2 therapies before and/or after HAART withdrawal are virtually identical to those of untreated cohorts. Thus, neither prolonged familiarity with HIV infection nor vaccine and/or IL-2 therapies significantly increase the strengths of the immune system's humoral or interferon responses to HIV infection. **An** important question to answer is why vaccine and/or IL-2 therapies all fail to measurably strengthen the immune system response to HIV infection. **Dynamically** modeling the rebound of HIV infection following HAART withdrawal leads to the conclusion that the immune system's inability to reduce the viral load to a value below threshold is not due a failure in its phagocytic arm but to the failure of its cellular arm to clear productively infected cells from the host at a rapidly enough rate. This failure is traceable to the existence of a pool of *productively infected* CD4<sup>+</sup> cells (T cells, natural killer cells, etc.) whose half-lives *in vivo* are uncharacteristically long so that their numbers are easily replaced by newly infected, similarly long-lived, CD4<sup>+</sup> cells. One compartment of this pool consists of CD4<sup>+</sup> Natural Killer cells that are HIV-1 infectable, have higher survival half lives than typically infected CD4<sup>+</sup> T cells, and are unaffected by HAART. This pool cannot be cleared by the immune system, primed by vaccines and/or IL-2 or not, and is responsible for the observed, characteristic, saturation in the viral density curve at the viral set-point. Thus, an HIV infected immune system cannot eliminate all sources of viral production on its own- outside antiviral intervention is needed. **Expanding** HAART to include fusion blockers and overcoming its current deficiencies is the most promising path to take in seeking an effective therapy for HIV infection. Moreover, achieving and using the ideal HAART as a prophylactic would *prevent infection in the uninfected*.

### Su2.21. Model for the Repopulating Capacity of Transplanted T Cells.

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Transfer of gene-modified T cells is a potent option for treatment of immune deficiencies. A major technical challenge is to preserve the repopulating capacity of T cells during *ex vivo* expansion and culture. A limited survival of transplanted gene-modified T cells was observed in several clinical trials. This shows that the transplanted *ex vivo* expanded T cells may have a competitive disadvantage to the non-manipulated cells in the recipient, especially if the transgene does not confer a strong selective advantage. We established a transplantation model in the lymphocyte deficient mouse strain Rag-1<sup>-/-</sup> to analyze the repopulation capacity of transplanted T cells. Donor T cells were collected from Ly-5.1 C57BL/6 and Ly-5.2 mice. One population was treated with an *ex vivo* expansion and transduction protocol, the other population served as unmanipulated competitor cells. Both populations were mixed in several variations and transplanted into Rag-1 recipients. The competitive repopulation between treated and untreated cells was analyzed by distinguishing surface antigen expression. The data predict that not only high levels of gene marking, but also the

'fitness' of transplanted cells determine the effectiveness of therapies with gene-modified T cells.

### Su2.22. Apoptosis and the Emergence of Clonal Dual Positive CD4CD8 Cells in a Young Boy with CD4 Lymphopenia.

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BW presented at age 2 yrs with tonsillitis, pneumonia, lymphadenopathy, oral thrush, anaemia and hepatosplenomegaly. Investigations revealed lymphopenia  $wbc - 5.2 \times 10^9 /L$  (neut: 4.5, lymph: 0.5, mono: 0.1). Throat and mouth swabs revealed a  $\beta$  haemolytic group C streptococcus, pseudomonas aeruginosa and candida, chest radiography showed right middle lobe pneumonia. He was treated with a IV erythromycin and cefuroxime and made a good recovery. Within 3 months he presented with right sided pneumonia complicated by empyema, treated effectively with intravenous azlocillin and tobramycin. Four months later he presented with streptococcal pneumonia treated with intravenous penicillin and cefotaxime and was commenced on long term penicillin prophylaxis. At the age of 3 years he presented with a vasculitic rash on lower limbs and buttocks compatible with *pityriasis lichenoides*. At this stage he was assessed immunologically, this revealed normal serum IgG (13 g/l), IgM (6 g/l) and IgA (1.7G/L) and a poor response to pneumococcus and HIB vaccinations. Immunophenotyping of blood lymphocytes showed reduced CD3, CD4 and CD8 positive cells (0.16, 0.07 and  $0.09 \times 10^9 /l$  respectively), normal CD19+ B cells ( $0.27 \times 10^9 /l$ ) with a poor response to PHA and anti-CD3. Bone marrow examination was unremarkable. EBV serology was positive for IgG consistent with recently acquired infection. An apoptotic defect of both CD4 and CD8 cells was shown with excessive surface expression of CD95 on CD4 and CD8 cells, together with enhanced apoptosis in cell cultures as judged by propidium iodide and annexin V staining. At age 5 yrs BW mounted a good antibody response to pneumococcus and HIB and lymphocyte response to PHA was normal. However, his CD3, CD4 and CD8 lymphopenia (0.24, 0.11 and  $0.11 \times 10^9 /l$  respectively) persisted. BW was monitored routinely and at age 10, his lymphocyte count improved and a population of CD4 CD8 dual positive cells was found for the first time (lymphocytes  $1.2 \times 10^9 /l$ , CD3 0.76, CD4 0.24, CD8 0.18, CD4 CD8 dual positives 0.4). A TCRVB7 clone was identified in association with CD4 CD8 double positive cells confirmed by cell sorting analysis. This clone was not identified in earlier populations of cells and there was no evidence for TCR or IgH clones in earlier cell preparations. BW is currently well without prophylactic antibiotics. We present a child with an apoptotic defect and low CD4 count who developed a clonal CD4CD8 dual positive TCRVB7 T cell population with clinical improvement. While there is no evident cause for this population, viral aetiologies remain a possibility.

### Su2.23. Hypogammaglobulinemia and Lymphoproliferation in Two Patients with Heterozygous Deleterious Mutation of the Caspase-9 Gene.

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Fas triggering induces apoptosis by activating a caspase cascade composed of caspase-8, -10, -7, and -3. A second pathway induces mitochondrial release of cytochrome c that induces assembly of Apaf-1 and several procaspase-9 molecules, that are then activated, and activate caspase-3. In lymphocytes, Fas-induced apoptosis is involved in shutting off the immune response. Inherited deleterious mutations impairing Fas function cause the autoimmune lymphoproliferative syndrome (ALPS), characterized by autoimmunities, lymphadenopathy/splenomegaly, and expansion of CD4/CD8 double-negative (DN) T-cells. ALPS can be caused by heterozygous mutations of Fas (ALPS-Ia), FasL (ALPS-Ib) or caspase-10 (ALPS-II), whereas the mutated gene is unknown in other patients. Intriguingly, the parent transmitting the mutation is often healthy, which suggests a role for other complementary factors.

Looking for genes involved in ALPS, we detected a heterozygous single nucleotide mutation (A/C in position 710 of cDNA) of the caspase-9 gene in two non-consanguineous patients; one (patient-1) displayed an ALPS-like pattern associated with severe hypogammaglobulinemia, the other (patient-2) displayed mild hypogammaglobulinemia and developed Burkitt lymphoma. The mutation was not detected in 140 healthy donors and changed histidine 237 to proline at a site involved in the catalytic activity. In patient-1, it was inherited from the father and carried also by a sister, who were both healthy; similarly, it was carried by the healthy sister of patient-2 (his parents were not available). All mutated subjects displayed defective lymphocyte response to mitogens, with decreased proliferation, expression of activation antigens, and cytokine secretion; all of them displayed defective mitochondrial-induced apoptosis (induced by etoposide) and caspase-9 activation. Transient cotransfection of the wild-type and the mutated caspase-9 into 293T cells showed that the mutated form has a dominant negative activity. Moreover, stable transfection of the mutated form in Jurkat cells expressing an endogenous wild-type form showed that the mutated form inhibits not only etoposide-induced apoptosis, but also cell proliferation and CD25 expression. Similar results were obtained using the specific caspase-9 inhibitor Z-LEHD-FMK on PBMC activated with anti-CD3 mAb; cells treated with Z-LEHD-FMK displayed lower cell proliferation and CD25 expression than untreated cells. These data suggest that mutations of the caspase-9 gene may favor development of immunodeficiency and lymphoproliferation. It is noteworthy that a similar pattern has been previously described by other authors in a family carrying mutations of the caspase-8 gene.

### Su2.24. Haplotypes, Mutations and Phenotype Genotype Correlation in Turkish A-T Patients.

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The ataxia-telangiectasia mutated (ATM) gene was screened for haplotypes and mutations in 57 unrelated AT families of Turkish origin. Four microsatellite markers localized to an interval



spanning 1.4 cM were used to determine the haplotypes. Eight haplotypes were observed more than once across the families (47 % of all cases). Of the 27 probands carrying the recurring haplotypes, the most common, haplotype [A] carrying 3576G>A, was present in 11 patients (19% of all cases). Mutation screening was done by SSCP, PTT, dHPLC, and direct sequencing of cDNA, with subsequent sequencing of the abnormal regions. Twenty eight different mutations were identified in 46 patients, 14 of which were new; most were aberrant splicing. In all, eight founder mutations were found in the 92 alleles. Phenotypic features of the patients (presence of ATM protein, ATM kinase activity, CSA, serum AFP level, serum Ig levels, antibody production against pneumococcal polysaccharides, frequency of sinopulmonary infections, rate of progression of ataxia and outcome) were compared to genotypes. In general, disease outcome did not correlate with genotype; however, differences in disease progression were noted. Patients with mutation 3576G>A had milder disease with infrequent sinopulmonary infections, longer life span, and positive antibody production against pneumococcal polysaccharides. ATM protein was undetectable in those tested. Five of the 57 patients developed lymphoma, two developed leukemia and one developed thyroid carcinoma. This survey demonstrated that haplotyping is an efficient initial screening technique to identify mutations in Turkish AT patients and other ethnic populations.

#### Su2.25. Mechanisms of Immunotoxicity of Mycotoxins.

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**Background.** The fungi have been implicated in inflammatory reactions with immune and non-immune mechanisms, such as rhinitis, asthma, hypersensitivity pneumonitis, allergic bronchopulmonary mycoses, allergic fungal sinusitis, extrinsic allergic alveolitis etc. The exact prevalence of fungal allergies is not known. Studies based on skin tests suggest that at least 3–10% of adults and children worldwide are affected by fungal allergy (Bush RK, Portnoy JM, 2001; Homer WE et al., 1995). Apart from their direct allergenic effects, fungi may carry mycotoxins in their spores or produce volatile metabolites (Miller JD et al., 1998). Trichothecene mycotoxins are a group of structurally toxins produced mainly by *Fusarium* fungi found on many crops. A variety of *Fusarium* fungi produce a number of different mycotoxins of the class of trichothecenes. Three of the better known toxins are T-2, HT-2 toxin and deoxynivalenol (DON, vomitoxin). Inhalation of mycotoxins such as aflatoxins, secalonic acid D, zearalenone and trichothecens produced by *Aspergillus*, *Penicillium* and *Fusarium* fungi, may affect the immunological response of the lung tissue or present other hazards to human health.

**Aim of investigation.** To evaluate the possible mechanisms of immunosuppressive effects of T-2 toxin and DON on different cells and mechanisms of immune system.

**Results.** T-2 toxin and DON dose-dependently increase synthesis of anti-ovalbumin-IgE-Ab and this effect was not genetically restricted. Both mycotoxins dose-dependently inhibited stem cell proliferation in mice and PHA-induced MNC proliferation. T-2 toxin and DON suppress the activity of peritoneal, spleen, bone marrow and alveolar macrophages. The production of IL1 under the influence of toxins decreased as well as the activity of different hydrolases. Most sensitive were enzymes in bone marrow macrophages. T-2 toxin as well as DON suppressed the activity of all enzymes, especially thiol proteinases-catepsins B, C and H. Less sensitive were hydrolases of spleen and peritoneal macrophages.

**Conclusion.** T-2 and DON trichothecenic mycotoxins induce immunotoxic, mainly immunosuppressive effects on different immune cells via multiple mechanisms. Bone marrow cells are most sensitive to immunosuppressive effects of studied mycotoxins.

#### Su2.26. Immunotherapy by Cytogenes in Patients with Acquired Neutropenia.

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It is known that acquired neutropenia is disorder of neutrophil production predisposing patients to acute or recurrent bacterial infections.

The purpose of our study was to examine the effect of new cytogenes- synthetic short peptides on the base of naturally originated brain and bone marrow immunoregulatory peptides, designed in our laboratory on blood count and the development of infections in patients with acquired neutropenia of different genesis. It was provided hematological and clinical analysis using standard methods of patients' objective investigation. 37 healthy volunteers with previous cytotoxic chemotherapy or radiotherapy were under daily observation. Healthy individuals without cytogenes treatment or with normal blood counts served as controls.

It was found that peptides completely restored quantitative and qualitative index of blood neutrophil counts, and stimulated their functional activity. During all time it was not observed any complications deviation of blood count. Only 2 of 37 patients, observable during 4 months after immunotherapy had been illness with the easy form of a simple herpes virus.

The results of the presented study indicate that cytogenes may to restore differential blood count, avert the development of infections and to put in complete recovery in patients with acquired neutropenia of different genesis.

#### Su2.27. Evaluation of Humoral Immune Response in Children with Otitis Media and Control Group.

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##### Introduction:

Acute otitis media is acute inflammation of middle ear with signs and symptoms such as fever, otalgia, redness of tympanic membrane and accumulation of fluid in the middle ear. The most important bacteria that cause otitis media are streptococcus pneumonia and hemophilus influenza, both of them have polysaccharide capsule. Acute otitis media is one of the major childhood infection and cause temporary hearing losses that may affect language development of the patient.

We have not any information about level of immunoglobulin and complement in patient with otitis media in Iran. this along with frequent complications of otitis media encourage us to evaluate the level of immunoglobulin and complement in patients and help them if there is defect in immunity of them.

##### Materials and Methods:

In our study 75 children with otitis media and 75 healthy children under age of 6 year were evaluated. Serum gamma

globulins were determined with serum electrophoresis. IgM, IgG, IgA, C3, C4 in serum were determined with single radial immunodiffusion. Level of IgG<sub>2</sub> in serum was determined with Sandwich ELISA through our Homemade ELISA.

#### Result:

Show that otitis media occurred in male 1.3 times more than female. The level of gammaglobulins, IgM, IgA have increase in patient group versus health group in 1–2 year age group ( $P < 0.05$ ), but no meaningful increase in 3–4 and 5–6 years age group was noted ( $P > 0/05$ ). No statically difference was noted in the level of IgG, C<sub>3</sub> and C<sub>4</sub> between patient and health groups in all age groups ( $P < 0.05$ ). IgG<sub>2</sub> has decreased in group versus health group in 3–4 year age group ( $P < 0.05$ ) but no statistical difference was noted in 1–2 year and 5–6 year age groups ( $P > 0.05$ ).

### Su2.28. New Composition Immunotherapy in Treatment of Secondary Immunodeficiency Accompanied by Viral Infectious Syndrome.

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Among different clinical syndromes accompanied the secondary damage of immune system—the viral syndrome is leading one. Our studies last years had demonstrated facts: persons having few recurrent episodes, from 4 till 24, of acute viral respiratory infections (RAVRI) by the year may have also persisting viral infections such as CMV, VEB, HSV (I, II types), VH6. Often their clinical manifestations are similar “syndrome of chronic fatigue. We had studied 325 patients with RAVRI (75M, 50 F) in age from 20 till 72 years. All of them had clinical markers of secondary immunodeficiency accompanied by viral infection syndrome. We detected: the system of IFN (plasma’s IFN, IFNa, IFNg); immune system (CD3+, CD4+, CD8+, CD16+, CD19+, RBTL, IgG, IgA, IgM, neutrophilic phagocyt); herpes viral infection (HSV I/II types, CMV, VEB, VH6) by means of PCR and there were tested specific antibodies-IgG and IgM classes against HSV I/II types, CMV, VEB. 100% patients had have combine defects of IFN’s system, different deficiencies of T-cell’s immunity, NK cells, phagocytes. More than 90,0% patients had have suggesting herpesviral infections. All of this patients were treated following complex immunotherapy in different combination: 1) local and systemic IFN therapy (recombinant IFNa-viferon-1, -2, -3, -4, viferon oil), using high-, middle- and low-dose therapy during 2,5-3,5 month-base therapy; 2) specific and non specific passive intravenous immunoglobulins (cytotect, pentaglobin, intraglobin, octagam) only if it was necessary; 3) immunomodulation therapy thymus’s factors (tactivin, tymogen, imunofan) was directed to restoration of T-cell’s immunity; 4) synthetic preparation with polyvalent effects as polyoxidonium and licopidum (reconstruction of NK and neutrophilic leucocyt). At present it’s possible to combine immunotherapy and therapy with synthetic antiviral drugs. This kind of immunoreconstruction may be accompanied by “support therapy”. Using new kinds of complex immunotherapy in treatment of secondary immunodeficiency accompanied viral syndrome we had obtain positive clinical and immunological effects. Those effects had shown decreasing frequency of acute episodes of recurrent viral infections from 4-24 till 1-2 per year, elimination of viral pathogens (81,5% patients), reconstruction of destroy different immune mechanisms (88,8%).

### Su2.29. Therapeutic Efficacy of Gc Protein-Derived Macrophage Activating Factor for HIV-Infected/AIDS Patients.

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Although human immunodeficiency virus (HIV) is reported to be infective to macrophages, the activated macrophages do not support growth of HIV due to lack of DNA replication. Macrophages, when highly activated via inflammation, can recognize (development of receptors for viruses) and destroy a variety of viruses and their infected cells. Inflammation-primed macrophage activation process is the principal macrophage activation cascade that requires serum vitamin D<sub>3</sub>-binding protein (known as Gc protein) and participation of B and T lymphocytes. Stepwise hydrolysis of Gc protein with the membranous β-galactosidase (*Bgl<sub>1</sub>*) of inflammation-primed B cells and the *Neu-1* sialidase of T cells yields a potent macrophage activating factor, the protein with N-acetylgalactosamine as the remaining sugar (Yamamoto 1996. Mol Immunol 33: 1157-1164). Thus, Gc protein is the precursor for the principal macrophage activating factor (MAF). However, the MAF precursor activity of serum Gc protein of HIV-infected patients was lost or reduced because Gc protein is deglycosylated by serum α-N-acetylgalactosaminidase (Nagalase) secreted from HIV-infected cells (Yamamoto et al. 1995. AIDS Res Hum Retrovirus 11: 1373-8). Thus, deglycosylated Gc protein cannot be converted to MAF. Since macrophage-activation for enhanced phagocytosis and antigen presentation to B and T lymphocytes is the first indispensable step in both humoral and cellular immunity development, lack of macrophage activation leads to immunosuppression. Exogenously given MAF, however, can bypass the deglycosylated Gc protein and directly act on macrophages for activation. Stepwise treatment of highly purified Gc protein with immobilized β-galactosidase and sialidase generates the most potent macrophage activating factor (termed GcMAF) ever discovered but it produces no side effect in humans. Efficacy of GcMAF for several HIV-infected/AIDS patients was assessed by HIV-specific serum Nagalase activity because the serum Nagalase level is proportional to the amount of HIV-infected cells (or virus load). After approximately 16 weekly administrations of 100 ng GcMAF (once a week intramuscular injection), these patients exhibited insignificantly low serum Nagalase levels equivalent to healthy controls. Eradication of HIV and HIV-infected cells was confirmed by complete clearance of viral antigens (i.e., 24p and gp120) in their blood stream. The disease did not relapse four years after completion of GcMAF therapy.

### Su2.30. Pathogenic Significance of α-N-Acetylgalactosaminidase Activity Found in the Fusion Protein gp160 of Human Immunodeficiency Virus Type 1.

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Serum vitamin D<sub>3</sub>-binding protein (known as Gc protein) is the precursor for the principal macrophage activating factor (MAF). The precursor activity of serum Gc protein was lost or reduced in HIV-infected/AIDS patients. These patient sera contained α-N-acetylgalactosaminidase (Nagalase) that deglycosylates serum Gc protein (Yamamoto et al. 1995. AIDS Res Hum Retrovirus 11: 1373-8). Deglycosylated Gc protein cannot be converted to MAF, thus loses the MAF precursor activity. Since macrophage

activation for enhanced phagocytosis and antigen presentation to B and T lymphocytes is the first indispensable step in both humoral and cellular immunity development, lack of macrophage activation leads to immunosuppression and opportunistic neoplasm. Therefore, the Nagalase activity level of HIV-infected/AIDS patient sera regulates the degree of immunosuppression. Nagalase in HIV-infected patient blood stream was found to be complexed with patient immunoglobulin G, suggesting that this enzyme is immunogenic, seemingly a viral gene product. In fact, Nagalase was inducible by treatment of HIV-infected patient peripheral mononuclear cells with a provirus inducing agent, Mitomycin C. This glycosidase should reside on an outer envelope protein capable of interacting with cellular O-glycans. Although cloned gp160 exhibited no Nagalase activity, treatment of gp160 with 0.01% trypsin for 30 min expressed Nagalase activity suggesting that proteolytic cleavage of gp160 to generate gp120 and gp41 is required for Nagalase activity. In fact cloned gp120 exhibited Nagalase activity while cloned gp41 showed no enzyme activity. Since proteolytic cleavage of the protein gp160 is required for expression of both fusion capacity and Nagalase activity, Nagalase seems to be an enzymatic basis promoting fusion for initiation of infection. When cloned gp120 was treated with 0.001% trypsin for 30 min, Nagalase activity further increased significantly. This suggests that a proteolytic cleavage site of V3 loop of gp120 may be required for further enhancement of Nagalase activity for infectivity. Dipeptidylpeptidase IV (DPPIV) activity of CD26 is also able to cleave immediately adjacent (2 residues) to the tryptic site of V3 loop, supporting that CD26 is required for infectivity its enhancing capacity of Nagalase activity. Therefore, the Nagalase appears to play dual roles in viral infectivity and immunosuppression. The Nagalase activity has the pathogenic significance in HIV infection.

### Su2.31. Clinical and Laboratory Findings in Twenty Eight Patients with a Primary Immunoglobulin Deficiency Associated with Lymphadenopathy, Hepatosplenomegaly and Pulmonary Infiltrates.

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**Background:** Some patients with common variable immunodeficiency (CVID) present with adenopathy and hepatosplenomegaly. Tissue biopsy shows the presence of sarcoid-like granulomas.

**Objective:** To evaluate the underlying pathology of the lymph nodes, liver or lungs in patients with humoral immunodeficiency. The pathology will be correlated with clinical presentation, immunoglobulin levels, T and B-cell counts, and microbiology studies.

**Design:** Retrospective chart review of patients evaluated at the Primary Immune Deficiency Clinic, Mayo clinic Rochester between 1998 and 2004.

**Setting:** Large tertiary care medical center.

**Patients:** Patients with an immunoglobulin deficiency including CVID, IgG subclass deficiency and selective IgA deficiency who have adenopathy, pulmonary infiltrates and hepatosplenomegaly are included. Twenty eight patients were identified out of a total of one hundred and fifty with a humoral antibody deficiency. The electronic charts of the 28 patients were reviewed.

**Evaluation:** Patients underwent clinical and peripheral blood laboratory studies. The results presented are based on pathologic evaluation of lymph node, lung or liver biopsies. Tissues from

biopsies and bronchioalveolar lavage were also cultured for the presence of bacterial, fungal and mycobacterial infections.

**Results:** Sixteen of the 28 patients were males. The average age of the 28 patients was  $43 \pm 14$ , with a median of 44 and range from 16-23. Generalized adenopathy affected 89%, splenomegaly 71% and hepatomegaly 50%. 50% had sarcoid-like granulomas and 40% follicular lymphoid hyperplasia. Two patients developed lymphoma and one chronic lymphocytic leukemia. One patient had nocardia lung infection, another atypical mycobacterium, one osteomyelitis and two sepsis. Twelve (43%) had a significant autoimmune disease; this includes hemolytic anemia (21%), idiopathic thrombocytopenic purpura (32%), and inflammatory arthritis (7%). Other autoimmune disorders included Celiac disease, systemic lupus, type-1 diabetes, hypothyroidism and anti-phospholipid syndrome. All patients with autoimmune disease had adenopathy, 93% splenomegaly, 57% granulomas and 50% lymphoid hyperplasia. Compared with 75% of those without autoimmunity having adenopathy, 47% splenomegaly, 40% hepatomegaly, 33% lymphoid hyperplasia and 27% granulomas. Patients with granulomatous disease had significantly higher hepatomegaly ( $P = 0.044$ ) and hemolytic anemia ( $P = 0.035$ ). Average CD-19 cell count was 217 with a median of 83. Mean CD4 count was 676 and median 573, and mean CD8 count was 394 and median 278. No subgroup difference in immunoglobulin levels or T and B-cell counts was noted.

**Conclusion:** Adenopathy and/or hepatosplenomegaly in patients with immunoglobulin deficiency are not all granulomatous in nature but also include nodular lymphoid hyperplasia. Auto-immune disease is also prevalent among those patients. No difference in the immunoglobulin levels or the T and B-cell count between the two groups is noted. Larger number of patients and genetic mutation analysis might differentiate between the different subgroups of patients.

### Su2.32. Thirteen Years of *M. bovis*-BCG Culture-Positive Infection in an IL-12R $\beta$ 1 Deficient Patient: Treatment and Outcome.

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The IFN $\gamma$ /IL-12 pathway plays a critical role in controlling mycobacterial infections. Patients with mutations in this pathway show an exquisite susceptibility to these germs. Patient FP was BCG-vaccinated at birth, and at 6 months of age he developed a right arm regional/localized BCGitis. Thereafter, and until he was 2 years old, he presented with recurrent lung infections partially responsive to antibiotics. Pyrazinamide-resistant *M. bovis*-BCG was isolated from sputum at age 2. FP received multiple anti-mycobacterial drug regimes without clearance of mycobacteria or clinical improvement. At 9 years of age he was referred to our unit. He presented with a mild pulmonary insufficiency, and mycobacterial suppurative cervical adenitis. Traditional primary immunodeficiencies and HIV were ruled out. Recombinant (r) IFN $\gamma$  was added to his treatment (SC, 40 $\mu$ g/m<sup>2</sup>, 3 times a week), with resolution of the suppurative lesions. His sputum persisted culture-positive for another 6 years despite combined anti-mycobacterial treatment (enteral route; according to mycobacterial susceptibility), plus rIFN $\gamma$  (upon availability). IL-12R $\beta$ 1 deficiency was con-

firmed at age 14 (homozygous deletion-insertion GC->TT 1687-1688; leading to V541V-Q542X). At the age of 15, *FP* showed a profound clinical deterioration with severe respiratory insufficiency and a 10×5×5 cm thoracic mycobacterial mass. The patient's sputum and mass grew a multidrug resistant *M. bovis-BCG* (resistant to isoniazid, rifampicin, pyrazinamide, rifabutin, and ethionamide), and his serum showed no mycobactericidal activity *in vitro*. Protein-losing enteropathy and steatorrhea was also detected at that time. He initiated treatment with streptomycin/ciprofloxacin/clarithromycin/amoxicillin-clavulanic acid/clofazimine/linezolid (all sensitive by antibiogram), and rIFN $\gamma$  was raised to 100 $\mu$ g/mt<sup>2</sup>. Two months after this therapeutic scheme was started, his sputum turned culture-negative and his serum showed a positive bactericidal activity *in vitro*. At 8 months of treatment he presented with a sensitive peripheral neuropathy and optic neuropathy. Because of this complication, streptomycin and linezolid were withdrawn, and capreomycin was introduced. After 1 year of anti-mycobacterial treatment, the whole regime was suspended. Four months later, and under weekly azythromycin prophylaxis, the patient remains culture-negative (14 months), the thoracic mass has completely resolved, the peripheral and optic neuropathy show a significant but not complete recovery, and he leads a normal life. A combination of parenteral and enteral anti-mycobacterial treatment, including linezolid and rIFN $\gamma$ , helped to clear a 15 years-long/13 years culture-positive disease caused by *M. bovis-BCG* with acquired multidrug-resistance, in an IL-12R $\beta$ 1 deficient patient with impaired intestinal absorption.

### Su2.33. Equivalent Performance of VACUTAINER® CPT™ to Ficoll-Hypaque Gradient Separation in Maintaining the Quality and Function of PBMC from HIV Seropositive Blood Samples.

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For immune monitoring studies during HIV vaccine clinical trials, whole blood specimens from HIV seropositive patients may be collected at multiple sites and sent to a central location for PBMC isolation, cryopreservation and evaluation. Clinical researchers prefer to use cryopreserved PBMC because they enable batch analysis of multiple time points, and the samples can be archived for future evaluation. Typically, investigators have two sample collection options: (1) whole blood can be collected in a VACUTAINER® tube and sent to a central location for isolation of PBMC using Ficoll-Hypaque density gradient separation, or (2) whole blood can be collected in a VACUTAINER® CPT™ (Cell Preparation Tube), and PBMC isolated by centrifugation at the collection site, and shipped to a central location in the collection tube. The CPT option has some advantages over the Ficoll-Hypaque option such as ease of use, and less hands-on sample manipulation. For functional studies, it is desirable to know that PBMC samples are handled in a manner that will not compromise the ability of the cells to respond to activation stimuli. It is likewise important that sufficient recovery and viability of PBMC be retained in cryopreserved preparations. Therefore, it would be of value to know that PBMC collected and processed by either method perform in a similar manner. In this study we show a comparison of both PBMC collection options, using whole blood specimens from 19 HIV seropositive patients (CD4 > 350, viral

load < 50). The performance of samples collected by these two methods was compared by assessment of antigen-specific CD8+ and CD8- T cell immune responses (using cytokine flow cytometry), and cellular viability and recovery (using a hemacytometer and Trypan Blue exclusion). Antigen-specific IFN $\gamma$  expression was measured by stimulating PBMC with CMV pp65 and HIV p55 peptide mixes for 6 hours *in vitro*, followed by fixation, permeabilization, and intracellular staining. For data comparison, non-parametric paired t-test analysis of the intracellular expression of IFN $\gamma$ , viability, and cellular recovery was performed. The results indicate that after PBMC had been frozen and thawed, samples tested for CMV- and HIV- specific IFN $\gamma$  expression performed comparably to fresh PBMC under both collection conditions. The viability was significantly different between fresh and frozen PBMC derived using Ficoll-Hypaque ( $p = 0.0005$ ), although it was never less than 90% for PBMC harvested by either collection method. There were no significant differences in the post-freezing IFN $\gamma$  response, viability, or recovery between PBMC derived by Ficoll-Hypaque and by CPT, suggesting that either collection method would result in comparable yields of the viable cells needed to perform equivalently in functional studies. Overall, these data suggest that CPT is an efficient system for the collection and cryopreservation of functionally active HIV seropositive PBMC, as well as a viable alternative to Ficoll-Hypaque.

