UNIVERSITY OF CALIFORNIA, SAN DIEGO

B cell co-receptors CD19 and CD21 in tolerance and auto-immunity.

A dissertation submitted in partial satisfaction of the requirements for the

Doctor of Philosophy

in

Biological Sciences

by

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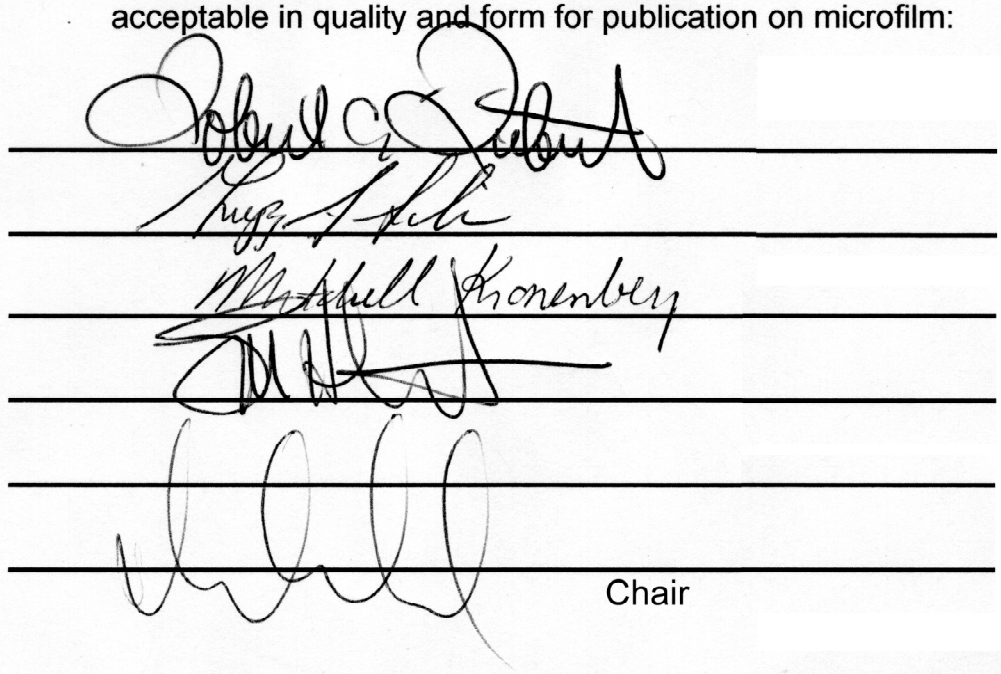

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DEDICATION

The dedication of onseself to the enduring task of a Ph.D. is a conquest not fathomable without the contributing support of your family, friends and peers. It is their strength and support that creates the environment that enables one to succed in such a strenuous task. The support and generosity of my family, friends and peers has provided me the strength to continue in this effort over these last few years of my life. I dedicate this work to them in thanks for their commitment, support and generosity.

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ABSTRACT OF THE DISSERTATION

B cell co-receptors CD19 and CD21 in tolerance and auto-immunity.

by

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The focus of this thesis is on the role of the B cell co-receptors CD19 and CD21 in the induction of B cell dependent autoimmunity. B cell dependent autoimmunity can be described as a process consisting of three fundamental steps; first is the generation of B cells that target self, second is the activation of these cells to produce pro-inflammatory soluble antibody (sIg), and third is the deposition of this antibody on the target tissue where it initiates inflammation against self. Collectively, the first two steps in this process are functionally defined as the “inductive phase” as they result in the production of auto-antibody that is sufficient to induce disease symptoms in a secondary recipients. The third step is referred to as the “effector phase” of disease as it is the manifestation of the symptoms of inflammation that result from the downstream effector functions of auto-antibody production. This work addresses the roles that these two B cell co-receptors and their downstream phosphlipid signaling play in both the generation and the activation of auto-specific B cells; their roles in the inductive phase.

General Introduction

B cell Development

The one gene one protein theory dictates that for every gene locus there is only one single gene product produced. However with the seemingly unlimited diversity of BCR/antibodies specificities available to the mammalian immune system comes the requirement for an unlimited amount of genetic material to encode all these receptors. Quantitatively, the amount of DNA required to individually encode such gene product diversity exceeds the total genomic content. Breaking with the one gene one protein theory, it was discovered that the vast BCR/antibody repertoire was instead derived from a limited amount of genomic content that was rearranged and modified through a variety of recombinatorial and diversifying mechanisms to produce novel non-germline sequences that encode the diversity of BCR/antibody specificities.[1]

Mature lymphocytes are generated from a common hematopoetic stem cell (HSC) precursor found in the bone marrow of adult mice. It is during this process of lymphopoeisis in the bone marrow that most B cell precursors rearrange and modify their genomic content to produce these novel BCR/antibody sequences in a tightly regulated stepwise process of

differentiation that occurs in concomitant with a progressive stepwise recombinational building of the gene sequence encoding a functional BCR.[2]

First in this process comes the rearrangement of the heavy chain genes in early bone marrow B cell progenitors pro-B cell subsets; Hardy fractions A-C, $B220^{+}$ $CD43^{+}$ population of cells. This is followed by the rearrangement of the light chain genes in the pre-B cell subsets; Hardy fraction D, $B220^{+}$ $CD43^{-}$. It is the successful production of both a functional heavy and light chains that defines the transition into the immature B cell fraction which migrates out from the bone marrow; Hardy fraction E, IgM^{+} $B220^{+}$ $CD43^{-}$.[3, 4] Importantly, although the production of a heavy and light chain BCR is a critical component to the progression of a B cell through the early developmental stages, this progression is antigen independent. Antigen specific binding and subsequent antigen dependent signal transduction is only possible when the ligand specificity of a BCR is finally determined by the assembly of a fully functional surface BCR composed of both a heavy and light chain together at the immature B cells stage.

During development, fraction A cells are known as pre-pro B cells, and they are not entirely committed to the B lineage. These cells can be subdivided into the AA4.1 positive and negative subsets, where the positive subset is the B cell committed population. The developmental progression

into the fraction B cell group coincides with the onset of CD19 and CD79α/CD79β (Igα/Igβ) expression, in addition to the Rag and TdT enzymes responsible for the rearrangement of the heavy chain locus.[5] These cells are the true pro-B cells, and they initiate the progressive rearrangement of the Ig loci. First is an attempt to rearrange the diversity heavy (DH) loci to junctional heavy (JH) loci; DH➔JH, followed by the variable heavy (VH) loci to the prior rearranged DHJH; VH➔DHJH. The transition to fraction C coincides with the successful production of a complete VHDHJH rearrangement. A functional VHDHJH can produce a heavy chain that pairs with the surrogate light chain composed of Lambda 5 and VpreB. This assembly is defined as the pre-B cell receptor (pre-BCR), and its membrane expression with Igα/Igβ is critical for the cell progression from the pro-B cell stage to the pre-B cell stage.[6] The pro to pre-B cell transition is marked by a burst in proliferative expansion and the turning off of Rag and TdT enzymes to prevent the further rearrangement of the other heavy chain allele; a process called allelic exclusion.[7]

Prior to the pre-B cell stage, pro-survival signals were provided by the availability of both Interleukin 7 (IL-7) and c-kit, as well as through bone marrow stromal cell surface factor.[8] However, with the arrival of surface pre-BCR expression, comes a decrease in the availability of these pro-survival signals; Hardy fraction D cells decrease the surface expression of the IL-7

Receptor (IL-7R). In addition to the loss of this pro-survival receptor, these cells also lose both CD43 and importantly, lose pre-BCR surface expression as the surrogate light chain locus is also turned off.[9] Pre-B cells re-express Rag to initiate light chain rearrangement while maintaining the intracellular expression of the previously rearranged heavy chain without surrogate light chain to stabilize its surface localization. As the IL-7R expression drops, these cells begin to lose their IL-7 dependent survival signals, and thus rely on the timely production of an alternative survival signal. This comes in the form of restored BCR surface expression.[10] The successful rearrangement of a functional light chain replaces the role of surrogate light chain by pairing with the intra-cellular heavy chain allows for the re-trafficking of the heavy chain to the surface, thus resulting in the surface expression of a complete BCR. As the light chain doesn't include a diversity region, its rearrangement effort is simplified to the rearrangement of the variable light (VL) chain segments to the junctional light (JL) chain segments; VL➔JL. If the rearrangement of one locus is ineffective, then the effort proceeds in an attempt to rearrange another kappa or lambda locus.[11]

The expression of a functional BCR on the cell surface identifies a cell as being a part of Hardy fraction E. These BCR positive B cells are known as immature B cells and have finally gained the capacity to bind epitopes with the antigen binding site derived from contributions by both the heavy and light

chain components of the antigen receptor. Prior to the immature B cell stage, the pre-BCR being composed of only the heavy chain with surrogate light chain does not normally bind antigen.

Associated with both the pre-BCR and the BCR complex on the surface are two proteins containing intracellular tyrosine activation motifs (ITAMs) CD79α and CD79β, also known as Igα and Igβ. These receptor associated ITAM motif containing proteins are important for promoting B cell development.[12] A loss in their expression abrogates B cell development through these stages, while the constitutive surface expression of the ITAM motifs using a chimeric protein LMP2 containing Igα and Igβ ITAM sequences restores the B cell developmental progression in the absence of pre-BCR and BCR components seen in Rag deficient animals.[13] The BCR association of these ITAM motifs containing proteins are believed to be required for weak "tonic" signals that drive B cell developmental stage progression; termed positive selection. The pre-BCR provides only positive survival and differentiation signals, while new antigen dependent signals through the immature B cell Receptor can alternatively produce both positive and inhibitory cell outcomes.[14] The binding of overt antigen with the immature BCR amplifies the phosphorylation of the ITAM motifs in Igα and Igβ, and is known to generate inhibitory signals that result in the negative selection of immature B cells. These early and strong signals are surmised to instigate negative

outcomes by either attenuating B cell survival signals or by directly promoting negative signals that arrest B cell differentiation.[15]

After transitioning to the immature B cell stage, these cells egress from the bone marrow mesenchyme and migrate through the parenchyme to enter the peripheral circulation in the blood stream. These cells are commonly referred to as early transitional type 1 B cells, (T-1), and are characterized as being B220+ CD19+ CD43- IgM+, IgD-, CD21-, CD23-, HSAhi. Once these peripheral immature T-1 B cells reach the spleen, they undergo a second set of differentiation steps, which involves a transition from the T-1 stage to a transitional type 2 (T-2) stage and then either terminally into a transitional type 3 (T-3) stage or transiently through the T-3 stage into the mature naïve B cell pool.[16] This peripheral maturation predominantly takes place in the spleen because it is believed to be dependent on the availability of the B cell survival factor BAFF that is highly expressed there.[17, 18] However, mature B cells can be produced in splenectomized or BAFF deficient mice, but with a dramatically reduced efficiency.[19, 20] The T-1 B cell to T-2 B cell transition is marked by the co-expression of IgM with an alternatively spliced IgD form of the BCR. It also coincides with the initiation of the expression of both the Complement Receptor 2 binding subunit, CD21, and the low affinity Fcε Receptor, CD23. The subsequent transition from T-2 to Mature B cells may occur after the a transient T-3 B cell stage and is characterized by an increase

in cell size and the expression of C1q receptor (493/AA4.1), with the final progression into the mature resting naïve B cell pool being marked by a down-regulation in the expression of surface IgM with retained IgD expression, a decrease in the expression of HSA and a decrease in cell size.[16, 21] The T-3 stage may also represent a subset of activated cells that are being neglected during their attempt to find T cell help as they are larger possibly antigen activated B cells. This T-3 population possibly includes a portion of self-reactive auto-specific B cells that have received antigen receptor signals but cannot acquire T cell help due to the auto-specific nature of the antigen encountered. Without the acquisition of T cell help they could be excluded from the follicles, and instead should be shunted into the outer PALS where they would await apoptosis and are therefore short-lived.

Alternative Peripheral B cell subsets

Alternative subsets of peripheral mature B cells exist, which can be split into two major groups, the B-1 cells and the marginal zone (MZ) B cells. B-1 cells are spread through many lymphoid tissues of the body in addition to being the predominant B cell type in the peritoneal cavity. They commonly express BCRs derived from canonical VDJ rearrangements created by the direct conjoining of somatic encoded regions without the inclusion of modified or randomly generated intervening sequences called P- or N-nucleotide additions.[22, 23] These germ-line encoded specificities are generally skewed

towards conserved poly-reactive antigens such as membrane phosphoryl-choline, carbohydrates and lipids antigens and possibly some intracellular antigens such as DNA and RNA (interestingly these antigens are typically have exposed charged residues).[24] Although the exact origin of B-1 cell development remains unclear, it is commonly believed that they initially are derived from neonatal lymphopoiesis in the fetal liver, and are maintained in the periphery through long-term survival and slow self-renewal. Although recent evidence shows that some clones can contain secondary rearrangements indicative of antigen selection.[25] Functionally, B-1 B cells are believed to secrete low levels of IgM and produce the majority of natural antibody present in the blood of naïve mice.[26, 27] It is clear that the conserved antigenic poly-reactive specificities is a distinguishing characteristic of B-1 cells and that these specificities are commonly cross-reactive with self antigens, however it is unclear as to how antigen specificity selects the cells into this subset.

MZ B cells have many of the same characteristics as B-1 cells as they are IgMhi, IgD-, CD23-, CD21+, and are also comprised of cells of a larger size than resting naïve B cells. However unlike B-1 cells that are spread through many tissues of the body, the MZ B cells are localized to a specific anatomical location within the spleen and are not found in other secondary lymphoid tissues of the mouse.[28] Evidence suggests that like B-1 cells, MZ

B cells also are low-level secretors of natural IgM antibody, in addition to producing immediately available antibody after antigenic exposure.[29] However unlike B-1 cells they predominantly express BCRs derived from both canonical and non-canonical rearrangements, including sequences that frequently show evidence of prior affinity maturation indicating that they are selected for during a prior immune response.[30] As such, MZ B cells may be the adaptive form of IgM memory that results in the long-term production of antigen selected natural antibody in the serum.

Complement Receptor 2 Complex (CR2)

The complement receptor 2 complex (CR2) on B cells consists of multiple protein subunits: CD21, CD19, CD81 and Leu-13. The function of CD21 in the complex is primarily as the ligand binding subunit, while CD19 participates as the prominent transmembrane signaling subunit. Although CD81 and Leu-13 probably participate in the complex they are not a specific component of the CR2 complex as they are broadly expressed and probably participate in signal transduction from multiple other signaling complexes.[31]

CD21

CD21 receptor binds to multiple breakdown products derived from the C3 component of complement, iC3b, C3dg, and C3d.[32] The human CD21 protein is a 140kDa transmembrane glycoprotein consisting of ~15 or 16

extracellular SCR domains, a ~28 amino acid transmembrane domain and an ~34 amino acid cytoplasmic tail. It is expressed on B cells, some epithelial cells, follicular dendritic cells, thymocytes, some peripheral blood T cells and astrocytes. In mice, CD21 is composed of 15 SCR domains and is ~150kDa. Dissimilar to human, mouse CD21 is not expressed on erythrocytes. As such, complement-dependent immune complex clearance in mice instead depends on complement receptors present on circulating platelets.[33] In B cells, CD21 expression occurs only in the periphery, beginning at the immature transitional Stage 2 for B-2 B cells and is constitutively present on both B-1 and MZ subsets.[16] In addition to the CD21/CD19/CD81/Leu-13 CR2 complex formed on B cells, CD21 can independently and exclusively associate with the Complement Receptor 1 (CR1) protein CD35 in a CD21/CD35 complex.[34]

The human *cd21* gene is located in the RCA cluster on chromosome 1 (1q32), and is located adjacent to the human Complement Receptor 1 (CR1) gene cd35. In humans, a duplication event followed by crossover recombination in the highly repetitive short cytoplasmic repeat domain (SCR) encoding sequences probably gave rise to these two genes and their polymorphic allelic variations.[35] However, in mice the CD21 and CD35 proteins are encoded in a single locus, *cr1/cr2* on chromosome 1, and are the differential products of an alternative RNA splice event.[36]

The human CD35 gene product is a highly polymorphic ~235kDa glycoprotein, and is expressed on monocytes, polymorphonuclear cells, erythrocytes, netutrophils, eosinophils, kidney podocytes, follicular dendritic cells, brain astrocytes B cells and some T cells.[37-39] Interestingly, the expression level is highest on B cells at 20-40K copies per cell. The human CD35 glycoprotein contains usually 30 but up to 37 extracellular SCR domains depending on the inherited allele, followed by a transmembrane domain and a short cytoplasmic domain. CD35 in mice contains 27 SCR domains, is ~190kDA and like CD21 in mice, is not expressed on erythrocytes.[40]

While the ligation of human CR1 on polymorphonuclear cells and macrophages can enhance phagocytic clearance of C3 and C4 labeled immune complexed antigens from tissues, its expression on erythrocytes functions to filter these immune complexed antigens from the blood into the spleen and liver.[41, 42] CR1 can also abrogate the continued activation of the complement cascade by participating as a decay-accelerating factor for both the alternative and classical pathway C3 convertases (C3bBbP and C4b2a). CD35 (CR1) inhibits the C3bBb(P) alternative pathway C3 convertase by binding the C3b portion and assisting in the cleavage of C3b to iC3b by serum complement factor I (fI). Subsequently CD35 can bind to iC3b and further assist in its cleavage to C3dg.[43] Similar to its inhibition of the alternative C3 convertase, CD35 also binds to C4b, and inhibits the C4b2a classical pathway

C3 convertase by assisting in the cleavage of C4 to iC4b and then to C4d by fI.[37, 44] Importantly, the C3dg and C4d break down products remain covalently linked to the activating antigen surface materials, and the C3dg by-product subsequently participates in opsonizing and enhancing the immune recognition of tagged antigens.

Complement binding by CD21 (CR2) is however more restrictive in that it can bind only iC3b, C3dg and C3d.[45] This differential recognition of C3 products is derived from the additional SCR domains in the N terminal region of CD35 that also provide CD35 with its C4 binding specificity. Unlike CD35, CD21 does not act as an inhibitor of the complement cascade since it does not bind to C3b or C4b of the C3 convertases.[46, 47] As C3dg and its partial product C3d are selectively capable of binding CD21 expressed on FDCs in secondary lymphoid organs, CD21 plays a key role in antigen trapping by Follicular Dendritic Cells (FDC). CD21 dependent FDC antigen trapping assists in the development of adaptive immune responses by retaining these C3d immune complexed antigens for recognition by cells of the adaptive immune system such as B cells.[48, 49]

Although C3dg and C3d labeled antigens trapped by CD21 on FDCs are likely to enhance B cells activation by increasing the chance of encounter, B cells activation can be dramatically enhanced by CD21 in two more ways.

The antigen specific coligation of the BCR with the CD21 in the CR2 complex expressed on B cells by complement C3dg/C3d tagged antigen can increase the amount of CD19 associated with the BCR due to surface co-localization of the BCR with CD19.[50] CD19 association with the BCR enhances B cell activation signals.[51] The short 34 amino acid cytoplasmic tail of CD21 suggests that it may have only limited CD19 independent B cell signaling functions. However CD21 can also enhance B cells activation by opsonizing antigens for increased B cell phagocytosis and antigen presentation to CD4+ T helper cells that can then provide further secondary B cell activation signals.[52] As C3d has been shown to increase phagocytosis in both B cells and in macrophages; which unlike B cells do not express CD19, it is unclear as to what role if any that CD19 or the rest of the complex may play in the phagocytosis and antigen presentation aspects of CR2.[53] As such, it is possible that direct CD21 specific B cells activation signals are CD19 dependent, while phagocytic enhancement and the recruitment of T cell dependent B cell activation could be intrinsic to CD21 and independent of CD19.

CD19

The *cd19* loci is located on the short arm of chromosome 16 (16p11.2) in humans and on chromosome 7 in mouse, and encodes a transcript consisting of 13 exons in humans and 15 exons in mice. CD19 is expressed in

a B cell restricted fashion in both mouse and humans.[54] The *cd19* locus is transcriptionally regulated by the Pax5 transcription factor, and as such its onset in expression occurs after the initiation of D-J gene rearrangement of the Ig heavy chain locus in the early pro-B cell progenitor stage (CD45R/B220+, IgM-, NK1.1-, ckit+, IL-7R+, CD25-, CD43+, BP1-).[55, 56] As a definitive B cell marker, its expression is maintained in all peripheral B cells subsets except for plasma cells, where it is downregulated in expression during plasma cells differentiation.[57, 58] The CD19 gene product is a 95 kd transmembrane glycoprotein and member of the (Ig) immunoglobulin superfamily. Its extracellular composition consists of two C2 –type Ig like domains interceded by a potential disulfide-linked domain.[59] Although the extracellular region of CD19 is glycosylated, any role for this glycolytic modification has yet to be delineated.

As a transmembrane protein, CD19 contains a single transmembrane spanning sequence, and an approximatelly 247 amino acid intracellular portion with 9 tyrosine residues conserved between mouse and human; Y330, Y360, Y391, Y403, Y421, Y443, Y482, Y490, Y513.[60] Each of the intracellular tyrosines in CD19 have been investigated and found to recruit signaling moieties such as GRB2/SOS, Vav, PLCγ, Fyn/Lyn/Lck, c-abl, and the p85 subunit of PI3-Kinase.[61, 62] Although the specific interaction sites between these molecules and CD19 have been carefully delineated, the clarification of

the timing and order of such complex interactions is complicated by the inter-molecular interactions between binding partners and the overlap of the binding sites for these individual molecules on CD19. Further work is needed in order to clarify the elaborate nature of CD19 signaling.

In addition to the intra-cellular molecular interactions of CD19 with soluble signaling subunits, there are also direct interactions of CD19 with a multitude of other surface and membrane proximal molecules that contribute to a wide range of signaling complexes in which CD19 participates. It is the direct but transient association of CD19 with the CD79α/CD79β (Igα/Igβ) signaling subunits of the BCR and the pre-BCR complexes using the membrane proximal cytoplasmic domain of CD19 encoded in exon 6 that makes its role in sIg signaling so prominent.[31, 63] As mentioned previously, a partial pool of CD19 binds in a 1:1 ratio with the complement binding protein CD21 using both its transmembrane and extracellular domains.[64] CD19 can also associate directly with the tetraspanin protein CD81 (TAPA-1) exclusively through a CD19 extracellular domain interaction.[31] It is the association of CD81 with Leu-13 bridges Leu-13 into a complex with CD19 and CD21 creating the Complement Receptor 2 (CR2) complex consisting of CD21/CD19/CD81/Leu-13.[65] A CD21 independent complex of CD19/CD81/Leu-13 may participate in CD19's association with the BCR/Pre-BCR in the absence of CD21/BCR co-ligation by complement tagged antigens.

Recently, a tertiary role for CD19 in MHC class II signaling has recently been elucidated. As the Igα/Igβ heterodimeric signaling subunits of the BCR were identified to participate in a secondary role in MHC class II signaling, it was also found that CD19 is phosphorylated upon MHC class II ligation.[66, 67] As such, CD19 may participation in yet another important B cell surface signaling complex involved in long term B cell activation during T cell conjugation. Interestingly, CD81 has previously been reported to associate with MHC class II, although possibly independent of CD19, as CD81 is expressed in excess to CD19.[68] CD19 has also been implicated as a potential signal transducer for several other surface receptors on B cells including CD38, CD40, CD72, VLA-4 and FcgRIIB.[69-73]

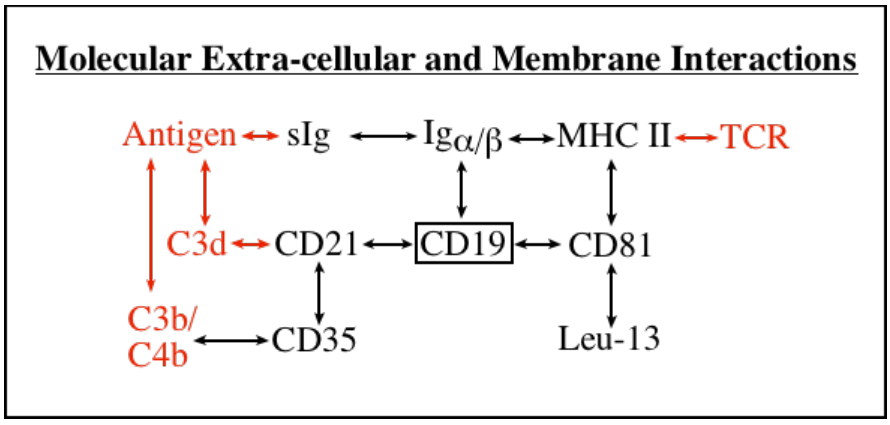

Figure 1 Molecular Interactions of CD21 and CD19

Complement C3

Serum contains many proteins involved in the recognition, opsonization and clearance of potentially dangerous material. The innate system of complement is one collective group of such proteins that are essential for fighting pathogenic infections. Complement is a cascade of highly regulated serum derived protein-protein interactions events that are initiated by the recognition of foreign materials. When activated, the complement system contributes to host defense through multiple endpoint outcomes: the production of soluble immune cell chemo-attractant by-products, the tagging of foreign materials with stable breakdown complement by-products, and the lysis of membranes with the pore forming MAC complex.

The MAC complex functions to lyse and kill microbes that may be present at the local site of complement activation.[74] The soluble by-products of complement activation assist in the recruitment of phagocytic immune cells to the site of cascade activation and potential danger through their chemo-attractant properties,[75] while the stable complement by-products that tag local antigens participate in immune function on two levels. First, stable complement tagging can opsonize local antigens for increased phagocytosis by non-adaptive immune cells thereby increasing their clearance from the system,[76] and second they can increase antigenic recognition and

subsequent activation of cells of the adaptive immune system promoting adaptive immune responses.[77, 78] Importantly, if the former non-adaptive phagocytic mechanism is sufficient to effectively clear the dangerous materials from the system, then the latter activation of the adaptive immune system does not occur as the antigen is removed from the system prior to the availability for adaptive immune system stimulation. It is only under the condition of failed antigenic clearance that the antigens are exposed to the adaptive immune system cells.

There are three major pathways that lead to the activation of the complement cascade; the classical pathway (CP), the alternative pathway (AP), and the lectin pathway. The classical pathway is antibody directed in its recognition of instigating materials. This inducing antibody can be derived from either naturally occurring antibody from innate B cell subsets or serum antibody sourced from previous antigen-driven responses.[79] The alternative pathway is however antibody independent and is initiated instead by the binding of serum active complement produced under the weak but constitutively activation of complement in the serum at all times that. Host cell surfaces are however protected from complement attack due to the production and expression of inhibitory receptors and secreted molecules that are normally not expressed on foreign cell surfaces or produced by pathogens.[80, 81] The lectin pathway is instigated by the binding of complement instigating

host sugar binding lectins to a diverse group of sugar moieties not exposed on host cell surfaces, but commonly expressed on common pathogen surfaces.[82, 83]

As mentioned, the classical pathway is antibody directed.[79] In humans, IgM pentamers efficiently activate the complement cascade, while IgG isotypes require antigenic aggregation.[84, 85] Among the IgG isotypes in humans, IgG3 is the strongest complement activating isotype, followed by IgG1 and IgG2, however IgG4 does not activate the complement cascade. In mice IgM pentamers perform similarly, and IgG3, IgG2b and IgG2a can activate the complement cascade, while IgG1 does not.

The binding of complement fixing antibody to antigen mediates the linkage of the C1 complex to the antigen.[86] C1 complex consists of 2 molecules of each C1r and C1s, along with C1q, which is itself a macro-complex built of 6 copies of an A, B, and C polypepetide complex.[87] This macromolecular complex when linked to antibody bound antigen can act as a C4 convertase, in that it cleaves the next component in the cascade, C4 into the two breakdown byproducts C4a and C4b. The C4b by-product contains a thioester group that binds to available hydroxyl and amino groups on local antigenic surfaces creating a covalent bond.[88, 89] C4b then binds to the next cascade component C2 and presents it for cleavage by C1 complex.[90]

The C2a byproduct remains linked to the stable C4b on the antigen, this complex acts as the C3 convertase of the classical pathway. Subsequent C3 cleavage by this complex leads to the production of C3b and the release of the soluble chemoattractant C3a. C3b fulfills multiple roles; like C4b it can covalently attach to available hydroxyl and amino groups on the local antigenic surface through a thioester group,[91] as well as help form of a larger complex of C4bC2aC3b.[92] This larger complex acts as the classical pathway's C5 convertase that cleaves C5 into the soluble C5a chemoattractant and C5b that seeds the formation of the MAC complex.[93]

The alternative pathway of complement also requires the formation of both a C3 and a C5 convertase, however these convertases are composed of different components of the complement cascade. Importantly as mentioned, the alternative pathway is activated by the presence of foreign surfaces and unlike the classical system, is antibody independent. In serum, C3 has a natural "tickover" conversion to C3a and C3b that occurs at a low rate. This C3b can potentially form a covalent attachment to both local foreign and host membranes. C3b binding to membranes is inhibited by the presence of sialic acids typically present on the surface membranes of host cells, and it's further activation of the complement cascade is typically inhibited by host cell surface complement inhibitory receptors.[94] In the absence of these inhibitors on foreign cell surfaces, C3b can bind to serum factor B of the complement

cascade and enable factor B cleavage by the serum factor D, forming the C3bBb complex. This complex is the alternative pathway C3 convertase. Similar to the classical C3 convertase, it produces the soluble C3a chemoattractant as well as result in both the covalent attachment of C3b to the local antigens and the further building of a C5 convertase composed of C3bBbC3b. At this point, the alternative and classical pathway intersect in the ability to cleave C5 into the soluble C5a chemoattractant and the initiation of the MAC complex by C5b.[95]

The third mechanisms of complement activation is that of the lectin pathway. The complement cascade can be initiated by serum sugar binding lectins such as C-reactive protein (CRP) that binds bacterial C-polysaccharides and the mannose-binding protein (MBP) that can bind either mannose or N-acetyl glucosamine residues present on bacterial and yeast cell surfaces but are typically hidden on host cell surfaces.[96, 97] CRP and MBP can initiate the complement cascade by associating with either C1r and C1s like C1q does, or by binding to serum serine proteases like MBP associated serine protease (MASP).[98] These lectin associated serine protease complexes converge with the classical pathway through the cleavage of C4 into C4a and C4b.

As mentioned, there are many important outcomes that result from the activation of the complement cascade. First is the formation of the pore forming MAC complex that can lyse foreign cell membranes. Second is the chemo-attraction of phagocytic cells to the site of complement cascade initiation by the soluble chemo-attractants C3a and C5a. Third, the stable covalently attached breakdown product iC3b, formed by the cleavage of C3b by other serum factors of the complement system, assists in the opsonization of antigens for increased recognition and uptake by the recruited phagocytes. These phagocytic Macrophage, Myeloid, and NK cells, can bind and uptake iC3b labeled antigens using the complement receptors CR3 and CR4 composed of CD18 with either CD11b or CD11c respectively.[99] The recruitment and activation of these phagocytic cells can result in the effective clearance of dangerous complement activating materials from tissues. As such the innate immune system may be able to effectively combat infections. However, if a dangerous infection evades effective innate clearance or becomes systemic, then complement tagged antigens can be trafficked or trapped in secondary and primary lymphoid tissues such as lymph nodes and the spleen where the adaptive immune system can be potently engaged and activated by complement labeled antigens.[100]

One critical feature of the complement cascade in the activation of the adaptive immune response is in the enhancement of antigenic filtration and

presentation of complement tagged antigens trafficked to the lymphoid tissues. Importantly, iC3b on tagged antigens can be further converted to the stable C3d/C3dg byproducts by the serum factor I in the presence of complement receptor CR1 that is expressed predominantly on B cells and follicular dendritic cells (FDCs).[39, 101] The resulting C3d/C3dg is stable and selective for further recognition by the complement receptors CR1 and CR2. Using complement receptors, FDCs can efficiently trap and filter complement tagged antigens. FDC's can then present them to adaptive immune cells, thereby increasing the activation of the adaptive immune system. The resulting FDC antigen presentation is a mechanism that is elementary to the generation of germinal centers reactions where adaptive immune cells proliferate and differentiate.[102]

A second feature of the complement cascade in the activation of the adaptive immune system is through the direct activation of B cells using the CR2 complex composed of CD21/CD19 expressed on B cells. The co-ligation of the BCR with the CR2 complex on B cell surfaces, results in the enhanced activation of B cells and stronger B cell antibody responses.[78]

B cell signaling

B cell receptor ligation can result in a diversity of cellular responses ranging from positive outcomes such as activation, proliferation, differentiation,

and antibody secretion to negative outcomes such as anergy and apoptosis. It is clear that as a mitogenic receptor, BCR ligation can instigate the activation of multiple signaling pathway outputs including NFAT, MAPK/ERK, NFKB and MAPK/JNK activation.[103, 104] It is not well understood however as to how the temporal and spatial integration of these multiple signal pathways work into establishing differential cellular responses to antigen. Of particular confusion is the nature of transduction pathways that constitute “tonic” pro-survival signaling by the BCR, as opposed to that of overt BCR ligation that can result in both activation and apoptosis. Like most mitogenic receptors, it is clear that BCR ligation activates proximal tyrosine kinases that initiate signal propagation. In particular, the src and syk family of tyrosine kinases are two families of tyrosine kinases known to be activated during the ligation of the B cell receptor and participate in the initiation of its signal transduction pathways.[105]

One major pathway activated by BCR induced tyrosine kinases is the activation of the lipid kinase phosphatidyl-inositide-3-Kinase (PI3K).[106] PI3K activation downstream of the BCR is dependent on the recruitment of the PI3K catalytic subunit p110, to a membrane proximal location.[107] The binding of the PI3K adapter subunit p85 via its two SH2 domains to receptor proximal phospho-tyrosines produced during BCR activation of the aforementioned tyrosine kinases is responsible for p110 recruitment. In particular BCR

induced phosphorylation of tyrosines 482 and 513 on CD19 have been shown to be an efficient recruitment site for p85 and functions to promote PI3K activation.[60] Upon membrane localization of this lipid kinase, it acts to produces a PIP3(3,4,5) product from the PIP2(4,5) substrate by phophorylating the 3' hydroxyl group of the inositol ring. This lipid product functions participates in further BCR signal propagation by functioning as a docking site for subsequent signaling proteins containing pleckstrin homology (PH) domains that bind to the phopho-inositol head groups containing these 3' phosphate residues.[108]

Another critical phospholipid modifying enzyme that is dependent on membrane proximal BCR activated tyrosine kinases is the phosphatidyl-inositol-lipase enzyme PLCγ. The activation of this enzyme in B cells is Syk and Btk kinase dependent. Syk kinase phosphorylates the tyrosine residues on the B cell linker protein (SLP-65/BLNK), which then act to recruit both PLCγ and Btk to a complex through SH2 binding domains. The Syk kinase further phosphorylates Btk leading to the induction of its autophosphorylation and subsequent phosphorylation of the BLNK linked PLCγ enzyme. This phosphorylation enhances the PLCγ activity.[109, 110] Independent of the BLNK adapter functioning to link these two proteins together, Btk is localized to the membrane by the binding of PIP3(3,4,5) with a PH domain and PLCγ is membrane localized by its PH domain binding to PIP2(4,5). Interestingly,

activated membrane localized PLCγ uses the same PIP2(4,5) phosphatidylinositol-phosphate substrate as PI3K, but rather than phosphorylating it to produce PIP3(3,4,5), this phospho-lipase cleaves PIP2(4,5) into two types of second messengers; a lipid insoluble product diacylglycerol (DAG), and the soluble inositol-phosphate product IP3(1,4,5).[111]

Secondary Messengers

The IP3 second messenger derived from BCR induced PLCγ activation, is a soluble ligand for receptors regulating the opening of (Ca2+) channels located on both surface membranes and on the endoplasmic reticulum (ER).[112] The subsequent Ca2+ flux from extracellular and intracellular stores is necessary for the activation of a multitude of Ca2+ dependent enzymes including those involved in transduction pathways that activate NFAT, JNK and NFKB activation.[113]

Ca2+ flux initiates the activation of a signaling cascade that results in the nuclear localization of the NFAT transcription factor. In resting cells, the transcription factor NFATp/NFATc is constitutively phosphorylated, masking the NFAT nuclear localization sequence and thus keeping NFATp/NFATc in the cytoplasm. At cytoplasmic concentrations of 200ηM, Ca2+ can efficiently bind to a regulatory protein called calmodulin, which enables it to form a

complex with a phophatase called calcineurin facilitating the activation of it's phosphatase activity. Calmodulin activated calcineurin dephosphorylates NFAT exposing its nuclear localization sequence and promoting its nuclear importation.[114]

The DAG second messenger also derived from BCR induced PLCγ activity participates in the activation of a group of serine/threonine protein kinases belonging to the diverse PKC family. One subset of PKC members requires the Ca2+ flux promoted by the IP3/IP4 byproducts; PKCα, β1, β2, and γ making up the conventional PKC family cPKC. Alternatively, the PKC δ, ε, η and θ family members are DAG dependent but can be activated in the absence of Ca2+ flux and are part of the novel PKC family nPKC. A third family of PKC members, PKC λ, ι, ξ and μ are able to be activated without DAG and Ca2+ flux and are part of the atypical PKC family aPKC, however their activation may be regulated by other lipid products.[115]

The PIP3(3,4,5) product produced by PI3K activity is necessary for the activation of some of the novel PKC family members, in particular δ, ε, and θ.[116] However, PKCμ is the only family member containing a PH domain (and interestingly a putative transmembrane domain). As such, there must be an indirect contribution of PIP3(3,4,5) to the regulation of these PKC family members. PKC has however been shown to be activated by phosphorylation

in the activation loop by an ABC family kinase called PDK-1. This PDK-1 induced phosphorylation enhances the activity of some PKC family members and is absolutely critical to the activity of others, in particular PKCδ.[117] The PDK-1 kinase contains a PH domain that allows for PDK-1 binding to PIP3(3,4,5) products on the membrane and may localize PDK-1 membrane proximal to DAG bound membrane localized PKC family members. Thus it may be the PIP3(3,4,5) dependence of PDK-1 that makes the activation of some of the novel PKC family members dependent on PIP3(3,4,5).

Some PKC family members inactivate the GSK-3β kinase that phosphorylates the c-Jun transcription factor family at thr231/243/249 inhibiting its function. In addition to blocking this inhibitory kinase, these PKC family members simultaneously promote the activation of a c-Jun phosphatase that promotes the dephosphorylation of these sites on c-Jun thereby enabling c-Jun's ability to form complexes with c-fos, forming the AP-1 complex that works with NFAT to promote transcription. Thus PKC removes an inhibitory restriction on c-Jun. Complicating matters, the N-terminal phosphorylation of c-Jun by the Janus N-Terminal Kinase family (JNK) facilitates the formation of the AP-1 complex. It has been well established that the JNK kinase family is activated by PKC family members, but recently it has been found that this is performed through the assistance of a RACK1 linker.[118] As such PIP3(3,4,5) dependent PDK-1 activation leading to the activation of certain PKC family

members represents a second BCR pathway involved in the activation of nuclear signal inputs. Without it, only NFAT can be activated and not AP-1 complex.

BCR signaling promotes the activation of another family of transcription factors termed (Nuclear Factor of Kappa B) NFkB. Currently our understanding of how BCR ligation leads to the activation of this pathway is considerably vague. However it is known that NFkB transcription factor complexes are held in the cytoplasm by inhibitory proteins such as the Inhibitor of Kappa B protein (IkB), blocking their nuclear translocation. An inducible IkB kinase complex (IKK) phosphorylates IkB causing its release of NFkB transcription factors and their subsequent nuclear re-localization so that they can function towards regulating transcription.[119] IKK complex activation has been shown to occur through various methods, however the BCR specific mechanism of induction remains unresolved.

Inhibitors of PKC family of kinases have been shown to block mitogen induced NFkB nuclear translocation and activity, while synthetic activators of PKC family members such as PMA have been shown to synthetically induce NFkB activity, implicating that there is a potential role for the PKC family in the activation of NFkB.[120-124] Currently it is also believed that BCR induced IKKβ activation involves a pathway containing an IKKβ associated complex

consisting of a Caspase Recruitment Domain-containing MAGUK kinase protein-1 (CARMA-1), a caspase recruitment domain protein called Bcl10, and another protein called MALT1 that associates but appears dispensable to IKK activation.[125-127] Interestingly, the PKC family has also been shown to regulate the activation of this CARMA-1 containing complex and in doing so may regulate the activation of NFkB pathway through the downstream activation of IKKβ.[103, 128-131] Recently been suggested that PDK-1 activation may contribute directly to NFkB activation through the direct phosphorylation of IKKβ on ser181 in the activation loop by PDK-1.[132]

In addition to the release of the IkB inhibitor, the phosphorylation of the NFkB transcription factor subunits themselves regulates the transcriptional transactivation potential of the NFkB transcription factors. This phosphorylation can occur through the AKT kinase that is downstream of PDK-1 activation.[133] As PDK-1 activity is regulated by PIP3(3,4,5) binding through its PH domain, the activation of the NFkB transcription factors by the BCR may also be downstream of PI3K activity through both PDK-1 activation of PKC family members, direct IKKβ activation and AKT induced phosphorylation of NFkB itself.

The ERK pathway is a MAP kinase cascade known to be activated downstream of the B cell receptor. However like NFkB activation, ERK

activation remains confusing due to the fact that there are potentially overlapping mechanisms that may be downstream of the BCR that may be capable of activating the ERK cascade of kinases. The first known pathway is through the activation of some PKC family members. PKC members have been shown to phophorylate and activate a MAP kinase kinase kinase (MAPKKK) called Raf-1 that phorphorylates and activate a MAP kinase Kinase (MAPKK) called MEK, that is known to activate the MAP kinase (MAPK) ERK by phosphorylating it on both a tyrosine and a threonine residue.[134, 135] It is at the level of Raf-1 activation that the second potential BCR induced ERK activation pathway may intersect. BCR induced tyrosine phosphorylation can lead to the recruitment of a protein called Son of Sevenless (SOS) through a linker protein called Grb2 that binds to receptor induced tyrosine phosphorylations through SH2 domains and to SOS by SH3 domains. SOS is a guanine exchange factor that once localized to the membrane can take an inactive membrane associated complex containing the small guanine binding protein RAS bound to GDP and exchanges the GDP for GTP producing an active RAS/GTP complex. RAS/GTP can bind and recruit Raf-1 to the membrane where it is capable of inducing the MEK, ERK signaling cascade described.[136] The result of ERK activation is the induction of a diverse pool of new transcription factors involved in the induction of transcription for early response factors such as the expression of c-Fos and c-Jun that can form the AP-1 complex following JNK induced activation.[137]

Together, BCR induced activation of tyrosine kinases leads to the recruitment and activation of phosphatidyl-inositol lipases and kinases that produce second messengers capable of activating Ca2+ flux and serine/threonine kinases that lead to the induction of nuclear transcription factors involved in potentiating BCR antigen recognition to nuclear signal inputs. Here I present only a few of the pathways found downstream of the BCR induced signaling, and although a minimalist view, it still remains a complicated scheme of events that result in multiple signaling outputs derived from a single input. Importantly, BCR signaling does not happen in a vacuum, and other cell surface contextual co-receptors may modify and regulate the incoming signals to further signal potentiation. CD21/CD19 co-receptor engagement is one such signal modifier in that it efficiently enhances the PI3K pathway downstream of the BCR.