### Su2.34. Binding of the Green Tea Polyphenol, Epigallocatechin Gallate, to the CD4 Receptor on Human CD4+T Cells Resulting in Inhibition of HIV-1-gp120 Binding.

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**Background:** HIV-1 infection ultimately results in impaired specific immune function by virtue of the initial binding of the HIV-1 virion envelope glycoprotein, gp120, to the CD4 receptor. Epigallocatechin gallate (EGCG), a component of green tea, binds strongly to many biological molecules and is responsible for multiple health benefits. EGCG inhibits HIV-1 replication in human peripheral blood mononuclear cells *in vitro* by inhibiting the biochemical activity of HIV-1 reverse transcriptase resulting in a subsequent decrease in HIV p24 antigen concentration. We present evidence that EGCG (at physiologically relevant levels) binds to the CD4 molecule at the gp120 attachment site, thus establishing the potential use of EGCG as a therapeutic treatment for HIV infection. **Methods:** CD4<sup>+</sup>T cells were positively selected by immunomagnetic separation from platelet-depleted human leukopaks to obtain a highly purified CD4<sup>+</sup>T cell population. Nuclear magnetic resonance (NMR) spectroscopy and saturation transfer difference (STD) experiments examined the binding of EGCG to CD4. Inhibition binding studies were assessed by flow cytometry. **Results:** NMR titration studies revealed that addition of CD4 to 100  $\mu$ M EGCG voided the NMR signal from EGCG but not from the control, (+)-catechin. Addition of an IgG to EGCG had no effect. In STD experiments, addition of 21 mM CD4/binding site to 520 mM EGCG showed strong saturation at rings B and D of EGCG, while catechin or IgG had little effect. To investigate whether the binding of EGCG to the CD4 molecule on human lymphocytes is capable of inhibiting the binding of gp120 to CD4, we analyzed the binding ability of gp120

to the EGCG-treated and untreated CD4<sup>+</sup>T cells. EGCG markedly inhibited the binding of gp120 to CD4<sup>+</sup> T cells in a dose-dependent manner (0% at 20nM, 25% at 200nM  $P < 0.01$ , and 45% at 2000nM,  $P < 0.01$ ). Thus, at physiologically relevant levels of 200nM, EGCG exerted an inhibitory effect. The control catechin did not alter the binding capacity of gp120. Molecular modeling studies suggested a binding site for EGCG (Phe 43, Arg 59, Trp 62) in the D1 domain of CD4, the pocket that binds gp120. By calculation, we find that concentrations of EGCG, which are achievable by drinking green tea (170 nM), could reduce the binding of gp120 to CD4 by 10- to 20-fold. **Conclusions:** We have demonstrated clear evidence of high affinity binding of EGCG to the CD4 molecule. Along with this we, conclude that EGCG at concentrations equivalent to those obtainable by the consumption of green tea is able to significantly reduce the attachment of gp120 to CD4. It is reasonable to suspect that EGCG has potential use as adjunctive therapy in HIV infection. The potential competitive binding properties of EGCG for the CD4 binding sites by gp120 may translate to an HIV-1 preventative strategy. It is possible that green tea or its extracts may be involved in the future treatment of HIV-1 infection.

### Su2.35. Defect in CRAC Ca<sup>2+</sup> Channel Function Associated with Altered K<sup>+</sup> Channel Gating Properties in T Cells from Immunodeficient Patients.

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Activation of the T cell receptor (TCR) triggers a rise in intracellular Ca<sup>2+</sup>, essential for a productive immune response. Influx of Ca<sup>2+</sup> occurs through store-operated Ca<sup>2+</sup> release activated Ca<sup>2+</sup> (CRAC) channels, the molecular identity of which remains unknown. We identified a congenital human immunodeficiency (SCID) characterized by a complete absence of ion permeation through CRAC channels resulting in virtually no Ca<sup>2+</sup> influx following active or passive depletion of intracellular Ca<sup>2+</sup> stores. While a minimal Ca<sup>2+</sup> influx was seen in the SCID T cells at high extra cellular Ca<sup>2+</sup> concentrations, this influx could be blocked by 2-APB, La<sup>3+</sup>, Gd<sup>3+</sup> and SKF96365, respectively and is therefore likely to be mediated by CRAC channels. In the absence of CRAC mediated Ca<sup>2+</sup> influx, no other significant sources of Ca<sup>2+</sup> influx were detected in the SCID T cells suggesting that CRAC channels provide the predominant, if not the sole Ca<sup>2+</sup> influx pathway following TCR stimulation in human T cells. Intriguingly, the voltage-gated K<sup>+</sup> channel Kv1.3, instrumental for setting a negative membrane potential and providing the driving force for Ca<sup>2+</sup> entry, showed altered activation properties in the SCID T cells which were not secondary to reduced intracellular Ca<sup>2+</sup> levels. Potential candidate genes for the CRAC channel pore protein, particularly transient receptor potential (TRP) channel genes, were tested for their involvement in the Ca<sup>2+</sup> defect, but no alterations were observed. TRPV6 and TRPV5, two TRP channels with electrophysiological properties similar to those of CRAC channels, were able to restore Ca<sup>2+</sup> influx in the CRAC deficient T cells but this influx was not dependent on prior store depletion indicating that these channels form constitutively open Ca<sup>2+</sup> channels when heterologously expressed. A comprehensive complementation cloning approach was initiated to localize genes, which are able to restore Ca<sup>2+</sup>

influx in CRAC deficient T cells. To this end, human chromosomes derived from a monochromosomal somatic hybrid panel were introduced into Ca<sup>2+</sup> deficient T cells using micro-cell-mediated chromosome transfer (MMCT). We identified two chromosomes, which can functionally complement the Ca<sup>2+</sup> signaling defect in the CRAC deficient T cells. A subchromosomal fragment (SCF) representing a ~25 Mb region of one of the chromosomes defined by FISH mapping was sufficient for complementation of Ca<sup>2+</sup> influx in CRAC deficient T cells. Homozygosity mapping of both chromosomes revealed an autozygous region within the borders of the SCF defining a potential candidate gene region of 3 Mb. Genes in this region are under investigation for their contribution to Ca<sup>2+</sup> influx in T cells. From our studies in the CRAC deficient SCID T cells we conclude that CRAC currents provide the paramount Ca<sup>2+</sup> influx pathway in human T cells and that the defect in the SCID patients resides in a channel component itself or a protein involved in its regulation.

### Su2.36. Proposal for Diagnostic Criteria for Immunodeficiency Associated with DiGeorge Syndrome.

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DiGeorge syndrome is a congenital disorder characterized by developmental defects usually associated with immunodeficiency due to the hypoplasia or pathologic migration of the thymus. ESID/PAGID diagnostic criteria for DiGeorge associated immunodeficiency can be found on [www.esid.org](http://www.esid.org). We tested our cohort of patients with FISH proven del22q11 deletion against these currently valid and available criteria. As majority of patients failed the diagnosis despite genetically proven status of syndrome DiGeorge, we propose revised criteria for this disorder.

DiGeorge syndrome have genetic (del 22q11.2 or deletion on chromosome 10p) or non-genetic causes (eg. CHARGE association or alcoholic embryopathy). For the definition of immunodeficiency following situations apply:

#### 1. Immunodeficiency associated with del22q11.2

Male or female patient with reduced number of CD3 ± Tcells (less than 1500/mm<sup>3</sup>) in the first 3 years of life and two of the three following characteristics:

- 1.1. Conotruncal cardiac defect (exceptionally other cardiac)
- 1.2. Laboratory and/or clinical signs of hypoparathyroidism
- 1.3. Deletion of chromosome 22q11.2

#### 2. Immunodeficiency associated with DiGeorge syndrome del22q11.2 neg.

No criteria available due to extremely large variation in phenotype.

#### 3. Complete syndrome DiGeorge

3.1. Athymia documented as fewer than 50 recent thymic emigrants (CD3 ± CD45RA ± CD62L ± cells/cubic mm) and/or TRECs <100/100 000 T cells

3.2. Hypoparathyroidism

3.3. Heart defects supported by phenotypic features associated with DiGeorge syndrome.

These criteria represented solid diagnostic base for all our patients. Immune parameters vary greatly with age in DiGeorge syndrome, therefore, age-dependent changes must be considered in the diagnostic procedure.

This proposal of diagnostic criteria is widely open for discussions and revision.

### Su2.37. Immunological Characterization of Children with Transient Hypogammaglobulinemia of Infancy (THI): In Vitro Immunoglobulin Production Is a Candidate Predictive Marker.

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Transient hypogammaglobulinemia of infancy (THI) may represent a self-limited maturational delay of normal immunoglobulin synthesis or an early manifestation of a primary immunodeficiency. We evaluated distinct immunological parameters to predict the natural outcome and highlight their relevance in the pathogenesis of this disorder. B cells subsets, stimulated in vitro immunoglobulin production (sIVIP) and specific antibody response to tetanus toxoid, HiB and Streptococcus pneumoniae vaccines have been evaluated in 15 patients (mean age 12–36 mo) with a) IgG values <2SD below the mean for age associated to low values of IgA and/or IgM; b) intact cellular immunity; c) absence of clinical and immunological signs of other immunodeficiencies. No abnormalities in distinct B cell subsets have been documented except for 1/15 patients with a low expression of B memory unswitched cells (CD22+/CD27+/IgD-/IgM-) compared to age-matched normal controls (1.8% versus normal range of 6.9–11.2%). Stimulated in vitro immunoglobulin production (sIVIP) in baseline conditions and after addition of IL-10 revealed a normal response to all isotypes in 8/15 (53%) patients, while low values of sIVIgGP, sIVIgMP, sIVIgAP were identified respectively in 6/15 (40%), 3/15 (20%) and 3/15 (20%). sIVIP patients didn't correlate with serum Ig concentrations. Antibody response to vaccine antigens showed a low antibody response to Streptococcus pneumoniae in 2 children with altered sIVIgGP. These preliminary data emphasize the role of sIVIgGP and sIVIgAP as biological predictive and/or prognostic markers of this disorder and make us advance the hypothesis that a defect of class-switching mechanism can contribute to the pathogenetic scenario of THI. Extensive clinical and immunological evaluation is needed to better define this condition.

Acknowledgements: This study is in the contest of the Italian THI CSS-ID/AIEOP.

### Su2.38. Health-Related Quality of Life in Patients with Primary Immunodeficiencies in North America Receiving Home-Based Subcutaneous Immunoglobulin Replacement Therapy.

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**INTRODUCTION:** Intravenous infusions of immunoglobulin (IVIG) every 2–6 weeks either as hospital- or home-based therapy are the mainstay treatment for patients with primary immunodeficiencies diseases (PID) in North America. Weekly

self-administered subcutaneous immunoglobulin (SCIG) infusions at home have been proven to be a feasible, safe and effective alternative. However, data regarding health-related quality of life (HRQL) in PID patients is limited, even though self-administered infusions at home confront the patient with an array of challenges. In the present study we assessed the HRQL in patients switching from hospital- or home-based IVIG therapy to self-infused SCIG at home. **METHODS:** In a prospective longitudinal study in the US and Canada, 44 adult PID patients self-infused weekly SCIG (Vivaglobin®, ZLB Behring) at home for 12 months. HRQL was assessed at baseline, 6-, 9-, and 12-months using a generic instrument (SF-36), a questionnaire addressing treatment satisfaction (Life Quality Index), preference questions and the Health Status (assessed on a visual analogue scale ranging from 0-100 points). **RESULTS:** 28 patients had received hospital- ('pre-hosp group') and 16 home- ('pre-home group') based IVIG replacement therapy prior to study entry. After 12-months, 81% of the patients in the pre-hosp group and 69% in the pre-home group preferred SCIG replacement therapy; 90% and 91%, respectively, of the patients preferred home therapy. The pre-hosp group reported significant improvements ( $p \leq 0.05$ ;  $\geq 10$ -points difference) with respect to the SF-36 scales Vitality, Role-Physical, General Health and Health Transition. For patients in the pre-home group the General Health scale was improved ( $p \leq 0.05$ ; 10-points difference). Treatment satisfaction was significantly ( $p \leq 0.05$ ) improved in 13 of 15 items in the pre-hosp group, whilst patients in the pre-home group did not report notable changes after 12-months. Patients' perception of their health status increased from 71-points at baseline to 85-points at 12-months in the pre-hosp group and from 73 to 76-points in the pre-home group. **CONCLUSIONS:** Subcutaneous, home-based immunoglobulin replacement therapy significantly improved key parameters of the health-related quality of life and a wide range of aspects addressing treatment satisfaction of patients, who had received IVIG replacement therapy at the hospital. For patients already experienced with IVIG replacement therapy at home, no great changes were observed.

### Su2.39. Host MHC Influences Susceptibility to Hypersensitivity Reactions to Two Drugs Commonly Used in HIV Therapy.

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Host genetic factors influence the outcome of HIV infection at multiple levels including susceptibility to infection, rate of decline of immune function, the adaptation of the virus to the host's immune response and the development of AIDS related illnesses. Genes within the major histocompatibility complex (MHC) predispose to the development of an immune mediated hypersensitivity response to two active antiretroviral drugs. Abacavir hypersensitivity is strongly associated with the 57.1AH characterised by the presence of HLA-B\*5701 and a particular HSP70 polymorphism (HSP70-hom M493T). The presence of these markers is associated with a positive predictive value of >90% and a negative predictive value of >99%.

In contrast susceptibility to the multisystem or hepatotoxic reactions to Nevirapine are associated with the MHC class II region. *HLA-DRB1\*0101* was associated with such hyper-sensitivity but specifically in those with more than 25% CD4+ cells (OR 17.7;  $P = 0.0006$ ), suggesting the HLA class II molecules are likely to be directly involved in the pathogenesis.

Thus immunogenetics laboratories are likely to be involved increasingly in pharmacogenomic studies, both in providing a practical clinical application in preventing drug hypersensitivity reactions and for providing insights into the pathogenesis of immunologically mediated drug hypersensitivity reactions.

#### Su2.40. Multisystem Disease in CAEBV Infection.

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We describe a 16 year old Caucasian male with a 7 year history of chronic active EBV infection. At the age of 5 he developed uncomplicated glandular fever. He remained well until 9 years of age, when he presented with a history of headaches, hemiplegic migraine and splenomegaly. Ultrasound scan confirmed splenomegaly without hepatomegaly or lymphadenopathy. A head CT scan was normal. One year later he developed a left sided Bell's palsy and abnormal optic disc appearances. Ophthalmic review diagnosed bilateral pseudo optic disc swelling with retinal periphlebitis. Immunoglobulin analysis showed a raised IgG of 24.7g/L. At the age of 12 his height had fallen from the 10<sup>th</sup> centile at age 5 to the 0.4<sup>th</sup> centile and an endocrinology assessment was undertaken. A bone scan showed a bone age delay of 3 years but no other abnormalities.

At the age of 15 he was admitted with a 2 week history of pyrexia, lethargy and hepatosplenomegaly and an immunology opinion was requested. He had accompanying neutropaenia and thrombocytopenia. Investigations revealed 7000 EBV DNA copies per ml with serology consistent with past infection. Ultrasound revealed a 20cm spleen of uniform echotexture with additional large intra-abdominal nodes. Bone marrow examination was normal. Liver biopsy showed no evidence of lymphoma but some evidence of acute necrosis and chronic inflammation.

Immunological testing revealed hypergammaglobulinaemia with an IgG of 19.3 G/L (normal 6–16) and normal IgA and IgM. Anti neutrophil antibodies were detected but no other autoantibodies were identified. Cellular immunophenotyping was as shown below: CD3 = 1.91 (0.8–3.5), CD4 = 0.75 (0.4–2.1), CD8 = 1.14 (0.2–1.2), CD19 = 0.15 (0.2–0.6) and NK = 0.08–1.2). Currently his immunophenotype shows a CD3 = 1.12, CD4 = 0.6, CD8 = 0.47, CD19 = 0.02 and NK = 0.05.

TCR  $\beta$  immunophenotyping showed an expansion of V $\beta$ 17 T cells, accounting for 40% of the T cells. This population was mainly CD8 positive (>95%). TCR  $\beta$  clonality studies were consistent with a clonal population. A protein blot for SAP was unremarkable. These changes persisted after clearance of EBV which occurred by day 20, following treatment of the acute illness with Rituximab (anti CD20 MoAb) and intravenous immunoglobulin. Further testing outside of the acute illness has shown a low EBV count of 140 copies/ml in blood, a reduction in V $\beta$ 17 T cells to 9% and the demonstration of EBV DNA in a liver biopsy.

This individual exhibits some of the diverse, multisystem features that have been previously recognised in chronic active EBV infection. The underlying immunological defect of such patients is poorly understood. A clearer understanding of the underlying defects in such patients will advance our understanding of how chronic latent viral infections are immunologically controlled and improve therapeutic approaches to this difficult to manage condition.

#### Su2.41. Infants with Recurrent Infections and Low Immunoglobulins: Analysis of Immunoglobulin Normalization.

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**RATIONALE:** Infants presenting with recurrent infections and low immunoglobulins (Igs) lacking evidence of other immune deficiency disorders are often diagnosed with Transient Hypogammaglobulinemia of Infancy (THI). Although THI is considered a primary immunodeficiency, it is not well defined, and long term follow-up not reported. The purpose of this study is to: 1) Characterize infants with recurrent infections and low Igs. 2) Analyze characteristics to correlate them with time elapsed to Ig normalization. 3) Provide long term follow-up. **METHODS:** Patients evaluated between 1977-2004 were eligible if they: 1) Were term born and <24 months at presentation. 2) Had one or more Ig class 2 SD below normal. 3) Produced antibody to diphtheria and tetanus. 4) Had evidence of intact cell mediated immunity. 5) Lacked features of other immunodeficiency syndromes and 6) Had at least one follow-up set of Igs. Forty-nine patients had the following collected: Igs at presentation and follow-up, age at presentation and Ig normalization, and gender. The data was analyzed utilizing parametric survival analysis which assumes a logarithm of time to normalization has a logistic distribution. The time to normalization indicates the time from age at diagnosis. **RESULTS:** Of those included, 33/49 were male and 16/49 female. Mean age of presentation was 9.6 months. When the data was evaluated by gender, it took longer for the females Igs to normalize when compared to males. Twenty-five percent of the males had normalized by time of 0.3 years, while 25% of the females demonstrated normalization at 4 years. Likewise, 50% of the males had normalized at 1.1 years post-diagnosis, where it took the females 10.4 years for 50% to normalize. This difference was statistically significant ( $P < 0.001$ ). When the data was evaluated with respect to gender and age at diagnosis, it was found that the younger the females at diagnosis the longer time to normalization, but for males the earlier diagnosis the shorter time to normalization. This gender by age at diagnosis interaction was a strong trend ( $P$ -value = .083). Overall the course of these patients was benign. None had serious infections, however 2 female non-identical twins met criteria for IgA deficiency. Over half (59%) had a history of wheezing and 26% were atopic. Several have been followed to age greater than 20 years. **CONCLUSIONS:** 1) Females presenting with diminished immunoglobulins during infancy require longer time to normalization compared to males. 2) Females presenting younger take a longer time to normalize 3) Males presenting with low Igs during infancy display a strong trend toward longer time to normalization the older at presentation. 4) Children with decreased Igs in infancy are a heterogeneous population. In many, especially females, the hypogammaglobulinemia is neither transient nor

limited to infancy and thus diagnosis of THI can only be made retrospectively. 5) Children with low Igs and demonstrated ability to produce specific antibody have a generally benign course and fewer infections as they grew older, but excess atopy.

#### **Su2.42. The Role of Wiskott Aldrich Syndrome Protein in T Helper Cell Function.**

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Wiskott Aldrich Syndrome (WAS) is characterized by immune deficiency, thrombocytopenia, eczema, and increased incidence of autoimmune disease. The protein deficient in this syndrome, WASp, is required for actin-based cytoskeletal rearrangement and T cell activation. Recent data suggest that the Tec kinase, Itk, is required for WASp activation and actin polymerization. Additionally, Itk-deficient mice show defective T helper cell differentiation. Regulation of T helper cell differentiation and cytokine production is essential for mounting appropriate responses against pathogens, while dysregulation of these pathways has been implicated as a possible cause of hypersensitivity and autoimmunity. Given the increased incidence of eczema and autoimmune disease in WAS patients, we asked whether WASp might also be involved in T helper cell differentiation and function.

We have found that murine WAS<sup>-/-</sup> CD4<sup>+</sup> T cell cultures exhibit marked reductions in secretion of both the Th1 cytokine IFN-gamma and the Th2 cytokine IL-4. Nonetheless, intracellular staining revealed that WASp-deficient CD4 cells produce normal to elevated levels of IFN-gamma and IL-4. These data suggest that WASp may be involved in the secretion, but not production of IL-4 and IFN-gamma in CD4<sup>+</sup> T cells.

Surprisingly, following challenge with the Th2 inducing agent, *Schistosoma mansoni* eggs, WAS<sup>-/-</sup> mice show heightened responses including larger pulmonary granulomas and higher production and secretion of Th2 cytokines. Moreover, 30 days post-infectious challenge with the Th1 inducing parasite *Toxoplasma gondii*, WAS<sup>-/-</sup> mice show fewer numbers of brain cysts and increased production and secretion of IFN-gamma. Thus, in response to parasitic challenge, WASp-deficient mice can mount both a Th1 and Th2 response in vivo that may be exaggerated. Together, these findings suggest that WASp-deficiency has complex effects on T helper cell effector function, which may provide insight into the development of autoimmunity in patients with Wiskott Aldrich Syndrome.

#### **Su2.43. Cyclic CD4 Lymphopenia and Absence Interleukin-2: A Novel Immunodeficiency Presentation.**

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**Introduction:** Immunodeficiency with CD4<sup>+</sup> lymphopenia in the absence of HIV infection has been described and represents a heterogeneous condition. Interleukin-2 (IL-2) is a potent T cell growth factor produced primarily by activated CD4<sup>+</sup> T cells, and its synthesis is tightly regulated at the mRNA level. A closely related cytokine, Interleukin-15 (IL-15), shared two of the IL-2 receptor (IL-2R) subunits, but utilizes a unique IL-15 receptor  $\alpha$  chain

(IL-15R $\alpha$ ) instead of IL-2R $\alpha$  (CD25). Both cytokines appear to generate identical intracellular signals; however, they may have distinct in vivo properties. There are few reports related to altered regulation of IL-2 in primary immunodeficiency in human, and one case of human immunodeficiency arising from a mutation in the IL-2R $\alpha$ . We report here a novel immunodeficiency accompanied by cyclic CD4<sup>+</sup> lymphopenia, undetectable IL-2 and high IL-15. **Case report:** The patient is a 20 year old male, without underlying HIV infection, with recurrent mucosal candidiasis and cyclic CD4<sup>+</sup> lymphopenia (CD4<sup>+</sup> <250 cells/ul) since infancy. His mother died at the age 41 from *Pneumocystis pneumonia* with no HIV infection. Immune evaluation shortly before her death showed an IgG level of 440 mg/dl and a low CD4<sup>+</sup> T cell count. The patient presented with recurrent oral and esophageal candidiasis preceded or accompanied by a low CD4<sup>+</sup> T cell count and anergy to candida. His clinical course has been stable between the episodes of lymphopenia. In a recent exacerbation the patient presented with dysphagia and oral thrush that resolved with oral fluconazole, but was followed by severe diarrhea and weight loss. Colonoscopy revealed severe descending colon colitis with deep ulcerations. All bacterial, fungal, and mycobacterial cultures were negative. Total CD3<sup>+</sup> T cells count was low at 630 cells/ul (63%), CD4<sup>+</sup> T cells count was low at 200 cells/ul (24%). Immunoglobulin M (IgM) was low at 29 mg/dl (normal 48–271 mg/dl), IgG subclass 4 was low at 1.3 mg/dl (normal 13.5–73.9 mg/dl). IgG response to pneumococcal polysaccharide antigens was normal. Mitogen induced lymphoproliferative response was decreased to Pokeweed mitogen. Anti nuclear antibody was positive. The colitis resolved with intravenous antifungal and antibiotics. Repeat total CD3<sup>+</sup> T cells count doubled to 1,410 cells/ul, and the CD4<sup>+</sup> T cells count increased to 296 cells/ul. Lymphocyte phenotyping revealed low CD4<sup>+</sup>CD45RA<sup>+</sup> naive T cells at 3%, and proportionally increased CD45RO<sup>+</sup>CD4<sup>+</sup> memory T cells to 17%. The percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells was constantly low at 3% and 6%, and CD19<sup>+</sup>CD25<sup>+</sup> were undetectable (0%). IL-2 and mRNA for IL-2 were undetectable while IL-15 levels were markedly increased. **Conclusions:** We present a familial immunodeficiency in a patient with cyclic CD4<sup>+</sup> lymphopenia, recurrent mucosal candidiasis and ulcerative colitis. The finding of IL-2 deficiency with a concomitant increase of IL-15 may suggest a compensatory mechanism by which IL-15 mounts incomplete adaptive immune responses through an IL-2-independent pathway with a persistent susceptibility to fungal infections.

#### **Su2.44. Novel Severe T-Cell Immunodeficiency Syndrome Involving Absent Thymus, Coloboma, Bullous Dermatitis, Eosinophilia, and Elevated IgE.**

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**Objective:** To report a novel severe immunodeficiency of absent thymus, coloboma, bullous dermatitis (BD), eosinophilia, elevated IgE, and monoclonal T cell expansion with a CD4<sup>+</sup> defect. **Methods:** 10wk WM with hx of coloboma, eczema and otitis presented with BD and *Pseudomonas* superinfection involving trunk and eyes. Due to the unusual skin findings and ocular pathogen, an immune assessment was done as delineated in results section. Clinical course included skin improvement on antibiotics and 6d of immunosuppressants. Based on immunofluorescence studies, immunosuppressants were stopped. He then developed eczema herpeticum and recurrent BD, hypertension, HSM, and



lymphadenopathy. Despite IVIG and aggressive antibiotic therapy, he deteriorated clinically with HSV-1 viremia, *Parainfluenza* infection, *Pseudomonas* bacteremia, respiratory failure, and died with *Enterococcus* and CONS bacteremia at age 16wk. **Results:** Skin cultures grew *Pseudomonas* and CONS. While skin biopsy initially showed nonspecific dermatitis, classic features of pemphigus vulgaris were seen on 2 later biopsies. Direct and indirect immunofluorescence studies for pemphigus were not conclusive and serum was negative for desmoglein Ab. Initial CBC showed ANC 8960, ALC 1344, AEC 1991. Eosinophilia persisted (8–18%). Serum Ig levels prior to IVIG were IgG 1070mg/dL, IgA 16.1, IgM 120, IgE 39,900 IU/ml. HIV Ab and DNA PCR were negative. NBT and complement screens were normal. Isohemagglutinins were negative with blood type O. Lymphocyte phenotyping showed CD3+CD4+ 8.4% (#538/mm<sup>3</sup>), CD3+CD8+ 61.9% (#3963), CD3-CD19+ 20.4% (#1024), CD56+ 4% (#256), CD45RA+ 0% (#0), CD29+ 5.6% (#359), and a CD4:CD8 ratio of 0.14. Mitogen studies 10d off immunosuppressants showed very poor response to PHA, ConA and PWM. Karyotype was 46 XY, indicating neither maternal engraftment nor aneuploidy. Echocardiogram and abdominal ultrasound at age 11wk were normal. EBV PCR and CMV antigenemia were negative. No deletions were found by FISH at 22q11 and 10p13. RAG-1 and -2 mutational analysis is pending. Two monoclonal T cell expansions were detected by PCR of TCR gamma locus. HSV IgM was present and HSV IgG was not detected until after IVIG was given. Tracheal aspirate silver stain was negative. Autopsy revealed an absent thymus, HSM, and diffuse massive lymphadenopathy with lack of normal architecture. Interfollicular zones were depleted with scattered dendritic cells and plasma cells, few T lymphocytes, rare primary follicles, and increased stromal elements without increase in histiocytes. No evidence of GVHD was found. **Conclusion:** We describe a severe immunodeficiency syndrome consisting of CD8+ T cell predominance, CD4+ and nave T cell deficiency, absence of thymic tissue, poor mitogen responses, 2 distinct monoclonal T cell expansions, eosinophilia, and elevated IgE. Although possibly representing a new form of combined immunodeficiency, this group of findings is most consistent with a novel presentation of atypical DiGeorge syndrome.

#### Su2.45. Isolated PCP Infection in Children-A Systemic or Specific Immunodeficiency?

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We report 3 cases of pneumocystis carinii (PCP) pneumonia occurring in Caucasian male infants in the absence of other typical clinical or laboratory features of immune incompetence.

##### Case 1

A 5 month old male infant presented with a short history of poor feeding and respiratory distress. Following a CXR which was characteristic of PCP a bronchoalveolar lavage (BAL) was undertaken which confirmed the presence of PCP. The child responded to high dose steroids, Septrin and IVIG and was discharged on day 24. Immunological investigations on admission were as follows: CD3 = 0.23 (2.3–6.5), CD4 = 0.19 (1.5–5), CD8 = 0.05 (0.5–1.6) CD19 = 0.69 (0.6–3) and NK = 0.07 (0.1–3). Currently these are: CD3 = 0.27, CD4 = 0.20, CD8 = 0.07, CD19 = 0.99 and NK = 0.29.

The stimulation index following PHA was 156. Lymphocytes were low on admission at 1.7 (Normal = 6–9). HIV testing was negative. CD40 ligand was expressed upon activated T cells and CD132 (common  $\gamma$  chain) was demonstrated by protein blotting. TCR  $\alpha\beta$  immunophenotyping was normal. He is currently well on prophylactic Septrin and SCIG.

##### Case 2

A 4 month old infant was admitted with a 3 day history of poor feeding and respiratory distress. A CXR was consistent with PCP and a subsequent BAL confirmed the presence of PCP. He was treated and discharged home on day 14. Immunological investigations on admission were as follows: CD3–2.18, CD4 = 1.5, CD8 = 0.44, CD19 0.99 and NK = 0.24

The stimulation index following PHA was 2 on admission and 50 at his most recent review. Immunoglobulins were normal. HIV testing was negative. CD40 ligand was expressed upon activated T cells and protein blotting confirmed the presence of CD132. TCR  $\alpha\beta$  immunophenotyping was normal. Interestingly, this child's mother had been previously diagnosed with hyper IgE syndrome but he has not yet been shown to have a raised IgE or other typical features of his mother's condition. The child has had no further clinical episodes associated with immune incompetence and remains well on Septrin and SCIG.

##### Case 3

A 3 month old infant presented with a respiratory distress, weight loss and an enterocolitis. A CXR showed a bilateral hilar infiltrate and a BAL sample confirmed PCP. Immunological investigations on admission were as follows: CD3 = 4.79, CD4 = 4.07, CD8 = 0.61, CD19 = 0.39 and NK = 0.22.

The stimulation index following PHA was normal. Immunoglobulins were normal. HIV testing was negative. TCR  $\alpha\beta$  immunophenotyping was normal. CD40 ligand was expressed upon activated T cells.

All 3 children demonstrated a susceptibility to PCP without other typical features of immunodeficiency that usually accompany such a presentation in children with SCID or HIGM syndromes. The long term management of such children with apparent single pathogen sensitivity is of interest with regard to the use of a watch and wait approach with imperfect prophylactic treatments or bone marrow transplantation (BMT).

#### Su2.46. Combined Immunodeficiency with Defective Expression in MHC Class II Genes.

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The bare lymphocyte syndrome is a member of the relatively heterogeneous class of severe combined immunodeficiency and is associated with lack of expression of HLA antigens on some cells of hematopoietic origin. We report cases with BLS who had protracted pneumonia, repeated severe infections of the upper and lower respiratory tract, persistent diarrhea, candidiasis for several months. Laboratory tests revealed hypogammaglobulinemia, mild anaemic syndrome and leucocytosis. Peripheral blood immunophenotyping showed prominent decrease of CD4+ T-cells with inverted CD4/CD8 ratio. Antigen-induced lymphocyte proliferation and cell-mediated lymphocytotoxicity were absent in vitro. The detection of MHC molecules expression by flow cytometry using monoclonal antibodies to HLA-DR, DQ and DP antigens as well as to HLA class I antigens revealed lack of MHC class II

molecules expression on non-stimulated and IFN $\gamma$  stimulated PBMC. In contrast the expression of MHC class I molecules was conserved. SBT typing did not show any unknown polymorphisms in HLA-DRB1, DQB1 and DPB1 loci. Since the genes coding for class II polypeptides seemed to be unaffected, the genetic defect in these patients must have concerned the regulation of the expression of the HLA-DR genes. By studding mutations in transacting genes MHC2TA, RFX5, RFXAP we were able to detect genetic defects responsible for a failure to express class II genes in our patients.

**Su2.47. Efficacy and Safety of Subcutaneous Immunoglobulin Replacement Therapy at Home in Patients with Primary Immunodeficiency Diseases: Combined Analysis of Two Clinical Studies, One in North America and One in Europe.**

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**INTRODUCTION:** Intravenous infusions of immunoglobulin (IVIG) every 2–6 weeks have been the standard therapy for patients with primary immunodeficiency diseases (PID). Complications of IVIG therapy limit the use of IGIV at home. Weekly self-administered subcutaneous immunoglobulin (SCIG) infusions at home are becoming an alternative therapy regime. We evaluated a 16% pasteurized, preservative free, liquid, human IgG preparation intended for subcutaneous use with regard to safety and efficacy.

**METHODS:** In two prospective studies, one in the US and Canada (NA study) and one in Europe and Brazil (EU study), 125 PID patients between 3 and 74 years of age self-infused SCIG (Vivaglobin<sup>®</sup>, ZLB Behring) on a weekly basis at home. The patients began the SCIG therapy one week after their last IVIG infusions, and entered a 3-month wash in/wash out period followed by a 12-months efficacy period in the NA study and a 6-months efficacy period in the EU study. Clinical endpoints included the rate of serious bacterial infections (SBI), rates of all types of infections, as well as serum (S) IgG levels observed during the study. Safety variables comprised local and systemic reactions, laboratory investigations and vital signs.

**RESULTS:** A total of 5,953 infusions were administered to 125 patients in the course of the two studies. The patients received a weekly man dose of 158 mg/kg in the NA study and 89 mg/kg in the EU study during the efficacy phase of the studies. Only three SBIs (pneumonias) were reported during the efficacy phase in the two studies; two in the NA study and one in the EU study, resulting in an identical annualized rate of 0.04 SBI per patient. The annualized rate for any kind of infection was similar in both studies with 4.4 episodes / patient year in the NA study and 4.3 episodes/patient year in the EU study. Upper respirator infections were the most frequently reported types of infection. Mean S-IgG levels increased from 837 mg/dL to 922 mg/dL at 101% of the previous IVIG dose in the EU study and from 786 mg/dL at start of SCIG to 1040 mg/dL at 136% of the previous IVIG dose in the NA study. No study drug related serious adverse events were reported in any of the studies. Local injection site reactions, of mostly mild or moderate intensity, dropped rapidly in both studies from initially

85% to about 40% during the course of the NA study and from 65% to about 20% in the EU study.

**CONCLUSIONS:** Two major clinical trials have demonstrated that weekly self-administration of SCIG with a 16% IgG preparation is safe and effective in patients with PID, resulting in normalized stable S-IgG levels and providing satisfactory protection against severe bacterial infections.

**Su2.48. Abstract Withdrawn.**

**Su2.49. Association of IgA Deficiency and Ciliary Dyskinesia: Report of a Familial Case.**

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**Rationale:** Selective IgA deficiency is the most common form of primary immunodeficiency. However, there is a difference in frequency between the Caucasian and Asian populations (approximately 1 in 700 Caucasians and 1 in 18500 Japanese being affected). In addition some IgA deficiency individuals have increased susceptibility to upper respiratory tract or gastrointestinal infections. Primary ciliary dyskinesia is a phenotypically and genetically heterogeneous condition in which the primary defect is in the ultrastructure or function of cilia, highly complex organelles that are structurally related to the flagella of sperm and protozoa. Its clinical features include recurrent sinopulmonary infections, subfertility and laterality defects; the latter due to ciliary dysfunction at the embryological node. Then here we describe for the first time a familiar case of association of IgA Deficiency and Ciliary Dyskinesia.

**Methods:** Clinical and Laboratory evaluation of two siblings who presented to our division with a history of bronchiectasis and recurrent infections.

**Results:** We describe 2 male siblings, 35 and 39 years old, which started to present recurrent infections in childhood, mainly sinus and lung infections. They evolved with progressive loss of pulmonary function, and one of them had already been submitted to pulmonary transplantation. Family history included a father and older brother with similar symptoms who died because of disseminated bronchiectasis. Laboratory findings showed IgA deficiency with normal IgG and IgG subclasses and normal response to polysaccharide antigens. Pulmonary CT scans confirmed bronchiectasis. Further evaluation showed oligospermia with reduction of sperm motility. Electronic microscopy of respiratory mucosa was compatible with ciliary dyskinesia.

**Conclusion:** This is the first report of the association of IgA Deficiency and Ciliary Dyskinesia which reinforces the importance of a complete laboratory evaluation of patients with recurrent infections.

**Su2.50. Atypical Mycobacterial Infection and Chronic Granulomatous Disease: Experience of One Center.**

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Increased incidence of tuberculosis has been reported in CGD patients who live in endemic countries. There are multiple reports of complications following Bacillus Calmette-Guerin (BCG)

vaccination in affected patients, manifesting with axillary lymphadenitis and local ulceration. Also known are several cases of disseminated BCG infection in the setting of CGD. Less commonly, atypical mycobacteria other than the vaccine strain, have been recognized as causes of pulmonary infection in CGD.

We describe and discuss three CGD patients from one center followed between 1975 and 2004, who have been diagnosed with atypical mycobacterial infection.

Patient 1: A 27 year old man with a family history of autosomal recessive CGD, was diagnosed with CGD following *Mycobacterium fortuitum* pneumonia (previously reported). Patient 2: A 7 year old boy, one of three affected siblings with p47phox deficiency, was referred for *Mycobacterium avium* pneumonia and bronchiectasis, but had had numerous bacterial pneumonias previously. Patient 3: A p22phox deficient 7 year old boy was diagnosed with CGD shortly before presentation with disseminated *Mycobacterium chelonae* infection. All three patients responded to anti-mycobacterial antibiotics and have not had recurrent mycobacterial disease to date.

In our series, atypical mycobacterial infection either roughly coincided with or preceded the diagnosis of CGD. Surprisingly, all of our patients had recessive forms of CGD, 2 with p47phox deficiency, and one with the rarer p22phox deficiency, despite the fact that recessive CGD is less common and generally thought to be less severe than X-linked gp91phox deficiency. None of the patients had been on interferon gamma (IFN $\gamma$ ) or antibacterial prophylaxis at the time of development of their nontuberculous infection. Unlike patients with severe defects in the interferon gamma receptor, none of these patients had recurrences of mycobacterial disease. Although limited in-vitro data have demonstrated no difference in the inhibition of intracellular mycobacterial growth within PMNs and monocytes derived from either patients with CGD or normal volunteers, there appears to be a predisposition to infection with atypical mycobacteria based on our experience. We suggest ruling out CGD in patients with pulmonary or disseminated atypical mycobacterial disease and considering mycobacteria as possible pathogens in infections in CGD patients

#### **Su2.51. Use of Subcutaneous Immunoglobulin for Treatment of Chronic Warts in Patient with Common Variable Immunodeficiency.**

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We describe a 19 year old man with a diagnosis of common variable immunodeficiency (CVID) and a two year history of chronic warts. At eighteen years of age, he was diagnosed with CVID following a history of chronic sinusitis, chronic otitis media, and persistent cervical lymphadenopathy. His serum IgG levels were very low (37 mg/dL) although B and T cell subsets were within normal limits. Monthly infusions of intravenous immunoglobulin and continuous oral antibiotics led to improvement of infectious episodes and decreased lymphadenopathy, but warts on all his fingers and elbows persisted and became very disfiguring. He attempted laser therapy, cryotherapy, and imiquimod for removal of warts but met with little success. Difficult-to-treat warts are common in patients with immune deficiencies. Most cases are described in patients with cell-mediated immunodeficiencies, and immunomodulatory treatment is often focused on enhancing lymphocyte function. This patient had normal

delayed hypersensitivity responses but topical immunotherapy using dinitrochlorobenzene (DNCB) was also not effective. One syndrome, called WHIM (Warts, Hypogammaglobulinemia, recurrent bacterial Infections, Myelokathexis) is characterized by low serum gammaglobulins, failure of leukocytes to leave the bone marrow, and chronic neutropenia. Treatment of warts with intravenous immunoglobulin for these patients have been met with poor success. Our patient did not suffer from chronic neutropenia. His therapy for CVID changed from monthly infusions of immunoglobulin to weekly subcutaneous immunoglobulin infusions. Two months into subcutaneous immunoglobulin therapy, his warts resolved completely with no scarring, even though the patient did not receive any specific therapy for the warts while on subcutaneous immunoglobulin. The successful resolution of cutaneous warts with subcutaneous immunoglobulin treatment suggests subcutaneous immunoglobulin may provide a more steady-state level of IgG to allow for an enhanced ability to combat human papillomavirus in hypogammaglobulinemic patients.

#### **Su2.52. Distribution and Clinical Aspects of Pediatric Primary Immunodeficiencies in Taiwan.**

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Recent advances in immunologic techniques have lead to increased recognition of primary immunodeficiencies (PID). A review of pediatric patients with suspected immunodeficiencies in Taiwan from Jan. 1985 to Jan. 2005 and molecular/genetic analyses done on some patients were investigated. Based on the *International Classification of Disease, Ninth Revision* (ICD-9) and published articles, 99 patients with PID (22 females and 77 males) were identified: 59 (60%) with antibody production deficiencies, 15 (15%) with defective phagocyte function, 8 (8%) with combined B and T cell immunodeficiencies, 16 (16%) with T cell deficiencies, and one (1%) with primary complement deficiencies. Those with secondary immunodeficiencies were excluded from the study. Recurrent sinopulmonary infections (62%) were the most common clinical manifestation, followed by sepsis (57%), severe skin infection (40%), splenomegaly/hepatomegaly (27%), central nervous system dysfunction (22%), chronic diarrhea (22%), and failure to thrive (19%). Ten (10%) patients died, seven of infections, one of disseminated intravascular coagulopathy, one of hepatocellular carcinoma and one of lymphoma. Six novel mutations were found from 22 selected patients. This is the first report on PID in Taiwan, based on Chinese population.

#### **Su2.53. Experimental Study of the Complex Bi-Directional Interactions between Human Immunodeficiency Virus Type 1 (HIV-1) and the Protozoan Parasite *Leishmania*.**

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As a result of overlapping geographical distribution, coinfection with human immunodeficiency virus type 1 (HIV-1)

and protozoan parasite *Leishmania* is becoming a common event and presents an extremely serious clinical problem. The two diseases produce cumulative deficiencies of the immune response as both pathogens destroy the same immune cells, exponentially increasing disease severity and consequences. On one hand, *Leishmania* increases the risk of progression to acquired immunodeficiency syndrome (AIDS) and AIDS-related deaths. On the other hand, HIV-1 accelerates the dissemination of *Leishmania* infection and quickens the natural course of the parasitic diseases. The optimal therapeutic approach to HIV/*Leishmania* co-infected patients is still uncertain, due to the complex pathogenesis of the co-infection and the lack of literature and information. This study was aimed at exploring (1) the putative effect of *Leishmania* on the process of HIV-1 replication and transmission, and (2) the potential role played by HIV-1 in *Leishmania* infection. The experiments were performed with physiological experimental models, namely a human lymphoid tissue *ex vivo* culture system, human primary monocyte-derived macrophages (MDMs), as well as dendritic cells that include human primary monocyte-derived dendritic cells (MDDCs) and a DC-SIGN transfected cell line. The results show that (1) *Leishmania* enhances HIV-1 replication in both primary human macrophages and in human lymphoid tissues, (2) the *Leishmania*-directed increase in HIV-1 production is associated with a complex network of proinflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\alpha$ , and IL-6, (3) *Leishmania* also modulates the process of HIV-1 transmission through competition binding to DC-SIGN in dendritic cells, and (4) HIV-1, on the other hand, is also able to promote the intracellular growth of *Leishmania* in human primary macrophages through an enhanced uptake of the parasite. These findings help to unravel the molecular cellular mechanisms through which the two microorganisms interact, provide novel insight into the complex relationships between both human pathogens, and, most importantly, offer information that may be useful for the design of effective therapeutic strategies to control disease progression in persons dually infected with HIV-1 and *Leishmania*.

#### Su2.54. Hereditary Angioedema: A New Mutation and a Search of Gravity Factor?

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Hereditary angioedema (HAE) is an autosomal dominant disease, it manifests as recurrent attacks of intense, massive, localized edema without concomitant pruritus. In case of laryngeal edema, the attack can be life-threatening with the risk of asphyxiation if not treated adequately.

AIMS: Our objectives are to study the place of a new intronic mutation (640 G> A) as a molecular basis of HEA type I, and to investigate the effect of sequence variation within the coding region of C1 inhibitor gene (polymorphism 566 T > G) on disease expression as it has been reported recently (S-A Cumming al, J Med Genet 2003, 40: e114).

METHODS: The studies were performed on 15 members of an Algerian family, five patients with HAE type I and the others are

healthy. PCR products were purified using a QIA quick PCR purification kit (QIAGEN, CRAWLEY, UK) and the fragments were then sequenced by direct sequencing of PCR amplified DNA according to the ABI Prism 3700 DNA-Analyser (Biosystems). Fonctionnel tests were performed in HepG2 and Hep3B transfected cells by C1 inhibitor minigene (all exonic regions and only intron 2 and 3) followed by reverse transcription of RNA and agarose electrophoresis of cDNA.

RESULTS AND DISCUSSION: Five members of our family have HAE type I and all of them present a 640 G> A mutation within the intron2 in position+3 (IVS2+3) which isn't a canonic splice-site region. Fonctionnel tests reveal a presence of 02 different bands, the first one migrates in position 186pb (as W.T band which includes exon2) and the second one in 107pb (as 638 G> A mutation which causes total splicing defect of exon 2). We concluded that our new mutation 640 G> A, never described right now, causes a partial splicing defect of exon2. Nevertheless, only 02 patients with HAE have a 566 T > G mutation (inherited from the mother who's healthy and already described as a polymorphism). Fonctionnel tests have shown no difference in C1 inhibitor synthesis depending of the presence or no of 566 T > G mutation, and the severe clinical expression of the disease was observed in one patient without that mutation.

CONCLUSION: We describe a new intronic mutation in C1 inhibitor gene associated with HAE: 640 G> A that will cause a partial splicing defect of exon2, and we conclude that clinical severity of HAE in our family cannot be explained by the presence or the absence of a 566 T > G polymorphism.

## Immuno-dermatology

### Su2.55. Bexarotene Gel 1% vs. Vehicle Gel in Combination with Narrow-Band Ultraviolet B Phototherapy for Moderate to Severe Psoriasis Vulgaris.

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**Background:** Psoriasis is a chronic, autoimmune dermatological disorder characterized by erythematous, hyperkeratotic plaques. Ultraviolet B (UVB) phototherapy is FDA-approved for psoriasis and acts by inducing apoptosis in lesional T-cells. However, phototherapy is often inconvenient and less effective for thick plaques. Oral retinoids are teratogenic and rarely clear psoriasis as monotherapy. Retinoids thin the epidermis in thick plaques, allowing for better penetration of UVB; since limited UVB penetration through thick plaques inhibits efficacy, combining retinoids with UVB would be expected to yield synergistic improvement of efficacy. Bexarotene is a topical retinoid that is FDA-approved for the treatment of cutaneous T-cell lymphoma and is being clinically evaluated in the treatment of psoriasis. **Objective:** To determine whether bexarotene gel 1% plus narrow-band UVB (NBUVB) phototherapy is more effective for moderate to severe psoriasis than NBUVB plus vehicle gel. **Materials and Methods:** This was a single-center, double-blind, vehicle-controlled, bilateral comparison of bexarotene gel 1% vs. placebo, in combination with NBUVB, for moderate to severe psoriasis. Nine subjects were randomly assigned gels to be applied to target lesions on the right and left sides of the body. For 10 weeks, subjects applied bexarotene and placebo to the assigned sides. Subjects received NBUVB 3 times weekly for 8 weeks, beginning 2 weeks after the start of topical

treatment. Evaluations were done at week 0 (baseline), then weekly for 8 weeks, starting at the initiation of NBUVB. Efficacy was assessed using target lesion scoring and photography. **Results:** Bexarotene gel 1% plus NBUVB was significantly more effective than placebo plus NBUVB for moderate to severe psoriasis. The changes in target lesion scores were compared for active drug- and placebo-treated sides. The mean decrease in score from baseline for drug-treated lesions was 67.6% (95% CI 50.9%–84.3%), while that of placebo-treated lesions was 48.2% (95% CI 24.0%–72.5%). Because of the small sample size, the nonparametric Wilcoxon rank-sum test was used to analyze differences in score changes between two groups. The score improvement with drug was significantly greater compared to placebo ( $P = 0.04$ ). Scaling, erythema, and induration were reduced to a greater extent with drug. Adverse events were mild and included rash and skin irritation. **Conclusions:** Compared to placebo, bexarotene gel 1% appeared to increase the efficacy of NBUVB phototherapy with minimal toxicity. Further studies are warranted which include a larger number of subjects.

### Su2.56. The Higher pH Level Present in the Skin of Patients with Atopic Eczema Stimulates the Release of *Malassezia sympodialis* Allergens.

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Atopic eczema (AE) is a chronic inflammatory skin disorder. The pathogenesis of AE is not fully understood, but defects in the immune system and the ruptured skin barrier are of importance. The pH of both lesional and non-lesional skin in patients with AE is higher (pH 6) as compared to healthy skin (pH 5.5). The yeast *Malassezia* belongs to the normal human skin micro flora and can induce IgE and T cell reactivity in patients with AE. Previously, we have identified several IgE-binding components, including a 67-kDa protein, in *M. sympodialis* extract. Furthermore, based on amino acid sequencing and screening of IgE-binding clones from a *M. sympodialis* phage display cDNA library, we could isolate a clone with partial sequence of the above mentioned 67-kDa allergen. We have also shown that the 67-kDa allergen is exposed on the cell surface of *M. sympodialis*. The aim of this study was to investigate whether the 67-kDa allergen can be released under different pH conditions mimicking those of AE skin and healthy skin. *M. sympodialis* was cultured in Dixon broth pH 5.5 or 6.1 at 32 °C. Culture supernatants were analysed for the presence of IgE-binding components by immunoblotting using serum from an AE patient with specific IgE to *M. sympodialis*. The result showed that the release of the 67-kDa allergen was substantially enhanced in the culture supernatants with the higher pH. RACE-PCR, cloning and sequencing were used to find the complete coding sequence of the 67-kDa protein which showed similarity to the glucose oxidase family. This sequence was expressed in *Escherichia coli* as a 6-histidine tagged recombinant protein. The IgE-binding frequency of the recombinant allergen was 59%, to 22 sera from AE patients with serum IgE-antibodies to *M. sympodialis*, indicating that the 67-kDa protein is a major *M. sympodialis* allergen. In conclusion, we have cloned a major *M. sympodialis* allergen which is released to a higher extent at pH 6. The data suggest that the skin barrier in AE patients provides an environment that can enhance the release of allergens from *M. sympodialis*, which can contribute to the inflammation.

### Su2.57. Tuberculin Skin Testing Widely Used as a Diagnostic Aid for Tuberculosis, False Negative Outcome Has Questioned Its Specificity & Sensitivity In spite of Tuberculous Infection.

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**Purpose:** Skin test to exclude/include infection by Mycobacterium tubercle bacillus had been popular in the good olden days, but now has limited its wide spread significance.

**Methods:** Tuberculin skin testing an evidence of tuberculous infection had been in practice since years as diagnostic tool for epidemiological study of the whole/random population, to judge the degree of control of tuberculosis & exclude/include indications for BCG vaccination. Problems had arisen for its limiting value in patients having imminent tuberculous infection as confirmed by isolation of organisms in the sputum smear/culture, a conclusive chemotherapeutic response or clinical evidence of tuberculous infection. Since 1998 to early 2002 tuberculous conditions as under with false negative outcome had been documented.

Infection by atypical Mycobacterial infection.

Advanced age with tuberculosis.

False techniques of testing/ improper storage of tuberculin solution.

Overwhelming tuberculosis.

Early stages of sarcoidosis.

\*Miliary tuberculosis.

Hodgkins diseases.

Malnutrition.

Aids/Immunodeficiency.

Tuberculosis with hyperpyrexia.

Tuberculosis with generalized skin xanthoma.

In some cases no induration had been observed even to 100TU, despite the active tuberculous infection.

**Results:** In face of its limitations as an ideal diagnostic aid in the detection of tuberculosis, its role had been now a supporting test in the absence of BCG with conclusive evidence on radiological & clinical grounds.

**Conclusions:** The cellular mechanisms responsible for skin test reactivity are related mainly to previously sensitized CD4+ population of lymphocytes that are attracted to the site of skin test.

\*Tuberculin test had been negative, reactivity restored during the continuation of chemotherapy.

**Clinical Implications:** Tuberculin test despite short of ideal to conclude the diagnosis of tuberculosis still had been an important place in the diagnostic tool list.

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### Su2.58. Absence of Draining Lymph Nodes Results in Th2 Immune Deviation and Loss of Resistance to *Leishmania major* Infection.

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Lymph nodes (LNs) are important sentinel organs which harbour circulating lymphocytes and APCs. Antigen specific cellular and humoral immune responses are induced in LNs.

**Objective:** We investigated if LNs are required for the induction of protective immunity against *Leishmania (L.) major* infection.

**Materials and Methods:** We utilized mice lacking peripheral LNs due to in utero blockade of membrane lymphotoxin (LT)(peripheral (p)LN null). Mesenteric LNs were present in pLN null mice. In utero antagonism of membrane LT is transient and the organogenic defects in LN development are irreversible. The secondary lymphoid architecture in the adult progeny of mice undergoing gestational treatment is intact.

We also investigated the course of leishmaniasis in mice with genetic deletion of LT ligands (LT- $\beta$ -/-) or of the LT- $\beta$  receptor(R) which lack skin draining LNs (LT- $\beta$ -/-) or all LNs (LT- $\beta$ -R-/-). To distinguish between the role of LT and LNs in anti-*L. major* immunity, we also infected wild type  $\rightarrow$  LT- $\beta$ -R-/- bone marrow chimera which express the LT- $\beta$  receptor on hematopoietic cells. In addition, C57BL/6 wild type mice were treated with neutralizing LT- $\beta$ -R-IgG during *L. major* infection. Splenectomy experiments were performed in order to investigate the role of the spleen in resistance against *L. major*.

**Results:** pLN null mice of the resistant C57BL/6 strain developed systemic infection with increased IL-4 and reduced  $\gamma$ -interferon secretion in the presence of mesenteric LNs. Similarly, in gene deficient mice without local draining LNs (LT- $\beta$ -/-) or lacking all LNs (LT- $\beta$ -receptor-/-) leishmaniasis was disseminated and accompanied by increased secretion of IL-4 in CD4<sup>+</sup> T cells. The clinical course of infection was similar in LT- $\beta$ -receptor-/- mice and wild type  $\rightarrow$  LT- $\beta$ -R-/- bone marrow chimera, which similarly secreted more IL-4. Treatment of wild type mice with LT- $\beta$ -R-IgG during *L. major* infection did not abrogate resistance against *L. major*. Splenectomized C57BL/6 mice cleared the infection at the same rate as sham operated mice.

**Conclusions:** Peripheral LNs are critical for immunity against *L. major* in a genetically resistant mouse strain. These LNs provide a milieu which stimulates the induction of a Th1-anti-*Leishmania* response and prevents Th2 immunity. In the absence of pLNs mesenteric LNs and/or the spleen prime for a Th2 response. The spleen is dispensable for the induction of resistance against *L. major* in the presence of pLNs. Interaction between LT ligands and the LT-R is not required for control of *L. major* in C57BL/6 mice.

### Su2.59. Dissociation of Transactivation from Transrepression Activity by a Selective Glucocorticoid Receptor Agonist (SEGRA) Leads to Separation of Therapeutic Effects from Side Effects.

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Glucocorticoids (GCs) are the most commonly used anti-inflammatory and immunosuppressive drugs. Their outstanding therapeutic effects, however, are often accompanied by severe and sometimes irreversible side effects. Thus, the goal of GC pharmacological research is the development of new drugs which show a reduced side effect profile while maintaining the anti-inflammatory and immunosuppressive properties of classical GCs. GCs affect gene expression either by transactivation or transrepression mechanisms. The anti-inflammatory effects are mediated to a major extent via transrepression while many side effects are induced by a transactivation mechanism. Therefore, we

aimed to identify ligands of the glucocorticoid receptor (GR) that preferentially induce transrepression while avoiding or at least strongly reducing transactivation. Here we describe a selected non-steroidal selective GR-agonist (SEGRA), ZK 216348, which shows a significant dissociation of transrepression and transactivation activities both *in vitro* and *in vivo*. In a murine model of skin inflammation ZK 216348 is anti-inflammatorily active comparable to prednisolone after both systemic and topical application. A remarkable superior side effect profile was found with regard to blood glucose induction, spleen involution and to a lesser extend skin atrophy but not to ACTH suppression. Accordingly ZK 216348 should have a lower risk e.g. for induction of diabetes mellitus. Thus, the SEGRAs represent a promising new class of drug candidates with an improved effect/side effect profile in comparison to classical GCs. Moreover, they are attractive tool compounds for further investigating the mechanisms of GR-action.

### Su2.60. The Effect of Burn Trauma in Rats on Neutrophil Recruitment and Antioxidant Enzymes Activity in Epidermis.

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The results of autodermoplasty after severe burns may depend on inflammatory changes of epidermis in donor sites. This study was aimed at evaluation of myeloperoxidase (MPO) and antioxidant enzymes activity in unburned epidermis after burn trauma as well as after burn trauma complicated by endotoxemia in rats.

Rats were scald burned (20% of total body area) and E.coli liposaccharide was administered at the 4<sup>th</sup> day postburn (p.b.). Probes of unburned epidermis were cut at days 1,4,5 p.b. Activity of MPO, catalase (Cat), superoxide dismutase (SOD), glutathione peroxidase (GPx) were assessed. Results: MPO was elevated from the 1<sup>st</sup> day p.b. SOD and Cat were lowered at the 1<sup>st</sup> day and returned to normal by the 4<sup>th</sup> day. GPx was slightly activated at the 1<sup>st</sup> day. Endotoxin (ET) injection gave 2-fold increase in MPO activity from the 4<sup>th</sup> to 5<sup>th</sup> day which was accompanied by significant increase of GPx and SOD activity. Conclusions: burn trauma is followed by raise of MPO activity in unburned epidermis (especially at endotoxemia) and by early (at the 1<sup>st</sup> day) decrease of SOD and Cat activity. Further growth of MPO activity is accompanied by activation of antioxidant enzymes.

### Su2.61. Quantitative Changes of Mast Cells Following Topical Application of Honey on Third Degree Burns in Rats.

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**Objectives:** Hypertrophy scar and keloid may be occurred after burning. Mast cells have important roles in pathogenesis of them. The purpose of present study was determination of number

changes of mast cells in a experimental model of third degree burn.

**Methods:** A third degree burn was made in 24 rats by direct contact of skin with boiling water for 8 seconds. Rats were divided randomly into four groups. Group 1-Burns of control, 2, 3- Burns of these groups were received topical application of unboiled commercial honey one-time per day and twice daily. Group 4-Burns of this group were received topical application of nitrofurazone cream daily. Samples were extracted from 3 rats at day 15 and of another 3 rats at day 30, for light microscopical study were stained with toluidine blue. Numbers of mast cells were counted and Data were analyzed by non- parametric tests.

**Results:** Group 4 had highest number of mast cells at day 15 ( $30.43 \pm 41.1$ ) and of at day 30 ( $31.52 \pm 41.1$ ). Control group had lowest number of mast cells at day 15 ( $11.9 \pm 15.43$ ). Group 2 had lowest number of mast cells at day 30 ( $17.19 \pm 22.85$ ).

**Conclusions:** It is concluded that topical applications of honey on 3 degree burns, didn't have significant effect on the number of mast cells in comparison with control and routine treatment groups.