Adaptive Immune Responses

There are two major types of B cell dependent adaptive immune responses in response to antigen; those that require the presence of T cells called T cell dependent responses (TD) and those that do not, called T cell independent responses (TI). The former TD immune response produce a true adaptive response to antigenic challenge with the development and selection of B cells displaying antigen-specific antibody of higher affinity than those

present in the repertoire in the beginning of the response. These antigen selected cells are also maintained in the periperal pool for extended periods of time leading to a stronger and faster recall immune response upon later antigen exposure, immunological memory.[138] The latter TI type reponses produce more rapid antibdody responses and can lead to a transient increase in the immune recognition of antigen by immediately increasing the representative proportion of antigen specific lymphocytes in the periphery after antigenic challenge (in particular in the Marginal Zone).[139] These TI responses are generally transient and non-adaptive, as they do not usually produce long lasting memory responses and do not induce the somatic hypermutation of the BCR in order to diversify the antigen specific B cell repertoire. Since TI responses do not induce the hypermutation of the genes encoding the BCR their selection of higher affinity B cells into the repertoire is entirely dependent on selectively increasing the representation of higher affinity pre-existing B cell clones.[140]

TI response can be subdivided based upon the two different forms of antigenic stimulation that induces them. T cell independent type I (TI-1) responses are induced by associative pathogenic materials such as that of lipo-polysaccharide that can lead to the contextual recognition of the dangerous epitopes. T cell independent type II (TI-2) responses result from the recognition of polymeric antigens containing dense repetitive epitopes

within an antigen. It is the ability to immediately produce antibody in response to antigen without the time consuming requirement of seeking out T cell dependent licensing signals that permits TI responses to function quickly towards neutralizing and clearing potentially dangerous materials. However, these B cell responses typically produce only IgM and some IgG3 isotypic class switched B cells antibody responses.[141] Importantly, antibodies of these isotypes can efficiently fix complement but are not recognized by Fcγ receptors on phagocytes. As such they promote antigenic clearance without promoting strong inflammatory responses seen by other isotypes.[142, 143] The marginal zone and B-1 cell subsets are considered to be major contributors to the T cell independent immune responses with rapid production of IgM in response to activation by these types of antigens.[144-147]

T cell dependent antibody responses are considered to be the conventional antibody response. In these responses, antigen activated B cells conjugate with antigen primed T cells in a collaborative effort to determine the nature of the stimulating antigen; whether it is foreign and should be responded to or whether it is self and should not be. BCR ligation on conventional B cells induces both an increase in the expression of activation markers such as the B7.1 and B7.2 surface antigens, and it induces the uptake and endosomal targeting of the ligating antigen.[148, 149] The proteolytic processing of protein antigens in the endosomal compartment

functions to load peptidic fragments derived from the antigen into the cleft of the MHC class II molecule, which is then subsequently transported to the surface membrane and presented for T cell scanning.

CD4+ helper T cells scan activated professional antigen presenting cells (pAPC) presenting peptidic antigens derived from their previous antigenic encounter. When a T cell clone recognizes the presence of a foreign peptide epitope in the MHC class II molecule on the pAPC with high affinity, it promotes the conjugation of the T cell and pAPC through a conglomerate of receptor-receptor interactions.[150] Importantly, the aforementioned induction in the expression of the co-receptors B7.1 and B7.2 (CD80 and CD86 respectively) on the pAPC, help to stimulate the T cells reactivity by co-ligating CD28 on the T cell. This ligation, in addition to TCR stimulation, induces an effective T cell response; contributing to the production of cytokines and the expression of a new surface ligand CD40L that can stimulate the pAPC to respond positively towards an immunogenic outcome through the CD40 receptor.[151] These B7 and CD40 cross-stimulation between B cells and T cells is critical for the generation of Germinal Center (GC) reactions as shown by blocking studies.[152] During this conjugation, the CD4+ T cell receives activation signals from the pAPC and the T cell responds in kind, providing the pAPC with activation signals that license the pAPC to continue to be

responsive to antigen. These B cell-T cell interactions are critical to promoting stronger, adaptive immune responses.

T cell dependent immune responses are dramatically more stable and contribute more efficiently towards antibody production and the adaptive aspects of the immune system than do T cell independent responses. This is because antigen specific B cell clones that have received T cell licensing can more efficiently seed germinal centers (GCs), undergo class switch recombination (CSR), somatic hypermutation (SH) and affinity maturation, in addition to generating long lived memory B cells that are prepared to re-react to future antigenic challenge. In the GC, antigen specific B cells are activated and re-activated by retained antigen held by follicular dendritic cells. This FDC presented antigen provides a selecting target for the activation and positive selection of higher affinity B cells that go on to produce higher affinity antibody responses.[153] FDC antigen retention is mediated through both complement receptors and Fc receptors that filter immune complexes tagged with complement and antibodies respectively.[100] Both natural antibodies found in naïve serum and antibodies derived from either T cell independent immune responses or previous T cell dependent immune responses are capable of contributing to antigenic filtration by FDCs, however the natural IgM and T cell independent IgM and IgG3 isotype antibodies assist in filtration primarily through the activation of the complement system, while IgG1, IgG2a and

IgG2b can additionally assist in filtration directly through Fcγ receptors on FDCs.[154, 155]

Importantly, TD responses are delayed in time, requiring days before their outcome, while T cell independent responses result in immediate antibody production. In effect, the early recognition of antigen by innate B cells capable of generating T cell independent immune responses can promote the generation of T cell dependent immune responses by promoting FDC antigenic filtration, conjoining the early non-adaptive antibody production as a critical component to producing higher affinity antibody responses that are adaptive in nature.

Breaking B cell Tolerance Auto-immunity

The primary barrier for induction of antibody dependent auto-immune disease is the generation of auto-reactive B cells that can respond to self-antigen. The activation of these auto-reactive B cells so as to induce the production of pro-inflammatory antibodies is the second critical step towards the induction of auto-immunity. Together these two barriers constitute the inductive phase requirements for auto-immunity. Subsequent to the generation of auto-antibodies, their deposition in the tissues can effect in inflammation that causes the destructive outcome and pathogenesis; the effector phase.

Of long standing debate is how in a system of random recombinatorial BCR generation, can a system of selective elimination work efficiently enough to avoid the escape of auto-reactive lymphocytes into the constantly replenishing pool of peripheral lymphocytes. At a rate of 35 million large pre-B cells entering mitosis in the bone marrow of mice daily, only 10-15 million new immature B cells survive early BM selection in mice and exit into the periphery daily. Indicating that BM selection removes 20-25 million B cells daily. The peripheral pool once filled, maintains a steady number of B cells that contains 100-300 million B cells in it. Meaning that 10-15 million new immature B cells either are selected against in the periphery or replace those dying off daily. With an average naïve B cell surviving 2 months, this means that 5 million old B cells expire daily. Indicating that one half to two-thirds of all newly arriving B cells departing from the bone marrow daily don't survive; 5-10 million. Altogether, up to 35 million B cells are selected against daily by central (Bone Marrow) and peripheral mechanisms; maintaining a population containing an average of 100 million specificities at any one time. This means that every day of life, 10 percent of all the B cells in the body are eliminated.[156, 157] The necessary efficiency of tolerogenic selection against self-reactive B cells is mind-boggling in numbers. It is definitely not a nickel and dime process.

There are multiple mechanisms that contribute to the elimination of auto-reactive B cells and the prevention of auto-immunity, accounting for the great efficiency of B cell tolerance. As such, there are multiple potential points in which these mechanisms could fail, resulting in the production of the auto-immune state. As such, a single definitive identifiable source for the breakdown of tolerance leading to the generation and activation of auto-specific B cells and auto-immunity has been elusive. There are however three major plausible sources for the generation of auto-reactive B cells that could contribute to the inductive phase of auto-immunity. The first plausibility is the failure to delete auto-specific B cells in two compartments. First, during their development in the bone marrow; failed developmental deletion (central tolerance). Once in the peripheral mature B pool, these auto-specific B cells are at risk of being inappropriately activated to generate auto-antibodies if they can overcome a secondary set of selective pressures against them (peripheral tolerance). Importantly, it is known that auto-specific B cells can and do survive central deletion and exist in the periphery in an anergic state.[158, 159] However, as these auto-reactive cells are typically anergized and short-lived, the inappropriate activation of these anergized cells is one potential mechanism for the induction of auto-immunity.

A second potential source is the conversion of normally benign auto-specific neo-natal B cell subsets such as B-1 cells into producers of

pathogenic pro-inflammatory auto-antibodies.[160] B-1 cells frequently produce non-inflammatory auto-specific antibodies of the IgM isotype, and this “natural antibody” normally plays an important role in the clearance of cellular debris from the circulation, but IgM is not typically pro-inflammatory.[161] The inappropriate induction of B-1 B cells to Ig isotype switch could lead to the production of dangerous pro-inflammatory IgG isotypes that could instigate auto-immunity. Many auto-immune diseases display signs of this possibility, as B-1 like specificities are frequently the same as auto-antibodies found in Systemic Lupus Erythromotosis (SLE) among a group of various other auto-immune diseases.[162]

The third potential source for breaking the tolerance barrier is the generation of auto-specificity from a normal immune response to foreign antigen. During the immunogenic T cell dependent response to foreign antigens, the adaptive immune system attempts to increase antibody receptor affinity to foreign antigens through the process of somatic hyper-mutation and affinity maturation as discussed. This adaptive process in peripheral B cells may convert a B cell from initially being reactive exclusively against a foreign antigen to becoming cross-reactive with both a foreign and a self-antigen. A result made plausible if the affinity selecting foreign antigen contains secondary epitopes similar to a self; antigenic mimicry.[163]

T cell tolerance is paramount to the maintenance of B cell tolerance. The generation of auto-reactive T cell clones creates a permissive environment for auto-reactive B cell clone survival and activation. It has been shown that the linkage of an auto-specific B cell epitopes to an available auto-reactive T cell epitope can subvert the tolerogenic outcome by providing the inappropriate availability of signal two that is sufficient for breaking B cell tolerance and inducing disease.[164] Supporting the assessment of T cell dependence for the development of auto-immunity, animal models of auto-immune arthritis such as the Collagen-induced arthritis (CIA) in DBA/1 and B10 mice show a dependency on both class II MHC haplotype and on the presence of CD4+ T cells.[165-169]

Complement and Auto-immune arthritis

Rheumatoid Arthritis (RA) is an autoimmune syndrome of largely unknown etiology that affects joint and bone tissues. Some types of RA however may be antigen-specific, and thus suggest the involvement of the adaptive immune system in disease induction. As the presence of pathogenic auto-specific IgG antibodies is a key feature to RA, the activation and differentiation of B cells with the capacity to recognize and respond to joint specific antigens may be a key factor in the onset of arthritic lesions. In the recently described K/BxN mouse arthritis model, the B cell dependent production of an antibody component is necessary for the spontaneous

induction of arthritic inflammation and sufficient for the transfer of disease to secondary recipients.[170] In the Collagen-induced arthritis (CIA) mouse model of inflammatory arthritis, rheumatic cartilage and synovium contain antibodies to type II collagen, which arise from an exogenous antigen-driven response.[171, 172] Similar to the K/BxN model, the passive transfer of auto-antibodies is sufficient to initiate the development of arthritis. In the case of CIA, these auto-antibodies are derived from a monoclonal anti-collagen source.[173] Even with such a dependency on the humoral component of the immune system, little is known of the mechanisms that govern the activation and persistence of auto-reactive B cells in rheumatic disease.[174, 175]

As described, the innate complement system not only promotes inflammatory reactions downstream of antibody deposition, but also enhances the immuno-reactivity of labeled antigens leading to increased antibody responses. As such, the participation of serum complement in the generation of auto-immune responses and the initiation of disease is of significant importance. Components of the complement pathway have been shown to be critical in both the CIA and K/BxN mouse models of inflammatory arthritis. C5 or C3-deficient mice are resistant to CIA, while a deficiency in the alternative pathway factor B decreases the severity of clinical symptoms of inflammation.[176-178] In the K/BxN model, a C5-deficiency in the NOD background protects the TCR transgenic NOD mice from disease, while an F1

cross onto the C57Bl/6 strain becomes susceptible because of its genetic provision of a C5 allele. In addition, the passive transfer of K/BxN disease serum into Factor B, C3 or C5-deficient recipients attenuates the transfer of disease in this model.[179] Although it remains clear through these experiments that complement participates in the effector stage of inflammation induced by provision of auto-antibodies, the true nature of complement dependency for the initiation phase of disease remains unclear. Specifically, complement may participate in enhancing the generation and activation of auto-specific B cells, producing auto-antibody against joint antigens.

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Chapter 1: CD19 and Tolerance

Introduction:

Tolerance Modalities

Tolerance allows for the establishment of a state of immune homeostasis in which the immune repertoire consists of a pool of B cell clones capable of recognizing an infinite diversity of antigenic epitopes either previously seen or unseen, and is simultaneously prohibited from reacting to self.[1] This homeostatic pool of B cells is developed through a process of positive and negative selection that promotes the generation of B cells expressing functional antigen receptors (BCR) and concomitant elimination of auto-reactive B cells.

According to the “temporal” model of tolerance, a general mechanism for eliminating auto-reactive B cell clones is based on the temporal timing of BCR engagement during the progressive development and differentiation of B cells from hematopoietic stem cell precursors in the bone marrow to mature B cells in the periphery. This model assumes that any antigen available to a newly rearranged BCR is probably a self-antigen, and surmises that early engagement of the BCR will instigate B cell deletion. If a B cell evades this early BCR engagement, it matures and enters the peripheral pool. In accordance with this “temporal” model, B cell development is blocked by the cross-linking of B cell receptors on immature B cells,[2] while the cross-linking

of B cell receptors on mature B cells is mitogenic.[3] A major implication of these findings is that signaling from the immature BCR and the mature BCR may be fundamentally different in mechanism and hence outcome. On the surface, this model seems satisfactory to meet the minimum requirements for negative selection of auto-reactive B cells. As an aside, any newly arising pathogen-specific B cell in the bone marrow during the time of an infection would be eliminated and would not contribute to the adaptive immune response to that pathogen; only pre-existing B cells could respond.

Importantly, the surface expression of both the pre-BCR and immature BCR in addition to signaling components of the antigen receptor complex are critical for the positive selection of developing B cells during early transitional stages of development in the bone marrow.[4, 5] As surface B cell receptor signaling competence is required for positive selection during these transitions, it raises the question of how do developing B cells decide between deletion or positive selection from the same signaling receptors under the temporal model of tolerance? It is theorized that the strength and duration of BCR signaling can differentially effect the selection of developing B cells. Positive selection is the result of weak “tonic” signals derived from the surface expression of CD79α and CD79β (Igα/β) coassociated with the pre-BCR on pre-B cells or BCR on immature B cells.[6] This positive selection signal is believed to be BCR dependent, but antigen ligation independent and requires

the presence of the ITAM containing sequences such as those in Igα/β, which have also been shown to be sufficient to direct developmental progression when present in a chimeric LMP2 molecule.[7, 8] Negative selection on the other hand, is the result of antigenic cross-linking of the BCR on immature B cells leading to a developmentally early and “strong” signal that attenuate further positive selection and can even promote the negative selection of such antigen reactive clones.[9, 10] As a mature B cells can be activated by antigen ligation of the BCR, this means that at some point in maturation there must be a conversion of what was once a strong negative signal into a strong positive outcome; hence a temporal dependent strength of signal model of tolerance is more appropriate.[11]

Sufficient amounts of all self-antigens may not however be immediately available to every developing B cell in the bone marrow to be screened against due to the restricted location and limited level of expression of various potential auto-antigens in the organism.[12] The limited exposure to potential auto-antigens for centrally developing B cells in the bone marrow would affect the availability of effective receptor ligation strength needed for providing tolerizing signals. In fact, the restricted expression of an auto-antigen in peripheral tissue has been shown to be insufficient to delete all auto-specific B cells generated from the bone marrow.[13] These auto-specific B cells that have escaped from central tolerance are subsequently disseminated into the

periphery, but they do not however instigate disease, implicating the existence of other restrictive mechanisms in the periphery that can function to meet this central tolerance shortfall. Immature B cells leaving the bone marrow still must evade tolerogenic stimuli to be allowed to complete their developmental maturation. It is not until a B cell reaches the mature B cell stage that it is capable of effectively responding in a positive manner to antigenic encounter. As such, a central model for the developmental timing and strength of antigen encounter is insufficient to fully explain the induction of tolerance, and the participation of peripheral tolerance mechanisms must play an additional role in tolerizing immature B cells.

It has also been shown that mature B cells can be either activated or inactivated by antigenic encounter depending on the avidity of the antigen.[14, 15] As such, BCR ligation can induce tolerogenic signals in both immature and mature B cells, indicating that BCR signaling in both immature and mature B cells may not be that mechanistically different, but rather immature B cells may just be more sensitive to tolerogenic signal outcome. Since mature B cells can also be tolerized, the developmental timing of strong antigen encounter model as defined by a temporal conversion point in which previous negative signals instead produce a positive outcome is insufficient to explain the induction of tolerance. Additional contributing factors must work towards producing the homeostatic state of tolerance.

Another potential modality that could work towards promoting a tolerogenic signal is the duration of signaling model. Although a self-antigen may be low in concentration or restricted to a remote location, a true auto-antigen will typically persist in exposure to a lymphocyte, either mature or immature. In accordance with this theory, the persistence of an auto-antigen has been shown to be either necessary for the induction of tolerance or as a means to reinforce continuing inhibitory signals that maintain the tolerogenic state.[16-20]

The strongest detractor from the duration of signal model is that not all foreign antigens can be quickly cleared from the system. If pro-longed BCR ligation promotes the deletion of foreign-reactive mature B cells rather than their expansion during an infection, there would be a timing limit after which all the antigen reactive cells would be functionally deleted from the repertoire. By this mechanism of pro-longed BCR ligation, pathogen exposure would result in the selective clonal deletion of reactive cells, leaving a foreign-antigen specific hole in the repertoire that would then permit the pathogen to escape further immune attack; a very undesirable outcome for the host.

The conjoining of the two-signal hypothesis with the duration of signal hypothesis eliminates this theoretical problem. The two-signal hypothesis

requires that the activation versus inactivation of a lymphocyte be regulated through the composition of two associated signals being required for cell activation.[21] Although an auto-antigen may provide sufficient pro-longed activation signals through antigen ligation of the BCR (signal one), without the availability of a secondary pro-inflammatory contextual signal (signal two), the antigen-reactive clone will die by negative selection.[22, 23] Under this theory, the association of an antigen with the context of encounter becomes a primary factor towards the choice of activation or inactivation for the B cell.

Two major types of contextual “signal two” are believed to be capable of rescuing antigen activated B cells from a tolerogenic outcome, either a classical or a non-classical form.[24] Classical signal two is T cell dependent help, and rescues B cells from antigen induced inactivation by promoting a delayed but more robust, pro-longed and higher affinity B cell response in addition to resulting in the acquisition of long-term memory. Non-Classical signal two is derived from the co-recognition of innate danger signals in addition to antigen specific recognition. Non-Classical signal two also provides a rescue from inactivation, but promotes more rapid antibody response, however these responses can contribute only minimally to long-term memory with produce an overall lower affinity response than the classical response.[21] These two different sources of signal two are however not necessarily exclusive within the activation of a B cell. An activated B cell may

be able to receive both non-classical and classical signal two during the course of its antigenic activation.

Non-classical signal two can be defined as the provision of innate indicators of danger either pathogen associated or derived from the host innate immune system. Pathogen Associated Molecular Patterns, (PAMPs) may provide signal 2 through the conjugation of both the BCR and secondary mitogenic receptors such as Toll Receptor family members and RP105.[25-28] Within the group of innate indicators is the co-recognition of dangerous materials labeled with complement break down by-products C3b/C3d/C3dg that tag foreign antigens during the activation of the complement cascade. These complement products can provide sufficient signal 2 as a tagged antigen can be co-recognized by both the BCR and the complement receptor 2 complex (CR2) composed of CD21/CD19 expressed on the B cell surface.[29, 30] Interestingly, various host serum proteins can assist in enhancing the recognition of PAMPs by surface receptors, such as lipid binding protein (LBP) that binds to CD14 and helps to activate the TLR family members by forming a stimulatory complex.[31] In effect, this shows that mechanistically the PAMP and complement pathways are similar in design; a mechanism of serum opsonization of dangerous materials followed by recognition by surface receptors.

The provision of non-classical signal two has been shown to promote immediate B cell activation by inducing Ig secretion and B cell proliferation with limited class switch recombination.[32] These responses are T cell independent immune responses (TI), and these responses do not contribute to the generation of higher affinity B cell clones or to a great deal of antigen specific memory.[33] The number antigen reactive clones in the subsequent repertoire is higher, giving rise to a more sensitive secondary response to antigen encounter, but the affinity of these clones for the antigen remains equivalent to the primary response as the process of affinity maturation does not occur with TI antigens.[34, 35] The long term survival of TI activated B cells is not believed to be greater than that of naïve B cell clones, so although there is an immediate increase in antigen specific B cell represented in the peripheral pool, there is no long term memory.[36]

The avoidance of negative selection by the acquisition of classical signal two is a T cell dependent response.[37] T cell dependent responses are delayed, but more robust, they are sustained for longer and develop higher affinity B cell clones through affinity maturation. Importantly, there is also the generation of both class switch recombination and memory that occur in T cell dependent B cell responses.[38] Classical signal two is the availability of T cell help derived from the conjugation of antigen activated B cells with antigen specific T cells, initiating a T cell dependent immune responses (TD). This

provision of signal 2 comes in the form of cytokine exposure and the ligation of secondary B cell signaling receptors such as CD40 by CD40 ligand (CD40L) expressed on T cells. These signals are made available by T cells in response to their recognition of B cells presenting peptidic components of the encountered antigens in the major histo-compatibility complex II, (MHC II).[39-41] B cell Receptor engagement induces B cells to uptake, process and present antigen, which coincides with their induced and migration to the T/B border of the white pulp, to screen for available antigen specific T cells.[42, 43] The failure to find available T cell help results in B cells being restricted to the outer PALS and their exclusion from further differentiation in the germinal centers of the follicles.[44, 45] Furthermore, antigen specific B cells from BCR transgenic mice transferred into mice expressing the specific antigen as a self-antigen are not only excluded from the follicles as described, but are subsequently eliminated in the outer PALS by a fas mediated apoptotic mechanism.[46] This fas mediated deletion can be attenuated by the provision of sufficient signal 2.[47]

These classical and non-classical secondary context signals promote cell survival and proliferation. Without the presence of signal 2, signal 1 will be tolerogenic in outcome. Initial B cell encounter with antigen can occur under the context of antigen alone, or antigen co-ligation with non-classical signal two. As classical and non-classical secondary context signals are not

exclusive, the provision of early non-classical signals can actually promote the ability to process, present, and activate T cells leading to the further attainment of classical signal two.[48] These non-classical signals opsonize antigen and enhance its uptake and presentation in addition to inducing the expression of secondary activation markers such as B7.1, B7.2, and MHC II on B cells to assist in the conjugation-dependent activation of T cells.[49, 50] The major restriction to the acquisition of classical signal two for B cells is the provision of an effective peptidic T cell stimulating epitope to present in MHC II, without it the B cell is limited to a TI response. Antigen activated B cells are destined for tolerance unless they can acquire signal 2 prior to establishing the anergic state[51]. Once anergy is established, B cells are no longer capable of mounting T cell dependent immune responses.[52] Non-classical signal 2 may enable, prolong or enhance the ability of B cells to respond to classical signal 2. Without the early non-classical signal two, it may still be possible to acquire classical signal two, however the ability to evaluate this experimentally is difficult due to the diverse effects of adjuvant, complement and the PAMP receptors. It would be difficult to eliminate all non-classical forms of signal two prior to the provision of T cell conjugation.