### Su2.62. Anti-IFN-gamma as a Universal Treatment for Th1 Mediated Skin Diseases.

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We pioneered anticytokine therapy (AT), proposing removal of hyperproduced IFN (Skurkovich, *Nature*, 1974) and TNF- $\alpha$  together with certain IFNs (Skurkovich, *J IFN Research*, 1989) to treat various autoimmune diseases (AD). Cytokines induced by IFN-gamma (IFN-g), such as TNF- $\alpha$ , IL-1 and their receptors, often work similarly. We conducted the 1st AT in 1975. We had good results treating Th-1 AD, e.g., RA, MS, and corneal transplant rejection with anti-IFN-g. Here we treated Th-1 skin diseases having Th1 cytokines or autoreactive T cells that induce IFN-g and other Th1 cytokines in the lesions. **Objective:** To test removing IFN-g in these diseases. **Method:** Anti-IFN-g was given topically or IM for 3–5 days. **Results:** *Acne vulgaris*. Propionibacteria and estrogen in adolescence, pregnancy and premenstrual period are IFN-g inducers. By day 2 after treatment, most pustular elements had dried. By day 4, infiltrated elements remained but had paled. *Psoriasis vulgaris*. Group A strep. antigen-reactive T cells and IFN-g are found in skin lesions of psoriasis. Patients (pts.) had a rapid reduction of the erythema leading to disappearance of papular infiltrates and after 2–3 weeks, clearing of plaques and complete remission in most pts. Some with small infiltrates on the legs were given UV treatments, after which they went into remission or completed therapy with 80% plaque reduction. *Psoriatic arthritis*. Th-1 cytokines are found in the synovial fluid. Pts. responded as in psoriasis vulgaris. *Seborrheic dermatitis*. Expression of IFN-g mRNA has been detected in skin biopsies. After treatment, itchiness and peeling of skin decreased, and the skin turned paler. *Herpes simplex virus type 1 (HSV1)*. Autoreactive T cells in lesional skin induce IFN-g. 3 to 3.5 hours after treatment, pts. noted rapid reduction of burning and pain in the lesion. After 2 days, scabs formed, which faded in 5 days. *Dystrophic epidermolysis bullosa* (a genetic disease but with some AD features). After the 2nd injection of anti-IFN-g, temperature normalized. On day 2, pain, swelling and hyperemia of the ulcerative lesions at the neck disappeared as did signs of infectious damage to the skin on the

back. By day 5, erosive lesions on the mouth epithelialized. By day 7, active epithelialization of the ulcerative skin lesions was observed. *HSV2 genital lesions*. Dysregulated production of IFN-g may exert a pathological effect by increasing virus replication. Within hours after the 1st topical application, itching and pain disappeared. By day 3–4, the eroded area epithelialized. **Conclusions:** These diseases are connected with immune disturbances in which IFN-g plays a key role and could also be treated, besides with anti-IFN-g, with anti-TNF- $\alpha$  and anti-IL-1 alone or together and soluble receptors to IFN-g, TNF- $\alpha$ , or IL-1, an IL-1 receptor antagonist or anti-CD20. Anti-IFN-g may have fewer complications than anti-TNF- $\alpha$ , such as infection, SLE, demyelination, and others. Rosacea may also be treated with anti-IFN-g.

### Su2.63. Buschke's Scleredema: Atypical Onset and Evolution. Case Report.

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In August 2004, a 58-year-old female patient presented to the Emergency Department of the Clinic for significant facial edema and erythema, generalized pruritus, dyspnea, altered general condition. The manifestations appeared after a diet rich in fats and spices. The patient reported that she had had two previous similar episodes for which she had received antihistamine and cortisone treatment on an outpatient basis. The case was interpreted as Quinke's edema and the patient was administered intravenous and oral cortisone and antihistamine preparations. During hospitalization, laboratory investigations were performed, which showed an extremely low ESR (2-5), along with leukocytosis that reached shortly  $48,500/\text{mm}^3$ . The patient developed during this interval Buschke's scleredema located in the cephalic extremity and upper thorax, which was confirmed by both histopathological examination and antitopoisomerase I antibodies. Blood examination excluded the possibility of malignant hemopathy. Ten days after admission, the patient became confused, presented severe headache, impaired vision and temporospatial disorientation. CT examination showed no changes. The patient was transferred to the Clinic of Dermatology with the suspicion of tertiary syphilis (in spite of a negative VDRL test), and penicillin treatment was initiated. After 3 weeks, the patient was discharged in an improved condition, but with manifestations of spastic paraparesis. The case is unusual in terms of onset and evolution and no direct causal connection can be established between Quinke's edema, Buschke's scleredema and spastic paraparesis manifestations.

### Su2.64. The Characteristics of Mucosal Immunity in Chronic Adult Periodontitis Patients.

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**Background.** Chronic periodontitis is the result of the immune response to specific bacterial infections in relation to oral flora. The present study investigates the quantitative and qualitative aspects of immune cells in the oral mucosa with correlation to disease progression.

**Methods.** We investigated 30 patients (23–58 years) with chronic adult periodontitis. The diagnosis was verified by traditional clinical and radiologic examinations. The control group was 6 healthy individuals without symptoms of periodontal disease.

Informed consent was obtained and biopsies of periodontal tissues were taken after approval from the Ethics committee. Serial frozen sections of gingival mucosa were assessed using the avidin-biotin-peroxidase technique with monoclonal antibodies against HLA-DR, CD3, CD4, CD8, TCR  $\gamma\delta$ -chains and CD20. All data were assessed using non-parametric statistics.

**Results.** This investigation demonstrated an interaction between antigens and immune cells which moved from the epithelium to the lamina propria related to the severity of the periodontitis. This process paralleled the loss of the protective potential of the epithelium.

Specific immunological features for the early stage of periodontitis include an increase in antigen pressure related to an increase in antigen-uptake by dendritic cells and subsequent persistent inflammation in the epithelium. Further immunological events occur in the lamina propria, with cytotoxic response reduced. The intensity of inflammation in this site was promoted by autoantigens inducing T cell sensitization. CD3+ and CD8+ T cells were the major cell populations associated with tissue damage, while B cells (CD20+) were the most significant cells in the progression of chronic adult periodontitis.

**Conclusion.** Chronic adult periodontitis caused by pathogenic microorganisms may in part be related to host response in an immune-mediated event. Therapy of severe chronic periodontitis should target not only microorganisms which should be eradicated but also the immune response in oral mucosa which may enhance disease progression.

#### Su2.65. Acquired Angioedema and Coagulopathy Several Years after Syphilis.

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Angioedema (AE) is a rare condition, which is characterized by recurrent, episodic, nonpruritic, subcutaneous or submucosal swelling that primarily affects the face, extremities, upper airways and gastrointestinal tract. AE results from unrestrained activation of the complement system due to an inherited or acquired deficiency of C1 inhibitor (C1-Inh). Acquired AE (AAE) manifests in the adulthood, usually after the fourth decade with low serum levels of C1, C1q, C2, C4 and C1-INH activity and can exist in two forms. Type I AAE is mostly associated with lymphoproliferative or neoplastic disorders that result in exuberant complement activation, which overwhelms normal C1-Inh reserves by accelerated consumption. Type II AAE is defined by the presence of autoantibodies against the C1-Inh, which interfere with its function, thus allowing unopposed complement activation.

We report the case of a 67-year old man with a distant history of syphilis treated with penicillin, who experienced his first episode of facial edema in 4/02 after ingestion of sweet myrrh root that subsided spontaneously. In 11/02 he developed diffuse swelling of his upper extremities and tongue. No triggers could be identified and a minimal work-up at that time was unrevealing. He remained asymptomatic until 1/04 when he experienced two episodes of tongue swelling that were preceded by minor trauma from poorly fitting dentures. He was treated in a local emergency department with diphenhydramine, prednisone, and epinephrine on both occasions with gradual resolution of edema over several days. He was not taking any medications that could cause angioedema, and did not experience concom-

itant urticaria or pruritus. Subsequently, his primary care physician discovered a suppressed CH50, and referred him to our clinic for further evaluation and management of recurrent angioedema.

On presentation to our office, he was asymptomatic, and had a normal physical exam. He had not had any swelling until after age 65 and no family history of angioedema. He has never had any bleeding tendency or thrombotic events. His laboratory evaluation revealed markedly decreased complement levels, CH50, C1q, C2, C4 (all below range of detection), depressed C1-Inh level and function, reactive RPR at 1:2 and *T. pallidum* particle agglutination, elevated lupus anticoagulant, anti-cardiolipin IgA and IgM but not IgG. His PT and PTT were significantly elevated, which did not correct even at 1:9 mixing with pooled normal serum. Factor XI and XII were 19% and 45%, respectively (50–150%). Fibrin split product was 320 (0–10) and D-dimer was beyond the assay range, even after serial dilutions.

Our case demonstrates interconnection between the complement and coagulation cascades at several levels. It also indicates that multiple autoantibodies can coexist in the same individual and suggests that syphilis might have been the inciting culprit, which resulted in AAE. This case exemplifies the diagnostic and therapeutic challenges associated with AAE, which will be presented along with a review of the literature.

#### Su2.66. Expression of the Costimulatory Molecules on Antigen-Presenting Cells in Atopic Dermatitis.

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**Background.** Atopic dermatitis (AD) is a common dermatologic condition that is characterized by pruritic and eczematous lesions which can be chronic and persistent. Skin lesions are histologically characterized by infiltrating activated T-cells, but the mechanism of this activation remains unclear. IgE-mediated facilitated antigen presentation by IgE-bearing dendritic cells (DC) to T-cells may be a key event in the pathogenesis of AD.

**Methods.** Punch biopsies were obtained from 9 patients with chronic AD after obtaining signed informed consent with the approval of the local ethics committee. AD was diagnosed according to the criteria defined by Hanifin and Rajka. Human skin biopsies taken from patients undergoing cosmetic surgery ( $n = 9$ ) served as normal controls. The expression of CD80 (B7-1) and CD86 (D7-2) was demonstrated on CD1a+ epidermal dendritic cells (DC) in AD lesions by immunohistological analysis.

**Results.** Cryosections of inflammatory skin were immunostained to localize the CD80+ and CD86+ cells. CD80+ as well as CD86+ cells were identified in the lesional epidermis and dermis. In the epidermis, cells expressing CD80 and CD86 were found at the suprabasal as well as the basal level, scattered throughout the epidermis in an LC-like distribution pattern, being dendritically shaped and exhibiting a membranous staining pattern, suggestive of DC. In this study, we were able to demonstrate that DC are the major epidermal cell population expressing the costimulatory molecules CD80 and CD86 *in situ*, with AD patients showing the greatest expression.

**Conclusion.** Costimulatory molecules are an essential factor in the generation of an effective immune response, as failure to deliver costimulatory signals during antigen presentation leads to



T-cell anergy. The in situ enhanced expression of CD80 and CD86 molecules on epidermal DC may be relevant to the pathogenesis of inflammatory skin lesions in AD.

### Su2.67. Differential Distribution of Lymphocyte Subpopulations in Peripheral Blood and Muscle Biopsies in Untreated Juvenile Dermatomyositis (JDM).

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**Rationale:** Juvenile Dermatomyositis (JDM) is a chronic inflammatory disease that is characterized by a distinctive pattern of skin rash and muscle weakness. Untreated children with JDM have significantly lower total lymphocytes and CD8 cells in their peripheral blood than healthy age-matched controls (O'Gorman & Pachman, *Clin Diag Lab Immunol*, 1995, 2000). The current study used immunohistochemistry to examine the distribution of infiltrating lymphocyte subsets in muscle biopsies from untreated JDM patients and flow cytometry to examine the concurrent distribution of circulating lymphocyte subsets. The purpose of this study was to demonstrate a redistribution of lymphocyte subsets between blood and muscle, which may support the conjecture that these immune cells are activated and migrate from circulation to the affected tissue in untreated JDM.

**Methods:** Five children aged from 3 to 10 with untreated JDM were enrolled with IRB-approved consent signed. Fresh frozen muscle biopsies were sectioned and tissue sections were immunostained with McAb against CD3, CD4, CD8, CD14, CD19 and CD56. Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and DAB substrate (Biogenix) were used in combination to detect the target antigens. Positively stained cells were examined under the microscope and counted within the measured area defined by the computer software. Peripheral blood samples of these children were analyzed on a Becton Dickinson FACScan flow cytometry (Mountain View, Calif.) using a scheme of four-color (FITC, PE, ACP and PerCP) three panel reagent system.

**Results:** Immunohistological studies of JDM muscle biopsies demonstrate a pattern of focal lymphocyte infiltration. For each mm<sup>2</sup> of muscle tissue, subsets of lymphocytes ranged in number from 36–87 for CD4, 10–28 for CD8, 40–52 for CD14, 10–40 for CD56 and 2–12 for B cells. Corresponding flow cytometry absolute counts were 474–1629 for CD4, 308–850 for CD8, 61–258 for CD56 and 339–992 for B cells. The relationship between flow cytometry and immunohistological results were evaluated by regression analysis using correlation coefficients. NK cells (CD 56) in blood is inversely correlated with the number of NK cells in the muscle biopsy from the same patient ( $r^2 = 0.5848$ ,  $P < 0.045$ ). Both CD4 and CD8 also showed an inverse association ( $r^2 = 0.3593$  and  $r^2 = 0.2063$  respectively), but not as statistically significant ( $P > 0.05$ ).

**Conclusion:** JDM is the most common pediatric inflammatory myopathy involving the immunological activation of several lines of immune cells. The current study indicates that while CD4, CD8, CD14 and CD19 positive cells are present in the muscle biopsies, there is a decrease of NK cells in the blood and increased cells in the same patient's muscle. The inverse relationship between NK cells in blood and muscle suggests their influx, trafficking from the circulation to the inflamed site which implies that they may play an important role in the pathogenesis of the target damage seen in JDM.

Supported by R01 AR48289, The Arthritis Foundation and The Myositis Association (to LMP).

### Su2.68. Thalidomide, an Anti-Inflammatory Medication, Inhibits the Induction of Tumor Necrosis Factor- $\alpha$ (TNF $\alpha$ ) in Ultraviolet B (UVB)-Irradiated Human Keratinocytes by Destabilizing the TNF $\alpha$ mRNA.

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UVB induces TNF $\alpha$ , a cytokine that contributes to UV-induced apoptosis of keratinocytes (KCs). Exposure of apoptotic antigens may then trigger an immune response in photosensitive disorders, such as subacute cutaneous LE (SCLE). Thalidomide (Thal) is beneficial in TNF $\alpha$  associated diseases, and our prior studies demonstrate that it inhibits UVB-induced TNF $\alpha$  mRNA and protein. The goal of the current study was to understand the mechanism of this inhibition and to define the role of IL-1 $\alpha$  in the induction of TNF- $\alpha$ .

All experiments were conducted with neonatal KCs. Cells were pre-treated for 2h with 50  $\mu$ g of Thal/ml and irradiated with 30 mJ UVB/cm<sup>2</sup>  $\pm$  IL-1 $\alpha$ . TNF- $\alpha$  protein in culture medium was quantified by ELISA. Real-time PCR (RT-PCR) was used to quantify KCs TNF- $\alpha$  mRNA.

As previously reported, TNF $\alpha$  gene expression 3 h post-UVB was 7.6 (range 7.4–7.9) times the level in sham-irradiated controls. Thal reduced this induction by 54% ( $P < 0.001$ ). TNF $\alpha$  gene expression 3h after treatment with UVB + IL-1 $\alpha$  increased to 116 (range 112–120) times the level in the no-IL-1 $\alpha$ , sham-irradiated controls. Thal treatment of KCs + IL-1 $\alpha$  reduced this induction by 70% ( $P < 0.005$ ).

By RNA protection assay, we found that Thal selectively inhibits TNF- $\alpha$  mRNA relative to other human cytokines (TNF $\beta$ , LT $\beta$ , IFN $\beta$ , IFN $\gamma$  and TGF $\beta$ ) studied in a multi-probe set. We showed that Thal inhibited UVB+IL-1 $\alpha$ -induced TNF $\alpha$  mRNA by 61% similar to the results seen with RT-PCR.

To examine the mechanism of Thal inhibition of UV-induction of TNF $\alpha$ , we assessed the TNF $\alpha$  mRNA half-life by using actinomycin D, an inhibitor of RNA synthesis. KCs were pre-tx with Thal and irradiated with UVB, IL-1 $\alpha$  was added immediately after irradiation. In two separate experiments, we found that the degradation of TNF $\alpha$  mRNA was increased at least three-fold in the presence of Thal + IL-1 $\alpha$ .

Thus, Thal inhibits UV-induced TNF $\alpha$  protein secretion by enhancing the degradation of TNF $\alpha$  mRNA. This action may contribute to the drug's therapeutic effects in photosensitive disease.

### Su2.69. Gene Transcripts as Potential Diagnostic Markers for Allergic Contact Dermatitis.

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The standard procedure for diagnosing allergic contact dermatitis is to perform a patch test. Since this has several disadvantages, the development of a new *in vitro* test system would be of immense value. Gene transcripts that distinguish allergics from non-allergics may have the potential to serve as the molecular basis for such a diagnostic tool.

In this study, we use the high-density microarray technology in the identification of differentially expressed genes in allergen-stimulated peripheral blood mononuclear cells (PBMC) from chromium-allergic patients versus healthy controls.

To qualify for the gene expression study, chromium-allergic patients should show a positive patch test to both CrCl<sub>3</sub> and K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> and mononuclear cell cultures established from the patients should have a strong *in vitro* proliferative response to CrCl<sub>3</sub> assessed with the <sup>3</sup>H-thymidine assay. Non-allergic controls would only be accepted for the study if they had no clinical reactions to both chromium compounds and no cellular *in vitro* response to CrCl<sub>3</sub>. Using these criteria, 3 out of 6 patients and 3 out of 6 controls were selected for the study.

Using an Affymetrix GeneChip®, the gene expression was analysed in PBMC cultures grown with 100 mg/ml CrCl<sub>3</sub> or in media alone for 24 h.

A total of 26 genes were differentially expressed by more than 2 fold ( $P < 0.01$ ) in allergen-activated PBMC from patients compared to controls. 18 of these were upregulated whereas 8 were down-regulated. Using real-time PCR, the differential expression was confirmed for three selected genes: *CISH*, *ETS2*, *CASP8*. This was statistically significant ( $P < 0.01$ ) for *CISH* and *ETS2*.

The 26 differentially expressed genes identified in this study may potentially function as diagnostic markers for contact sensitivity.

#### Su2.70. Potent Anti-Inflammatory Effect of Topical Nuclear Factor kappa B Decoy in Skin Inflammation.

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**Rational:** Treating chronic skin inflammation is an ongoing challenge in children and adults. The long-term use of topical calcineurin inhibitors and corticosteroids raises concerns about immunosuppression and malignancy. Nuclear Factor kappa B (NFkB) transcription factor plays a central role in the progression and maintenance of chronic skin inflammation. This study explores the possibility of using topical NFkB decoy (NFkBBD) as a safer therapeutic alternative for skin inflammation. **Methods:** Efficacy and skin penetration of a high affinity, specific NFkBBD was examined in nonclinical animal models. Since pig skin is similar to human skin, topical NFkBBD penetration was tested in porcine skin. A dustmite antigen induced atopic dermatitis (AD) Nc/Nga mouse model, was employed to further evaluate the potency of NFkBBD with approved drugs including corticosteroids, tacrolimus and pimecrolimus. **Results:** 0.5% topical NFkBBD application to pig skin resulted in efficient nuclear localization. Nuclear localization of the NFkBBD was evident throughout the epidermis as well as the dermal layers. In a dustmite antigen induced AD mouse model, topical NFkBBD (0.1–1.0%) produced a dose-dependent reduction of ear swelling (up to 80%), similar to betamethasone treatment. Topical NFkBBD was more efficacious at both doses (0.25% and 1.0%) compared to topical calcineurin inhibitors, tacrolimus and pimecrolimus. NFkBBD therapy, resembling betamethasone, decreased expression of the pro-inflammatory cytokines IL-1beta, IL-6, TNF-alpha and TSLP in inflamed ears. In addition, topical application of NFkBBD decreased inflammation, epidermal hyperproliferation and cellular infiltration. Cessation of 0.1% topical betamethasone resulted in a severe rebound of inflammation, whereas NFkBBD efficacy was maintained for at least 15 days after treatment termination. Notably, unlike betamethasone that induces skin atrophy within 4 days, prolonged NFkBBD application fails to

show any such side effect. **Conclusion:** These results show anti-inflammatory steroid-like efficacy of topical NFkBBD in skin inflammation without the side effects of topical steroids. Hence, these observations illustrate the potential of topical NFkBBD as a novel effective and safe therapeutic agent of inflammatory skin diseases.

#### Su2.71. Dual Diagnosis of Pemphigus Vulgaris and Connective Tissue Disease.

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**Background:** Patients with a dual diagnosis of pemphigus vulgaris (PV) and autoimmune connective tissue disease (CTD) have previously been reported. These few reports lack long-term follow-up and clinical details on the relationship of the two diseases.

**Objective:** We report thirteen patients diagnosed with PV who had an additional CTD diagnosis of systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), or both.

**Methods:** We conducted a retrospective analysis of the clinical profile, serological data, treatment, and follow-up of patients seen at one tertiary academic referral center.

**Results:** The thirteen patients were Caucasian with a mean age of onset of PV of 47 years (range 23–71). Ten were female and three were male. In three patients both diseases occurred simultaneously and in the remaining ten PV preceded SLE/MCTD. PV was severe and difficult to treat in twelve patients, though it eventually responded to therapy and these patients were in remission or stable and controlled on tapering therapy. Long-term follow-up, mean 8 years (range 3–18 years), revealed that in six patients the CTD was stable and under control, with periodic need for symptomatic therapy. In seven patients the CTD was controlled but required corticosteroids or other systemic agents. Life-threatening systemic involvement observed in SLE or MCTD, such as renal, cardiac, and neurological manifestations, were absent.

**Conclusion:** The thirteen patients reported here may have a genetic predisposition to develop autoantibodies. Unknown triggering factors are likely to influence the levels of autoantibodies in such susceptible individuals and result in clinical disease presentation. The differences in response to therapy of the two diseases, PV and CTD, would suggest that both similar and different mechanisms influence autoimmunity, define the extent of disease, and regulate the immune response.

#### Su2.72. Production and Characterization of Human Monoclonal Antibody Against Desmoglein 3 from Pemphigus Vulgaris Patient.

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Pemphigus vulgaris is a potentially fatal autoimmune mucocutaneous disease associated with production of IgG autoantibodies to desmoglein 3, a 130 kDa epidermal protein. To further characterize the epitope(s) of pemphigus vulgaris antigen we established a human-human hybridoma by fusion of the peripheral blood mononuclear cells with a human and mouse heterohybridoma. This hybridoma designated as PVMAB706 and stable in culture and demonstrated yield of monoclonal antibodies specific

for pemphigus vulgaris. Immunofluorescence, immunoblot, ELISA assays demonstrated that the monoclonal antibody bind to the intercellular cement substance and to 130 kDa protein present in the skin and specifically binds to recombinant desmoglein 3 protein, but no to desmoglein 1 protein. The IgG subclass distribution study demonstrated that the antibody is of IgG4 subclass in nature. The antibody was pathogenic as demonstrated in vitro by their ability to produce acantholysis in normal human skin and mucous membranes in organ culture or in vivo by the induction of disease in neonatal BALB/c mice. The relevance and value of these monoclonal antibodies in the pathogenesis of pemphigus vulgaris is discussed.

### Su2.73. Identification of Epitope for Autoantibody and Its Role in Basement Membrane Separation in Oral Pemphigoid.

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Oral pemphigoid (OP) is a chronic autoimmune disease characterized by blisters and erosive lesions in the oral mucosa. We identified an epitope for OP Abs within the integrin alpha ( $\alpha$ )6 subunit, and designated four subunit fragments (A,B,C,D). Immunofluorescence studies demonstrated that all the fragments were present in the oral mucosa. Sera of 20 patients with active OP bound only to fragment A and its subfragment A2. The peptide A2.1, within fragment A2, accounted for the binding of all the test sera. Controls were sera samples from 10 healthy volunteers and 30 patients with other pemphigoid diseases. The OP patient sera and immunoaffinity-purified OP sera, rabbit antisera for fragments A and A2, and mAb GoH3 produced basement membrane separation of oral mucosa. Biopsies of oral lesions from OP patients showed that Ab to integrin  $\alpha$ 6 binds to the roof of the blister and that laminin 5 binds to the base. This in vitro study identifies a peptide in the integrin  $\alpha$ 6 molecule to which Abs in the sera of OP patients bind, and which may play an important role in the pathogenesis of OP.

### Su2.74. Effects of Electrical Microcurrent on Open Skin Wound Healing in Rabbit.

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**Objective:** With regarding acceleration of fractures healing by application of microcurrent electrical stimulation, in this study the effects of microcurrent (microampere) on the full thickness incisional wound healing of rabbits were studied.

**Methods:** 30 male adult rabbits were randomly divided into control and experimental groups. Each group divided into 3 subgroups, based on duration of study (4, 7, 15 days). Under general anesthesia and sterile conditions, one full thickness incision on skin of each rabbit was made. From day of surgery experimental group received electrotherapy daily for 2 hours (current intensity: 200 A/cm<sup>2</sup>, current density: 66 A/cm<sup>2</sup>, frequency 0.5 Hz). Polarity was negative at first 3 days and was positive at following days. At the end, rabbits were killed by chloroform and 2 samples were obtained from wound tissue and adjacent normal skin for histological and tensiometrical studies. Number of neutrophils and fibroblasts and cross

sections of vessels were counted. Data were analyzed by student T test.

**Results:** Number of fibroblasts of experimental group at seventh day (862.6 + 70.1) were higher significantly ( $P < 0.01$ ) than relevant control group (468.2 + 59). Tensile strength of experimental group at fifteenth day (2138.2 + 212) was higher significantly than relevant control group (1443.1 + 218.8).

**Discussion:** It seems the administration of microcurrent serves to boost the electromotive force behind the moving ions and radicals sufficient to allow entry into injured region so that favorable metabolism and repair can take place. Microcurrent by increasing ATP production could accelerate wound healing process.

**Key words:** electrical microcurrent stimulation, wound healing, histology, tensiometry, rabbit.

### Su2.75. Morphometric Assessment of Nitrofurazone Ointment on Healing of Infectious Second Degree Burns of Rat.

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**Objectives:** The therapeutic effects of Nitrofurazone ointment on healing of infectious and non-infectious second degree burns of rats were studied from morphometric and tensiometric and microbiological examinations point of views.

**Methods:** The type of investigation was experimental. 40 adult males and females rats distributed into infectious and non infectious groups. Each group divided into control and Nitrofurazone subgroups. Rats burned according to the standard method and 10% of total body surface of them contacted with boiling water (95°C) or 6 seconds. The day of burning was day zero. Half of burns were contaminated by a standard sample of pseudomonas aeruginosa. The burns of Nitrofurazone groups received Nitrofurazone ointment topically one time per day. At 15<sup>th</sup> and 30<sup>th</sup> day morphometric and microbiological examination and at 30 the day tensiometrical test performed. Data were analyzed by student test method.

**Results:** Results of the Nitrofurazone groups were significantly better than control groups. With regard of the results of present investigation it is concluded that topical application of Nitrofurazone had positive and significant effect on second degree burn wound healing of rats and in non-infectious burns it's effect was better than in infectious burns.

**Key words:** Nitrofurazone. Burn. Morphometry. Rat.

### Su2.76. Effect of Low-Power Gallium Aluminum Arsenide Laser Radiation on Mast Cells of Open Skin Wound Bed of Rats.

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**Objectives:** The effect of low power Gallium Aluminum Arsenide Laser (Ga. Al. Ar. Laser) radiation on numbers and degranulation of mast cells of open skin wound bed of rats from quantitative histological point of view were studied.

**Methods:** 46 male rats were randomly divided into experimental and control groups. Each group divided into 3 subgroups. Under general anesthesia and sterile conditions one full thickness skin circular wound were made on the dorsum of neck of each rat. The wounding day was day zero. From day one 1/2 doses of anesthetized drugs were injected to all rats and also experimental rats were received Ga. Al. Ar. laser which its energy density was

1.2 J/cm<sup>2</sup>. At the day 4, day 7, and day 15 after doing daily treatments, several rats were killed by ether and one sample was obtained from wound bed and normal adjacent skin from each rat. Samples were fixed in formalin saline and were prepared for routine histological study and sections were stained by % 1 watery solution of toulidine blue. Total number of mast cells and its grades (No. one, No. two, and No. three) were counted. In grade one, mast cell was intact and in grade two some granules have been extruded from the cell and in mast cells of grade 3, degranulation is more extensive and wide spread. Data were analyzed by Mann Whitney U test method.

**Results:** Total number of mast cells and its grades of control group at the day 4 was higher than experimental group, and difference of grade one mast cell with experimental group was significant ( $P < 0.01$ ). Total number of mast cells and its grades of experimental group at the day 7 and day 15 were higher than control groups respectively ( $P < 0.05$ ). Low power Gallium Aluminum Arsenide Laser radiation on open skin wound of rats were reduced significantly intact mast cells at inflammatory phase and increased total of them at proliferation and remodeling phases of wound healing process.

**KEY WORDS:** Laser, Mast cell, Wound Healing, Skin, Histology, Rat.

## Laboratory Immunology

### Su2.77. Influence of Atopic History on Cord Blood IgE.

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**Background:** Although the value of cord blood IgE in predicting the development of allergic diseases is unclear but it is widely used as a screening parameter for atopy. Therefore it is necessary to define factors affecting cord blood IgE values.

**Objectives:** To determine the influence of atopic history in relatives on cord blood IgE. **Methods:** Cord blood obtained immediately after birth from a total of 201 newborns, including 100 high risk infants (with history of atopy in relatives) and 101 newborns without family history of atopic disease. Mothers were recruited prenatally from the Obstetric ward of Hafez Hospital, Shiraz, Iran. Total IgE levels were determined by use of ELISA system.

**Results:** Family atopic history does not correlate with elevated cord blood IgE levels as 51% of newborns in case group (with family atopic history) and 64.3% of newborns in control group had values of cord blood IgE higher than 0.5 IU/ml ( $P = 0.158$ ). No significant association could be shown between cord blood IgE distribution and gestational age, birth weight, and age of mother. **Conclusion:** Family history of atopy does not play a significant role in determining the level of IgE in cord blood.