Mechanisms of Tolerance

The primary method for restricting the generation of auto-specific B cells is deletion by death. In the bone marrow, the elimination of newly

developing B cell clones by deletion typically occurs for one of two reasons; first is the inability to produce a functional BCR expressed on the surface, second is the strong ligation of this newly expressed BCR.[7] The former is “death by neglect” as the positive survival signals necessary for promoting the continuing development of the B cell into the periphery are lost without surface BCR expression, while the later is an antigen induced death (AID) mechanism.

Like central tolerance, peripheral tolerance can also utilizes the mechanism of deletion to remove auto-specific clones. Antigenic ligation of newly generated immature B cells promotes their death, and antigenic ligation of mature B cells in the absence of available signal two results in their deletion. This antigen-induced death can occur by either a fas dependent or independent AID mechanism.[53-55] As signal two inhibits the functional activation potential of the fas pathway, the absence of signal two in the presence of signal one results in the creation of an activation-induced fas sensitive cell. The subsequent fas mediated killing is performed by secondary cell types that recognize the antigen activated B cell and provide fas ligand that activates the B cell intrinsic apoptotic mechanisms of death.[56] Antigen-activated B cells may also die through a fas independent mechanism of necrosis that is instigated by the pro-longed consumption of critical metabolic resources that occurs with pro-longed B cell activation in the absence of signal two survival signals.[57]

Newly generated immature B cells may be incapable of responding to mechanisms of signal two either classical or non-classical, and therefore may behave much like mature B cells that are deleted in the absence of signal two. Unique to newly developing immature B cells departing from the bone marrow, they are capable of escaping tolerogenic signals that could result in their deletion by changing their antigenic specificity through continued or induced V gene rearrangement and therefore eliminating the source of signal one.[58] After the considerable effort and energy involved in creating a functional BCR through the rearrangement of both heavy and light chain genes, this “receptor editing” mechanism assists in the generation of functional non-self specific B cells from those that may have at one time been auto-specific. This mechanism involves shutting off the expression of the newly formed light chain, and initiating the further rearrangement of possible light chains. If these formerly auto-reactive B cells can rearrange the other light chain loci to create a functional light chain that can pair with the original light chain and is not capable of being ligated by an auto-antigen, then that clone may be able to survive and enter the peripheral mature B cell pool.[59]

In vivo, existing peripheral auto-reactive B cells are held in a state of anergy. In vitro, B cells that have undergone prolonged BCR ligation without the availability of signal two will also become refractory to further antigenic

stimulation; attributes similar to that of anergy.[60] Anergic B cells do not proliferate, secrete antibodies or up-regulate activation markers in response to further antigenic or mitogenic stimulation, it is a state of antigenic unresponsiveness towards a positive outcomes.[61] Anergy assures that peripheral antigen activated B cells that have not yet been deleted by fas dependent or independent mechanisms and have also failed to acquired signal two, do not contribute towards the generation of an immune response. As expected of a B cell on its way towards deletion, anergic B cells display a shorter life-span and restricted localization.[62]

Anergic B cells also express lower levels of surface antigen receptor IgM. This lower surface IgM could be either the result or the cause of the antigen unresponsive anergic state.[63] Decreased surface IgM may contribute to a loss in antigenic responsiveness in an anergic B cell, as there is less receptor to be stimulated. However the decrease in surface IgM may simply be the result of the pro-longed antigen encounter inducing the constitutive uptake and removal of the antigen receptor from the surface. Supporting the latter possibility, the removal of auto-antigen from anergic B cells permits the re-expression of higher IgM surface levels, yet the anergic state is maintained. Additionally, prolonged BCR ligation induces a decrease in surface IgM in naïve mature B cells similar to that of anergic B cells. Importantly, the continued flux of calcium in anergic B cells is dependent on

the continuous exposure to antigen, indicating that some level of constitutive BCR signaling is occurring in anergic B cells without the mitogenic outcome expected of a typical non-self antigen encounter.[64, 65]

Tolerance Models

As described, multiple types of signal inputs may be capable of determining the tolerogenic outcome; concentration of antigen, avidity of antigen, time of encounter in development, and duration of encounter with antigen. In addition, secondary rescue signals associated with the context of antigen specific BCR activation that may be capable of rescuing mature B cell clones from elimination include co-stimulation by either pathogen associated antigenic epitopes, complement, or T cell help.[61] Of primary importance to all of these selective mechanisms is the contribution of BCR signaling. As such, modifiers that confer either positive or negative impact on BCR signals may be of critical importance in determining the efficacy of BCR induced tolerance. CD19 is one such B cell specific co-receptor that contributes to the strength of BCR signaling by amplification of BCR signals, resulting in the enhanced activation of downstream signaling molecules.[66, 67] Early in BM development, CD19 participates in pre-BCR and immature BCR signaling without participating in the formation of the complement receptor 2 (CR2) complex, as CD21 is not expressed until after peripheral maturation.[68-70]

As CD19 is an enhancer to BCR signaling, early BCR associated CD19 signaling may participate in the generation of stronger BCR signals. Since negative selection results from strong BCR signals derived from antigen ligation, CD19 signaling may thus contribute to the induction of tolerance. Further the loss of CD19 may provide the opportunity for B cells to escape from negative selection and result in the generation of auto-reactive B cells in the periphery.

The ability to visualize bone marrow derived auto-reactive B cells in the periphery of both mice and humans is difficult however, as the generation of peripheral auto-specific B cells is a rare event. Transgenic mouse models that increase the frequency of auto-specific B cell generation are necessary to experimentally analyze such normally rare and transient events. A good model for measuring the generation of auto-reactive B cells is the soluble Hen Egg Lysozyme (HEL), anti-HEL transgenic mouse model created by Christopher Goodnow.[52] In this model, mice express the MD4 transgene encoding a pre-rearranged heavy and light chain that is specific to the protein HEL. This transgene is an efficient inducer of allelic exclusion with greater than 90% of developing B cells utilizing the transgene. Importantly this transgene is capable of producing both IgM and IgD isotypes, and is also capable of producing soluble antibody in response to B cell activation. However this transgene does not encode the IgG constant regions and does not undergo

class switch recombination (CSR) to the IgG isotypes. A second transgene used in the system is the ML5 transgene encoding a soluble form of hen egg lysozyme protein (sHEL) under the metallothionein promoter. In mice carrying both the MD4 and ML5 transgenes, B cells developing from the bone marrow are auto-specific and placed under tolerizing pressure. This model allows for experimental analysis of tolerance induction and the state of anergy seen in surviving auto-specific peripheral B cells.[52] As such, the mechanisms of deletion, receptor editing, and anergy can be measured.

In double transgenic WT mice, B cell tolerance remains intact with no measurable production of serum auto-antibodies, a high level of negative selection as displayed by a decrease in peripheral B cell numbers, and the induction of an anergic state in surviving peripheral auto-reactive B cells. These anergic B cells do not spontaneously activate to secrete soluble antibodies or form germinal centers in response to HEL immunization, and in vitro these B cells do not proliferate or display activation markers in response to mitogenic challenge. To address the plausibility that the CD19-dependent potentiation of BCR induced signals is an essential component for establishing tolerance, we crossed mice deficient for CD19[71] onto strains containing the MD4 and ML5 transgenes.

Results

The loss of tolerance can be measured through multiple means, however the production of auto-antibodies is a necessity for the generation of antibody dependent auto-immunity and is an absolute indicator of a failure in establishing tolerance. So the ability of CD19-deficient mice to produce auto-antibodies in this tolerance model was of primary interest. As such, the amount of serum HEL-specific and total serum IgM antibodies produced in CD19-deficient and WT mice expressing the anti-HEL (MD4+) transgene was measured and compared to CD19-deficient and WT mice expressing both the anti-HEL transgene and the sHEL transgenes (MD4+ML5+). The presence of serum IgM levels was measured by two types of sandwich Elisa assays. First being an antigen specific capture of serum IgM using HEL coated Elisa plates followed by visualization using alkaline phosphatase conjugated anti-mouse IgM antibody and PNPP substrate (Figure 1a). To eliminate the possibility that pre-bound HEL isolated with serum IgM may skew the quantification of serum antibody by competing with Elisa plate bound HEL for IgM binding, an antigen non-specific measurement was performed. . This sandwich Elisa was performed by capturing total mouse serum IgM using anti-mouse IgM coated plates followed by visualization using alkaline phosphatase conjugated anti-mouse Kappa light chain specific antibody and PNPP substrate (Figure 1b). Neither WT nor CD19-deficient tolerized double transgenic mice produced

measurable levels of auto-specific serum IgM, or significant amount of total serum IgM. Interestingly, CD19-deficient MD4+ transgene only mice produce lower serum IgM levels than their WT counterpart as analyzed by both methods. An average of 5 mice per group was analyzed. Immunization with exogenous HEL in CFA did not increase HEL specific IgM at 7 or 14 days after immunization in either WT or CD19-deficient tolerized mice, (Data not shown).

CD19-deficient animals appear to have a selective decrease in antibody secretion that could mask the ability to measure a break in tolerance through the presence of auto-antibody. As the ability to secrete antibody is only one read-out for measuring a break in tolerance, it was possible to employ other methods to evaluate the efficiency of tolerance. The efficiency of negative selection leading to a decrease in the generation of peripheral B cells is another measurement of tolerance that can be measured by quantifying the number of peripheral auto-specific B cells. The production of peripheral B cells in both CD19-deficient and WT transgenic mice was quantified by determining the percent of splenic cells staining positive for the B cell marker B220 by flow-cytometric staining, and extrapolating that percent to the number of total splenic cells. CD19-deficient double transgenic MD4+ML5+ mice produce a dramatically decreased number of B220+ cells in the spleen relative to CD19-deficient mice carrying the MD4+ transgene alone (Figure 2a). Like their WT counterparts, tolerized CD19-deficient mice show signs of intact negative

selection as determined by enumeration of peripheral B cells. Interestingly, CD19-deficient mice produce 30% fewer total splenic B220+ B cells than WT mice when carrying only the MD4 transgene, a sign of decreased bone marrow positive selection or, alternatively, decreased peripheral B cell survival. The penetrance of transgene expression was efficient in both CD19-deficient and WT mice, with an average of 98% and 97% of B cells staining respectively positive for the transgene haplotype IgM^a.

To evaluate the induction of central tolerance, comparative analysis of B cell numbers and percentages were determined for the bone marrow of transgenic mice either WT or CD19-deficient. Bone marrow was isolated from two femurs, and two shinbones per mouse, quantified and antibody stained. Once again, both WT and CD19-deficient tolerized mice both show a dramatic decrease in B cell numbers, indicating intact tolerance. CD19-deficient mice carrying only the MD4 transgene once again showed a marked decrease in the total number and percentage of B220+ B cells present (Figure 2b left side). B220+ IgM- bone marrow B cells include pre-pro, pro and pre-B cells not yet expressing an auto-specific BCR in addition to cells undergoing receptor editing. In transgenic mice these pre-pro, pro and Pre-B cells are usually decreased in number due to the faster developmental progression through these stages because of the available pre-arranged transgenic heavy and light chain. The decrease in B220+ B cells in the bone marrow could be a result of

either a decrease in positive selection or an increase in negative selection. In order to determine the contribution of negative selection, the number and percentage of B220+ IgM+ B cells were determined. These cells include recirculating matures and newly generated immature B cells B cells that are capable of antigen specific deletion. Both WT and CD19-deficient animals, IgM+ B cells are deleted even more efficiently deleted than total B220+ cells, indicating intact antigen specific negative selection (Figure 2b right side). MD4+ WT and MD4+ CD19-deficient mice have $5.5x10^6$ and $8.6x10^6$ B220+ IgM- B cells respectively, while tolerized MD4+ ML5+ WT and MD4+ ML5+ CD19-deficient mice respectively have $12.75x10^6$ and $10.5x10^6$ B220+ IgM- B cells, indicating that B cells not capable of recognizing antigen because they have not yet expressed the BCR are not deleted in either WT or CD19-deficient mice.

To determine if tolerance was intact in secondary peripheral lymphoid tissues, the percentages of B220+ B cells were determined in those tissues. Cells were isolated from popiliteal and inguineal lymph nodes (LN), Peripheral blood (PBL) and the Peritoneum (Peri) then stained. The percent of B220+ cells in these compartments were then quantified by flow cytometry. Both WT and CD19-deficient double transgenic tolerized mice have a dramatic decrease in B220+ B cells in all peripheral tissues evaluated. Interestingly, MD4+ CD19-deficient mice produce normal numbers of B220+ lymph node B

cells, however they have a dramatic decrease in peritoneal B220+ B cells and an increase in peripheral blood B220+ B cells when compared to their WT counterparts (Figure 2c).

The quantification of B220+ B cells in central and peripheral B cell compartments, both primary and secondary tissues, show that negative selection appears intact in both CD19-deficient and WT mice expressing both the MD4 and ML5 transgenes (Table 1). Interestingly, CD19 deficiency results in a general decrease in B cells in the bone marrow, spleen and peritoneum, but equivalent lymph node B cells and an increase in B cells in the peripheral blood; possibly an indicator of poor trafficking of CD19-deficient B cells.

Existing splenic B cells in both CD19-deficient and WT double transgenic tolerized mice display a typical decrease in surface IgM staining with retained IgD staining as visualized by flow cytometric staining for IgM and IgD (Figure 3a). Comparative evaluation of surface IgM expression on these splenic B cells from tolerant and non-tolerant CD19-deficient and WT B cells was determined by mean fluorescence intensity staining for IgM on B220+ gated B cells. Surface IgM expression on CD19-deficient tolerized B cells was higher than that of their tolerized WT counterparts; the first possible indicator of a selective decrease in tolerance induction in CD19-deficient mice. (Figure 3b) However, the surface expression of IgM on splenic MD4+ only transgenic

CD19-deficient B cells is higher than that of their WT counterparts as well, possibly indicating a higher set point for IgM down-regulation to start from. It was also possible that the CD19-deficient MD4+ only B cells expressing higher IgM were transitional B cell subsets that typically express higher IgM surface levels. Apart from this possibility, these B cells were found to stain similar to mature B cells (B22O+, IgD+, CD21+, CD23+, HSAlow, 493+ (Data not shown).

Bone marrow, lymph node, peritoneal and peripheral blood B cells from both CD19-deficient and WT double transgenic tolerized mice all possess the typical decrease in surface IgM staining as measured by flow cytometry, indicating that surviving auto-specific B cells appear tolerized in all tissues analyzed (Figure 4).

It was not possible to evaluate receptor editing by staining for IgM positive B cells that do not bind HEL, because endogenous HEL in tolerized mice blocks the ability to distinguish antigen specific from non-specific B cells by staining with labeled HEL. Therefore, peripheral splenic B cells were evaluated for receptor editing by the expression of endogenous lambda light chain usage on B220+ splenic B cells. The generation of B220+ positive cells in both WT and CD19-deficient tolerized mice that were positive for lambda light chain was found to be negligible at 0.04% and 0.07% respectively.

Lambda light chain expression was not seen in non-tolerized WT (0.11%) or non-tolerized CD19-deficient (0.08%) mice; importantly 5-10% of splenic B cells from both WT and CD19-deficient non-transgenic mice stained positive for lambda light chain expression indicating that the staining antibody was functional for light chain usage (Data not shown). It is distinctly possible that receptor editing occurs in this model, but that the endogenous kappa light chain is utilized instead of lambda.

If CD19 participates in the initiation or maintenance of tolerogenic signals, its activation must be induced or maintained in tolerized cells. To test if the activation of CD19 occurs in tolerized B cells in response to antigen ligation, splenic MD4+ and MD4+ML5+ B cells were purified by CD43 depletion and then stimulated for 15 minutes at 37^{o}C with either Medium alone, anti-IgD 10 μg/ml after applying Fc Block for 15 minutes, anti-IgM Fab"2 10 μg/ml, or 10 μg/ml HEL. The level of CD19 phosphorylation was determined by immuno-precipitating (IP) CD19 with and anti-CD19 antibody followed by western blot analysis for phosphorylation using an anti-phospho-tyrosine antibody 4G10 (Figure 5). Reprobing for CD19 confirmed that this phosphorylated protein was in fact CD19 (Data not shown). CD19 appears to be constitutively phosphorylated at a low level in tolerant B cells, and tolerant MD4+ML5+ B cells were still able to phosphorylate CD19 in response to antigenic rechallenge, albeit much less efficiently than MD4+ B cells.

The higher level of surface IgM on tolerized CD19-deficient B cells indicates that these cells may not be fully tolerized. To further evaluate the efficiency of tolerance induction, it was necessary to determine if these tolerized peripheral splenic CD19-deficient B cells were anergic. This was done by measuring the in vitro proliferation potential of purified splenic B cells (CD43 depletion) after the provision of either antigen specific stimulation with HEL or anti-IgM Fab"2 (Figure 6a), or general mitogenic stimulation with anti-CD40, anti-CD40 + IL-4 or LPS (Figure 6b). CD19-deficient and WT tolerant B cells failed to significantly proliferate in response to any of the stimulations, indicating that anergy is intact in auto-specific CD19-deficient B cells. Interestingly, MD4+ CD19-deficient B cells proliferated less than their MD4+ WT counterparts in response to all tested stimulants. As CD19-deficient B cells are less proliferative in to all antigenic and mitogenic stimulation in general, it could be assumed that this proliferative difference like the intrinsic antibody production defect may mask the ability to evaluate the loss of tolerance in the absence of CD19. It was therefore necessary to confirm the maintenance of anergy by other methods.

Anergic B cells will not upregulate the activation markers CD69 and B7.2 (CD86) in response to stimulation. Transgenic CD19-deficient and WT B cells were analyzed by flow cytometry 24 hours after antigenic (Figure 7a) and

mitogenic (Figure 7b) challenge for the expression of CD69 and B7.2 markers. MD4+ CD19-deficient and WT MD4+ B cells both upregulated these activation markers with equal efficiency, while tolerant CD19-deficient and tolerant WT B cells did not, indicating that anergy is maintained in both WT and CD19-deficient tolerized B cells.

By all methods employed thus far, it appears that CD19 deficiency does not contribute to the loss of tolerance induction in this mouse model. It is, possible however that the level of soluble HEL produced by the ML5 transgene is high enough to surpass the strength of signal even in the absence of CD19. Therefore, we postulated that a lower level of antigen might not be able to instigate the tolerogenic state in CD19-deficient B cells. To test this possibility, bone marrow B cell cultures depleted of CD23 positive cells (eliminating recirculating mature B cells) were established from MD4+ CD19-deficient and MD4+ WT mice. After 60 hours in culture with IL-7, these cultures were provided titrated amounts of exogenous HEL auto-antigen for 15 hours, and examined for their relative ability to down regulate surface IgM by flow cytometry (Figure 8a). Initial experiments determined that both WT and CD19-deficient B cells downregulated surface IgM in the concentration range between 1ng/ml and 100ng/ml of auto-antigen, it was then further narrowed to a range lower than 10ng/ml. The concentration of exogenous HEL necessary for surface IgM downregulation in both CD19-deficient and WT B cells appears

to be between 1.0ng/ml and 2.5ng/ml. In close comparison, CD19-deficient bone marrow B cells are actually more efficient than WT at down-regulating their surface IgM at 2.5ng/ml indicating that CD19 is not necessary for receptor downregulation by autoantigen.

These in vitro tolerized bone marrow cells were then tested for their ability to proliferate in response to additional antigen, or mitogenic challenge. Cultures were performed as above, but after 48 hours with HEL, cultures were provided with additional HEL and non-specific mitogen LPS and tested for proliferative capacity through their ability to incorporate BrdU. These cultured cells were then surface stained for B220 expression, followed by intracellular staining with an anti-BrdU antibody and visualized by flow cytometric analysis. The percent of B220+ gated cells staining for positive for BrdU was determined (Figure 8b). CD19-deficient and WT bone marrow cells are equally tolerogenic in response to this auto-antigen, but tolerance induction required at least 100ng/ml of HEL during pre-incubation conditions in order to establish anergic conditions. At 10ng/ml and lower, all cultured B cells still retained their proliferative capacity.

It is possible that the high affinity rather than the concentration of HEL overcomes the CD19 deficiency. To address this question, the same bone marrow culture system was applied to the lower affinity antigen Duck Egg

Lysozyme (DEL). The down-regulation of surface IgM (Figure 9a) and subsequent proliferative capacity (Figure 9b) was measured. Again, CD19 deficiency had no effect on the concentration of antigen necessary for surface IgM down-regulation or level of proliferative anergy.

Although CD19 specific signals do not appear to be necessary for inducing tolerance, they may be necessary for maintaining the state of anergy once cells are isolated away from tolerizing antigen. To address this possibility, peripheral splenic B cells from tolerizing CD19-deficient and WT animal were cultured without antigen for 12 hours and the upregulation of surface IgM was determined over time. Although tolerized splenic CD19-deficient B cells were previously shown to not down-regulate their surface IgM as efficiently as their WT counterparts, the re-upregulation of their surface IgM occurred at a faster rate than that of WT indicating that perhaps the maintenance of tolerance may be CD19 dependent (Figure 10a). However, the anergic state of in vivo tolerized peripheral B cells that have recovered their surface IgM expression after 24 hours of removal from auto-antigen was determined to be intact in both CD19-deficient and WT cells; both for antigenic specific (Figure 10b) and mitogen specific (Figure 10c) proliferative responses. Additionally, the upregulation of the activation markers CD69 and CD86/B7.2 in response to antigenic (Figure 10d) and mitogenic re-challenge (Figure 11e) was measured, showing maintained attenuation in both WT and CD19-

deficient recovered B cells. As such, anergy is retained in CD19-deficient and WT tolerized B cells removed from auto-antigen, indicating that CD19 is not necessary for the maintenance of the tolerance once cells are isolated from antigen.

Discussion

Clearly, the induction and maintenance of anergy does not require the B cell co-receptor CD19 in this model, even though CD19-deficient auto-specific splenic B cells in comparison to WT splenic B cells, do not downregulate their surface IgM as efficiently and re-upregulate surface IgM faster than WT tolerized cells after auto-antigen removal; a possible indicator of a break in tolerance induction. The higher surface IgM expression on tolerized CD19-deficient B cells may be a simple byproduct of the fact that CD19-deficient non-tolerized B cells have a higher surface IgM set-point level to begin with. It is important to point out that surface IgM expression appears to only be a read-out of antigenic presence and not of tolerance itself, since tolerized B cells that re-upregulate surface IgM after antigen removal are still maintained as anergic by both proliferation and activation marker expression. In addition, in vitro tolerized B cells that have down-regulated their surface IgM are not necessarily anergic by these same functional read-outs for anergy. The loss of surface IgM on tolerized B cells is not responsible for establishing the loss in antigenic responsiveness. Although CD19 deficiency leads to a

decrease in BCR downregulation, it does not confer a decreased susceptibility to the induction of tolerance as measured by antibody secretion, deletion, receptor editing or anergy. A deficiency in CD19 is therefore not believed to be a potential susceptibility factor towards the development of auto-immunity. CD19 is dispensable for the induction of tolerance according to these findings.

It should be pointed out that CD19-deficient non-tolerized B cells appear to be more like anergic cells than WT non-tolerized B cells, in that they have a reduced capacity to proliferate and secrete antibodies in response to antigenic and mitogenic stimulation. Against the original hypothesis, the loss of CD19 dependent signals may make a B cell more likely to be functionally anergic rather than less anergic.

It has previously been shown that the over-expression of human CD19 leads to a break of tolerance.[72] The observations that CD19-deficient B cells are in a semi-anergic state and that an increase in CD19 expression causes a loss of tolerance suggests that CD19 signaling actually functions in opposition to the induction of tolerance and instead promotes positive B cell responsiveness. As such, CD19 dependent BCR signals may provide a qualitative difference from CD19 independent BCR signaling, defining a qualitative difference in activating BCR signaling versus tolerizing BCR signaling as being CD19 specific. In order to establish a tolerogenic outcome,

auto-specific B cells may need to either attenuate the activation of CD19 or abrogate downstream signaling derived from CD19 specific signals. As CD19 signaling has been shown to occur predominantly through promoting the activation of phophatidyl-inositol-3-kinase (PI3K), the production of PIP3(3,4,5) downstream of PI3K activity may be essential in maintaining the state of immuno-reactivity rather than the induction of tolerance.

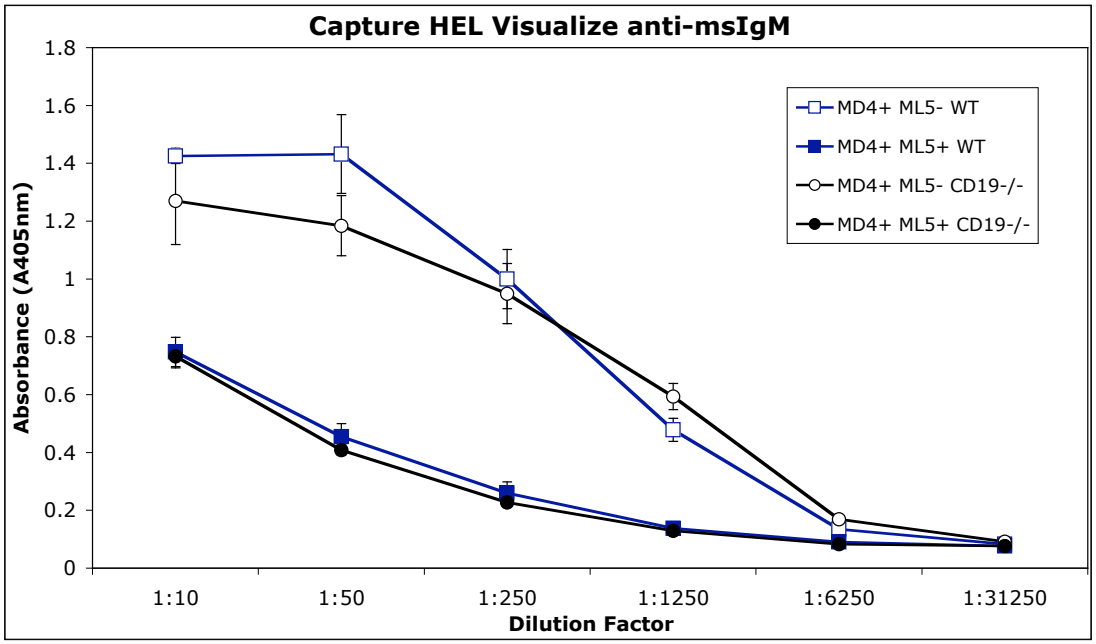
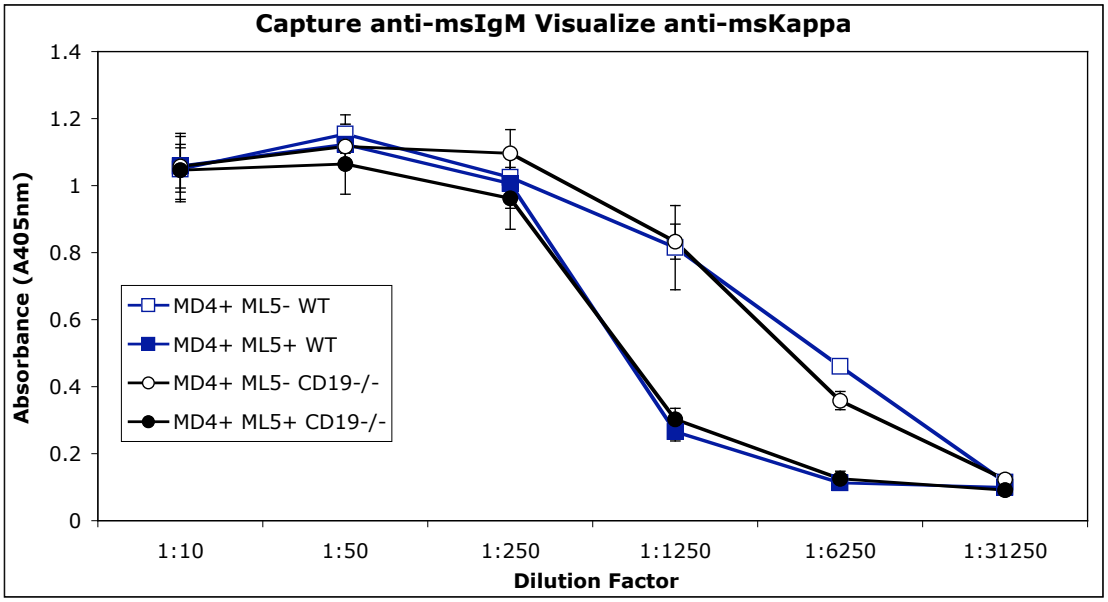


Figure 1a HEL Specific Serum IgM Levels



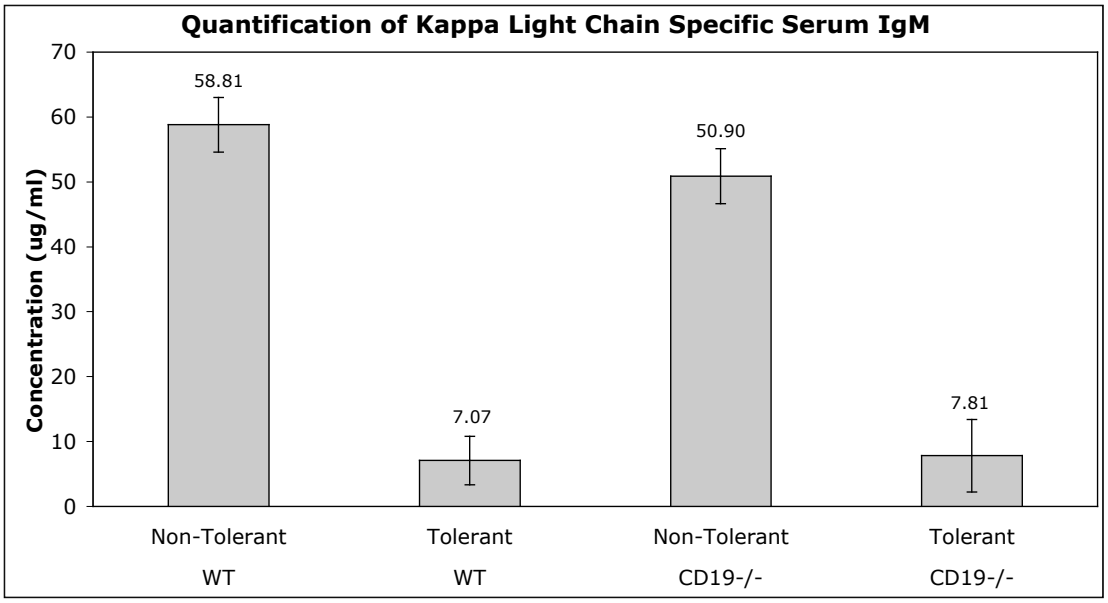


Figure 1b Kappa Light Chain Specific Serum IgM Levels

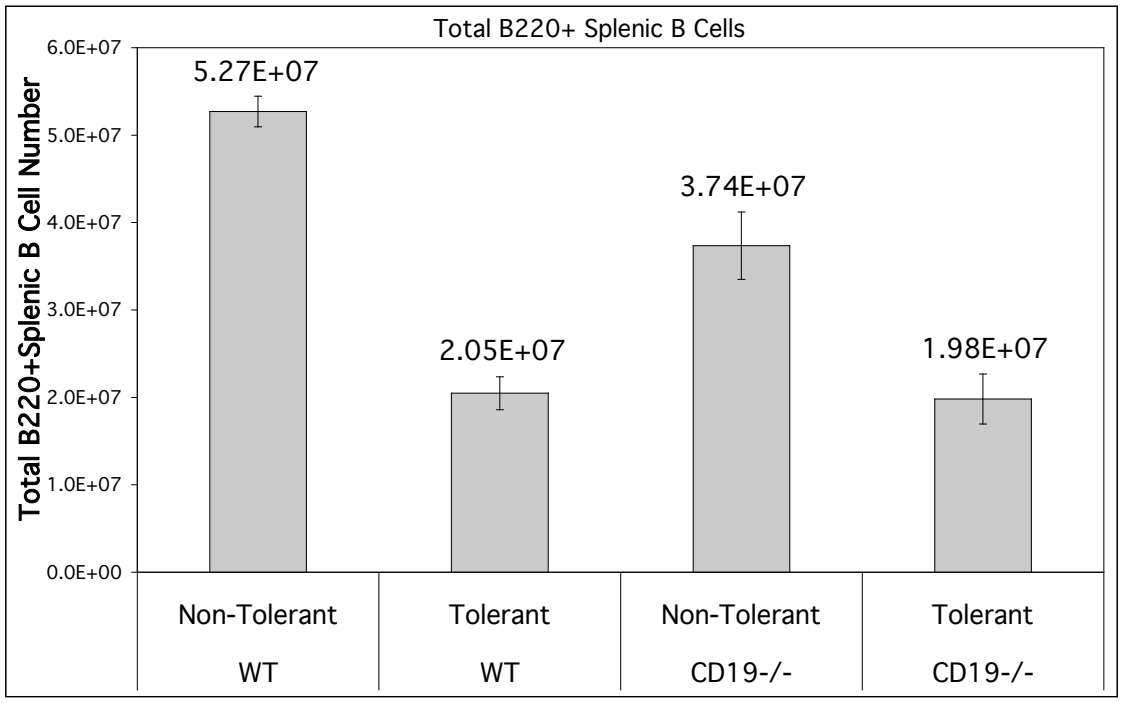


Figure 2a Total Splenic B220+ B cell Numbers

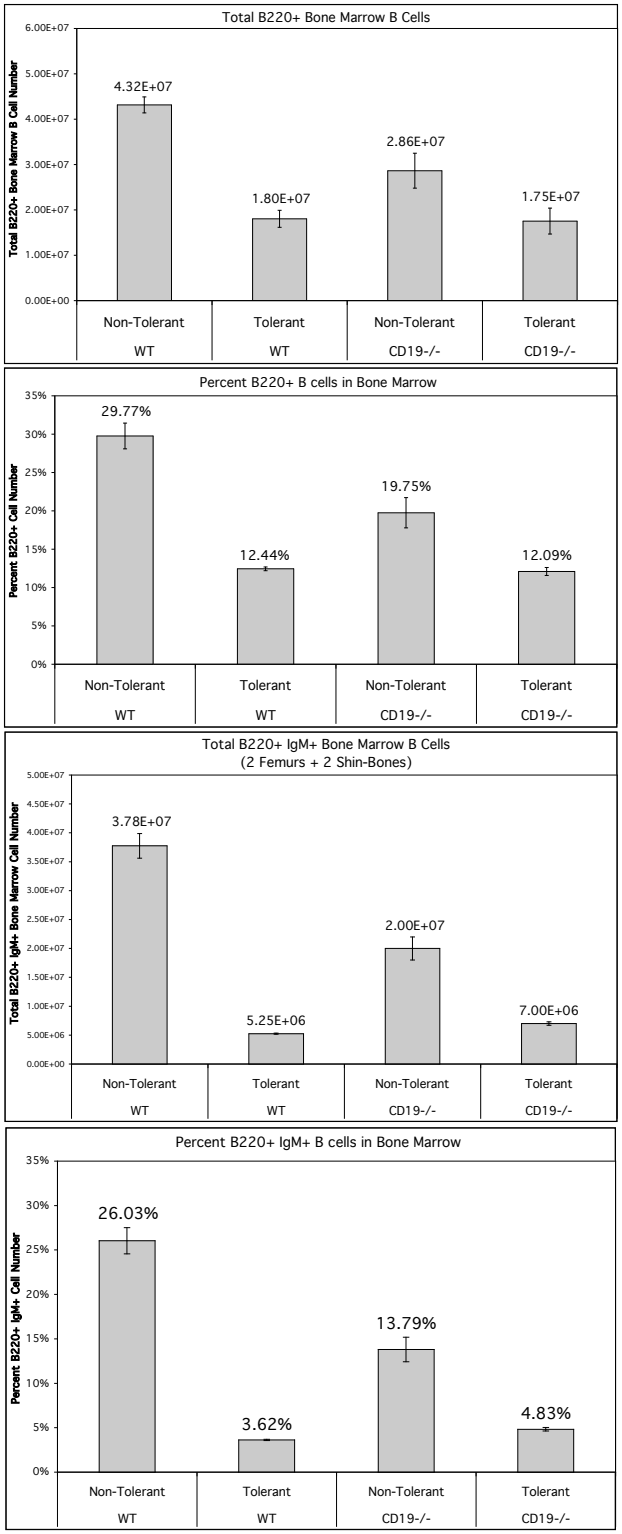


Figure 2b Enumeration of Bone Marrow B cell Populations

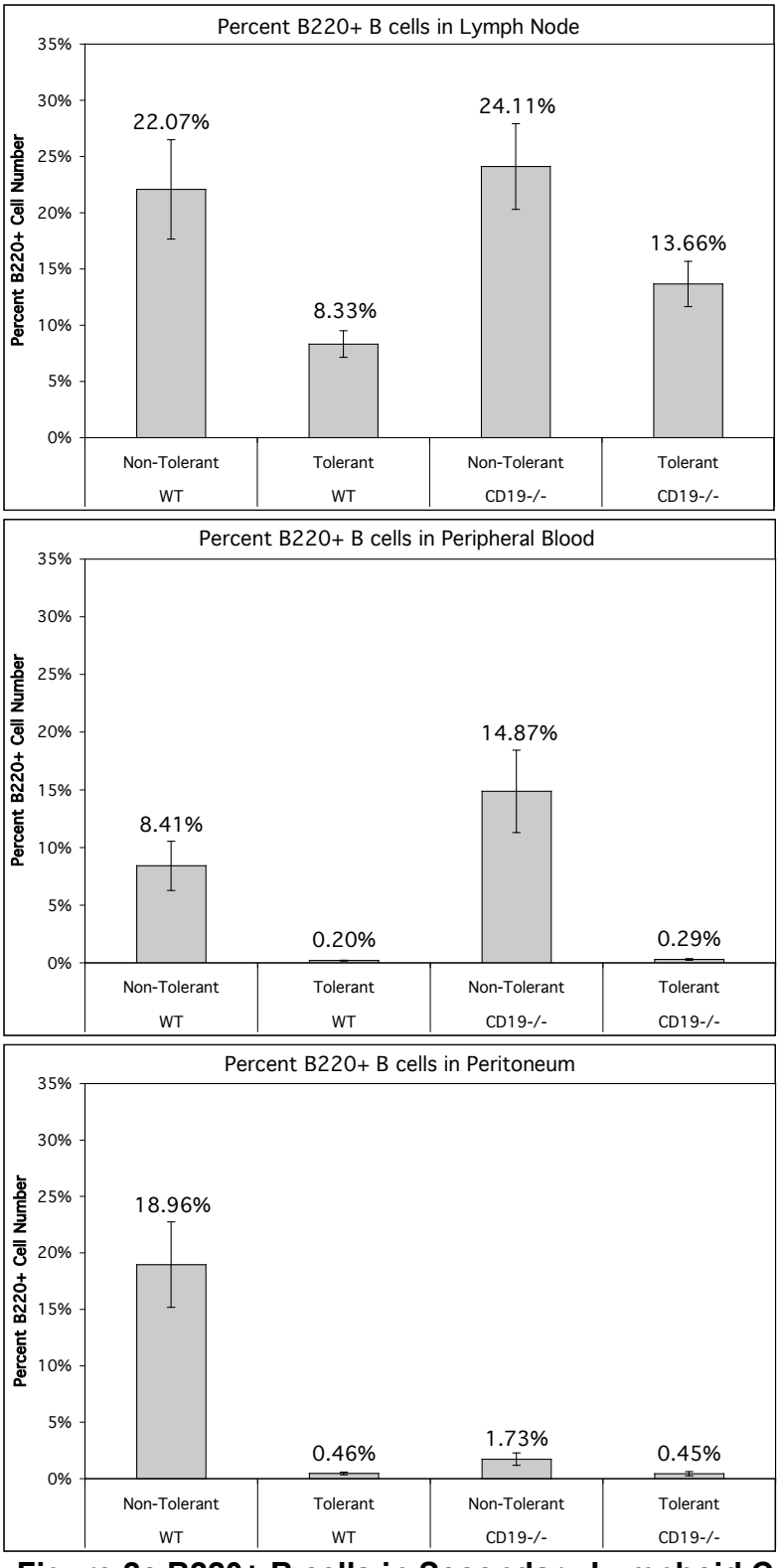


Figure 2c B220+ B cells in Secondary Lymphoid Compartment

Table 1 Summary of B cell Numbers

	Non-Tolerant WT MD4+CD19+/+	Tolerant WT MD4+ML5+CD19+/+	Non-Tolerant CD19-/- MD4+CD19-/-	Tolerant CD19-/- MD4+ML5+CD19-/-	
Spleen	5.27E+07 ± 1.76E+06	2.05E+07 ± 1.88E+06	3.74E+07 ± 3.85E+06	1.98E+07 ± 2.86E+06	Total Number B220+ B cells
Bone Marrow	3.85E+07 ± 1.00E+06	5.25E+06 ± 1.00E+05	2.00E+07 ± 2.00E+06	7.00E+06 ± 3.00E+05	Total Number B220+ B cells
	26.55% ± 0.69%	3.62% ± 0.07%	13.79% ± 1.38%	4.83% ± 0.21%	Percent B220+ B cells
	4.40E+07 ± 1.14E+06	1.80E+07 ± 3.44E+05	2.86E+07 ± 2.86E+06	1.75E+07 ± 7.52E+05	Total Number B220+ IgM+ B cells
	30.36% ± 0.79%	12.44% ± 0.24%	19.75% ± 1.98%	12.09% ± 0.52%	Percent B220+ B cells
Lymph Node	22.07% ± 4.42%	8.33% ± 1.19%	24.11% ± 3.80%	13.66% ± 2.01%	Percent B220+ B cells
Peripheral Blood	8.41% ± 2.13%	0.20% ± 0.03%	14.87% ± 3.57%	0.29% ± 0.06%	Percent B220+ B cells
Peritoneum	18.96% ± 3.79%	0.46% ± 0.12%	1.73% ± 0.55%	0.45% ± 0.19%	Percent B220+ B cells