### Su2.78. Lymphocyte Adenosine Deaminase Activity in Children with Idiopathic Nephrotic Syndrome.

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Adenosine deaminase (ADA) activity, as a marker of cell-mediated immunity, was evaluated in the serum (S-ADA) and lymphocyte (L-ADA) of 47 children with idiopathic nephrotic syndrome and 23 healthy controls. The mean S-ADA and L-ADA levels were significantly raised in active nephrotic syndrome (ANS) and in its sub-groups in comparison to controls. The ADA activity was significantly more elevated in relapsers than the first attack of nephrotic patients and the frequent relapsers had the highest enzymatic levels both in serum as well as lymphocytes. A significant positive correlation was found between serum and lymphocyte ADA levels ( $r = 0.736$ ,  $P < 0.01$ ). In remission, the S-ADA showed significant fall in comparison to their corresponding ANS value ( $P < 0.001$ ) and reached the level of controls. The mean L-ADA also showed reduction but the difference was statistically insignificant and the value was significantly raised, when compared with controls. The enzyme activity in serum and lymphocytes normalized in long-term remission group. Thus, ADA activity was abnormal in ANS cases and L-ADA demonstrated change both in active as well as remission stage of the disease.

Key words: Nephrotic syndrome, cell mediated immunity, adenosine deaminase activity.

### Su2.79. ESN: A Potent Natural Inhibitor of Proliferating and Inducer of Apoptosis on K562 Cells.

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**Objective:** To study the effects of ESN on inhibition of proliferation and induction of apoptosis of K562 cells, ESN is a natural extraction isolated from Chinese Herb. **Methods:** The inhibitory rates of the proliferation were detected by colony formation method and cell growth curves. The apoptosis was analyzed by morphology, flow cytometry (DNA contents), agarose gel electrophoresis (DNA Ladder) and Annexin V-FITC /PI staining (the percent of apoptosis cells). **Results:** After cells were treated with ESN at the concentration of 30 mg.L<sup>-1</sup> for 24, 48 and 72h, the inhibitory rate were 26.6%, 45.4% and 60.3%, respectively. After cells were treated with ESN at the concentration of 50 mg.L<sup>-1</sup> for 24, 48 and 72h, The inhibitory rate were 41.5%, 71.2% and 89.6%, respectively. The inhibitory rate of cell growth were significantly increased compared with the control group ( $P < 0.05$ ). The inhibitory effects are dose- and time-dependent. After the cell cultured with ESN at the concentration of 10,30,50 and 70 mg.L<sup>-1</sup> for 24 h, The percent of apoptosis cells were 7.8%, 21.5%,48.7% and 32.1% respectively, it's peak at 48.7%. By methods of flow cytometry, agarose gel electrophoresis and morphology, the apoptosis peaks, DNA ladder and the body of apoptosis were found. **Conclusion:** ESN can strongly inhibit the proliferation and induce apoptosis of K562 cells with a time and dose related manner. It is suggest that ESN might be applied in leukemia treatment.

### Su2.80. An Automatic System for the Image Analysis and Interpretation of HEP-2 Image Pattern.

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The kinds of cells that are considered are Hep-2 cells, which are used for the identification of antinuclear autoantibodies (ANA).

ANA testing for the assessment of systemic and organ specific autoimmune disease has increased progressively since immunofluorescence techniques were first used to demonstrate antinuclear antibodies in 1957. Hep-2 cells allow for recognition of over 30 different nuclear and cytoplasmic patterns, which are given by upwards of 100 different autoantibodies. The identification of the patterns has up to now been done manually by a human inspecting the slides with the help of a microscope. The lacking automation of this technique has resulted in the development of alternative techniques based on chemical reactions, which have not the discrimination power of the ANA testing. We present a decision support system that can automatically inspect and classify the cell patterns [1][2]. We describe the image acquisition unit, the image analysis and the feature extraction unit as well as the classification unit. The system was evaluated on a data set of 600 cell image samples. Finally, we give result on the classification accuracy and describe the final system.

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### Su2.81. Determination of ANA Autoantibodies with Multiplexed Immunoassays.

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The diagnosis and monitoring of autoimmune disease has traditionally been done by a three-stage process: starting with immunofluorescence, followed by further testing of positive samples by immunoassays. This approach is labour intensive, requiring trained specialists, multiple testing systems and large reagent and sample volumes, and frequently results in false positives. A multiplexed immunoassay system was developed that allows these steps to be combined in one automated assay, thus reducing time to result, labour and sample requirements. Nine key antigens for detection of anti-nuclear antibodies (ANA) were each tagged with unique barcoded microparticles, enabling tracking of each antigen and therefore allowing mixing of all ANA reagents and controls. Antigens included Jo-1, Scl-70, Sm, SmRNP, Ro (SSA), La (SSB), U1snRNP, Centromere B, dsDNA and internal controls. Recombinant antigens were used, resulting in better clinical sensitivity and specificity, a necessary feature for accurate rheumatic disease autoantibody testing. All reagents were lyophilised in a 96-well plate for assay stability and convenience. Using automated liquid handling robotics, serum samples were added to each well, followed by a fluorescently tagged detection antibody. Analysis was performed on the UltraPlex™ Automated Plate Reader. This fully automated multiplexed ANA immunoassay was used to screen hundreds of patient samples for ANA autoantibodies. In one day, 160 patients plus controls can be screened simultaneously for 9 autoantibodies, requiring 66% less labour and 90% less sample and reagents, with an overall 2–3 fold improvement in turnaround time of sample to result over traditional immunoassays. Furthermore, this approach can eliminate the need for the labour intensive front-end immunofluorescence test, which can have a high degree of false positives. With validation and testing done on large panels of patient samples, resulting in

specificity and sensitivity over 90% and 80% respectively, this multiplexed ANA assay is recommended for the screening, diagnosis and monitoring of autoimmune diseases. The assay, called UltraPlex™ ANA, provides a fast, flexible and cost-effective means for measuring multiple disease markers from a single sample.

### Su2.82. Distribution of Killer Cell Immunoglobulin-Like Receptors Genes in Chinese Han Population.

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**Objective** To design a series of primers to detect the diversity of killer Ig-like receptor(KIR) gene content and the haplotypes in Chinese Han population in Zhejiang. **Methods** DNA were extracted, and samples were genotyped with PCR-SSP method. **Result** (1)All 17 known KIR genes were observed in the population. All individuals contain 3DL3, 2DL4 and 3DL2; 2DL3, 2DL1, 2DP1, 3DP1 \* 3, 3DL1, 2DS4 \* 1/2 are also very common; the frequencies of 2DS4 \* 3, 2DL5, 2DS1, 3DS1, 2DS5, 2DS2, 2DL2, 2DS3 and 3DP1 \* 1/2 were 0.23, 0.19, 0.18, 0.16, 0.11, 0.1, 0.1, 0.08 and 0.07, respectively. (2)13 haplotypes were detected, and the most frequent one is haplotyoeA2, followed by A1. (3)34 kinds of genotypes were detected, AJ(A2,A2) and AF(A1,A2) show higher frequencies. Among them, 20 have not been found in Caucasians so far, and 8 can not be divided into haplotypes. (4) Linkage disequilibrium analysis indicated that some pairs of KIR genes show remarkable linkage disequilibrium. **Conclusion** There are distinctive frequencies of KIR gene content, haplotype, and genotype in Chinese Han population in Zhejiang, and new KIR haplotype might exist.

### Su2.83. Problems of Immunodiagnosics of Hepatitis C in Senior Patients.

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Antibodies to hepatitis C virus (HCV) are found in 5% of Moscow population seeking medical aid through the city health care system. Significant part of the patients are senior citizens (18.5%) for whom some difficulties in lab diagnostics are encountered. Due to these difficulties, there is an urgent need to improve the immunodiagnosics of HCV infection C in this population. It is well established fact that among people over 60-years old, anti-HCV antibodies are found 2.6 times less frequently than in the younger patients by use anti-HCV ELISA (2.72% and 7.03% respectively,  $P < 0.001$ ). Among positive sera samples of senior patients, there are more samples with low optical density (signal-to-cutoff ratio is less than 3.0) compared to the group of samples of the younger patients (15.21% and 8.66%, respectively,  $P < 0.05$ ). When low-positive sera were tested using other systems in addition to the required (screen test and confirming test) some samples showed controversial results. These "problematic" sera samples from senior patients tested with strip immunoblot assay were negative, and no viral RNA were found using PCR. Trying to find out the reason for nonspecific positive reaction with ELISA, we could not detect elevated concentrations of circulating immune complexes, or monoclonal gamma-globulinopathy in any of these sera samples. The rheumatoid factor did not have any effect on ELISA results. When protein fraction was analysed, it has been found the increase in alpha1-globulins in the sera of senior patients with nonspecific positive reaction,

compared to the negative or anti-HCV antibodies positive samples.

#### **Su2.84. The Novel Docosatriene, Protectin D1, Produced by T<sub>H</sub>2-Polarization Promotes Human T Cell Apoptosis Via Lipid-Raft Clustering.**

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Docosahexaenoic acid (DHA), a major  $\omega$ -3 fatty acid in human brain, synapses, retina, and other neural tissues, displays beneficial actions in neuronal development, cancer, and inflammatory diseases by unknown mechanisms. In this study, using lipidomic analysis, we found that the novel 10,17S-docosatriene termed protectin D1 (PD1) is generated from DHA by T helper type 2 (T<sub>H</sub>2)-polarized peripheral blood mononuclear cells (PBMC) in a lipoxygenase-dependent manner. PD1 (100 ng/mouse) blocked T cell migration in vivo. It also potently (1–10 nM) inhibited TNF $\alpha$  and IFN $\gamma$  secretion by activated T cells, and promoted their apoptosis through lipid raft clustering. These results demonstrate novel anti-inflammatory roles for PD1 in regulating events associated with inflammation and resolution.

#### **Su2.85. FACS-Based Method To Evaluate Inhibitory Antibodies in Patients Receiving Enzyme Replacement Therapy.**

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Lysosomal storage disorders are genetic diseases characterized by the deficiency of an active enzyme, resulting in the accumulation of macromolecular substrates in the lysosome. This accumulation disrupts cellular and organ function, ultimately leading to disease pathology. Enzyme replacement therapy, where a patient is infused with a recombinant enzyme, has been shown to be an effective treatment for these disorders. The development of an antibody response to the therapy is a key safety factor and can also impact product efficacy. A cell based assay using flow cytometry has been developed to evaluate whether patient antibodies interfere with cellular uptake of the replacement enzyme, which is mediated by the mannose 6-phosphate receptor. This assay is a novel method to determine whether antibodies inhibit enzyme uptake without relying on enzyme activity and without interference from endogenous enzyme. Results suggest that this assay is a specific and reproducible way to detect inhibitory antibodies in patients receiving enzyme replacement therapy.

#### **Su2.86. Experimental (war) Type System of Hemaimmune Reaction Road Map.**

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**Objective:** To establish a detection method of IL-8, which is applicated in the study of hemaimmune reaction road map.

**Methods:** 0.2ml suspension of cancer cells (S180, 5 $\times$ 10<sup>6</sup>/ml) or yeast cells (5 $\times$ 10<sup>8</sup>/ml) (or 0.2ml NS as control) were added into 0.2ml fresh anticoagulant whole blood (or 0.2ml blood cells or 0.2ml white blood cells and 0.3ml plasma) treated by citric acid, and incubated for 1 h at 37°. Using ELISA method of IL-

8, we detected experimental system of hemaimmune reaction road map.

**Results:** It was found that cancer cells or yeast cells can activate hemaimmune reaction: in whole blood group with cancer cells and yeast cells added, level (138–126.3 pg/ml) of IL-8 was higher than that (3.906pg/ml) of whole blood group with NS added. And on the cancer cells or yeast cells activation time, level (138–126.3pg/ml) of IL-8 in white blood cell group with plasma added was significantly higher than that (55.99–3.90pg/ml) in white blood cell group without plasma added. Level (59.99–52.19pg/ml) of IL-8 in white blood cell group with red blood cell and plasma added was significantly higher than that (27.35–20.83pg/ml) in white blood cell group without red blood cell.

**Conclusion:** These results indicate that: there is a road map of blood immune reaction. And in the hemaimmune reaction, plasma and red blood cells is requisite. The complement (in plasma) and red blood cell plays a vital role (like white blood cells) in blood hemaimmune reaction road map.

#### **Su2.87. Activation of Red Blood Cell Innate Immune Reaction Main Road by Antigen.**

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**Objective:** To determine the activation of antigen in red blood cell innate immune reaction main road.

**Methods:** Cancer cells (5  $\times$  10<sup>6</sup>/ml) and/or Bacillus calmette-Guerin (BCG 0.1mg) or yeast cells (5  $\times$  10<sup>8</sup>/ml) were added in whole blood cells 0.2ml (or white blood cells 0.2ml) and fresh plasma 0.3ml (or NS 0.3ml) treated by citric acid, and incubated for 1h at 37° to see results. Main aut- come index: IL-8 (ELASA method).

**Results:** It was found cancer cells. BCG and yeast cells can activate hemaimmune reaction, but these antigen not can activate white blood cells immune reaction in not adding plasma group, activation Index (2.124  $\pm$  0.860) of IL-8 in antigen adding whole blood cells and plasma group was significantly higher than that (0.390  $\pm$  0.080) in antigen adding white blood cells and plasma group ( $P < 0.01$ ).

**Conclusion:** These results indicate that there is red blood cells main road map of hemaimmune reaction, The red blood cell and complement plays a vital role in hemaimmune reaction road map.

#### **Su2.88. Evaluation of the Effects of Different Freezing Procedures on the Function and Composition of Lymphocyte Subpopulations from Blood and Synovial Fluid.**

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**Background:** With the development of biological drugs it is important to perform immunological studies on patient samples, both to monitor the treatment, but also to increase our understanding of the mechanism of the drug. Thus, both for comparative analyses between samples from different laboratory sites but also for practical reasons in the laboratory, analyses of frozen cells is an option that should be more used. However, the freezing of cells is known to affect their survival and function, and selective loss of certain populations is at risk. This could significantly affect the outcome of various analyses, as compared to the usage of fresh cells. **Objectives:** To evaluate the effect of

different freezing media and different freezing and thawing procedures on the proliferative capacity and the composition of the lymphocyte populations. **Material and methods:** Mononuclear cells were prepared from blood or synovial fluid from patients with rheumatic diseases and from healthy control blood. Proliferation in response to B and T cell stimulation (PwM, PHA and anti-CD3) was measured by incorporation of  $^3\text{H}$ -thymidine. Subpopulations of B cells (naive, memory, activated and plasma cells) and T cells (helper T cells, CTL, activated T cells, regulatory T cells and CD28null T cells) were analysed by four colour flow cytometry. We also studied yield and viability. **Results:** Our first results indicate that both the yield and viability seem to differ markedly depending on the freezing and thawing procedure used. An increased background proliferation level was observed in frozen/thawed mononuclear cells as compared to freshly prepared, with a decreased stimulatory index as a consequence. It was also evident that the different freezing/thawing protocols affected the B and T cell subpopulations differently. **Conclusion:** Different freezing protocols affects the properties of mononuclear cells differently, stressing the importance of testing different protocols in order to get the best possible yield and to retain the specific properties of the subpopulations of interest.

### Su2.89. Does the CD203c Basophil Marker Improve the Flow-Cytometry Diagnosis of Immediate Allergy?

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**Background:** Flow cytometry tests based either on the detection of the induced-expression of CD63 or the upregulation of CD203c by activated basophils in response to allergens has been developed as alternative methods for *in vitro* diagnosis of IgE-mediated reactions. The CD63 test is now currently used and has been proved to be effective in the diagnosis of allergy to various allergens. However there is no consensus about the diagnostic reliability of the CD203c test. The goal of our study was to compare the two assays using whole blood in the diagnosis of immediate-type allergy to cat.

**Methods:** 22 patients characterized by positive skin test and positive specific IgE to cat dander as well as 26 non allergic individuals were examined. Heparinized whole blood, primed or not with IL3, was incubated at 37°C with optimal concentrations of the major purified cat dander allergen of *Felis domesticus* (Feld I). Anti-IgE and anti-FcεRI Abs or washing solution were used as positive and negative controls respectively. After defined incubation times, suspensions were stained either with an anti-IgE PE and an anti-CD63 FITC provided by Basotest Kit® (Orpegen Pharma, Germany) or an anti-CD203c PE (Coulter Immunotech, France) and an anti-IgE FITC. In both techniques, an optimal gating of the basophils (CD45<sup>low</sup>) was obtained with the addition of an anti-CD45 PerCP. Analysis was performed on a FACSCalibur flow cytometer. Results were given as the percentages of IgE+ basophils expressing CD63 or CD203c above the threshold defined by the negative control. Alternatively, induced upregulation of CD203c was calculated as stimulation index (SI) of MFIs (mean fluorescence intensities) obtained with stimulated and unstimulated cells.

**Results:** When performed on IL-3 primed whole blood, exposure to Feld I resulted in marked expression of CD63 by basophils in 96 % of the patients. By comparison, using similar

experimental conditions (IL-3 priming, multiple staining strategy) the sensitivity obtained with the CD203c protocol only reached 86 %. Of the non-allergic individuals, only one showed a positive CD63 test but none demonstrated a positive CD203c test leading to a specificity of 96 % and 100 % respectively. On the other hand, none of the five patients tested exhibited basophil activation in response to an irrelevant allergen (birch allergen) reflecting a 100 % antigenic specificity in both protocols. Interestingly, the CD203c test was clearly improved in terms of sensitivity (92 % vs 86 %) in absence of IL-3 priming of whole blood in response to Feld I allergen.

**Conclusion:** Taken together our results indicate that, when using well defined analytical conditions (whole blood, no IL-3, gating strategy, expression of results...), the measurement of the CD203c as well as the CD63 markers on allergen-activated basophils constitute reliable *in vitro* flow-cytometry tests for the diagnosis of immediate allergy. However, the CD203c test remains to be investigated in other types of allergy (drug, food.) using similar approaches before drawing definitive conclusions.

### Su2.90. Prospective Comparison of ELISA and Immunodiffusion for Detection of Antibodies to Extractable Nuclear Antigens.

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We recently performed an evaluation of 3 ELISA based assay systems for detection of antibodies to extractable nuclear antigens (ENA's; Orton, et al. CDLI. 2004). The ELISA systems consisted of a single well screening ELISA, using pooled ENA's (Sm, RNP, SSA, SSB, Scl-70 and Jo-1) followed by the use of a multiwell specificity ELISA employing individual ENA's to determine the identity of the ENA antibodies in screen positive samples. We identified the system with the best overall sensitivity and specificity in our selected test sample population and implemented it in our laboratory. After implementation, we noted a significant number of weakly positive samples, the clinical significance of which, was not clear. The current study reports the results of a prospective comparison of the ELISA ID assays, the objective of which, was to determine if we could predict the likelihood of a positive ID assay based upon ELISA results. Over a 3 month period we tested 399 consecutive sera with the ENA ELISA screen assay. All samples that demonstrated  $\geq 20$  ENA units were then tested with the specificity ELISA and an ID assay that identified antibodies to the same group of ENA's. Thirty-two of 399 samples were borderline positive (range 20–25 ENA units) and 97 were positive (range 26– $\geq 422$  ENA units). Only 1 of the 32 screen ENA borderline positive samples had a positive specificity ELISA antibody detected (Scl-70) and none had positive ID results. Twenty three samples had borderline (20–30 ENA Units) specificity ELISA results (1 RNP, 4 SSA, 2 SSB, 4 Scl-70 and 12 Jo-1). None of these had a positive ID result. Positive results for Sm, RNP, SSA, SSB, Scl-70 and Jo-1 by specificity ELISA was found in 7, 13, 38, 17, 13 and 2 sera, respectively while ID was positive with 4, 7, 20, 8, 4 and 0 sera, respectively. Seventy Five percent of specificity ELISA positive/ID positive samples had  $>100$  specificity ENA Units (one sample was ID positive for SSA and borderline specificity SSA ELISA positive). Eighty five percent of 47 specificity ELISA positive/ID negative samples had  $<100$  specificity ENA units. The results of this prospective evaluation confirm our previous results showing the increased sensitivity of ELISA compared to ID for ENA antibody detection. Additionally,

the data suggest that the screening ELISA borderline range could be increased and that we could eliminate specificity ELISA testing of borderline positive samples without missing a significant number of specificity ELISA positive samples. In our laboratory, specificity ELISA positive samples with <100 ENA Units were less likely to demonstrate ID reactivity than were samples with >100 ENA Units indicating that only relatively strong ELISA reactivity predicts positive ID results. This analysis has provided a link between ELISA and ID results facilitating interpretation of ENA ELISA results, particularly for weakly positive samples.

### **Su2.91. A Novel Proteomics Assay Employing Amplification of Oligonucleotide Tags from Monoclonal Antibodies.**

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Protein microarray technology represents a rapidly evolving branch of proteomics research. Antibody arrays, in which antibodies are attached to a surface, are potentially useful tools for a variety of experimental questions. Inherent difficulties in this approach, however, have limited their efficacy. To address this problem, we are developing an assay to detect multiple specific protein analytes in a complex mixture without attaching antibodies to a solid support. The assay is based on linear amplification of unique oligonucleotide tags from antibodies that are indirectly coupled to an oligonucleotide template. The oligonucleotide tag is a distinctive 30-mer sequence downstream of a common T7 promoter. The antibody binds the protein of interest and indirectly indicates the concentration of analyte. After unbound material is washed away, T7 RNA polymerase amplifies labeled RNA transcripts from the template. In order to interpret the profile, the labeled probe is then hybridized to an oligonucleotide microarray that contains the complementary 30-mer sequences. There are several advantages to this approach, including a greater degree of multiplexing capability than is possible with fluorescent tags, linear amplification of signal, and sensitive and specific detection of protein analytes. Further, this approach is potentially compatible with many experimental designs, including multiplexed analysis of cell-surface markers or lysate preparations of rare cell populations.

### **Su2.92. Identification of Major Histocompatibility Complex (MHC) Class I-Related Genes in Cattle.**

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Non-classical MHC class I and class I-related molecules do not generally present antigenic peptides but may have other important immunological roles. A number of functional class I-related genes have been identified in or near the human MHC region. These include the MHC class I chain-related (MIC) genes and HFE. MICA/B encode molecules important for the regulation of immune responses by functioning as ligands for a natural killer (NK) cell receptor, NKG2D, and a subset of  $\gamma\delta$  T cell receptors. HFE is involved in the regulation of iron metabolism. Studies of class I-related MHC molecules in species other than human and mouse could shed light on their evolution. The project aims to identify and characterise the MIC and HFE genes in cattle.

A small number of partial cDNA sequences from cattle, homologous to human MICA and MICB were identified in public databases, and used to amplify MIC cDNA from three cattle

epithelial cell lines. DNA thus generated was used as a probe on Southern blots of restriction enzyme-digested genomic (g) and bacterial artificial chromosome (BAC) DNA. RNA was extracted from five tissues from a calf and used for reverse transcription experiments with MIC and HFE primers. Human HFE cDNA was used as a probe on cattle gDNA Southern blots. Primers for PCR were designed based on published HFE sequences from human, mouse, rat and rhinoceros and Rapid Amplification of cDNA Ends (RACE) was performed on cattle epithelial cDNA.

Southern blotting and sequencing analysis provide evidence for the presence of at least two MIC genes. At least four different cDNA sequences have been found in total, and each cell line contains at least three different sequences. Sequences fall into two groups that differ by a 21 nucleotide indel in the  $\alpha 2$  domain. Both long and short sequences are approximately 60% similar to human MICA and MICB. The positions of polymorphic residues differ between cattle and human MIC sequences. MIC mRNA can be detected at low levels in cattle lung and heart, and at very low levels in the stomach, intestine and liver. A single putative HFE gene was revealed by Southern blotting and the full length cDNA sequence has been determined. In contrast to human HFE, cattle HFE has an extra 24 nucleotides in the  $\alpha 1/\alpha 2$  boundary, as in mouse and rat. Tissue expression studies in cattle demonstrate the presence of HFE in heart, liver, lung, stomach and intestine with the highest mRNA expression in the heart and liver.

Cattle orthologues of the human MIC and HFE genes have been identified in this study. MIC  $\alpha 1$ - $\alpha 3$  sequence data demonstrate the presence of at least two expressed genes in cattle. Southern hybridisation of cattle gDNA and BAC DNA from MHC homozygous animals suggests the presence of four or more MIC genes in cattle. MIC genes are known to be highly polymorphic in human but the significance of these polymorphisms to ligand interactions remains unknown. Cattle HFE cDNA is 80% similar to the human HFE suggesting a role in iron metabolism in cattle. Molecular modelling of the cattle molecule predicts that the extra eight amino acids in the  $\alpha 1/\alpha 2$  boundary form a loop, although its function has not yet been established.

### **Su2.93. Pathogenic Significance of $\alpha$ -N-Acetylgalactosaminidase Activity Found in the Hemagglutinin of Influenza Virus.**

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Serum vitamin D<sub>3</sub>-binding protein (known as Gc protein) is the precursor for the principal macrophage activating factor (MAF). The precursor activity of serum Gc protein was reduced in all influenza virus-infected patients. These patient sera were found to contain  $\alpha$ -N-acetylgalactosaminidase (Nagalase) that deglycosylates serum Gc protein. Deglycosylated Gc protein cannot be converted to MAF, thus loses the MAF precursor activity. Since macrophage activation for enhanced phagocytosis and antigen presentation to B and T lymphocytes is the first indispensable step in both humoral and cellular immunity development, lack of macrophage activation leads to immunosuppression and secondary infection. Therefore, the Nagalase activity level of influenza patient sera regulates the degree of immunosuppression. An influenza virus stock grown on embryonated eggs contained a large amount of Nagalase activity. A sucrose gradient centrifugation analysis of the virus stock revealed that the profile of the Nagalase activity corresponds to that of the hemagglutinating activity. When these gradient fractions were



treated with 0.01% trypsin for 30 min, the Nagalase activity of each fraction increased significantly, suggesting that the Nagalase activity resides on an outer structural envelope protein of the influenza virion and is expressed by a proteolytic process. After disruption of influenza virions with sodium deoxycholate, fractionation of the envelope proteins with mannose specific lectin affinity column along with electrophoretic analysis of the Nagalase peak fraction revealed that Nagalase is the intrinsic component of the hemagglutinin (HA). Since both the fusion capacity and the Nagalase activity of the HA protein are expressed by proteolytic cleavage, the Nagalase activity appears to be an enzymatic basis promoting fusion for initiation of infection. Thus, Nagalase has pathogenic significance in regulating infectivity and immunosuppression. Similar results were also found in HIV (Yamamoto et al. 1995. *AIDS Res Hum Retrovirus* 11: 1373-8) and other enveloped viruses (e.g., Sendai, rubella and measles viruses).

#### **Su2.94. Role of L-Arginine Transporter, Solute Carrier 7A2 (SLC7A2), in the Immune System.**

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**INTRODUCTION** Although nitric oxide (NO) is a major regulator of inflammation, little attention has been focused on upstream regulators of NO such as the solute carrier (SLC) family of molecules. SLC7A2 supplies L-arginine to the inducible NO synthase (iNOS) and is required for sustained NO production by macrophages. Bacterial lipopolysaccharide (LPS) and the cytokine interferon- $\gamma$  (IFN- $\gamma$ ) are strong inducers of iNOS in macrophages. **OBJECTIVES** Identify immune cytokines modulated in LPS and IFN- $\gamma$  stimulated SLC7A2<sup>-/-</sup> macrophages and examine T-dependent immune responses in SLC7A2 deficient mice.

**MATERIAL AND METHODS** Adult wild type, iNOS deficient and SLC7A2 deficient mice of both sexes in the C57Bl/6 strain were stimulated with thioglycolate for 72 h. Peritoneal macrophages (PM) were primed with 20 units/ml IFN- $\gamma$  in culture media for 2 h, and then 100 ng/ml LPS were added for an additional 17 h. Total RNA was isolated and after reverse transcription, cDNA products were used for amplification of SLC7A2, iNOS and several cytokines by real time PCR. In other studies, mice of the three genotypes were inoculated intraperitoneally with 100  $\mu$ g DNP-KLH in Freund's complete adjuvant on day (d) 0 and boosted on d21 using 100  $\mu$ g DNP-KLH in Freund's incomplete adjuvant. Serum was collected at d0, d7, d14, d21, d28 and d35 and tested by ELISA for immunoglobulin production. **RESULTS** Loss of iNOS did not significantly affect SLC7A2 expression nor did loss of SLC7A2 affect iNOS expression. IFN- $\gamma$ , IL-10 and IL-4 expression were all significantly increased in iNOS and SLC7A2 deficient PM compared with wild type control PM. IL-6 and TGF $\beta$ -2 expression was significantly decreased in iNOS<sup>-/-</sup> and SLC7A2<sup>-/-</sup> PM compared with wild type controls and TGF $\beta$ -1, TNF- $\alpha$  and phospholipase A2 were unchanged. Arginase-I, but not arginase-II, was upregulated specifically in iNOS<sup>-/-</sup> PM, suggesting that arginase-I activity may be increased in the absence of substrate usage by iNOS. Serum IgG2a levels were dramatically reduced in iNOS and SLC7A2 deficient mice during the primary immune response, but returned to wild type levels after the boost. Serum IgM, IgG1 and IgG3 levels were normal in both genotypes. **CONCLUSIONS** Overall, SLC7A2 deficiency in macrophages results in a similar phenotype to iNOS deficiency. Interestingly, when L-arginine metabolism by iNOS is absent, there is an

upregulation of arginase-I expression, presumably to utilize the excess L-arginine. Whether this effect is dependent on SLC7A2 remains to be determined. Despite increased INF- $\gamma$  expression by iNOS and SLC7A2 deficient PM, IgG2a production is reduced following immunization, suggesting that the increase in Th2 cytokines IL-4 and IL-10 dominate this response; so the absence of SLC7A2 and iNOS genes can modulate cytokines levels expression. Thus, in PM it appears that a major function of SLC7A2 is to supply L-arginine for use in the NO pathway. And defining this function in inflammation; that is, understanding molecular pathways controlling NO production in macrophages, is an important step towards developing improved therapies for inflammatory diseases and primary immunodeficiencies.