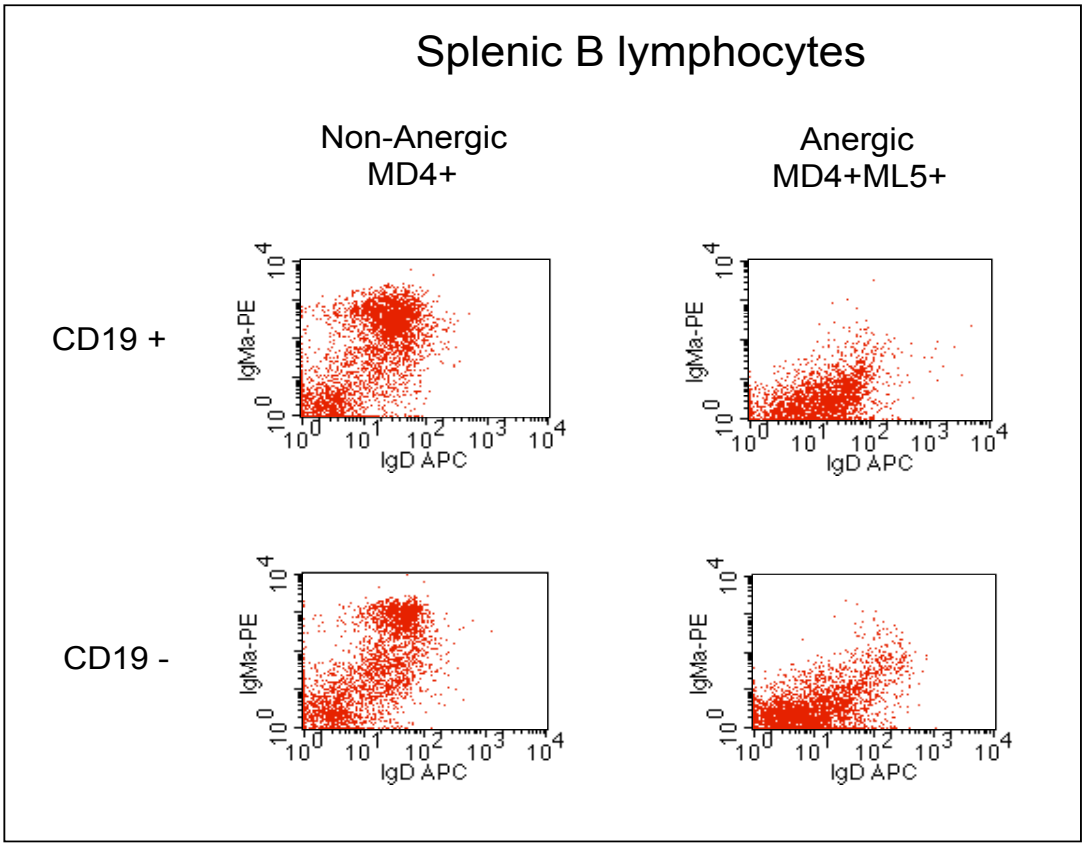


Figure 3a Ig Profile of Splenic B cells

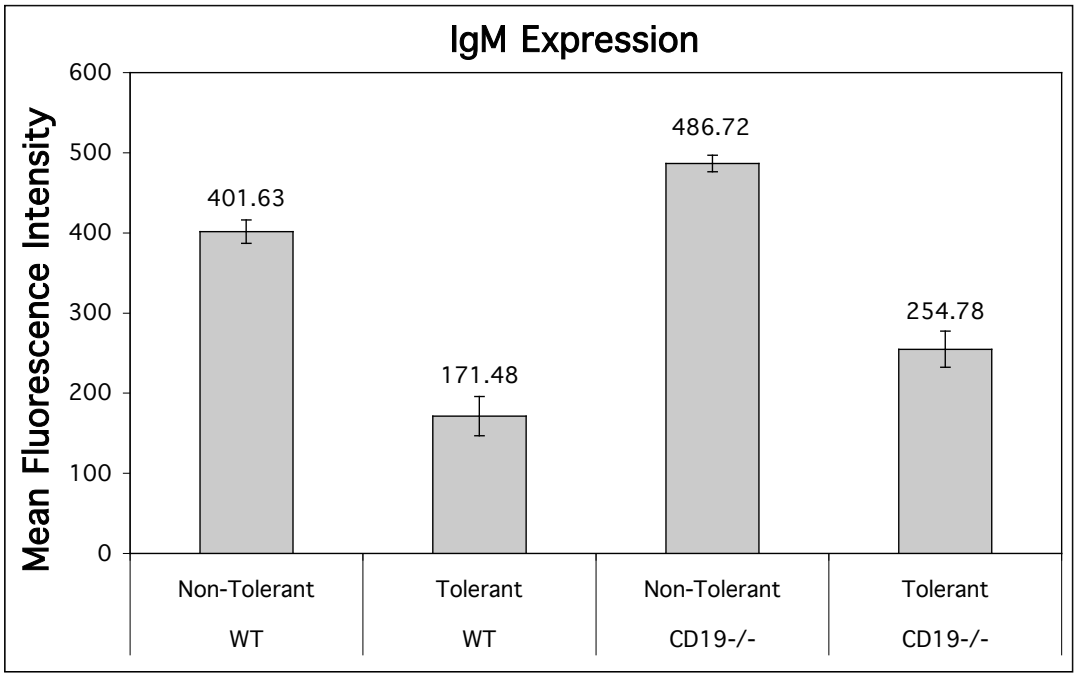


Figure 3b Surface IgM Expression levels in Splenic B220+ B cells

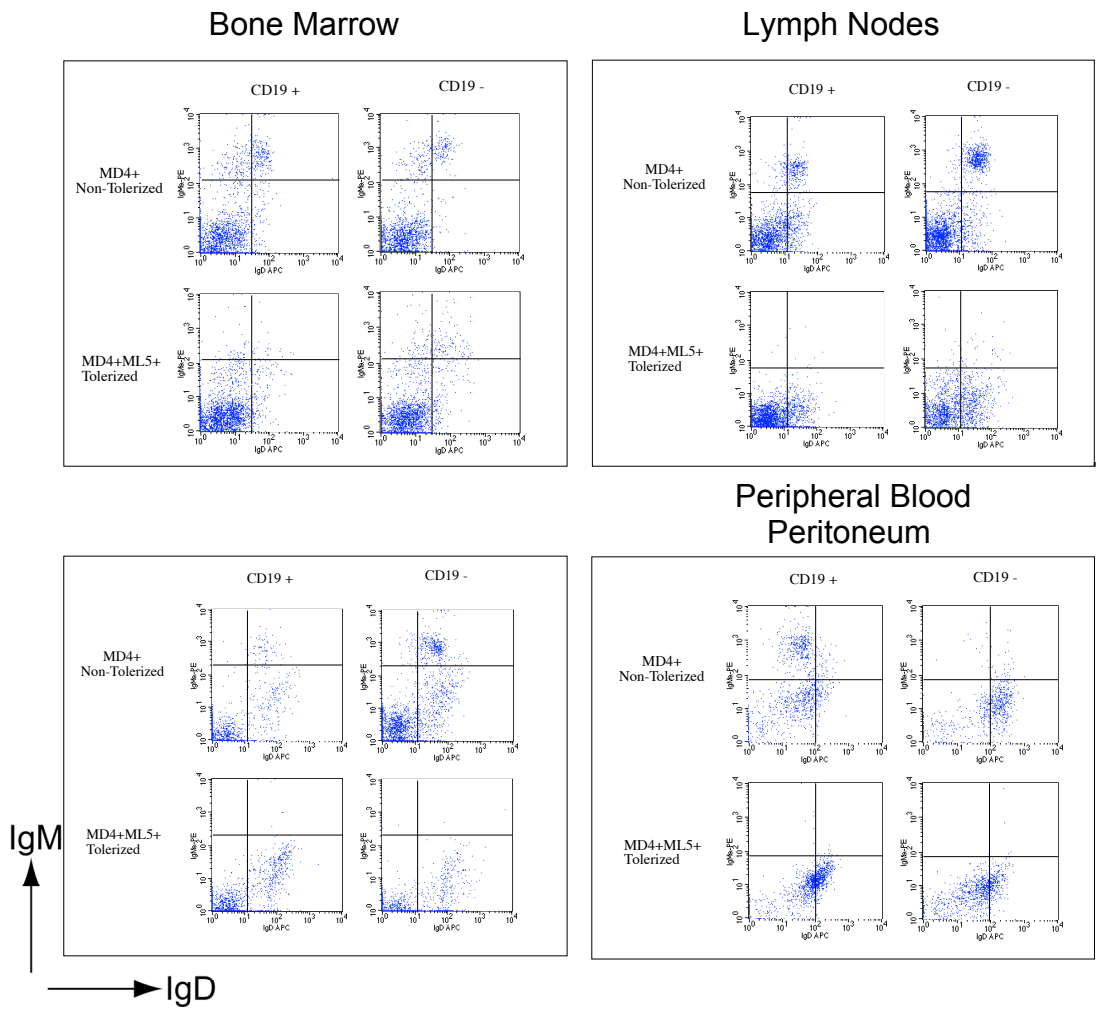


Figure 4 Ig Profile of Peripheral B cell Compartments

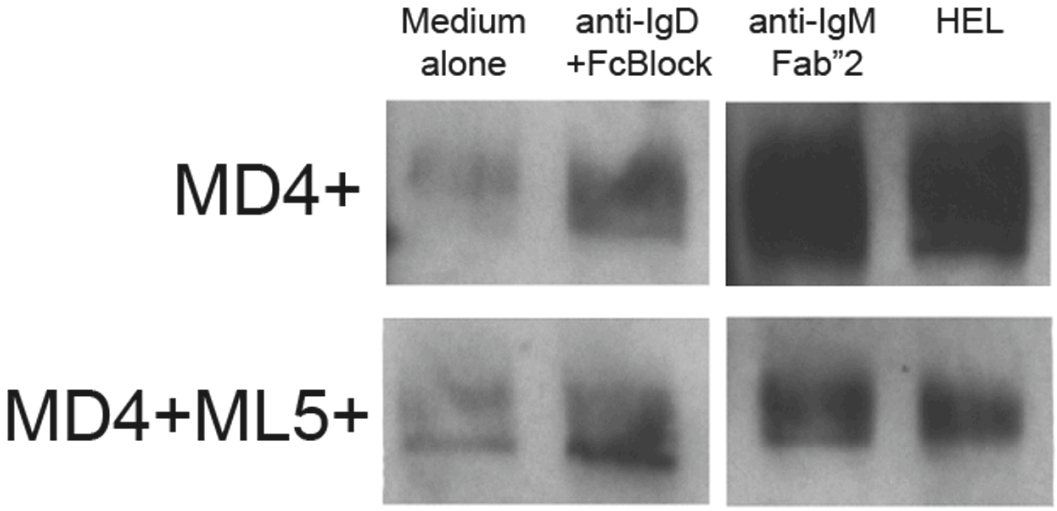


Figure 5 Antigen Induced CD19 Phosphorylation in Tolerized B cells

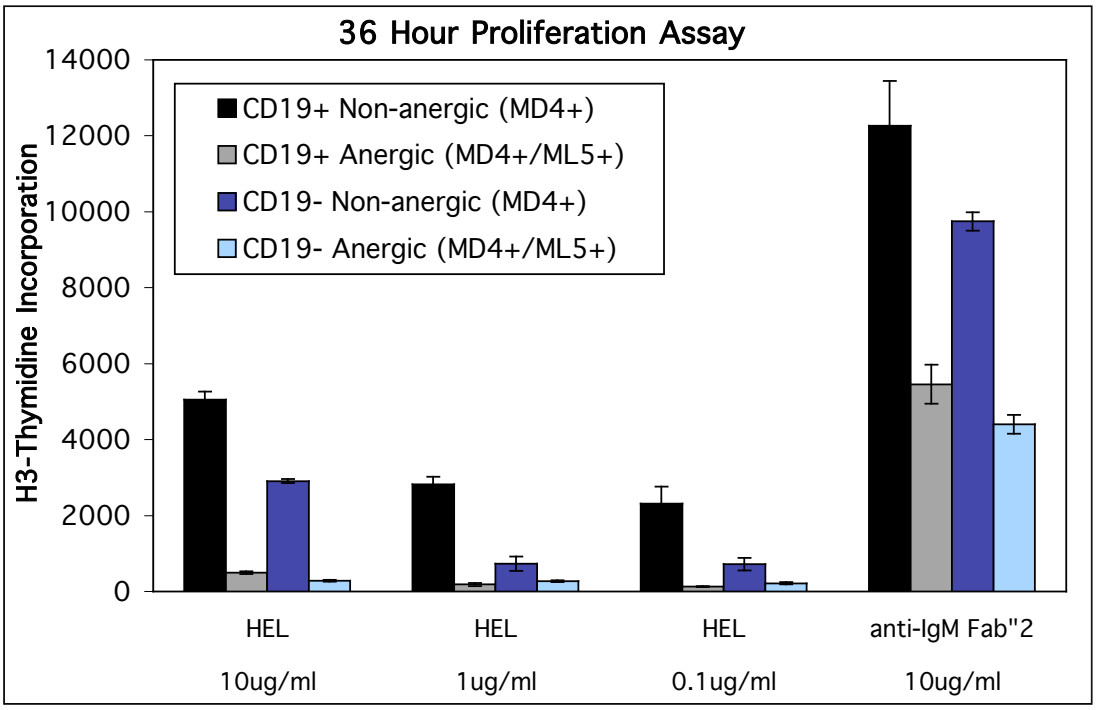


Figure 6a Antigen Specific Proliferative Anergy

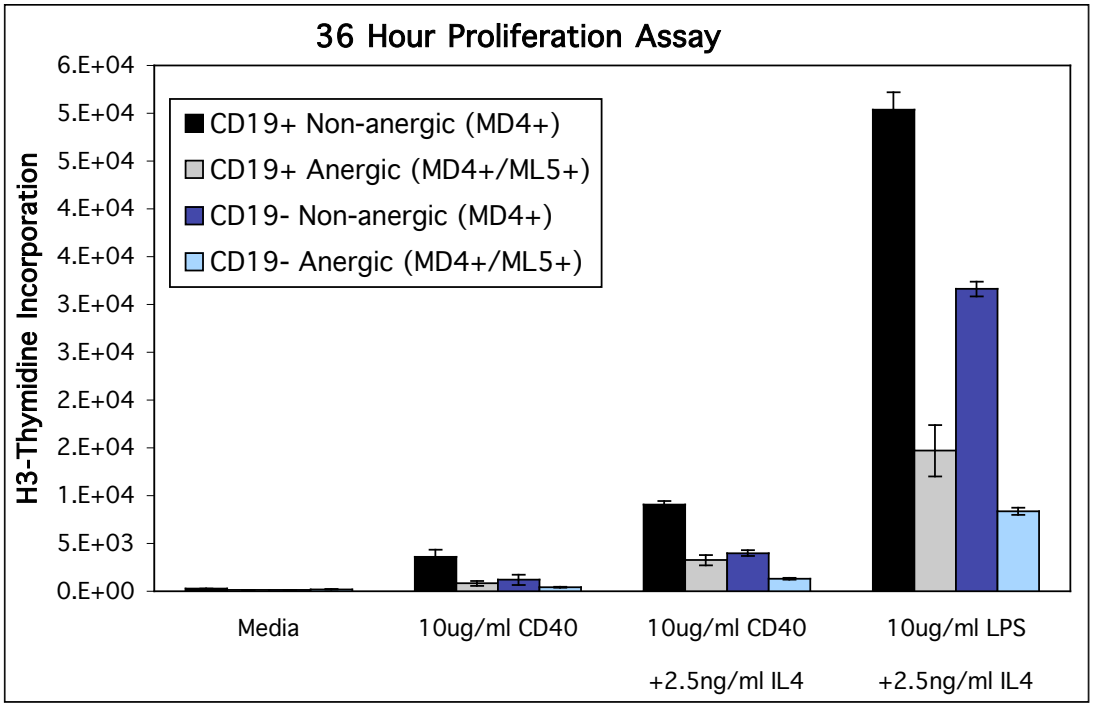


Figure 6b Proliferative Anergy to Mitogens

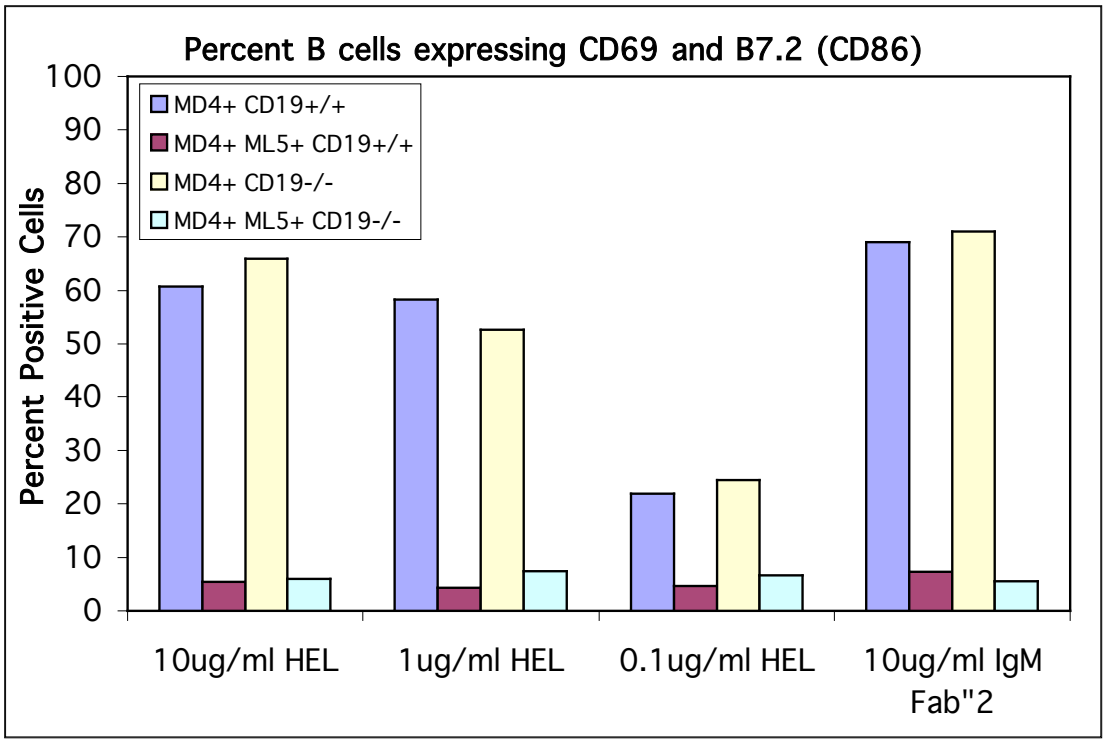


Figure 7a Antigen Induced Activation Marker Upregulation

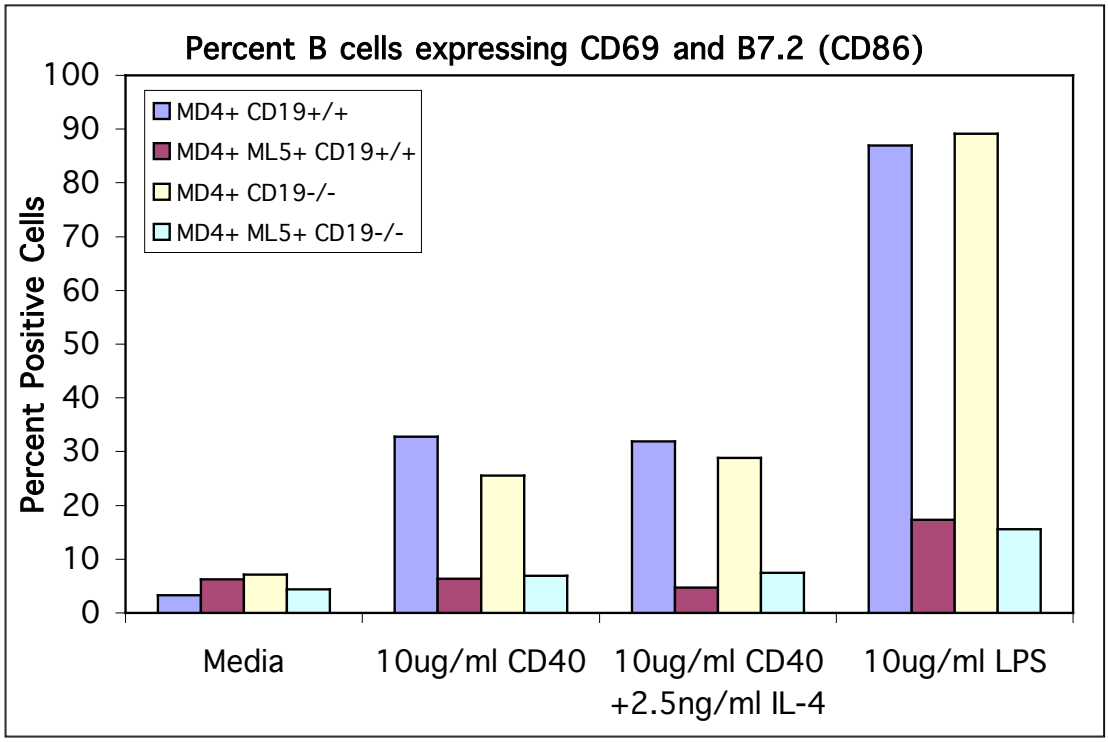


Figure 7b Mitogen Induced Activation Marker Upregulation

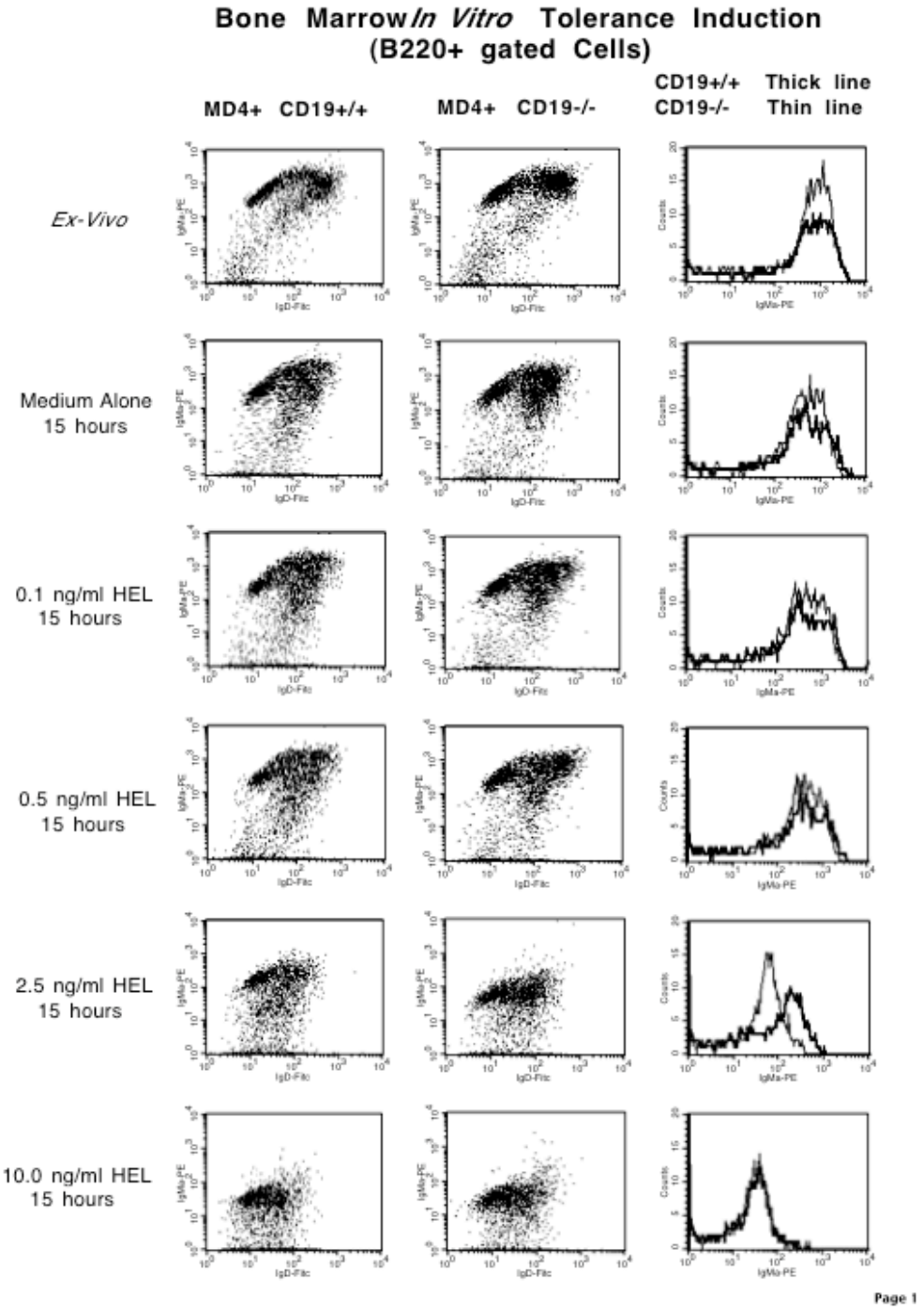
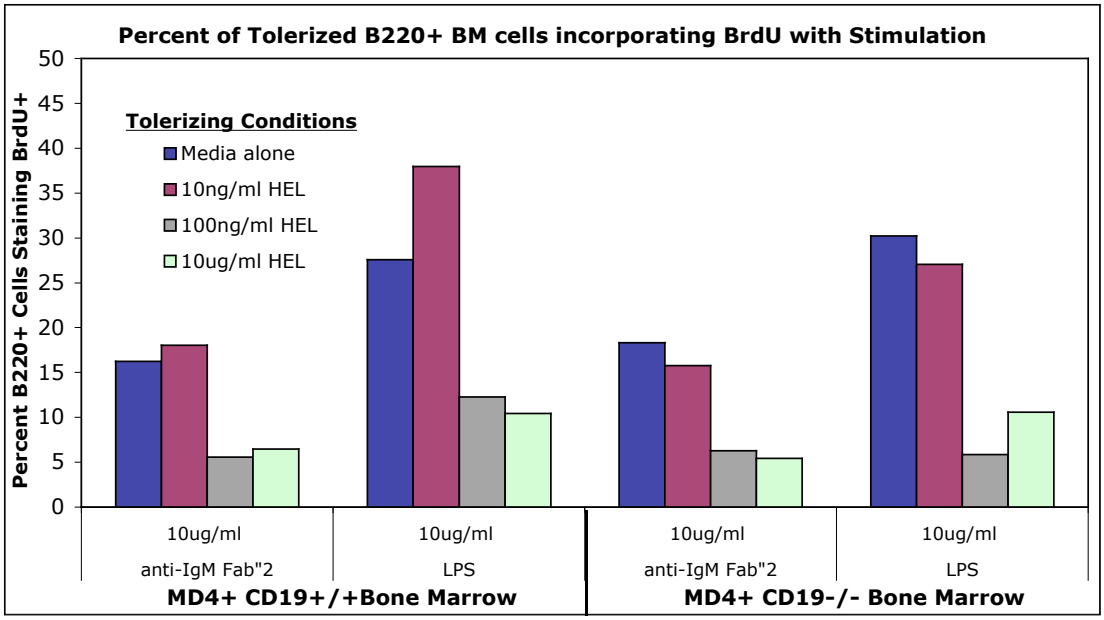


Figure 8a Developmental Antigen Induced IgM Down-regulation



HEL specific CD19-/- and WT bone marrow B cell cultures are tolerized with a concentration gradient of auto-antigen and subsequently measured for their antigen and mitogen induced proliferation capacity by measuring the incorporation of BrdU in order to determine the relative antigenic concentrations necessary for the establishment of anergy

Figure 8b Proliferation of In vitro Tolerized Bone Marrow B cells

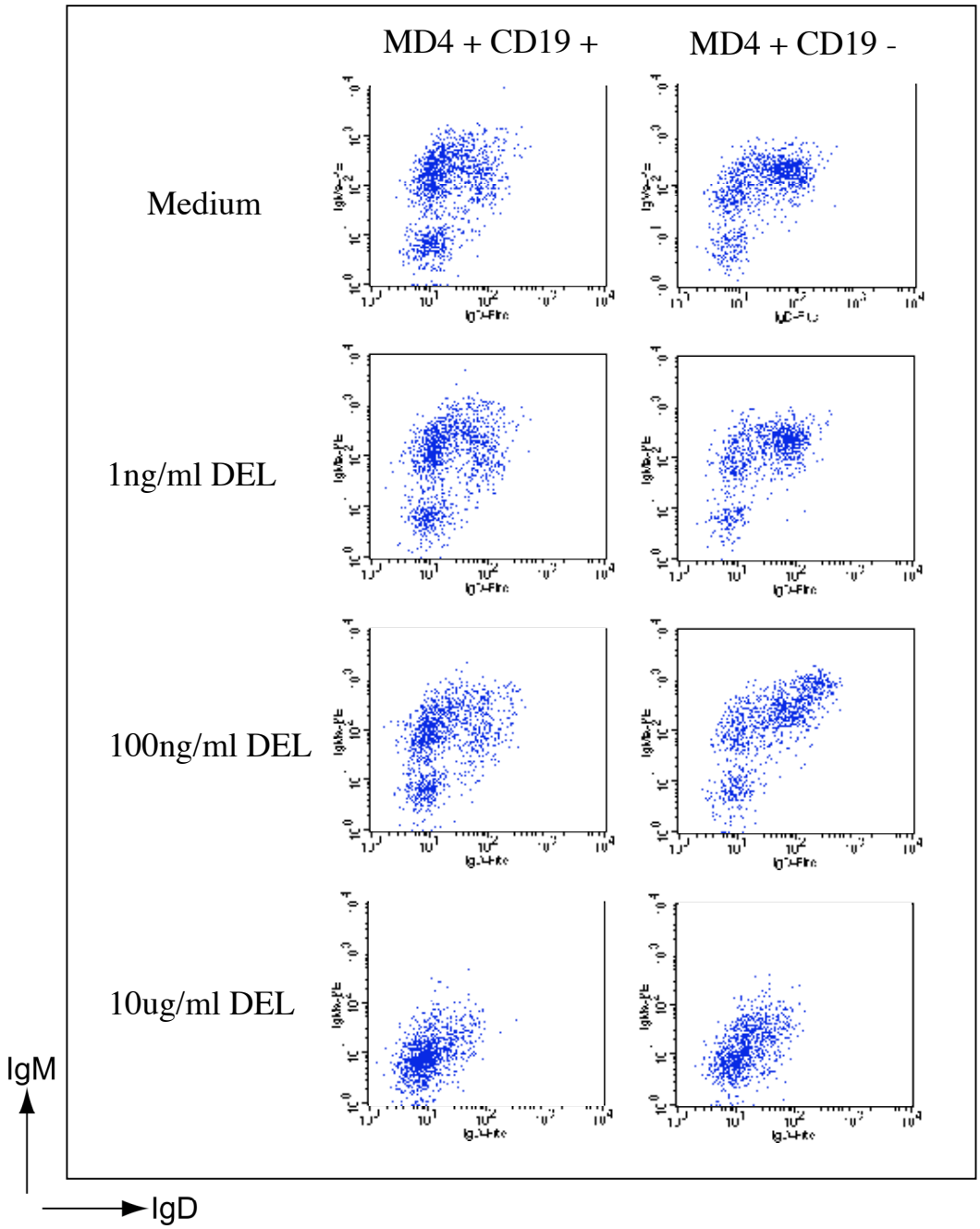
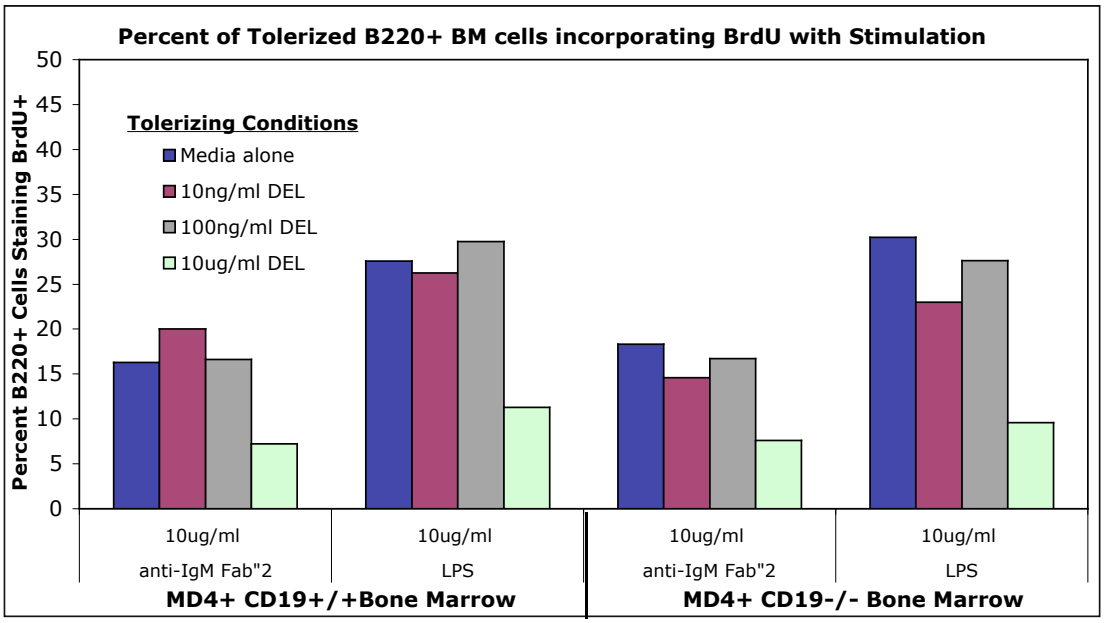


Figure 9a In vitro Low Affinity Antigen Induced IgM Down-regulation



HEL specific CD19-/- and WT bone marrow B cell cultures are tolerized with a concentration gradient of low affinity auto-antigen (Duck Egg Lysozyme) and subsequently measured for their antigen and mitogen induced proliferation capacity by measuring the incorporation of BrdU in order to determine the relative antigenic concentrations necessary for the establishment of anergy

Figure 9b Proliferation of Low Aff. In vitro Tolerized Bone Marrow B cells

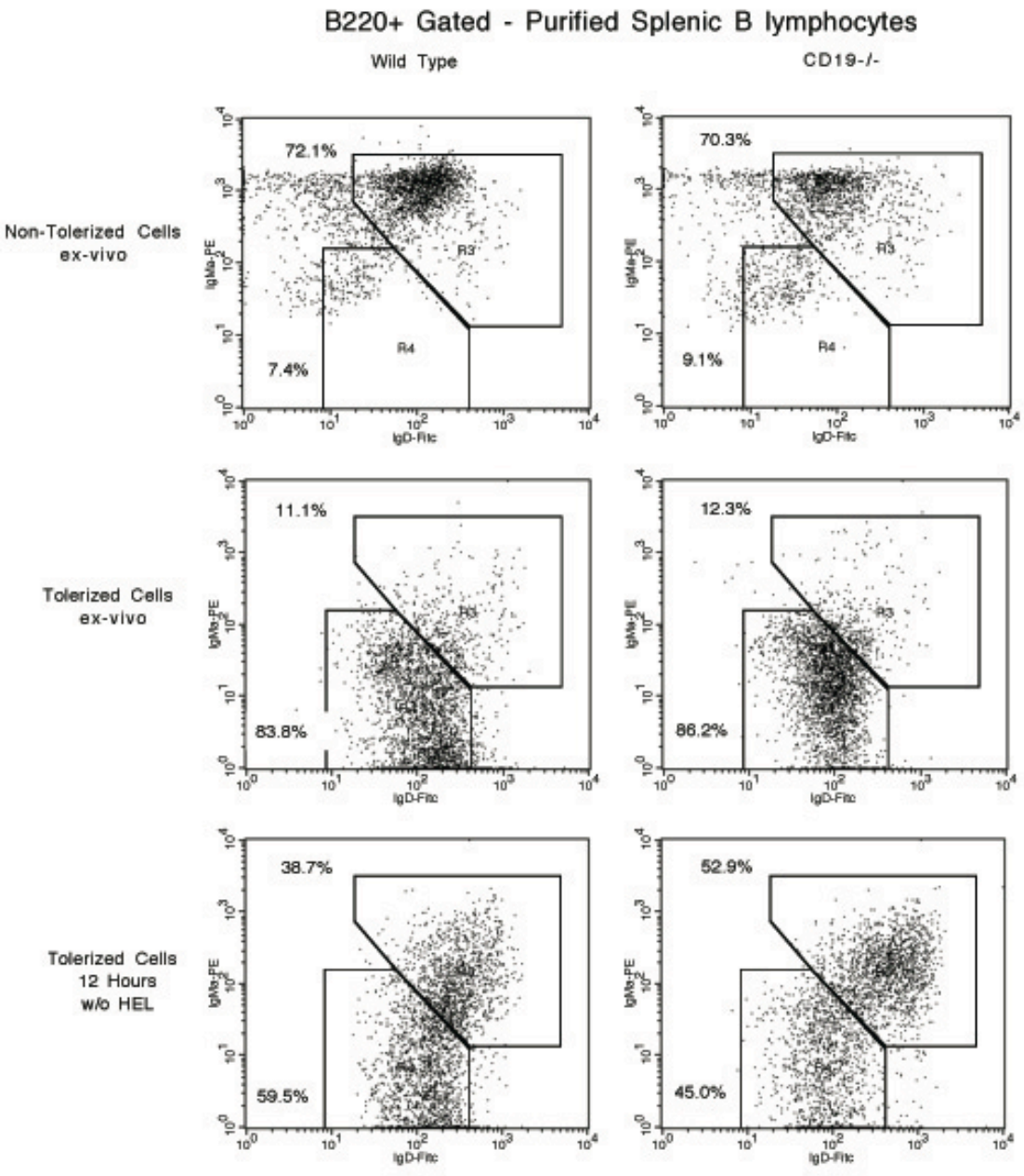
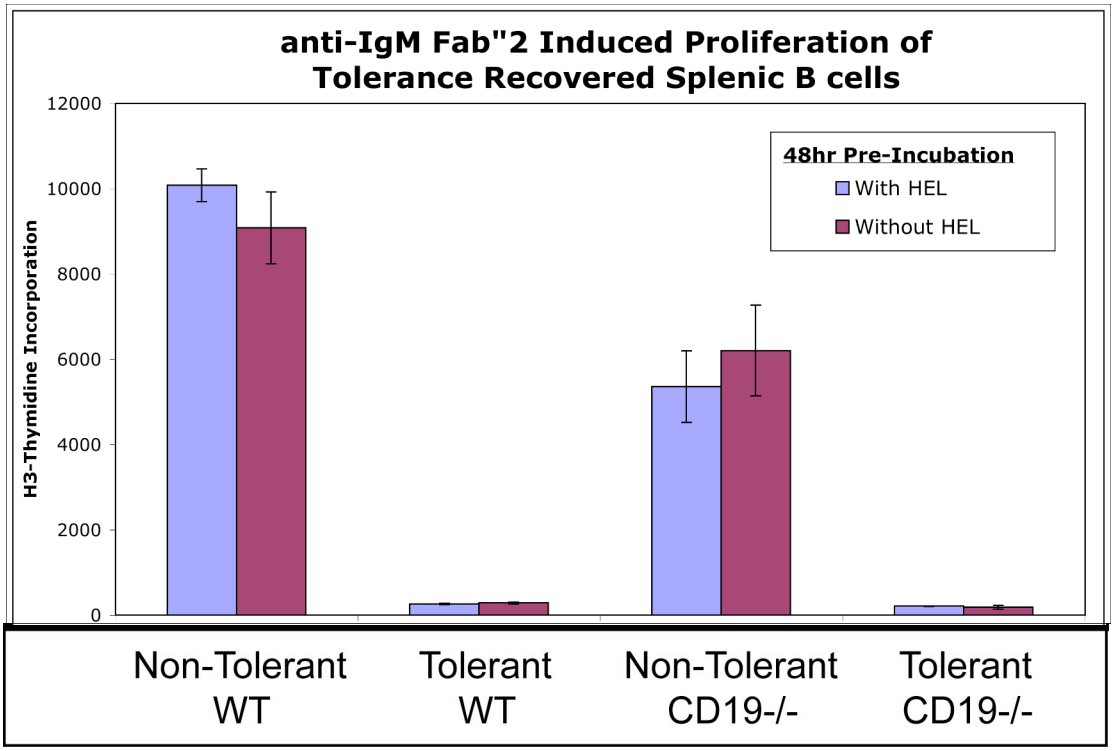
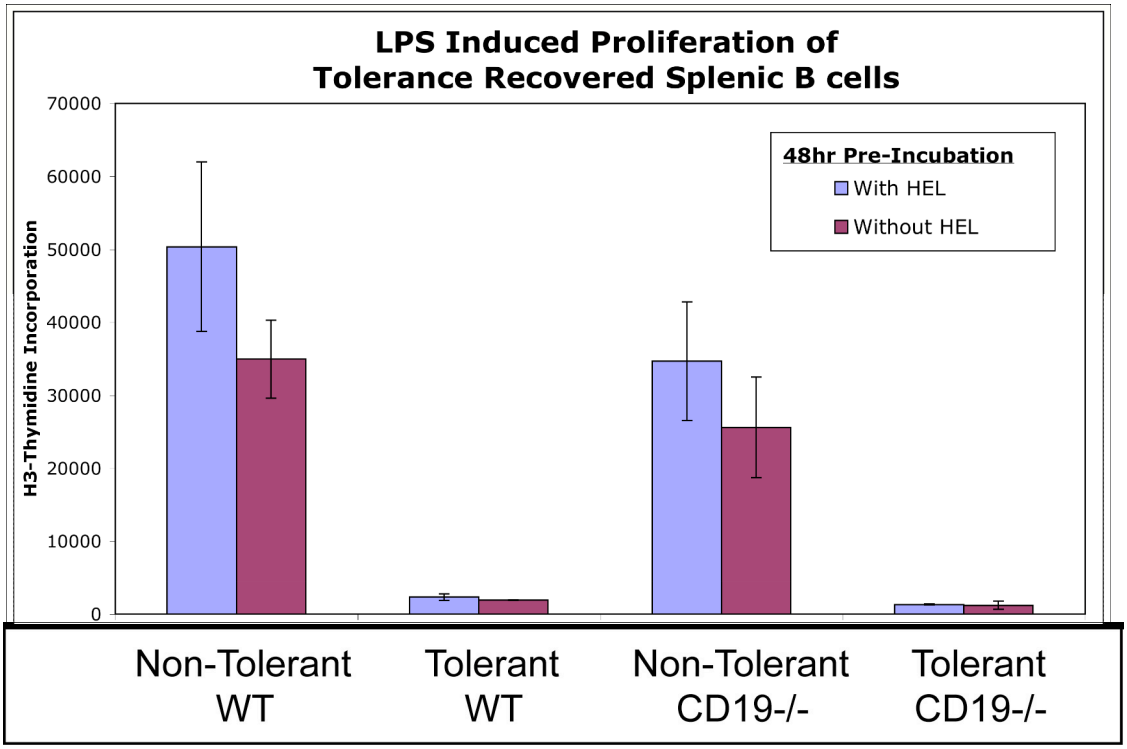


Figure 10a Recovery of Splenic B cell Ig expression without Antigen



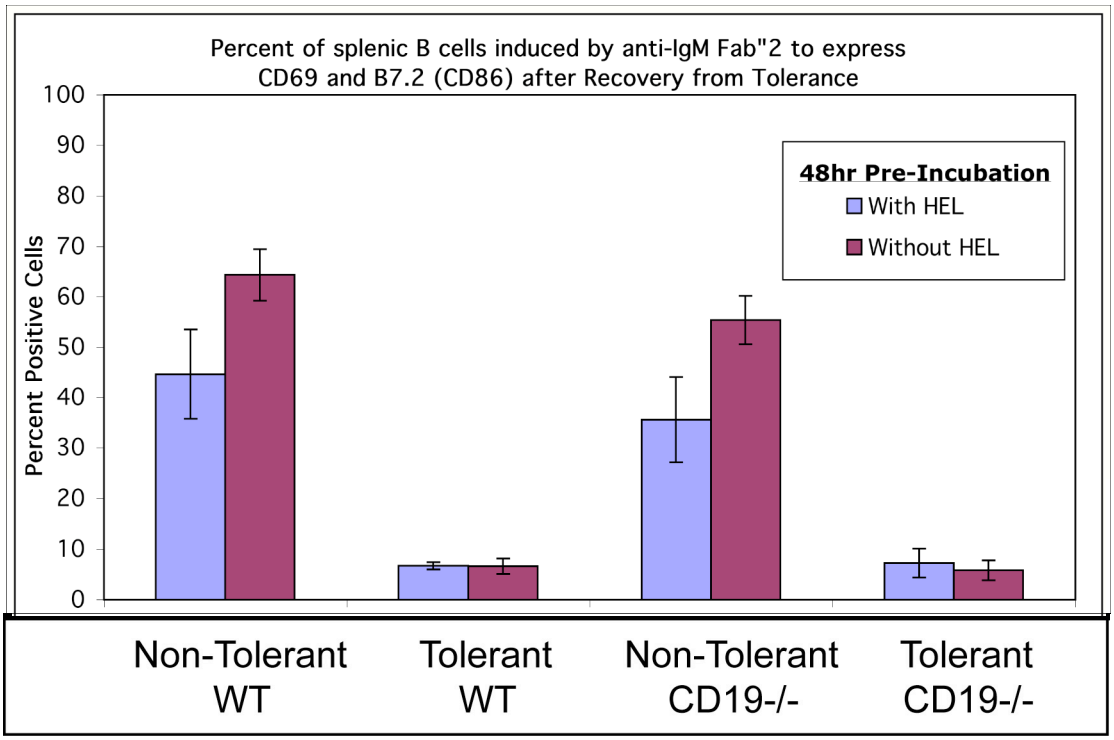
Tolerized splenic CD19-/- and WT B cells allowed to recover surface IgM expression for 48 hours without antigen in vitro, then their antigen specific proliferative anergy was measured by H3 Thymidine incorporation.