#### **Su2.95. Improved Immunological Methods Using Peptides: Western Blots, Peptide Arrays, Kinase Assays and ELISAs.**

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Peptides have become an instrumental tool in immunological research. Their use, however, in protein arrays is hampered by ineffective and variable binding efficiency of peptides that could result in low sensitivity, false positives and inconsistent signals; in Western blots and ELISAs their use is limited due to their small molecular mass. To overcome these hurdles, we apply intein-mediated protein ligation (IPL) in which a peptide possessing a N-terminal cysteine is linked to the carboxyl terminus of a reactive carrier protein via a peptide bond. The ligation products are then arrayed on to a membrane or used in Western blot analysis. In this poster, we demonstrate multiple applications of intein technology in immunological methods:

- a) Easy generation of carrier-peptide substrates suitable for Western blot analysis (1).
- b) Production of peptide arrays with improved sensitivity, including defining antibody epitopes (2).
- c) Generation of tailored substrates for kinase and phosphatase assays (3).
- d) Production of antigens for improved sensitivity in ELISA assays.

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#### **Su2.96. Characterization of Assay Variability in Real-Time and Batch Assays of Sequential Samples from the Same Donors.**

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**Background:** The enzyme-linked immunospot (ELISpot) assay is useful in measuring responses to vaccination and changes following immunotherapy. We have developed, optimized, and validated a customized BBI ELISpot kit for immunogenicity assessments in NIAID-sponsored HIV clinical trials, and compared its precision in fresh and frozen samples

collected at one month intervals, while measuring biological variability.

**Methods:** The capture antibody, detection antibody, Streptavidin-HRP, and substrate were titrated for optimal performance of the BBI kit and used according to kit instructions. Repeatability was characterized using PHA and CEF pool (CMV, EBV and Flu peptides) in a minimum of triplicate wells. The within donor variability was determined using 3 different donors with 5 time points collected one month apart. Samples were assayed by ELISpot using fresh cells in real time and by batch assay using frozen cells at the end of 6 months from the first time point. Within donor variability was presented as mean SFC per 200,000 PBMC  $\pm$  SD (%CV) for CEF for each of the 3 donors. PHA SFC were given per 100,000 cells.

**Results:** Repeatability within assay was similar whether CEF or PHA was assayed fresh (13% vs. 16%) or frozen (16% vs. 11%). For within donor measurements, CEF was  $283 \pm 58$  (20%),  $419 \pm 99$  (24%), and  $19 \pm 3$  (18%) for fresh; and  $172 \pm 60$  (35%),  $416 \pm 169$  (40%), and  $16 \pm 10$  (62%) for frozen. For PHA, the measurements were  $136 \pm 27$  (20%),  $184 \pm 75$  (41%),  $211 \pm 18$  (9%) for fresh, and  $252 \pm 72$  (28%),  $351 \pm 136$  (38%), and  $329 \pm 117$  (36%) for frozen. The CEF SFC values were higher for donor 1 in fresh vs. frozen; however there was no difference in the other 2 donors. The variability among samples from the same donor was higher in the batched frozen samples than in the individually run fresh samples. PHA showed opposite results, with all fresh samples having lower SFC values than frozen, and the variabilities were similar except for donor 3, which was higher in the frozen samples.

**Conclusion:** Both fresh individual and frozen batch assays demonstrated similar intra-assay repeatability. For CEF the mean values for 2 of 3 donors over 5 time points were essentially the same for fresh and frozen samples. Interestingly, the variability within a donor was greater in the batched frozen samples than the fresh samples. This suggests that day to day variations in freezing procedures may result in greater variability than testing real time on different days with donor samples drawn at different times. **Supported by NIAID- Contract # N01-AI-85341.**

### Su2.97. HLA-Typed PBMC Samples with Established Antigen/Peptide Reactivity for Accelerating and Standardizing Human Immunological Research.

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We have developed a protocol to cryopreserve human peripheral blood mononuclear cells (PBMC) while maintaining full functionality. The thawed PBMC display > 80% viability, and when tested for peptide or protein antigen-induced T cell recall responses in cytokine ELISPOT assays, the frequencies and per-cell cytokine productivities of the thawed cells approximate 100% of the fresh PBMC. Since serum is a highly variable "reagent" that affects the results, we developed serum free freezing and testing media towards standardization. We have started to build a PBMC library of HLA-typed healthy human donors. The PBMC of each donor has been characterized for reactivity to a panel of 23 individual peptides (common viral Class I-restricted determinants) and 5 protein recall antigens, recognized by CD8 and CD4 cells, respectively. Up to 1,500 vials of each of the characterized samples have been

cryopreserved and made available as positive and negative controls for T cell monitoring in ELISPOT, ELISA, cytokine bead array, tetramer/pentamer, and cytokine capture assays. Ready access to such highly characterized PBMC should facilitate human immunological research and offers reference samples for assay standardization within laboratories, and between different laboratories.

### Su2.98. Cell Proliferation Index. A Reliable and Validated Method That Quantifies Cell Proliferation According to CFSE Dilution.

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Cell proliferation is a mechanism intimately linked to the immune response. Several cellular activation-induced pathways converge in it, and it takes place synchronously to the acquisition of effector functions and phenotype. Thus, its measurement is essential because it is an important marker of response against a variety of stimuli. Several methods for the quantification of cell proliferation are available. The measurement of [<sup>3</sup>H]-thymidine uptake is probably the most widely used. However, it requires the handling of carcinogenic and radioactive materials. Moreover, when more than one cell population is being cultured, the method does not distinguish between them. An alternative approach is based on labeling T cells with carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE is membrane-permeable fluorescent dye that binds covalently intracellular molecules. When a cell divides, the fluorescence intensity halves in each of the two resultant cells. Thus, each round of cell division produces a population of cells that have one half of the fluorescence intensity than the cells they arose from. Using a FACS, cells that have not proliferated can be easily distinguished from cells that have proliferated, and according to the dilution of the dye, one can assume how many division rounds each cell has gone through. Its principal drawback is that results are semi-quantitative and, when differences are subtle, comparison is difficult. In the present work we present an algorithm that translates the semi-quantitative data obtained from the FACs and yields a numerical result. **Materials and Methods.** PBMC (from healthy donors) and Jurkat cells were used. Cells were cultured during 72 hours and stimulated with either plate bound aCD3 plus soluble aCD28, or PHA. Cell proliferation was quantified by: a) manual count of live and dead cells (according to trypan blue exclusion); b) [<sup>3</sup>H]-thymidine uptake; c) Cell Proliferation Index (CPI). For the FACS studies, a histogram (FL1 [CFSE intensity] vs. cell number) was drawn with the target population. A first marker (M1) was set according to a negative control (non-stimulated cells). Its geometric mean was recorded. Next, markers (M2, M3, etc) were set progressively, each one including a daughter population (considering the geometric mean must halve in each population). Hence, the cells accounted within the M1 have not proliferated, the cells in M2 have undergone one round of proliferation, the cells in M3 three rounds, and so on. The data was incorporated into this algorithm:  $CPI = \sum M_n \times 2^{n-1}$ . The results obtained with each method were compared. **Results.** Cell proliferation was detected by the three methods. The results obtained with the CPI correlated closely to the measurement of cell proliferation with [<sup>3</sup>H]-thymidine uptake ( $R = 0.92$ ,  $P < 0.0001$ ). At low mitogen concentrations, CPI was more sensitive than [<sup>3</sup>H]-thymidine

uptake. **Conclusion.** CPI is a sensitive and reliable means of quantifying cell proliferation.

### Su2.99. Evaluation of CellPrep, an Automated Cell Washing Instrument for Analysis of Surface Markers on Leukocyte Subpopulations Via Flow Cytometry.

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The use of flow cytometry-based assays in leukocyte subset analysis is a routine practice in basic research and clinical research settings. There have been significant improvements in reagents, procedures, and instrumentation that have allowed for the introduction of automated techniques for the preparation of cell samples prior to flow cytometric analysis. An invaluable advantage to automation is the standardization and reduced "hands-on time" as compared to manual techniques.

The current and most commonly used technique of centrifugation to wash cells by removing contaminating materials prior to flow cytometric analysis, does not lend itself well to automation. Additionally, subjecting cell populations to both g-force and intense cell-to-cell contact through centrifugation may alter sensitive activation and signal transduction expression. To simplify and automate cell washing in tubes, the non-centrifugal CellPrep instrument was designed and developed to wash cells using polysulfone hollow fibers.

The present study was carried out to compare scatter profiles, surface antigen expression, and recoveries of rare and non-rare events in unwashed, centrifuge-washed, and CellPrep-washed cell samples. It has been shown that the CellPrep instrument can be used successfully to wash peripheral blood cell suspensions with no adverse effects on the phenotypic and scatter profiles, as well as cell recoveries.

### Su2.100. Optimization of the Aspiration Dose of IL-1ra Preparation To Stop an Inflammation in the Mouse Respiratory Tract.

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Hyperproduction of interleukin-1 (IL-1) is a main factor to provoke inflammation. The interleukin-1 receptor antagonist (IL-1ra) may serve as a promising therapeutic and prophylactic agent for stopping inflammatory processes, including the infectious ones. In this study the prophylactic efficacy of IL-1ra was assessed after aerosol application to mice, in which inflammation was induced by intranasal instillations of LPS. The efficacy of IL-1ra was studied after two routes of application- the aerosol and the injection one. Based on a theory of multifactor analysis, the aspiration dose of IL-1ra and the particle size distribution in aerosol were optimized.

It was demonstrated that IL-1ra applied in a form of aerosol caused the decrease of inflammation in the respiratory tract of a mouse. The efficacy of this decrease depended on the aspiration dose of IL-1ra inhaled and on size of particles. 50% decrease of inflammation was registered for the aspiration dose of IL-1ra 150 mcg/mouse. The same effect for the injection IL-1ra was registered

at a dose of 270 mcg/mouse of IL-1ra. The anti-inflammatory effect of the fine-dispersed fraction of IL-1ra (particles of 2 µm in size) was 30% higher than that of the coarse-dispersed fraction (particles of 10 µm in size). The resultant data demonstrated the advantage of aerosol route of administration of IL-1ra over the injection one.

### Su2.101. The Use of Arginine-Rich Peptide Conjugated Antisense Oligomers To Alter Immune Function.

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Phosphorodiamidate Morpholino Oligomers (PMOs) are effective antisense agents for inhibiting gene expression; however limited uptake into populations of immune cells without the use of mechanical and chemical procedures often detrimental to cellular functions, restricts their usefulness. A strategy shown to enhance cellular uptake of PMOs into fibroblasts is the conjugation of an arginine-rich peptide to the oligomer. To examine uptake of these peptide-conjugated PMOs into lineages of primary T cells, B cells, and NK cells, as well as bone-marrow derived macrophages, and dendritic cells, we used flow cytometry to measure the presence of a fluorescein-linked PMO in treated cells. Uptake of the oligomer into these cell types is greatly enhanced by the addition of the arginine-rich peptides compared to unmodified PMO. Furthermore, differential uptake into different cell types was observed and found to be dependent on the amino acid composition of the peptide and the activation status of the cell. Demonstration of an antisense-specific effect is shown by targeting the expression of CD45 in cells treated with the PMO peptide conjugate CD45-(RxR)4. Antisense efficacy was also demonstrated by forcing alternative splicing of the CD45 mRNA using antisense PMO conjugates targeting the splice junctions of exons 4, 5, & 6. Successful uptake of PMO peptide conjugates into immune cells and target inhibition of gene expression suggests that PMO modified with arginine-rich peptides could potentially be used as an immune modulating therapeutic strategy.

### Su2.102. A Multi-Level Approach to Analyzing Immune Responses in Targeted Cells by Combining Cytomic and Proteomic Techniques of Cell Sorting and Protein Fractionation.

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In order to simplify the study of "Systems Biology" and facilitate the transition to "Cytomics" i.e. linking of genomics and proteomics to functionality of the cell, a multi-level and multi-pronged approach needs to be pursued. This requires that cell-based events that have so far been interrogated in isolation, for e.g. gene expression, protein synthesis, phenotype and function, signal transduction etc. now need to be integrated to better understand the "complex" cellular response. This continuum can to some extent be achieved by integrating cellular analysis techniques (for e.g., flow cytometry, imaging, multiplex assays) with genomics and proteomics techniques to understand the various aspects of such a response, thus accomplishing the unified evaluation of the cell. In the current study, the authors have attempted such an evaluation by integrating well-known techniques of flow cytometry-based phenotypic analysis and cell sorting with protein fractionation and analysis to better understand the complex nature of an immune response.

Peripheral blood mononuclear cells or T cell lines were subjected to "restricted polyclonal" or "true polyclonal" activation for 24hrs. Cells were then fluorescently labeled with antibodies to distinguish activated and non-activated cells and isolated using flow-based sorting techniques. Lysates of sorted-activated and sorted-non-activated cells were fractionated by two-dimensional liquid chromatography. The first dimension separation was achieved by chromatofocusing wherein intact proteins were separated by their isoelectric points and fractions collected by pH. These fractions were further separated by hydrophobicity on a second-dimension with high resolution, reversed-phase chromatography. The net result was the generation of high-resolution protein profile of the complex mixture. Qualitative and quantitative differences in protein profiles in activated and non-activated cells could be easily identified. The gel-free and intact nature of the fractions of interest allows for further interrogation and identification of the differentially expressed proteins in activated and non-activated cells to accomplish a thorough analysis of an activation profile.

The combination of flow-cytometry-based cell sorting and protein fractionation enables a more refined and targeted profiling and analysis of complex events associated with an immune response. Further combinations of genomic, proteomic and cytomic profiling will accomplish the unified evaluation of such a response.

#### **Su2.103. Notch Modulation of Peripheral Immunity: A Novel Approach to Antigen-Specific Immunotherapy.**

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Notch signalling plays an important role in the fate decisions made by multiple cell types during development. Using recombinant human Notch ligands, we have been evaluating the effects of Notch signalling on peripheral T-cell function both *in vitro* and *in vivo*. With murine CD4<sup>+</sup> T-cells *in vitro*, we have shown that Notch signalling mediated via the ligand Delta1 promotes a dose-dependent inhibition of effector cytokine production, along with an up-regulation of IL-4 and IL-10 synthesis. Similar modulation could be seen with Jagged1 and Delta4 ligands, and the balance between cytokine up-regulation and down-regulation was dependent upon both TCR and Notch signal strengths. Similar cytokine modulatory effects are also observed with human peripheral blood CD4<sup>+</sup> T-cells. Using the  $\gamma$ -secretase inhibitor DAPT to block cleavage of Notch, which is required for prototypic CBF1-dependent Notch signalling, we show that Delta-induced IL-4 and IL-10 upregulation involves this well-described pathway. By contrast, the Delta-mediated inhibition of effector cytokine production occurs more rapidly and involves a different, cleavage-independent signalling mechanism. Together, these activities are associated with a Delta1-modified differentiation of the cells: inhibited development of a Th1 phenotype and enhanced acquisition of a shared Th2/T<sub>reg</sub> profile. These effects are operative even under conditions for promoting Th1 or Th2 effector differentiation *in vitro*. Using both DNA gene-delivery and recombinant protein based treatments, we have also been able to down-modulate immune responses to antigens *in vivo*. Notch signalling may, therefore, provide a unique therapeutic approach to the treatment of immune disorders.

#### **Su2.104. Serum Heat-Inactivation and Granulocyte Presence Exerts a Differential Effect on the Antigen-Induced Proliferation of Lymphoid Subsets.**

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The results from an *in vitro* lymphocyte proliferation assay (LPA) can provide valuable information on the functional capabilities of an individual's immune system. However, the assay lacks standardization and the procedure and outcome can vary dependent on the laboratory's protocol. Here, we assessed via BrdU uptake and lymphocyte surface marker staining how heat-inactivation of autologous serum affects the *in vitro* lymphocyte proliferative response to *Candida albicans* antigen (CA) in the presence and absence of granulocytes. **Method:** Peripheral blood mononuclear cells (PBMC)  $\pm$  granulocytes (PBMC+) were cultured for 6 days with either fresh (FS) or heat-inactivated (HIS) autologous serum. BrdU was then added for an additional 24h to assess cell proliferation. The cells were harvested and stained on day 7 to determine the extent and phenotype of the proliferating cells. **Results:** A comparison of cultures containing FS vs. HIS revealed a significant difference in the degree of BrdU incorporation for the PBMC+ cultures only, with the FS group containing a greater number of BrdU<sup>+</sup> cells (CD3<sup>+</sup>,  $P < 0.05$ ; CD3-4<sup>+</sup>,  $P = 0.03$ ). Serum treatment did not significantly affect the PBMC cultures. Comparing PBMC vs. PBMC+ cultures, PBMC cultures overall incorporated more BrdU compared to the PBMC+ cultures. The presence of granulocytes significantly reduced the percentage of BrdU<sup>+</sup> cells in FS (CD3<sup>+</sup>,  $P = 0.027$ ; CD3<sup>+</sup>4<sup>+</sup>,  $P = 0.016$ ; CD3-4-,  $P = 0.029$ ) or HIS (CD3<sup>+</sup>,  $P = 0.005$ ; CD3-4-,  $P = 0.013$ ). Interestingly, the subset most affected by the presence of the granulocytes was the CD3- subset, independent of serum treatment (for PBMC vs. PBMC+: FS, 34% vs. 3% BrdU<sup>+</sup> cells; HIS, 45% vs. 0.5% BrdU<sup>+</sup> cells). **Conclusion:** *In vitro* assays are used as a measure of the *in vivo* state. Both complement and granulocytes are present in the *in vivo* situation, but they are often missing in *in vitro* assay protocols. While our initial interest was in determining whether heat-inactivation could significantly affect the *in vitro* immune response, the most interesting results were obtained with cultures containing granulocytes. Our results demonstrate that the selective removal of *in vivo* immune response components significantly affected the outcome of an *in vitro* assay. The outcome of assay variables such as those explored here should be further evaluated to improve the predictive scope of the *in vitro* proliferation assay and to determine the protective role of heat-sensitive serum protein against granulocyte suppression.

#### **Su2.105. Provision and Integration of HLA Alleles into Scientific Research.**

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The class I and class II human leukocyte antigens (HLA) mediate most adaptive immune responses. Disease susceptibility, disease resistance, vaccine development, autoimmunity, and clinical transplantation all represent immune responses in which HLA molecules play a vital role. A new generation of assays, including tetramers, ELISpots, cytokine-based flow cytometry (CFC), and more traditional assays such as chromium release and <sup>3</sup>H-thymidine incorporation, are poised to reveal the contribution of HLA molecules to various immune responses.

However, the application of these assays for identifying a specific immune response or lack thereof is complicated by the fact that most individuals have different HLA alleles and haplotypes. Furthermore, the many HLA alleles in the population tend to differ functionally. One must realize an HLA type in order to determine which class I or class II MHC molecule presented a particular peptide epitope in a positive ELISpot experiment. There are now 349 HLA-A, 627 HLA-B, 182 HLA-C, 394 DRB1, 80 DRB2-9, 28 DQA1, and 61 DQB1 alleles at the respective loci. The goal of our laboratory is to provide immunologists with a two-pronged HLA resource. **Our first HLA resource** is to provide researchers with a definitive class I and class II HLA type using DNA sequence-based typing. We have typed samples from populations throughout the world, identifying new and rare HLA alleles and haplotypes in a racially unbiased and precision manner. Of the MHC Class I and Class II linkage disequilibrium haplotype determining loci, we have typed 100 HLA-B and 55 HLA-DRB1 alleles. Our HLA typing laboratory is ASHI/CLIA accredited and is directed by ABHI certified laboratory director. **Our second HLA resource** is the HLA Ligand Database (<http://hlaligand.ouhsc.edu>) through which we provide an online resource whereby all known HLA class I and class II peptide ligands and motifs are catalogued. The HLA Ligand Database provides a resource whereby scientists can find or predict peptide epitopes that bind to particular HLA molecules. The database now contains 3724 peptide epitopes including those from A-C pathogens. In summary, our laboratory provides precision HLA typing and a catalogue of peptide ligands/motifs such that the functional role of human MHC molecules can be more easily elucidated.

#### **Su2.106. Potassium Channels IR, Kv1.5, and Kv1.3 Are Expressed on Human Dendritic Cells and Have a Functional Role in Maturation.**

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Potassium channels on immune cells have gained attention recently as promising targets of immunotherapy. We therefore turned our attention to potassium channels on antigen-presenting cells, specifically human dendritic cells, whose K<sup>+</sup> channel profile has not yet been described in the literature. We generated a population of immature dendritic cells by culturing monocytes from the blood of healthy human donors *in vitro* with GM-CSF and IL-4, as previously described, and then stimulated these cells with LPS or TNF- $\alpha$  to induce maturation. Whole-cell patch clamp analysis of these cells revealed an inward-rectifying K<sup>+</sup> current at early timepoints after stimulation, replaced by a mix of voltage-gated Kv1.3 and Kv1.5 channels at later stages of maturation. The identity of these channels was established by characteristic inactivation curves and pharmacological blockade. Further, immunofluorescent staining confirmed the presence of Kv1.3 and Kv1.5 on the surface of stimulated cells, colocalizing with HLA-DR. In order to determine whether these channels have a functional role in DC maturation, we then analyzed DCs stimulated in the presence of pharmacological Kv1.3 blockers, and found that both CD83 and CD80 upregulation and production of IL12 and IL6 were significantly impaired (28% decrease in CD83, 46% in CD80, 16% in IL6, 32% in IL12). To validate these results with a more

stable means of blocking Kv1.3 and Kv1.5 function, we turned to a dominant-negative Kv1 adenovirus construct, an ecdysone-inducible and GFP-tagged virus coding for a Kv1.3 molecule with a mutation in the pore-forming region. When induced, this mutant protein associates with other Kv1 family members in membrane tetramers and blocks channel function. We compared DCs infected with this Kv1 dominant-negative construct to DCs infected with a control virus coding for luciferase, and found that maturation of DCs expressing the Kv1.x viral product was highly reduced (53% less CD83 expression than control). Overall, our data are the first to report Kv1.5 and Kv1.3 expression on mature human DCs, and further indicate that these channels have a functional role in DC maturation. Our results therefore bolster the argument for K<sup>+</sup> channel blockade as an immunotherapeutic strategy targeting mature antigen presenting cells, which has implications for the treatment of a wide range of immune-mediated diseases. The therapeutic mechanism of Kv1.3 blockade in EAE, for example, has not yet been fully explored, and may include an effect on antigen presentation and priming of T-cells in conjunction with a direct suppression of T-cell function. Indeed, immunohistochemical staining of plaques in the brains of MS patients reveals Kv1.3 and Kv1.5 on microglia, offering a first piece of evidence that targeting these channels may affect antigen presentation to T-cell infiltrates within the brain.

#### **Su2.107. Monitoring the Effects of Immuno-Modulating Therapies.**

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As immune modifying therapies continue to be employed, developed and expanded to treat diseases such as rheumatoid arthritis, inflammatory bowel disease, HIV, HCV and cancer, a need exists to assess their effects on the immune system. Cylex<sup>®</sup> has developed several cell-based assays to measure the functional activity of lymphocytes from a small amount of whole blood. These assays utilize *in vitro* stimulation of a patient's blood sample, followed by magnetic isolation of specific sub-sets of lymphocytes. ImmuKnow<sup>™</sup>, an FDA-cleared assay, measures the global immunological response of CD4<sup>+</sup> cells and the T-cell Memory assay is a research test that measures antigen-specific responses of CD3<sup>+</sup> cells. By examining both global and antigen-specific cell mediated immunity, the Cylex platform is useful for monitoring the immune status of patients receiving immune modifying therapies. In this study, immune responses of apparently healthy individuals and drug-induced immunosuppressed transplant recipients to foreign antigens including Influenza, CMV, Tetanus and EBV as well as global immune responses to PHA were measured. Transplant recipients undergoing lymphocyte depleting therapies showed a marked decrease in global and antigen-specific responses. Recovery of these immunological responses occurred gradually over the next 6–12 months and was largely independent of the absolute lymphocyte count. Healthy volunteers with known natural exposure or vaccination showed significant responses to vaccine antigens whereas those not exposed or unvaccinated showed no response. In a study of response to tetanus, 90% of apparently healthy adults had an *in vitro* response to tetanus toxoid, but only 18% of transplant recipients and 28% of HIV<sup>+</sup> individuals had positive antigen responses. By examining both global and antigen-specific immune responses, the Cylex platform is useful for monitoring the effects of immuno-modulating therapies, whether they are designed to enhance or suppress the patient's immune response.

### Su2.108. Development of a Versatile Murine T Cell Expander Bead: Combining Quality with Practicality.

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Murine models of human disease are established immunological tools, prompting the need for a product to expand murine T cells *ex vivo*. The antigen CD28 provides an important T cell costimulatory signal. Dynabeads® coated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) were used in short-term (<14 day) cultures of BALB/c CD4+ or CD8+ T cells *in vitro*, in order to define ratios of the mAbs and of beads:cells allowing optimal upregulation of activation markers, increase in cell volume, and expansion of T cell numbers. The performance of the beads in both short and long-term cultures of various lymphocyte populations was then evaluated. Over 35-fold expansion of BALB/c CD4+ T cells was observed over 18 days; robust proliferation of CD8+ T cells, mononuclear cells and antigen-specific T cell clones could also be achieved, without loss of function. CD4+ T cells could be expanded for periods of ≥6 weeks; CD8+ T cells could not be expanded for >3 weeks. Following initiation of the cultures, restimulation was necessary at 7–12 day intervals. In summary, we have thus developed and rigorously validated a product allowing the expansion of diverse populations of murine T cells *in vitro*.

### Su2.109. Characterization of Endogenously Loaded Rhesus Macaque MHC Class I Peptides.

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The SIV-infected Indian Rhesus Macaque (*Macacca mulatta*) is an often used animal model for the study of HIV infections in humans. SIV, a retrovirus, is closely related to HIV-1 in nucleotide sequence and causes an AIDS like syndrome in the Rhesus Macaque. In terms of anti-viral immune responses, both macaques and humans mount strong cytotoxic T lymphocyte (CTL) driven anti-SIV and anti-HIV immune responses, respectively. In order to compare human and macaque anti-viral immune responses, to test SIV vaccine strategies, and to interpret viral escape mutants, human and macaque class I major histocompatibility complex (MHC) peptide binding properties must be elucidated. Our laboratory is focused upon the amino acid sequencing of pooled motifs and of individual peptide epitopes that are endogenously generated, trafficked, loaded, and class I MHC presented. In this study we transfected 4 macaque class I molecules (Mamu A\*02, A\*11, B\*01, and B\*12) into a human cell line and harvested 10–20 milligrams of each macaque class I molecule. Eluted peptides were initially subjected to 14 cycles of Edman degradation and the resulting data shows that macaque class I molecules are endogenously loaded with nonamers demonstrating P2, P9, and other ancillary anchors. At least 10 individual ligands were sequenced by MS/MS for each of the macaque class I, demonstrating variability in length as well as variability in sequence as compared to the pooled motifs. Finally, anchors and motifs not detected by other methods are apparent through the characterization of endogenous ligands. In summary, the characterization of endogenous macaque class I peptide

epitopes provide a more thorough understanding of immune responses in this animal model.

### Su2.110. NUSE and RLE: Quality Assessment of Oligonucleotide Microarray Data To Quantify Systemic Variation.

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**Introduction:** Measurement of differential RNA expression in multicenter clinical trials requires special attention to the quality in sample preparation. Sample collection and handling can adversely affect results; therefore, quality metrics are required to detect and assess the potential errors induced by these factors. We compared the reliability of newly devised quality metrics derived from fitted statistical models of probe level data from high-density oligonucleotide microarrays and compared them to standard GeneChip® microarray quality metrics. **Method:** We devised metrics using the Robust Multichip Analysis (RMA) process for deriving probe set summaries from GeneChip® microarrays. The first metric, Normalized Unscaled Standard Error (NUSE), provides a measure of relative chip quality derived from the residuals from the RMA model. The second metric, the Relative Log Expression (RLE), is an absolute metric that gauges variability of expression measures by summarizing the distribution of relative log expressions within a set of microarrays against a reference set. The RLE summaries are sensitive to technical sources of variability that are large compared to biological variation. These metrics were compared to standard quality metrics: Percent Present calls, GAPDH 3'/5', Background, and Scaling Factor. Two clinical trial sample sets were assessed: 368 microarrays from a Type I diabetes trial and 350 arrays from a ragweed allergy trial. A set of standard normal human control samples were sent blinded within patient sets during the course of ITN clinical trials and were used as the reference set against which RLE assessments were made. **Result:** NUSE and RLE metrics detected systematic variation within certain participant sets that were not detectable using the standard Affymetrix quality metrics. Elevated GAPDH 3'/5' ratios typically cited as an indicator of poor quality RNA showed no relationship to quality when applying the NUSE and RLE (cor, 0.09). Hybridization/washing artifacts were easily visualized by plotting NUSE residuals. While not always true, Percent Present calls provided the closest approximation to NUSE and RLE; in cases of extremely low Percent Present, NUSE and RLE are adversely affected (cor, -0.50). In both trials in which these metrics were applied, we identified chips within a participant time series that required exclusion from the analysis that would not have been discovered otherwise. **Conclusion:** In differentiating NUSE from RLE, NUSE values have no units and can only be used to assess the relative quality of arrays within an analysis set; RLE summaries provide a measure of reproducibility of gene expression data that can be compared across batches, experiments, or trials. Reflecting variability in expression measures, these proposed metrics provide a better basis for judging quality compared to standard metrics.

### Su2.111. High Resolution HLA Typing for Vaccine and Autoimmune Studies.

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The class I and class II Human leukocyte antigens (HLA) mediate most, if not all, adaptive immune responses. Since each individual has a different combination of class I and class II HLA molecules inherited from her/his parents, the immune response to infection and vaccination differs respectfully from person to person. In addition, many autoimmune diseases, such as arthritis and diabetes, are associated with particular class I and/or class II molecules. Knowledge of a patients/populations HLA molecules therefore becomes a key element in vaccine design and uncovering autoimmune triggering mechanisms. HLA DNA sequence-based typing (SBT) represents a method that identifies all polymorphisms in a racially independent manner. Our laboratory pioneered and continues to employ a precision HLA SBT method for studies of bone marrow transplantation, vaccine development, and autoimmunity. The class I and II HLA SBT process is split into three steps- PCR, DNA sequencing, & data processing-and here we report on the evolution of these 3 steps during the high resolution SBT of more than 20,000 individuals in the last 8 years. We describe the migration of our method from a solid phase sequencing chemistry to a capillary DNA sequencer, we discuss the location of PCR and DNA sequencing primers, we compare SSP and RSCA methods for the resolution of ambiguities, we discuss the software packages available for data processing, and we describe the costs now associated with HLA SBT. The robust nature and cost-efficiency of HLA SBT supports the continued application for studies of disease resistance, vaccine design, and autoimmunity.

## Organ Transplantation

### Su2.112. On the Possibility of Oral Tolerance To Be Used in Graft Transplantation.

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Oral tolerance has been studied for many decades. Some of its immunological mechanisms have been revealed. And oral tolerance has been used to treat autoimmune diseases, such as rheumatoid arthritis, uveitis, EAE, as well as some others, experimentally and/or clinically. Many of these seemed have obtained beneficial effects. Interestingly, oral tolerance has also been used in reproductive immunology, i.e. pregnant immunology. It has been used to induce maternal tolerance to paternal antigens, in order to establish an immune tolerance to the semi-allograft fetus by the mother to treat the abortions. But, as a way of being possible to establish an antigen-specific immune tolerance, why should not we use it to the transplantation immunology, trying to use this way to induce an immune tolerance to the transplant graft antigens, for the purpose to establish an immune tolerance to avoid the graft rejection. And this is what I would like to suggest strongly, and hope to draw some special attentions.

### Su2.113. Evidence for Naturally Occurring and Induced Regulatory T-Cells in Non-Human Primates.

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Non-human primates (NHP) are often used as preclinical model for the evaluation of tolerance inducing therapies. Regulatory T-cells (Treg) may be crucial for the maintenance of tolerance. Here we describe the identification and characterisation of Treg in NHP.

CD4+CD25+ cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy monkeys and from PBMC of 3 long-term drug free kidney transplant recipients (long-term trans-

plant survivors, LTS). One LTS monkey is 23 years post transplantation after treatment with pre-transplant bloodtransfusions and CsA for 1 year and two monkeys are 3 years after cessation of treatment with costimulation blockade and CsA. Several characteristics known to be specific for CD4+CD25+ Treg in humans and rodents were investigated.

Naturally occurring CD4+CD25+ cells were present in healthy monkeys as well as in the LTS monkeys. Similar to CD4+CD25+ T-cells in humans, CD4+CD25+ T-cells in NHP do not proliferate upon polyclonal or allogeneic stimulation. However, in contrast to humans, proliferation is only slightly increased upon addition of IL-2.

When CD4+CD25- cells are activated by a polyclonal (ConA) or allogeneic stimulus, CD4+CD25+ cells can suppress this proliferation and, although not proliferating themselves, the CD4+CD25+ cells cannot inhibit proliferation when they are irradiated. Inhibition cannot be blocked by anti-IL-10 or anti-TGF-beta.

CD25+ cells have, in accordance with human CD25+ cells more intracellular CD152 than CD25- cells.

CD4+CD25+ cells are present in the LTS monkeys in the same numbers as can be found in healthy NHP and they also do not proliferate upon ConA or allogeneic (donor specific and 3rd party) stimulation. To evaluate possible donor specific regulation in other subsets, anti-TGF-beta and anti-IL-10 were added to whole PBMC cultures. In the LTS monkey 23 years post transplantation, regulation seems to be TGF-beta mediated, which correlates with the presence of large amounts of latent TGF-beta in the kidney. In the other two LTS monkeys, neither anti-TGF-beta nor anti-IL10 seems to uncover proliferation, but the combination of both antibodies induces increased proliferation against the donor.

Naturally occurring Tregs, with similar characteristics as described in humans are present in NHP and can suppress CD4+CD25- cells. Although CD4+CD25+ cells in LTS monkeys are immunosuppressive, this is not donor specific, and therefore, donor specific regulation may be confined to another T-cell subset.

### Su2.114. Phenotypically and Functionally Distinct CD8+ Lymphocyte Populations in Long-Term Drug-Free Tolerance in Human Kidney Graft Recipients.

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**Objectives:** Kidney graft recipients with stable renal function in absence of immunosuppressive therapy are characterized by a skewed TCR Vbeta chain usage, essentially in the CD8+ subset. Therefore, the present study analyzes in more detail phenotypical and functional alterations of CD8+ lymphocytes in these drug-free tolerant patients (DF-Tol).

**Methods:** Peripheral blood CD8+ lymphocytes from DF-Tol, chronic rejection (CR), healthy controls (HC), and patients with stable kidney function under immunosuppression (StA) were analysed by flow cytometry for phenotypic and cytotoxic markers. Apoptosis was measured by annexin-V staining and proliferation by CFSE.

**Results:** Phenotyping revealed an increase of CD45RA-CCR7+ central memory and a decrease of CD45RA+CCR7-effector CD8+ lymphocytes in DF-Tol versus CR. The expression of CD28+ and CD27+ on effector and effector memory CD8+ lymphocytes was decreased in CR, with a high correlation between both markers. These profiles were stable over time and independent of treatment. The cytotoxic nature of CD8+ CD28- cells was indicated by the

higher expression of perforin and granzyme A in CR versus DF-Tol, the inverse correlation of these markers with CD28 and CD27 expression, and the increased expression of the cytotoxic marker CD57 on the CD8+ CD28- subset. The CD8+ CD28- lymphocytes expressed lower levels of Fas and were less sensitive to apoptosis than their CD8+ CD28+ counterparts. HC displayed the same profile as DF-Tol, indicating an increase of CD8+ CD28- effector lymphocytes in CR rather than a decrease in DF-Tol. Sta displayed a mixed profile, with some patients resembling DF-Tol and others mimicking CR.

**Conclusion:** A strong cytotoxic CD8+ CD28- signature differentiates CR from DF-Tol and HC, suggesting a suppression of pathological cytotoxicity in DF-Tol. Further investigation of the targets of these cytotoxic cells and evaluation of these profiles to identify patients at risk for CR are warranted.

### Su2.115. Could Early Posttransplant Allosensitivity Predict Patients at High Risk for Rejection and Graft Loss in Kidney Transplantation.

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While the relevance of pre-formed anti-HLA antibodies is well determined, less known is the role of alloantibodies produced after cadaveric kidney transplantation on graft outcome. The aim of this study was to evaluate the incidence, dynamics and profiles of developed post-transplant anti-HLA antibodies and their impact on graft outcome in kidney recipients. We retrospectively investigated 72 patients with no detectable alloantibodies prior to their first cadaveric kidney transplantation for the period October 1998-January 2004. All patients received triple immunosuppressive therapy. Biopsy-proven acute rejection was observed in 11.1% and chronic rejection-in 15.3% of the recipients. The alloantibody profile was determined with Flow-PRA Screening and Specific Tests (One Lambda, USA). Anti-HLA antibodies after transplantation were detected in 22.2% of the studied patients. Of them 56.25% had HLA class I-reactive only alloantibodies, 18.75%-class II-reactive only, and 25% both HLA class I and class II reactive. Donor-specific reactivity was determined in 75% of the alloantibody positive patients. A correlation between triplet mismatches and alloantibody production was observed. Most of the recipients (81.25%) produce alloantibodies in the early post-transplant period. Nine of 16 alloantibody-positive patients (56.2%) lost their graft due to immunologic cause compared to those with no detectable antibodies (7.1%). Alloantibody titre evaluation demonstrated that patients with high titers experienced acute rejections and early graft loss, while in those with low and stable titers chronic rejections were more common. In conclusion our data suggest that recipients with post-transplant HLA-reactive antibodies were more likely to develop allograft rejection that might be predicted early following transplantation. Algorithms, on the basis of our approach, could be tested for influence of post-transplant allosensitization on graft survival.

### Su2.116. Humoral and Cellular Response to Influenza Vaccination in Human Recipients Naturally Tolerant to a Kidney Allograft.

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**Background:** A rare cohort of kidney recipients continue to enjoy a normal renal function years after interruption of their immunosuppressive treatment and are considered as "tolerant". To assess whether this state of tolerance is specific to their graft and not the result of a state of immunodeficiency, we studied the immune response of these patients following influenza vaccination. We compared this response to that of kidney recipients under conventional maintenance immunosuppression and to healthy volunteers.

**Patients and Methods:** 4 tolerant recipients (TOL), 5 immunosuppressed-recipients (IS) and 9 healthy volunteers (HV) received a trivalent influenza vaccine (A/Moscow/10/99; A/NewCaledonia/20/99; B/HongKong/330/01) during the period of 2003–2004. The 3 groups were matched for age and renal function (mean age: 49 ± 22, 48 ± 14 and 48 ± 14 years for TOL, IS and HV respectively; mean creatinemia: 104 ± 7.2 μmol/l for TOL, 108 ± 16.8 μmol/l for IS). All IS recipients received a conventional immunosuppressive treatment, associating a calcineurin inhibitor with mycophenolate mofetil. The humoral response was measured by hemagglutination inhibition (HI) titers before vaccination and after 1 and 3 months. A positive response was defined as a 4 fold increase in HI titers. During the period of 2004–2005, 4 TOL, 8 IS and 9 HV received a trivalent influenza vaccine (A/Fujian/411/2002, A/Newcaledonia/20/99, B/Shanghai/361/2002). The cellular immune response was analyzed before and 1 month after vaccination. The frequency of specific T cells was determined by IFNγ-secreting T cells detected with an Enzyme-Linked-Immunesorbent Spot (ELISPOT) assay after a 24 hour *in vitro* stimulation with the vaccine.

**Results:** According to the viral strain, a positive humoral response was observed in 25 to 75% of TOL, in 0 to 40% of IS and in 33 to 89% of HV. Thus, IS recipients presented a poor humoral response as compared to HV, reaching a significant difference for the A/NewCaledonia strain ( $P < 0.05$ ), whereas the humoral response for TOL was not statistically different from HV. However, 1 month after vaccination, 87% of IS presented a strong cellular response to the influenza vaccination, whereas a comparable positive response was observed only in 50% of TOL and 55% of HV (not statistically different). Taken together, these data suggest that the patients who are tolerant to their kidney respond to the vaccination. In addition we show that the frequency of cells producing IFNγ following vaccination is unusually high, possibly due to repetitive stimulations.

**Conclusion:** TOL recipients present a humoral and cellular response to influenza vaccination similar to HV, suggesting that the tolerance state of this small cohort of patients is not related to a global immunodeficiency.

### Su2.117. Pharmacodynamic Monitoring of Calcineurin Inhibitors by Quantitative Analysis of NFAT-Regulated Gene Expression.

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With the introduction of calcineurin inhibitors (CNI) long-term allograft function has significantly improved. The problem of limited therapeutic margins and the toxicity of CNI remain unsolved. The quantitative assessment of inhibition of NFAT-regulated gene expression 2 hr after Cyclosporine A (CsA) intake



represents a novel approach to evaluate the biological effectiveness of CsA therapy and provides means to enable individualized immunosuppressive regimens.

In 55 patients carrying heart allografts we compared the degree of inhibition of IL-2, IFN- $\gamma$  and GM-CSF gene expression with the peak blood concentration of CsA. Functional immunosuppression as assessed by RT-PCR varies considerably among CsA treated individuals with stable graft function. Given the relatively constant level of inhibition over a broad range of drug concentrations, we felt that a considerable group of patients with unnecessarily high CsA doses might benefit from a reduced dosing of the drug without compromising the efficacy of the immunosuppressive therapy. Therefore, we started a clinical study reducing the dosage of CsA with close pharmacodynamic monitoring of the patients. Six patients after kidney transplantation enrolled in this study were monitored over the period of more than one year so far. In all patients the doses of CsA could be safely reduced without significantly changing the level of immunosuppression.

In conclusion, patients treated with calcineurin inhibitors might benefit from a reduced dosage of the drug, if they respond to CNI with a strong inhibition of NFAT-regulated gene expression.

#### **Su2.118. Profiling of "Operationally Tolerant" Kidney Recipients Using SELDI-TOF Mass Spectroscopy.**

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Despite the discovery of potent immunosuppressive agents, chronic rejection remains the main cause of graft loss after solid organ transplantation. In addition, exposure to immunosuppression may cause infections and malignancies which contribute to the high level of post-transplant morbidity. Achieving clinical tolerance would represent a major progress in transplantation. Operationally tolerant patients, accepting their graft in an immunosuppressive free environment after clinical organ transplantation, are still extremely rare but represent a unique opportunity of identifying tolerance fingerprints. Surface Enhanced Laser Desorption Ionization Time-of-Flight (SELDI-TOF) mass spectroscopy analysis was used here to profile sera from drug-free tolerant kidney recipients ( $n = 7$ ) compared to recipients undergoing chronic rejection ( $n = 8$ ) and control non-grafted patients with renal failure related to "non-immunologic" kidney diseases (uropathy, diabetes,  $n = 8$ ) and whose renal function match that of recipients with chronic rejection. Sera were fractionated into 6 fractions and each fraction was loaded on 2 different chromatographic surfaces (metal affinity IMAC30-Cu<sup>2+</sup> and cation exchange CM10). Results were cross validated by analysing serum samples from each individual in two independent experiments. Four protein peaks of interest were selected in 3 out of 6 fractions on the 2 chemistries. The 3 first protein peaks were found significantly increased in sera from patients with chronic rejection and renal failure controls compared to operationally tolerant patients ( $P < 0.05$ ) suggesting that these protein peaks may be related to renal failure. However, interestingly, the fourth protein peak was increased specifically in sera from operationally tolerant patients ( $P < 0.05$ ) but neither found in sera from patients with chronic rejections nor in renal failure controls. Considering the absence of specific tolerance markers, the identification of a non invasive and specific biological signature of tolerance would

open new perspectives for managing immunosuppressive drugs in long term recipients.

#### **Su2.119. Mega Dose Allogeneic Hematopoietic Stem Cell Transplantation, Natural Suppressor Cell Chimerism and Tolerance in Clinic- Ahmedabad Experience.**

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Aims:

Mega dose hematopoietic stem cell transplantation (HSCT) was performed in 150 living-related donor (LRD) renal allograft recipients under different conditioning protocol treated group (Tn) with equal number of controls (Cn), for chimerism associated tolerance.

Methods:

Unmodified HSCT (mean:  $20 \times 10^8$  cells/kgBW recipient) were administered in recipients' thymus, bone marrow (BM), portal, peripheral circulation with non-myeloablative conditioning. Transplantation was performed following negative lymphocytotoxicity cross matching. Immunosuppression regime in Tn included low dose cyclosporine (CsA), Prednisolone. Tn received standard triple drug immunosuppression. CsA doses in both groups were adjusted to maintain trough levels around 110 ng/ml. Immunologic monitoring included donor BM lineage subset chimerism measurement with CD epitope markers using flow cytometry at different time intervals in peripheral blood (PB) and BM.

Results:

Tn with mean follow up of 54.4 months, had adequate stable graft function with mean serum creatinine (SCr), 1.1 mg %. Chimerism was stabilized in PB, measured in terms of CD3<sup>dim</sup>CD4- CD8- (mean: 3.44%-PB, 5.82%-BM) and CD34<sup>+</sup> cells (mean: 1.49% -PB, 2.84 % BM). Tn had 100 % graft + patient survival with 8 rejection episodes on low dose CsA monotherapy; 11 patients are without immunosuppression for >3 years. Cn with mean follow up of 52 months, had mean SCr, 2.75 mg %. Chimerism was absent in PB and BM measured with same parameters. There was 68 graft, 72 patient survival and 105 rejection episodes on standard triple drug immunosuppression.

Conclusion:

This is the first report of reproducible cytoanalytic method identifying subset of donor chimeric cells in LRD recipients correlating with clinical tolerance.

### Su2.120. Analysis of HLA Class One Alloantibodies in the Sera of Sensitized Patients on Hemodialysis.

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**Introduction:** The specificity of HLA class one (HLA-A and B) alloantibodies was studied in 30 serum samples from antibody positive, potential kidney transplant recipients who had percent panel reactive antibody values (%PRA) of more than 5%.

**Methods:** Antibody detection was performed using the microlymphocytotoxicity technique. In this study, the specificity of antibodies was categorized as either private epitopes or cross reactive group (CREG) epitope clusters. A P value of less than 0.05 and R values greater than 3.841 indicated a significant association between a known antigen and an unknown serum sample.

**Results:** No specific antibody was defined in seven (23.33%) serum samples with %PRA values of less than 20%. At 88% to 100% PRA values (Seven patients = 23.33%), most of the serum reactions were positive because the patients had developed multiple antibodies against a large array of HLA antigens. Identifiable antibodies were found in 16 (53.3%) serum samples with % PRA values between 20% to 87%. Anti-CREG antibodies with or without antiprivates were identified in nine of the 16 samples (59.25%), whereas only six (37.5%) of these 16 samples (6.25%).

Contained two different private antibodies.

**Conclusion:** Antibody reactivity against CREG clusters was more common among patients with defineable antibodies. Knowing the specificity of HLA antibodies in patients sera, helps to define a suitable kidney with negative cross-matching for sensitized patients, among previously HLA typed donor banks. The records of these banks are maintained in computer programs at the Isfahan Transplantation Laboratory.

### Su2.121. IgG Monitoring Can Predict the Development of Infection in Heart Transplantation.

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**Introduction.** Infectious complication continue to represent a significant source of morbidity and mortality in heart transplantation. Among risk factors for 5 year mortality conditional on survival to 1 year, treatment for infection during the first year has been associated with a 28% increase in the risk of mortality.

**Objective.** To assess humoral immunity markers that can provide prognostic value for the development of infection in heart transplant recipients. **Patients and methods.** Between October 2002 and December 2004, we prospectively studied the clinical outcome of 32 consecutive heart transplanted recipients performed at a single center (84.4% men, aged 54 ± 9 years). Patients received induction therapy with daclizumab. Maintenance immunosuppression included mycophenolate mofetil, prednisone and either cyclosporine or tacrolimus based on side-effect profile. Humoral immunity studies included: Immunoglobulin (IgG, IgA, IgM) and IgG subclasses determined by nephelometry in serum samples obtained before transplantation, 7 days post-

transplantation and 1-month after transplantation. Potential clinical risk factors were evaluated, including recipient's age, pretransplant CMV serological status of donor (D) and recipient (R), and occurrence of treated rejection episodes before infection.

**Outcome measure.** Infection requiring intravenous (IV) drug therapy during the first year. Cox regression analysis was used to determine whether immunological parameters were associated with infectious events. Immunoglobulin levels were split into two groups using the median value [m.v.] observed as the cut-off. **Results.** During a mean follow-up of 12.6 months 12 patients had at least one episode of infection (37.5%); 9 of these were CMV infections treated with IV gancyclovir, 2 were episodes of bacterial pneumonia and one patient had pulmonary nocardiosis. Pretransplant IgG (below m.v. = 1160 mg/dl) (Relative Risk [RR] 6.31; 95% confidence interval [CI] 1.38-28.9; *P* = 0.018) and post-transplant IgG levels at day 7 (below m.v. = 676 mg/dl) (RR 8.84; CI 1.07-72.54; *P* = 0.042) were associated with an increase in the risk of developing infections. The decreased risk of development of infection associated with an increase of 100 mg/dl in the level of pre-transplant IgG was 27% (RH 0.71; CI 0.52-0.97; *P* = 0.036). D+/R- CMV serostatus was identified as significant risk factor of infection (RR 3.54; CI 1.11-11.26, *P* = 0.032). In the analysis that included clinical predictive variables, decreased values of pre-transplant IgG and post-transplant IgG levels at day 7 showed independent significant predictive values (RR 6.30; CI 1.38-28.9, *P* = 0.018 and RR 8.83; CI 1.07-72.54, *p* = 0.043, respectively). **Conclusions.** IgG level monitoring, a rapid and well standardized nephelometric determination, at baseline (pre-transplant) and 7 days post-transplant might help to identify the risk of developing infection in heart transplantation.

### Su2.122. New Insights in Mechanisms of Action of Different Immunosuppressive Drug Therapies by Assessing the Pharmacodynamics in Heart Transplant Recipients.

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**Objective:** Therapeutic drug monitoring (TDM) in heart transplanted (HTx) recipients for the calcineurin-inhibitors (CNI) cyclosporin (CsA) and tacrolimus (TRL) and the mTOR-inhibitors sirolimus (SRL) and everolimus (ERL) relies on the daily measurements of blood concentrations (pharmacokinetic, PK) to maintain drug concentrations within their respective target ranges. However, the unknown absolute bioavailability, the inter-individual variability regarding the biological effect and drug interactions are the limitations of TDM based on PK alone. Therefore, in this study we used our established T lymphocyte function assays to monitor the pharmacodynamics (PD) of a CNI based immunosuppression with a SRL based immunosuppression and compared both therapy regimes after conversion with a ERL based immunosuppression in HTx recipients.

**Methods:** 10 HTx recipients were converted from CNI to ERL, because of severe renal dysfunction (5 patients), and from SRL to ERL, because of the approval of ERL for HTx (5 patients). 24 or 48 hours after the last CNI or SRL dose, respectively, patients were treated with a fixed dosing regime: of 0.75 mg / BID ERL on days 1 to-3. PK measurements of CNI were done by EMIT and for SRL and ERL by LC-MS/MS. PD effects on adhesion molecules (e.g. CD11a, CD11b, CD54, CD62) and cytokines (e.g. IFN- $\gamma$ , TNF- $\alpha$ ,

IL-2, IL-4) of T lymphocytes in peripheral blood were analyzed by FACS.

**Results:** For all patients  $C_{\text{trough}}$  blood concentrations were within their respective target levels ( $\pm$  SEM): CsA:  $115.67 \pm 4.67$  ng/ml; SRL:  $10.52 \pm 1.52$  ng/ml; ERL:  $4.13 \pm 0.63$  mg/L. PD effects at trough (%expression  $\pm$  SEM) under CNI therapy compared to PD effects under SRL therapy were significant different for the following parameters ( $P < 0,05$ ): CNI: CD11a:  $45,56 \pm 5,67$ ; CD62:  $5,6 \pm 1,24$ ; IFN- $\gamma$ :  $32,40 \pm 4,23$ ; TNF- $\alpha$ :  $13,60 \pm 4,05$ ;  $28,78 \pm 8,11$ . SRL: CD11a:  $34,38 \pm 7,03$ ; CD62:  $16,88 \pm 7,89$ ; IFN- $\gamma$ :  $51,63 \pm 9,07$ ; TNF- $\alpha$ :  $47,43 \pm 10,56$ . PD effects at trough (%expression  $\pm$  SEM) in patients under CNI or SRL therapy before and after conversion to ERL (day-3) were significant different for the following parameters ( $P < 0,05$ ): TNF- $\alpha$ : CNI:  $13,60 \pm 4,05$ , ERL:  $28,34 \pm 6,03$ ; IL-2: CNI:  $25,25 \pm 3,94$ , ERL:  $47,78 \pm 5,05$ ; CD11a: SRL:  $34,38 \pm 7,03$ , ERL:  $51,48 \pm 3,37$ ; CD62: SRL:  $16,88 \pm 7,89$ , ERL:  $4,18 \pm 0,62$ .

**Conclusion:** For the first time, the assessment of PD effects on T cell functions showed new insights of mechanisms of action of different immunosuppressive therapies in HTx recipients. Furthermore, the results indicate that assessing PD effects on T lymphocyte functions may enhance the value of PK monitoring to avoid drug toxicity and to enhance drug efficacy.

### Su2.123. Assessment of Peripheral Blood Dendritic Cell Subsets and Their Functions To Monitor Immunosuppression after Heart Transplantation.

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**Objective:** Everolimus (ERL), the derivate of the mTOR inhibitor sirolimus (SRL) with a shorter half life, was recently approved for immunosuppressive therapy after heart transplantation (HTx). Dendritic cells (DCs) are potent antigen presenting cells that posses the ability to activate naive T cells. Earlier studies revealed an inhibitory effect of SRL on antigen expression in isolated DCs. However, nothing is known but the effects of either SRL or ERL on DCs in peripheral blood in vivo (pharmacodynamics, PD). Therefore, in this study we used DCs assays to monitor the conversion of a SRL based immunosuppression to a ERL based immunosuppression in HTx patients.

**Methods:** We assessed PD effects and the pharmacokinetics (PK) in 6 HTx patients before and after conversion of SRL to ERL. 48 hours after the last SRL dose (0,5 or 1 mg QD / day) patients were treated with a fixed dosing regime of 0,75 mg of ERL BID on days 1 to 3. All patients received mycophenolate mofetil / BID co-therapy with a dose range of 500-2000 mg / day. PK measurements of SRL and ERL were measured by LC-MS / MS. FACS analysis was used to characterized myeloid and plasmacytoid DCs by expression of CD11c<sup>+</sup> and CD123<sup>+</sup>, respectively, and to assess DCs cytokine production (IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-12).

**Results:** For all patients  $C_{\text{trough}}$ -values of SRL and of ERL on day 3 were within their respective target levels: SRL:  $4.38 \pm 0.17$  ng/ml and ERL:  $4.13 \pm 0.63$  mg/L. PD effects (%expression  $\pm$  SEM) on day-3 under ERL therapy were compared with PD effects under SRL at trough levels: CD11c<sup>+</sup>: ERL:  $51,26 \pm 6,34$ , SRL:  $48,90 \pm 3,41$ ; CD123<sup>+</sup>: ERL:  $22,08 \pm 2,83$ , SRL:  $16,18 \pm 2,65$

( $P < 0,05$ ); IL-1 $\beta$ : ERL:  $25,14 \pm 7,38$ , SRL:  $10,50 \pm 5,86$  ( $P < 0,05$ ); IL-12: ERL:  $9,72 \pm 4,52$ , SRL:  $5,45 \pm 1,88$  ( $P < 0,05$ ); TNF- $\alpha$ : ERL:  $24,44 \pm 7,21$ , SRL:  $28,68 \pm 9,55$ ; IL-8: ERL:  $27,62 \pm 10,49$ , SRL:  $25,48 \pm 8,13$ .

**Conclusion:** For the first time, the effects of SRL and ERL on DC subsets and cytokine production in peripheral blood were assessed in HTx patients. The results show that the mTOR-inhibitors SRL and its derivative ERL have different effects on DCs subsets and functions. Peripheral blood DCs monitoring (PD) may provide a new insight of mechanisms of immunosuppressants to improve the safety and efficacy of immunosuppressive therapy after HTx.

### Su2.124. T Helper Cells Produced Increased IL-6 and TNF- $\alpha$ Levels in Human Heart Transplant Recipients.

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**Objective:** Differential expression of cytokine production of T helper 1 (Th1) and 2 (Th2) cells is known to play a major role in allograft rejection. Furthermore, in earlier studies we show the importance to measure the pharmacodynamics (PD) of immunosuppressive drug therapy after heart transplantation (HTx) to enhance pharmacokinetic (PK) drug monitoring. Therefore, in this study we assessed the PD effects of the combination cyclosporin (CsA) plus mycophenolate mofetil (MMF) on the cytokine production of Th1 and 2 cells in human HTx recipients at different time points and compared these with the cytokine expression in untreated control groups.

**Methods:** Blood from 50 HTx recipients was drawn before (C0) and 2h after dosing (C2) with CsA and MMF. Untreated control groups consisted of 47 patients with dilated cardiomyopathy (DCM) and 20 healthy volunteers (HV). CsA and mycophenolic acid (MPA) concentrations were measured by EMIT. Cytometric bead array (CBA) assay was used to asses cytokine production of Th1 cells (INF- $\gamma$ , IL-2, TNF- $\alpha$ ) and Th2 cells (IL-4, IL-10, IL-6) in serum with FACS.

**Results:** Evening doses of CsA (25 / 50 / 75 or 100 mg) and MMF (250 / 500 or 1000 mg) produced C0-levels: CsA:  $160 \pm 11$  ng/ml and MPA:  $1.7 \pm 0.2$  mg/L. Morning doses of CsA (50 / 75 or 100 mg) and MMF (250 / 500 / 1000 or 1500 mg) produced C2-levels: CsA:  $533 \pm 53$  ng/ml and MPA:  $7.5 \pm 1.2$  mg/L. Cytokine production (pg/ml  $\pm$  SEM) in HTx recipients was significant different at C0 compared to C2 for TNF- $\alpha$  and IL-6 ( $P < 0,05$ ): C0: TNF- $\alpha$ :  $9 \pm 2$ ; IL-6:  $75 \pm 11$ ; C2: TNF- $\alpha$ :  $5 \pm 1$ ; IL-6:  $51 \pm 12$ : Production for both TNF- $\alpha$  and IL-6 was significant increased at C0 and C2 compared to DCM and HV ( $P < 0,05$ ): TNF- $\alpha$ : DCM:  $2 \pm 0,5$ ; HV:  $1 \pm 0,1$  IL-6: DCM:  $30 \pm 7$ ; HV:  $32 \pm 6$ .

**Conclusion:** For the first time, the CBA assay was used to assess differences in cytokine production of Th1 and 2 cells at different time points of CsA and MMF treated human HTx recipients and untreated control groups. Future studies in HTx recipients to show if a PD monitoring of IL-6 and TNF- $\alpha$  production could be biomarkers for clinical outcome like rejection or infection.

### Su2.125. The Expression of Survivin in T Cells and Its Possible Significance.

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**AIM:** To investigate the expression of survivin in T cells *in vitro* and *in vivo*. **METHODS:** Expression of survivin in splenocytes stimulated with ConA or alloantigen was detected by immunohistochemical staining. Anti-IL2 receptor antibodies were added to inhibit T cells proliferation. Flow cytometry was used to analyze CD3, CD25 and survivin expressions. Splenocytes from C57BL/6(H-2<sup>b</sup>) mice were infused into F1 mice (Balb/c×C57BL/6) to develop GVHR model, and expression of the survivin was observed by immunohistochemical stain *in vivo* after alloantigen stimulation. **RESULTS:** Expression of survivin was detected in stimulated cells. Survivin+ cells were CD3+ T cells, and lymphoblasts could simultaneously express CD3, CD25 and survivin. Anti-IL2 receptor antibodies inhibited the survivin expression, and the percentage of M phase cells decreased. From day 4 to 12 after infusion, the alloantigen-activated T cell infiltrating in livers of F1 mice expressed survivin. **CONCLUSIONS:** As a marker of activated T cells, survivin can be detected in T cells after *in vivo* and *in vitro* stimulation and divided the procedure of T cells response to alloantigen into activation and effect phase.

**Keywords:** T cells; survivin; CD25

#### Su2.126. Anti-CD132 Monoclonal Antibodies Inducing Activated T Cells Apoptosis after Alloantigen Stimulation.

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**Aim:** To investigate the mechanism of anti-CD132 monoclonal antibodies (mAbs) inhibiting T cells proliferation *in vitro*.

**Methods:** Balb/c and C57BL/6 mice splenocytes were harvested for two-way mixed lymphocyte culture (MLC)(control group). Anti-CD132 mAbs (final concentration 100mg·L<sup>-1</sup>) were added in MLC on day 0 (group 1) or day 3 (group 2). Fluorescence activated cell sorting (FACS) was used to measure the proliferation(CFSE), the apoptosis of T cells (PE-CD3, FITC-Annexin-v) and cell cycle (propidium iodide stain). The expression of survivin(a kind of protein highly expressed in the G2/M phase) in T cells was detected by immunochemical stain.

**Results:** Multi-generation of CFSE-labeled splenocytes were found dividing and their fluorescent strength decreased in MLC in the control group while there was no obvious change in fluorescent intensity in group 1 and group 2 implying no sign of division. On day 3, apoptosis induced by anti-CD132 mAbs was detected in partial T cells, but not in the former two days in group 1. In group 2, the number of cells in M phase (activated T cells) decreased and apoptotic cells increased on the fourth day. The phenomena were not observed in control group ( $P < 0.01$ ). Expression of survivin in T cells was detected in control group but not in group 1 and 2.

**Conclusions:** Our study shows that blockade of CD132 signaling pathway can inhibit T cells proliferation *in vitro* by means of inducing activated alloreactive T cells apoptosis but not affecting the resting T cells. Anti-CD132 mAbs may be valuable candidates for clinical applications.

**Key Words:** CD132, apoptosis, two-ways MLC

#### Su2.127. Cytomegalovirus-Specific CD4 T-Cell Immunity Is Associated with Protection from Human Cardiac Allograft Rejection and Negative Coronary-Artery Remodeling.

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Cytomegalovirus (CMV) infection in cardiac allograft recipients, particularly symptomatic disease with high levels of viral replication, has been associated with a greater risk of graft rejection and negative coronary-artery remodeling, a likely precursor of frank transplant arteriopathy (TA). The role of CMV-specific T-cell responses in modifying these risks in patients with asymptomatic CMV replication after transplant is unknown. **Methods.** We longitudinally studied post-transplant CMV-specific T-cell immunity by intracellular cytokine staining for IFN- $\gamma$ <sup>+</sup> cells using flow cytometry in 30 consecutive cardiac transplant recipients during the first year after transplant. All patients had pre-transplant acquisition of CMV based on antibody seropositivity, and received initial post-transplant CMV prophylactic therapy with ganciclovir/valganciclovir and a 3-drug immunosuppression that included cyclosporine, mycophenolate mofetil and prednisone. None of the patients had symptomatic CMV disease. CMV load in circulating PMNs was determined by real-time PCR. Allograft rejection was assessed by endomyocardial biopsy, and TA was evaluated by *intravascular ultrasound* (IVUS) of the left anterior descending coronary artery at baseline (within 6 weeks) and at 1 year after transplantation, by measuring the changes in intimal (plaque), lumen and whole vessel area. **Results.** Recipients with a detectable CMV-specific CD4 T-cell response by 3 months post-transplantation (early response group) were significantly protected from acute rejection and had significantly lower viral load compared to those who lacked detectable an early CMV-specific CD4 T-cell response (late response group). Despite intimal hyperplasia did not differ between groups, patients with a late CD4 response had a significant reduction of vessel area ( $P = 0.039$ ), and consequently loss in coronary lumen ( $P = 0.052$ ). This process suggests important variability in coronary geometry according to CD4 T-cell specific immunity. In contrast, an early CD8 T-cell response was not significantly associated with freedom from rejection, negative remodeling, or low viral load. **Conclusions.** The control of CMV replication by T-cell immunity, particularly by CD4 T cells, is important in limiting allograft immune rejection and the evolution of transplant vascular disease. This is demonstrable in a patient population with relatively low levels of post-transplant CMV viral load because of pre-transplant CMV immunity and early post-transplant ganciclovir/valganciclovir prophylaxis. Interventions to increase CMV T-cell immunity might provide a clinically useful adjunct for limiting the deleterious post-transplant effects of CMV.

#### Su2.128. Immunological Evaluation of Lymphocyte Activation, Cytokines and Apoptosis on Tacrolimus-Sirolimus-Induced Long-Term Allograft Survival in Nonhuman Primates.

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**Background:** We reported previously that a 60-day course of combined tacrolimus (FK506) and sirolimus (RAPA) therapy induced long-term survival of renal allografts after withdrawal of immunosuppressants in Vervet monkeys. The mechanism of drug-induced allograft survival was evaluated via Th1/Th2 cytokines, apoptosis and mixed lymphocyte reaction (MLR) activity in primates in the present study.

**Methods:** Phenotypes were analyzed by flow cytometric analysis (FACS). Cytokines were quantified by enzyme linked immunosorbent assay (ELISA). MLR and cytotoxic T lymphocyte assays were performed by incorporation of 72-h <sup>3</sup>H-thymidine and 4-h <sup>51</sup>Cr release.

**Results:** A 60-day course of combined FK506 and RAPA treatment resulted in long-term survival of kidney allografts (67% >100 days) without intermittent acute rejection. Low sensitivity to MLR was seen in long-term renal allograft survival among monkeys treated with FK506 and RAPA. Increased levels of CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>/CD56<sup>+</sup> natural killer (NK) T and CD86<sup>+</sup>CD8-CD11<sup>+</sup> dendritic cells were observed. High expression of CD4<sup>+</sup>FasL<sup>+</sup> was detected. In addition, Interleukin (IL)-2 and interferon (IFN)- $\gamma$  concentrations with long-term allograft survival were not increased significantly. Late-phase dominance of Th2, IL-4, IL-10 and transforming growth factor (TGF)- $\beta$  was found to result in long-term survival by combined FK506 and RAPA treatment of.

**Conclusions:** FK506 and RAPA-induced long-term allograft survival in primates is related to up-regulation of FasL expression, NKT cells and dendritic cells with down-regulation of MLR sensitivity. It is also associated with late-dominant expression of Th2 cytokines.

### Su2.129. Baohuoside-1, a Novel Immunosuppressive Molecule, Inhibits Lymphocyte Activation *In Vitro* and *In Vivo*.

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**Background.** Immunosuppressive effect of Baohuoside-1, a novel flavonoid isolated from *Epimedium davidii*, was evaluated *in vitro* and *in vivo* in this study.

**Methods.** The antiproliferative properties of B1 were evaluated by proliferation assay. Western blotting and fluorescence activated cell sorting were employed to investigate the expression of cyclins and cyclin-dependent kinase proteins.

**Results.** The major findings were: (1) B1 effectively inhibited the cell proliferation activated by interleukin 2 and mitogenic antigen, with a 50% inhibitory concentration in low mM and in a dose- and time-dependent manner. (2) B1 resulted in G<sub>1</sub>-S phase cell arrest. (3) It down-regulated the expression of cyclin A, D and p33 cyclin-dependent kinase-2 (p33cdk2) proteins. (4) B1 suppressed the growth of several tumour cell lines. (5) The synergistic immunosuppression between B1 and FK506 was observed a heart transplantation model *in vivo*.

**Conclusions.** B1 immunosuppression of IL-2-activated T cell proliferation occurs in G<sub>1</sub>-S transition. It may be associated with the expression of cyclin A, D and p33cdk2 proteins. B1 prolongs effectively heart allograft survival in rats. The mechanism of B1 is different from tacrolimus and sirolimus.

### Su2.130. Cellular Reactivity to Human Hsp60 in Murine Skin and Heart Transplantation.

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**Introduction:** The increased expression of Hsp60 in different kinds of graft tissues has been associated with rejection and suggests a proinflammatory role for Hsp60. On the other hand, there are reports in which treatment with Hsp60 delayed rejection of skin allograft. Our group has reported an association between Hsp60-induced T cell proliferation and rejection and Hsp60-induced IL-4 production and no rejection, in human kidney allotransplantation, suggesting the existence of both proinflammatory and potentially regulatory autoreactive T cells directed to Hsp60. The aim of this work was to functionally analyze the cellular response to human Hsp60, in different murine transplantation models. **Method:** Briefly, we studied T cell proliferative response and antigen-induced cytokine production by ELISA. Antigens used: 11 Hsp60 synthetic peptides, I-Hsp60 (intermediate region), C-Hsp60 (C-terminal region) and Hsp60 protein in different transplantation groups: (i) syngenic skin, (ii) allogenic skin, and (iii) vascularized heart transplantation. **Results** In the syngenic group, we observed a predominant IL-4 and IL-10 production induced by Hsp60 peptides in both BALB/c and C57BL/6 models. In the skin allogeneic groups, (BALB/c (H-2<sup>d</sup>) as recipients of C57BL6 (H-2<sup>b</sup>), ( $n = 4$ ) and C57BL6 mice as recipients of BALB/c, ( $n = 5$ ), we observed IFN $\gamma$  and IL-10 production induced by Hsp60 and different Hsp60 peptides, but IL-4 was not detected. In the heart allografting model, using BALB/c as recipients of C57BL6 mice, ( $n = 4$ ), all animals showed proliferative response to Hsp60. This group displayed IFN $\gamma$  production induced by recombinant Hsp60 in 4 out of 4 animals. IL-10 production was induced by Hsp60 in 2 out of 4 animals. **Conclusion:** These results show a predominant Th2 profile in T cell reactivity to Hsp60 in syngenic skin transplantation. In contrast, we observed predominantly Th1 proinflammatory cytokine production, induced by Hsp60, in both skin and heart allograft rejection, with a low frequency of IL-10 production and no IL-4 production. Further analysis of Hsp60 peptides and regions may allow the identification of potentially regulatory epitopes, which may be used for the induction of immune tolerance. **Supported by FAPESP and CNPq.**

### Su2.131. Evaluation of Skin Graft Survival Using the Encapsulated Hsp60 Peptide (p277) in a Murine Model of Minor Antigen Disparities.

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**Introduction:** T cell reactivity induced by Hsp60 has been frequently implicated in the inflammatory process in autoimmune diseases and graft rejection. Our group has reported an association between Hsp60-T induced cell proliferation and rejection in human kidney allotransplantation and Hsp60-induced IL-4 production and no rejection, suggesting the existence of both proinflammatory and potentially regulatory autoreactive T cells directed to Hsp60. We evaluated the capacity of a human Hsp60-derived peptide (p277) to induce tolerance in a murine model of skin grafting with minor antigen disparities. **Methods:** Briefly, BALB/c (H-2<sup>d</sup>, CD5<sup>1</sup>) mice were used as recipients of skin graft from DBA2 mice (H-2<sup>d</sup>, CD5<sup>2</sup>) mice. Six recipients received five intranasal inoculation of p277 peptide encapsulated in poly-lactic/glycolic-acid microspheres (50m g). As control group, animals immunized with empty microspheres ( $n = 6$ ) and non-immunized animals ( $n = 9$ ) were used. **Results** We obtained a slight increase in graft survival, with median survival time (MST) 15,5 days in p277 treated animals, in comparison with controls groups (15 days,  $P = 0.17$ ), but 2 animals from this group had a graft survival over 20 days. We did not observe proliferative response against p277 or Hsp60. We observed a predominant IFN $\gamma$  production induced by Hsp60 and recombinant fragment corresponding to the intermediary region of the Hsp60 (I-Hsp60), in animals from different experimental groups. In contrast, IL-4 production was induced predominantly by the C-terminal fragment of the Hsp60 (C-Hsp60) in 3 out of 6 mice from p277 treated group. Finally, IL-10 production induced by p277 peptide was observed in 4 out of 6 mice from p277 treated group. **Conclusion:** These results show that reactivity to Hsp60 may be used to prolong graft survival in a model of minor antigen disparities. The combination of the tolerogenic potential of the mucosal route and the capacity of different Hsp60 regions to induce regulatory cytokines may be further explored for down modulating inflammatory processes. **Supported by FAPESP and CNPq.**

### Su2.132. The Role of Indoleamine 2,3-Dioxygenase (IDO) in a Murine CTLA4Ig-Based Mixed Chimerism Model.

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**Background:** Apart from indirectly blocking the CD28 costimulation pathway by competing for ligation with B7 molecules, recent data suggest that the dimeric fusion protein CTLA4Ig can initiate a negative signaling pathway in APCs by inducing the tryptophan catabolizing enzyme IDO. Decreasing availability of tryptophan and the accumulation of catabolic byproducts such as kynurenine result in impaired T cell proliferation and apoptotic death. We therefore investigated whether IDO played a role in our allogeneic mixed chimerism protocol involving the use of CTLA4Ig.

**Materials and Methods:** C57BL/6 mice received non-myeloablative total body irradiation (TBI, 3 Gy, d-1), approx.  $20 \times 10^6$  fully allogeneic Balb/c bone marrow cells (d0) and costimulation blockade consisting of anti-CD154 mAb (1mg, d0) and CTLA4Ig

(0.5mg, d2). Additional groups were either treated with the same regimen without CTLA4Ig or were implanted with 7-day-release 1-methyltryptophan pellets on d-1 (1-MT, which is a competitive inhibitor of IDO, 200mg/pellet) or placebo pellets. Macrochimerism and deletion of donor-reactive T cells were followed by flow cytometry. Serum levels of tryptophan and kynurenine (trp, kyn) were measured at several time-points by high performance liquid chromatography (HPLC). Kyn to trp ratios were calculated, indicative of IDO activity.

**Results:** 15/18 mice which received bone marrow transplantation (BMT), TBI, anti-CD154 plus CTLA4Ig, but only 6/18 mice without CTLA4Ig treatment developed lasting (20 weeks) mixed chimerism (pooled data from two independent experiments,  $P < 0.01$ ). 10/17 mice with CTLA4Ig treatment accepted donor skin grafts for more than 100 days, whereas only 2/17 mice without CTLA4Ig accepted donor skin long-term ( $P < 0.01$ ). 3<sup>rd</sup> party grafts were promptly rejected in all groups. Thus, CTLA4Ig is critically important for chimerism and tolerance induction in this protocol. Serum kyn/trp ratios were similar in groups treated with and without CTLA4Ig at several time points post-BMT (e.g. on d3:  $11.8 \pm 2.7$  vs.  $12.1 \pm 3.3$ , d4:  $10.4 \pm 3.4$  vs.  $13.9 \pm 4.6$ , d11:  $16.2 \pm 4.3$  vs.  $17.4 \pm 6.3$ , d14:  $37.7 \pm 14.5$  vs.  $40.8 \pm 20.7$ ;  $P = n.s.$  for all time-points). 10 weeks after BMT 6/8 mice treated with 1-MT pellets showed multilineage chimerism, which was not significantly different from the group implanted with placebo pellets (4/4 chimeras;  $70.1 \pm 33.9$  vs.  $61.1 \pm 38.2\%$  myeloid chimerism,  $34.0 \pm 19.51$  vs.  $33.0 \pm 21.2\%$  B cell chimerism,  $P = n.s.$ ). Deletion of donor-reactive CD4 cells was not significantly affected by treatment with 1-MT.

**Conclusion:** CTLA4Ig is critically required for reliable chimerism induction in this model of fully allogeneic BMT. Its effect was not reversed by inhibition of the tryptophan depleting enzyme IDO.

### Su2.133. Evaluation of Immuknow-Immune Cell Function Assay as a New Parameter of Net State of Immunosuppression in Monitoring Renal Transplant Recipients.

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**Background:** The survival of transplanted organ is dependent on maintenance of continuous immunosuppression. However, even strictest adherence to the recommended drug levels does not prevent numerous complications associated with immunosuppression. The Immuknow assay measures cell-mediated immunity and is reportedly able to discern between profiles of over and under-immunosuppression. The aim of this pilot study was to evaluate this assay in characterization of immune status of renal transplant recipient in correlation with patient's clinical condition. **Method:** 13 kidney recipients were monitored for a period of first six post-transplantation weeks. The immune function was determined by quantitative measurement of intracellular ATP level in CD4<sup>+</sup> lymphocytes after PHA stimulation. FK or CSA blood levels and parameters of rejection, graft function and infection, were correlated with the assay results.

**Results:** ATP release and FK/CSA blood level values were evaluated at baseline (n 13), during quiescence (n 26), acute rejection (n 4) and infection (n 4). Rejection was positively correlated with ATP 25–100% elevation from baseline in 3/4 instances. Such correlation with CNI levels (20% decrease) could

be found in only 1/4 instances. Likewise, infection was correlated with 50–60% decrease in ATP release in 2/4 instances but not by CNI levels (0/4). In stable patients, the ATP deviation from the preoperative baseline, indicative of quiescence, was much smaller than that of CNI levels (means of deviations  $0.4 \pm 36\%$  vs.  $18 \pm 56\%$ ).

**Conclusion:** *Immuknow* is better correlated with the clinical status than CNI level and therefore could be recommended for post-transplant monitoring.

### Su2.134. CD80 but Not CD86 Costimulatory Molecules Suppresses Xenogeneic Humoral Rejection by Regulation of Complement C3dg Generation.

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**OBJECTIVE:** Acute vascular rejection (AVR) and cell-mediated rejection (CMR) remain the primary immunological barriers to successful xenotransplantation. While the CD86, but not CD80, costimulatory pathway has been shown to play a critical role in allotransplantation heart allograft rejection, the immunoregulatory roles of CD80 and CD86 have not been fully dissected in xenotransplantation.

**METHODS:** Using a concordant Lewis rat-to-mouse heterotopic heart xenotransplantation model we characterized the role of CD80 and CD86 in xenotransplantation using CD80 and CD86 knockout mice on the C57BL/6 background (Jackson Labs). Graft function was monitored by abdominal palpation. Xenoantibody levels were measured by flow cytometry by incubating sera from transplant recipients with Lewis rat lymph node cells, followed by staining with anti-mouse IgM, IgG1 or IgG2a -FITC or -PE conjugated antibodies. C3dg intragraft deposition was characterized by western blotting with a goat-anti-mouse C3 polyclonal antibody.

**RESULTS:** C57BL/6 recipients reject xenograft hearts on POD17-21 and show CMR/AVR histopathology. We now report that *CD80*<sup>-/-</sup> recipients reject xenograft hearts on POD5 and show AVR histopathology (an antibody driven process). In contrast, *CD86*<sup>-/-</sup> recipients reject xenograft hearts on POD17 but show predominantly CMR pathology (T cell driven process). We show that *CD80*<sup>-/-</sup> recipients have significantly increased serum levels of IgG1/2a xenoantibodies, as well as intragraft IgG deposition, than *CD86*<sup>-/-</sup> or C57BL/6 recipients at POD5-6. Furthermore, C57BL/6 but not *CD86*<sup>-/-</sup> recipients show an increase in IgG1/2a xenoantibodies on POD17-21. Furthermore, *CD80*<sup>-/-</sup> recipients showed deposition of complement C3dg protein in the graft on POD5, whereas *CD86*<sup>-/-</sup> and C57BL/6 recipients on POD6 or POD17-21 did not show any C3dg deposition.

**CONCLUSIONS:** This data demonstrates that CD80 suppresses xenogeneic antibody-driven AVR, while CD86 promotes CMR. Furthermore, this data suggests that CD80 costimulatory molecule suppresses xenogeneic humoral responses by inhibiting the generation of C3dg, a result of C3 activation. We contend that CD80 and CD86 play distinct roles in regulation of xenogeneic rejection by differentially regulating B cell responses and complement activation/degradation. These results highlight the importance of different mechanisms regulating xenogeneic and allogeneic graft rejection.

This work was supported by grants from CIHR, MOTS, ORDCF, and CANVAC.

### Su2.135. Lack of Correlation between the Presence of Anti-Human Panel Reactive Antibodies (PRA) and Anti-Swine Non-Galactose- $\alpha$ 1,3-Galactose (NonGal) Cytotoxic Antibodies in Patients Awaiting Life-Saving Organ Transplantation.

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**Background:** Until recently, hyperacute xenograft rejection (HXR) mediated by preformed xenoreactive natural antibodies against the Gal epitope, produced by the  $\alpha$ 1,3-galactosyltransferase (GalT) enzyme, appeared an insurmountable obstacle to clinical xenotransplantation. Recent studies demonstrate that use of organs from GalT-knockout (KO) miniature swine prevents HXR. It was not known whether highly-allosensitized patients unlikely to receive crossmatch-negative human organs are also xenosensitized against nonGal swine antigens.

**Aim:** To determine if correlation exists between PRA levels and anti-swine nonGal reactivity in patients awaiting renal allotransplantation.

**Methods:** Serum samples were obtained from 15 highly-allosensitized patients ("high PRA" denotes PRA  $\geq 80\%$ ) and 45 non-allosensitized patients ("low PRA" denotes PRA  $\leq 10\%$ ) on the waiting list for cadaveric renal transplantation. The %PRA was determined using a standard anti-human globulin-augmented cytotoxicity assay. GalT-KO PBMC were obtained from a GalT<sup>-/-</sup> miniature swine using standard density gradient centrifugation. Assays included FACS for serum IgM and IgG binding and complement-dependent cytotoxicity (CDC), in which reactions containing no test serum, normal human serum, and known sensitized serum were included as controls.

**Results:** Comparisons between high and low PRA sera for FACS and CDC results against GalT-KO PBMC are expressed as average % antibody binding  $\pm 95\%$  CI and average % cytotoxicity  $\pm 95\%$  CI. No statistically significant difference was found in: 1) serum IgM binding between high PRA ( $25.2 \pm 6.9$ ) and low PRA ( $30.1 \pm 6.0$ ) patients, nor in 2) serum IgG binding between high PRA ( $10.9 \pm 5.3$ ) and low PRA ( $8.2 \pm 2.0$ ) patients, nor in 3) serum cytotoxicity at 1:4 dilution between high PRA ( $21.5 \pm 5.7$ ) and low PRA ( $22.2 \pm 5.9$ ) samples. Of the 15 highly-allosensitized sera tested, only 1 patient's serum showed high IgM binding ( $>50\%$ ) and high cytotoxicity ( $>50\%$  at 1:4 serum dilution) against GalT-KO targets. None of the 15 highly-sensitized sera showed high IgG binding ( $<50\%$ ) against GalT-KO PBMC. Cytotoxicity levels showed a positive correlation with IgM binding ( $r = 0.657$ ), but not with IgG binding ( $r = 0.046$ ). Studies on target cells from additional GalT-KO animals are in progress.

**Conclusion:** No correlation was found between anti-human and anti-GalT-KO swine reactivity in patients awaiting life-saving renal transplantation. Xenosensitization against nonGal antigens is uncommon in highly-allosensitized patients, who may therefore be candidates for xenotransplantation of GalT-KO porcine organs in a future clinical trial.

**Su2.136. TIRC7 Is Expressed upon Immune Activation Early in Peripheral Blood Lymphocytes and Remains Induced in Inflammatory Cells during Rejection after Kidney Transplantation as Well as in Joints from Patients with Established Rheumatoid Arthritis.**

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**Aims:** TIRC7 is an activation induced cell surface molecule on T and B cells that negatively regulates their function. To analyze the potential role of TIRC7 expression associated with the early immune response, several human tissues including transplanted kidneys, joint samples and peripheral blood lymphocytes (PBL) were analyzed for TIRC7 expression.

**Methods:** Expression of TIRC7 was tested in clinical biopsies of renal allograft recipients treated with a calcineurin inhibitor-based immunosuppression by immunohistology using a FITC labelled anti-TIRC7 mAb following the biopsy-confirmed diagnosis of acute rejections. Joint tissues isolated from patients with Rheumatoid Arthritis (RA) were subjected to immunohistology using a directly FITC labelled anti-TIRC7 mAb. Flow cytometric analysis was performed using an anti-TIRC7 mAb on PBL activated with alloantigen. TIRC7 protein expression was determined on CD4+ and CD8+ T cells using a polyclonal TIRC7-specific antibody. Cells were stained with conjugated mAb (CD28, CD45RO) and the samples were analyzed by a FACScalibur flow cytometer.

**Results:** Immune fluorescent microscopy revealed that lymphocytes of human tissues obtained from patients with acute rejection under immunosuppressive combination therapy with various agents Calcineurin inhibitors; FK 506/Cyclosporin A (CyA), methyl prednisolone (MP), and mycophenolate mofetil (CC) strongly expressed TIRC7 ( $n = 5$ ) whereas normal kidneys did not show any TIRC7 expression. TIRC7 mAb staining revealed strong upregulation of TIRC7 protein expression in tissues obtained from knee joint samples as well as in mononuclear cells from joint fluid obtained from patients with established RA. No staining was observed in all experiments using control antibody for staining. On human PBL TIRC7 was predominantly induced within the first hour of activation on CD4+45RO+28+ and CD8+45RO+28+ but less on CD28-negative T cells suggesting primary expression on resting memory T cells but less on naïve and effector/memory T cells.

**Conclusions:** TIRC7 is strongly upregulated following in vitro activation of resting memory T cells but less on naïve (CD45RO-) and effector/memory (CD28-) T cells and also in vivo in patients with Rheumatoid Arthritis (RA) and in rejected kidney tissues. In summary, these studies suggest TIRC7 might be suitable for targeting of memory T cells and as a diagnostic marker of immune activation.

**Su2.137. Peripheral and Graft Infiltrating T Cell Responses to Self Heat Shock Protein 60 in Renal Transplant Patients.**

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Heat Shock Proteins (HSP) are highly conserved molecules with a wide range of functions including activation of B and T cell responses. The expression of these proteins is elevated in stress conditions, such as inflammation and tissue damage. In transplantation (Tx), it has been shown that T cells reactive to mycobacteria Hsp65 and to human Hsp70 infiltrate the graft and may have a role in pro-inflammatory activities. However, other findings suggest that self-Hsp60 T-cell reactivity is also part of a normal regulatory response that down-regulates inflammatory processes. Previous data from our group suggested that in the early period post-Tx the anti-Hsp60 peripheral T cell repertoires in renal transplanted patients were predominantly pro-inflammatory while later they were predominantly regulatory. In this context, our objective was to analyze the potentially regulatory response to Hsp60 in renal transplant patients in different clinical stages, and also to analyze whether human graft infiltrating lymphocytes recognize self Hsp60. PBMC from 17 long-term (LT) renal transplant patients (mean time post-Tx = 29 years, stable renal function) and from 12 renal transplant patients with recently diagnosed chronic rejection (CR) (mean time post-Tx = 4 years) were stimulated with Hsp60 or PHA as control. We analyzed cytokine production by ELISA. The production of IL-10 induced by Hsp60 was detected in 12% (2/17) of the stable LT patients and in 73% (8/11) of the patients with CR. IFN- $\gamma$  was induced by Hsp60 in 17% (3/17) of the stable LT and in 50% (6/12) of the CR patients. In contrast, TGF- $\beta$  was induced in 60% and 28% of the LT and CR patients, respectively. The production of IL-4 was uniformly low in all patients. To study the response to Hsp60 in graft infiltrating lymphocytes (GIL), we established GIL T cell lines from five renal transplant patients. The immunophenotype of T cell lines was characterized by FACS and proliferation and cytokine production induced by Hsp60 or PHA were measured. Our results showed a predominance of CD8<sup>+</sup> T cells in four of the five T cell lines analyzed, with only one pure CD4<sup>+</sup> T cell line. Only one T cell line proliferated in response to Hsp60. We observed IL-10 production induced by Hsp60 in four out of the five T cell lines studied. IFN- $\gamma$  was induced by Hsp60 in only one of the five T cell lines. These results show that autoreactive T cells to Hsp60 infiltrate the graft and suggest that these T cells may also have immunoregulatory functions. The analyses of the peripheral repertoire suggest that the putative regulatory IL-10 response to Hsp60 is present in a high frequency in CR patients, and that LT patients may have a regulatory mechanism induced by reactivity to Hsp60 mediated by TGF- $\beta$ . Supported by FAPESP/CNPq.

**Su2.138. MHC Class I Restricted EBV-Peptides Pulsed-DC1 Boost *In Vitro* IFN-g (Type-1) and IL-10 (Treg) CD8+ T Cells in Solid Organ Transplant Patients.**

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**Introduction:** Post-transplantation lymphoproliferative disorders (PTLDs) are potentially life-threatening complications of solid organ transplantation (SOTx), triggered by EBV infection in



chronically immunosuppressed (IS) recipients. Our goal is to establish dendritic cell (DC)-based protocols for vaccination and adoptive immunotherapy for refractory PTLD encountered in IS SOTx patients. In this study we analyzed the comparative ability of EBV-peptide pulsed Type-1 polarized DCs (i.e. DC1) generated from IS SOTx patients and healthy donors to boost Type-1 (IFN-g) EBV-specific CD8+ T cells *ex vivo*. **Methods:** EBV+/HLA-A02+ IS SOTx patients receiving chronic 2 drugs maintenance therapy ( $n = 9$ ) or healthy controls ( $n = 10$ ) were recruited for this study. The EBV-specific CTLs were generated *ex vivo* by co-culturing autologous T cells with DC1 loaded with a mixture of three peptides derived from EBV Ags. To assess the incidence and the functional polarization of CD8+ T cell responses to EBV epitopes, ELISPOT assays for IFN-g and IL-5, and ELISA assays for TGF- $\beta$  and IL-10 were performed on PBMC, or on *ex vivo* generated EBV-specific CTLs. **Results:** Patients' peripheral blood circulating CD8+ T lymphocytes preserved their functional Type-1 polarization against EBV Ags, as compared to healthy donors. After 10 days of *ex vivo* stimulation with EBV peptide-pulsed DC1, the patients' co-cultures contained lower numbers of CTLs, as compared to

healthy controls, suggesting a defect in patients' T cell proliferative potential. However, IFN-g producing EBV-specific CD8+ T cells were successfully boosted in both IS SOTx patients and normal controls, at comparable frequencies, and this appeared to reflect an expansion of pre-existing EBV "memory" T cells. In addition, the patients' CTLs produced significantly higher levels of IL-10 (but no TGF $\beta$ ) as compared to healthy donors, suggesting that some regulatory T cells may have been expanded *in vitro*. **Conclusion:** Our results show that IS stable SOTx patients display Type-1 immune (IFN-g) responses against EBV Ags in their peripheral blood, to a degree comparable to that observed in normal controls. These T cells can be further boosted *ex vivo* using EBV Ag-loaded Type-1 polarized DCs. However, only the patients' expanded co-cultures contained EBV-specific CD8+ T cells producing IL-10. These data suggest that patients' (but not normal donor) DCs might be partially impaired in their ability to become fully matured, since they elicited both IFN-g and IL-10 production from EBV-specific CD8+ T cells. We are currently investigating approaches to expand Type-1 biased T cells in DC-based co-culture conditions, for the optimization of adoptive immunotherapy of PTLD in SOTx patients.