Figure 10b Antigen Specific Proliferation of Recovered Splenic B cells



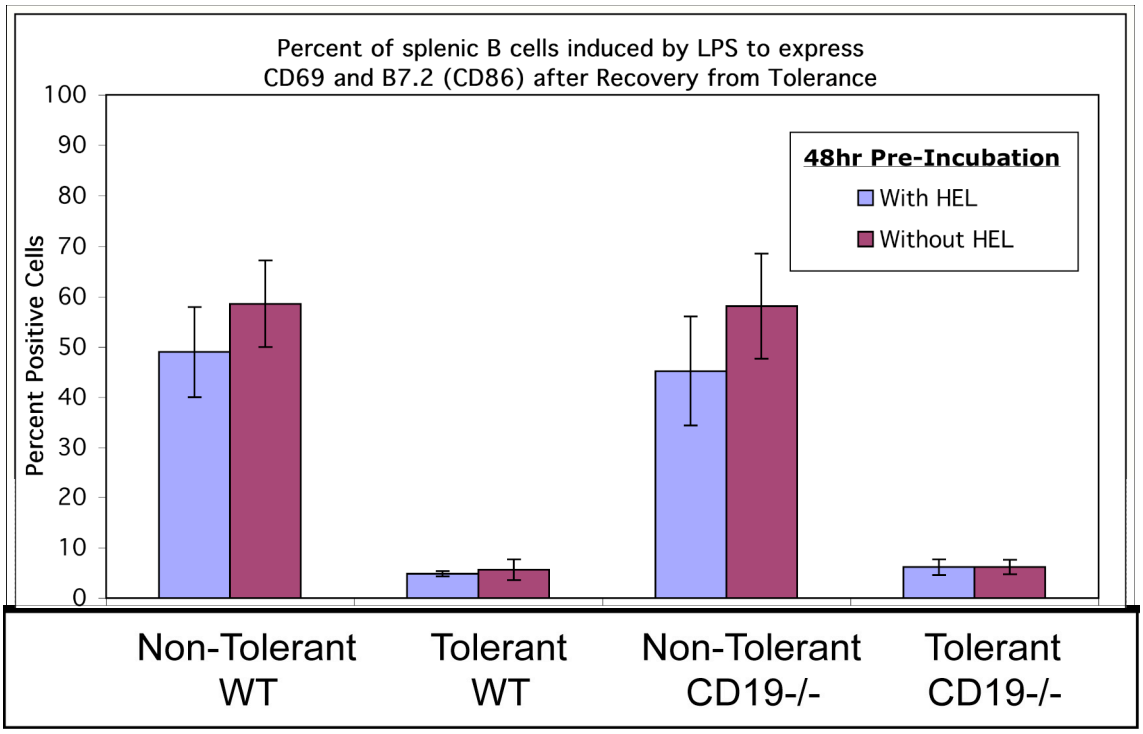
Tolerized splenic CD19-/- and WT B cells allowed to recover surface IgM expression for 48 hours without antigen in vitro, then their mitogen specific proliferative anergy was measured by H3 Thymidine incorporation.

Figure 10c Mitogen Induced Proliferation of Recovered Splenic B cells



Tolerized splenic CD19-/- and WT B cells allowed to recover surface IgM expression for 48 hours without antigen in vitro, then their ability to upregulate the expression of activation markers (CD69 and B7.2) in response to antigen was measured by flow-cytometry.

Figure 10d Antigen Induced Activation Markers on Recovered Splenic B cells



Tolerized splenic CD19-/- and WT B cells allowed to recover surface IgM expression for 48 hours without antigen in vitro, then their ability to upregulate the expression of activation markers (CD69 and B7.2) in response to mitogen was measured by flow-cytometry.

Figure 10e Mitogen Induced Activation Markers on Recovered Splenic B cells

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Chapter 2: PTEN and Tolerance

Introduction:

B cell Tolerance; Qualitative differences in signaling

Antigen recognition by resting mature B cells initiates the utilization of multiple signaling pathways that result in the positive outcomes of activation, proliferation, and differentiation. Anergic B cells are however incapable of responding to antigen activation in a positive manner. One plausible explanation for this difference, is that prior strong or sustained tolerizing antigen exposure modifies the signaling potential of anergic B cells, leading to subsequent qualitative differences in the signaling pathways utilized downstream of BCR ligation. As a result of these qualitative signaling differences, auto-reactive B cells are unable to produce positive outcomes in response to antigenic encounter and instead undergo deletion, receptor editing or anergy.

Signaling in Tolerized B cells

Antigen ligation of resting B cells induces a biphasic change in cytoplasmic Ca2+ levels characterized by a rapid ten-fold spike in intracellular cytoplasmic Ca2+ levels from resting levels of less than 100ηM spiked to 1μM, followed by a prolonged elevated cytoplasmic Ca2+ concentration of 500ηM. Anergic B cells do not flux Ca2+ upon the addition of more antigenic stimulation. Instead they retain a constant low level of cytoplasmic Ca2+ in

the 200-300ηM range, which is still two to three-fold higher than non-activated resting cells. In anergic B cells, this level of Ca2+ is sufficient to induce the nuclear translocation of NFATp/NFATc, but not sufficient for the function of NFKB and JNK pathways that require 500ηM, Ca2+. As such it has been shown that anergic B cells constitutively translocate NFATp/NFATc into the nucleus, but fail to induce NFKB or JNK kinases.[1] This difference in Ca2+ flux in naïve versus anergic B cells is, however, an issue of chronic BCR ligation and not a permanent hardwiring change in anergic B cells. The removal of antigen from anergic B cells restores their ability to flux calcium to normal levels upon antigen re-exposure. However, even with the restoration of Ca2+ flux, the ability to activate the NFKB and JNK pathways remains attenuated, indicating a second qualitative difference exists in the activation of these pathways. Interestingly, naïve B cells exposed long-term to antigen will establish a level of cytoplasmic Ca2+ similar to that of anergic B cells, and do not proliferate in response to increased antigen exposure.[2]

There are multiple possible causes for the lower Ca2+ level in anergic and chronically activated B cells; a decrease in the production of Ca2+ flux-inducing secondary messengers, the activation of a Ca2+ efflux pump in tolerized cells, or that the activation of a subset of Ca2+ channels is lost upon long term antigen activation. As Ca2+ flux is primarily derived from the release of Ca2+ from intracellular pools in the endoplasmic reticulum (ER), the

binding of the secondary messenger intermediate inositol-3-phosphate (IP3) to the IP3 receptor (IP-3R) that regulates ER Ca2+ channels is an essential component in inducing Ca2+ flux. IP3 is produced by the cleavage of PIP2(4,5) by phopholipase PLCγ activated by BCR ligation.[3, 4] Although reduced, the maintained ability to flux calcium at a lower rate in anergized cells indicates that the production of IP3 is intact, but may be reduced. Immature B cells express higher levels of the Src and Syk kinases that are upstream of PLCγ activation as well as an important PLCγ binding protein B cell Linker protein (BLNK) that helps to link PLCγ to the tec kinase Btk. Even though immature B cells are more susceptible to antigen induced tolerance, they activate these pathways much more efficiently than mature B cells as seen by enhanced Igα, Lyn, Syk, BLNK, and PLCγ phosphorylation and Ca2+ flux. At the same time, they produce lower levels of measurable IP3.[5]

Naïve B cells can activate the ERK and JNK kinases within 2-10 minutes of BCR ligation. The activation of ERK and JNK kinases leads to the phosphorylation of the immediate RSK kinase, which in turn leads to the induction of the transcription factor egr-1 within 30-60 minutes. Like the constitutive NFATp/NFATc activation, anergic B cells maintain constitutively activated but lower ERK and RSK kinases and maintain the expression of high levels of the egr-1 transcription factor. However the JNK kinase is not capable of being activated in anergic B cells.[1] This implies that the BCR is still

functionally capable of inducing ERK activation, which normally may proceed through either the Grb2/Sos/RasGRP/Ras/Raf-1/MEK/ERK signaling pathway or through the PKC/Raf-1/MEK/ERK pathway. Anergic B cells however are not capable of activating the PKC/JNK/c-Jun pathway, or the PKC and AKT pathways that lead to NFKB activation.[2] The implication of these findings is that in anergic B cells, ERK is probably kept activated by the Grb2/Sos/Ras/Raf-1/MEK pathway, and not through the PKC dependent pathway because there is a general loss of PKC activation pathways in anergic B cells. Interestingly, antigen induced deletion of immature B cells can be mimicked on mature B cells by providing PKC inhibitors to antigen activated mature B cells making them susceptible to antigen-induced death. PKC activation is a selective defect in anergic B cells that is of pivotal importance.

The mechanism accounting for the failed activation of PKC in anergic B cells is of vital interest as it identifies the difference between activating and tolerizing BCR signaling. Classically, the activation of PKC is regulated through three major mechanisms: 1) the presence of Ca2+, 2) localization to the membrane by DAG binding and 3) an essential phosphorylation in the activation domain by the kinase PDK-1. Although PKC is a Ca2+ dependent enzyme, the Ca2+ levels in anergic B cells are sufficient for activation and the addition of Ca2+ ionophore is not capable of restoring the activation of the

enzyme in anergic cells. This implies that there is a potential defect in one of the other two pathways that is responsible for the failed activation of PKC in anergic B cells.

The addition of the phorbol ester PMA is sufficient to recover both the activation of PKC and reconstitute both JNK and NFKB activation in anergic B cells.[1] PMA activates PKC family members by mimicking diacylglycerol (DAG), functioning as a linker attaching PKC to the membrane.[6] The suggestion from these findings is that a depressed level of DAG is a primary candidate for the origin of selective signal defects in anergic B cells. As such, a selective loss in the activation of PLCγ is of central focus as it produces the DAG second messenger critical for the activation of the PKC dependent signaling pathways. As such, another BCR signaling component necessary for the functional activation of the JNK and NFKB systems possibly also affecting the strength of PLCγ activity might be differentially activated in anergic versus naïve activated B cells. One such candidate is the production of a secondary messenger phosphtidyl-inositol-3 phosphate(PIP3(3,4,5)) product derived from the additional phosphorylation of the 3' hydroxyl group of the inositol ring of PIP2(4,5). The PIP3(3,4,5) secondary messenger is capable of indirectly enhancing PLCγ activity by promoting PLCγ phosphorylation and activation. The mechanism for this action is believed to be dependent on the binding of the PLCγ phosphorylating tec kinase Btk to PIP3(3,4,5) using its PH domain.

PIP3(3,4,5) dependent membrane localization of Btk places it proximal to both the PLCγ substrate and within proximity to receptor induced kinases that activate it.[7]

In criticism to the theory of depressed PLCγ activity in anergic B cells, it should be noted that the constitutive low level of Ca2+ flux in anergic B cells suggests that PLCγ signaling is however still intact in anergic B cells as PLCγ is concomitantly responsible for the simultaneous generation of the Ca2+ flux inducing IP3 secondary messenger as well as DAG (stochastic byproducts of PIP2(4,5) cleavage). An alternatively mechanism that could account for lowering the DAG levels in anergic B cells could be the hyper-activation of the DAG recycling enzyme DAG Kinase that would rapidly turnover any DAG being produced in anergic B cells. However there is not evidence of such a mechanism as of yet. Interestingly, tolerance sensitive immature B cells hyper-activate the PLCγ pathway resulting in strong Ca2+ flux, suggesting that a loss in PLCγ activation is probably not the qualitative signal difference responsible for the induction of tolerance, however it may still be quantitatively affected.

PTEN

We have shown that augmentation of BCR signaling by the B cell co-receptor CD19 is not necessary for the induction or maintenance of tolerance,

and that CD19-deficient non-tolerized B cells display multiple attributes similar to WT tolerized B cells (Chapter 1a). Normal CD19-deficient mice produce a decrease in peripheral B cell numbers that may be a result of either a decrease in positive selection or enhanced negative selection, and they produce decreased amounts of serum antibody in response to antigenic challenge. Additionally, CD19-deficient B cells have a uniform decrease in proliferative capacity in response to antigenic and mitogenic stimulation, an attribute similar to anergic B cells. Conversely, the over-expression of human CD19 in a mouse model of tolerance containing auto-specific B cells gives rise to a break in tolerance.[8]

These findings suggest that CD19 signaling works qualitatively in BCR signaling to promote positive outcomes and repress negative tolerogenic outcomes. Without CD19, B cells are more likely to be tolerized in response to antigen because of a decrease in producing these qualitatively positive signals. As CD19 signaling occurs predominantly through the recruitment and activation of PI3K, CD19 signaling promotes the production of the PIP3(3,4,5) product.[9, 10] It is possible that CD19 induced PI3K activation may specifically contribute to the qualitative differences in BCR signaling found between anergic and naïve activated B cells. If this is true, the generation and sustained levels of PIP3(3,4,5) may be of critical importance to block tolerance.

Although the generation of PIP3(3,4,5) can be induced downstream of BCR signaling by the activation of the PI3K recruited to the membrane by CD19, the levels of PIP3(3,4,5) are tightly regulated in B cells by the constitutive activity of phophoinositide 3-phosphatase and tensin homolog (PTEN).[11] Originally identified as a tumor suppressor, this inositol phosphatase catalyzes the reverse reaction of PI3K by specifically dephosphorylating the 3' hydroxyl group of the inositol ring, leading to the production of PIP2(4,5).[12-14] A deficiency in PTEN gives rise to an elevation in PIP3(3,4,5) levels in cells, mimicking the effects of an over-activated CD19/PI3K pathway.

Results

To evaluate the effects of PIP3(3,4,5) generation on establishing tolerance, we tested the effects of a B cell specific PTEN deficiency on the induction and maintenance of tolerance in the auto-specific anti-HEL/sHEL transgenic mouse model. This was accomplished by crossing mice carrying floxed PTEN alleles (PTENfl/fl)[15] onto the MD4+ML5+CD19+/- background. Since the *cre* recombinase gene was targeted into the CD19 locus during the generation of the knockout allele,[16, 17] the inheritance of a single targeted CD19 allele generates a method to specifically express Cre recombinase in a B cell specific manner and delete PTEN specifically in B cells.[18] Importantly,

CD19 heterozygosity itself does not contribute to a break in tolerance, as marked by peripheral cell numbers, BCR expression levels or the state of anergy noted in previous experiments. The following experiments were performed on four groups of age matched, sex matched mice: MD4+ CD19+/+ PTENfl/fl (PTEN+ Non-tolerized), MD4+ CD19+/- PTENfl/fl (PTEN- Non-tolerized), MD4+ ML5+ CD19+/+ PTENfl/fl (PTEN+ Tolerized), MD4+ ML5+ CD19+/- PTENfl/fl (PTEN- Tolerized). These experiments are preliminary as they have been performed once, but on multiple mice for each group.

The initial experiment performed on the PTEN knockout model of tolerance was to measure the generation of peripheral B cells so as to evaluate the efficacy of tolerance with respect to negative selection of auto-specific B cells. To do this, we isolated both spleen and lymph node tissues and enumerating cells by flow cytometry as described in Chapter 1. PTEN deficiency resulted in an increase number of B cells in both splenic (Figure 1a) and lymph node tissues (Figure 1b), indicating a possible break in negative selection. To determine whether central tolerance was intact, we isolated bone marrow and measured the relative percent of bone marrow cells in the Immature (B220+ IgM+ IgD-) population, the number of recirculating mature B cells (B220+ IgM+ IgD+) and B220+ Ig negative B cells that represent both pro/pre B cells and potential cells undergoing receptor editing (B220+ IgM- IgD-) (Figure 1c). This later population is typically increased in this tolerizing

model. As expected, tolerant WT mice produce more pro/pre B cells/receptor editing cells, and less recirculating mature cells. Auto-specific PTEN deficient mice however produce normal numbers of pro/pre B cells and more recirculating matures, indicating that negative selection in the bone marrow may not be intact without PTEN.

To determine if peripheral splenic tolerized PTEN deficient B cell populations were phenotypically consistent with tolerized WT B cells, we performed flow cytometric staining for surface IgM and IgD (Figure 2a). PTEN deficiency leads to the production of normal B cell populations. This was also confirmed by co-staining for CD23 and CD21 (Data not shown). Interestingly, PTEN-deficient tolerized B cells maintain lower levels of IgM expression and HEL binding than their non-tolerized counterparts (Figure 2b), however in the absence of PTEN, IgM expression is dramatically higher than tolerized WT B cells. Again suggesting that tolerance is not intact in PTEN deficient mice. Given this finding, it is surprising that binding to HEL remains dramatically decreased in auto-specific PTEN deficient mice when compared to IgM levels. This probably is due to competitive inhibition of HEL-bio staining by the presence of endogenous HEL isolated with the cells. Consistent with the IgM and IgD surface phenotype of PTEN deficient auto-specific splenic B cells, lymph node B cells also down-regulated surface IgM, however they are dramatically higher than that of their WT tolerized counterpart (Figure 2c).

To determine if peripheral auto-specific B cells found in B cell specific PTEN deficient mice were maintained as anergic, antigen and mitogen specific proliferation assays were performed on CD43 depleted splenic B cells from these mice. PTEN deficiency generated a loss in the establishment of proliferative anergy in this model. Both PTEN deficient MD4+ non-tolerized B cells and PTEN deficient MD4+ML5+ tolerized B cells were equally capable of proliferating in response to antigen specific stimulation with both HEL and anti-IgM Fab"2 in a dose dependent manner (Figure 3a), and to mitogenic challenge with either anti-CD40 or LPS (Figure 3b). Importantly, these experiments establish that PTEN activity is a critical element to establishing anergy in B cells. As PTEN deficiency results in the enhanced production of PIP3(3,4,5), the regulation of this inositol phosphatide product may be of critical importance for maintaining B cell anergy.

Discussion

In the absence of the PTEN inositol phosphatase, auto-specific B cells are capable of escaping negative selection and are not maintained as anergic in the periphery. Since PTEN deficiency produces an increase in PIP3(3,4,5), it can be inferred that tolerogenic signal outcomes are a result of a decrease in cellular PIP3(3,4,5) levels. As such, the difference between activation and tolerance induction may be derived from the production and consumption of

PIP3(3,4,5) as a limiting substrate. Conditions that promote PIP3(3,4,5) generation will promote cellular activation, while conditions that diminish PIP3(3,4,5) will result in tolerogenic outcomes. If the rate of PIP3(3,4,5) consumption is greater than the rate of its production during B cell activation, then tolerance will ensue.

If PIP3(3,4,5) dependent signaling is a qualitative difference between signaling in naïve activated B cells versus tolerized B cells, then PIP3(3,4,5) may function to prevent tolerance induction. Thus, PIP3(3,4,5) should participate in signaling cascades that promote B cell activation and block tolerance. Molecules such as CD19 and PI3K that together promote the generation of PIP3(3,4,5) during BCR signaling should specifically abrogate tolerance by inhibiting receptor editing, attenuating clonal deletion and anergy and promoting cell survival and clonal proliferation. All these mechanisms are in fact found to be directly regulated downstream of CD19/PI3K activity.

Receptor editing is enhanced in auto-specific B cells without PI3K activity, either blocked by the addition of the PI3K inhibitor wortmannin or because of a specific loss of the PI3K promoting CD19 co-receptor in another model of tolerance.[19, 20] PI3K activity and CD19 have also been shown to promote cell survival and proliferation through the activation of the serine/threonine kinase AKT that further promotes the activation of survival signals such as Bcl2 and represses the activity of cell cycle inhibitors such as

FoxO3 and FoxO1.[21, 22] As a result, CD19-deficient B cells have poor survival signals making them more likely to be deleted and are less proliferative to antigen and mitogenic stimulation.

CD19 is necessary for the efficient recruitment and activation of PI3K downstream of BCR ligation, nonetheless there are CD19-independent BCR induced mechanisms for the recruitment of PI3K to the membrane. As such, the production of PIP3(3,4,5) is only attenuated not completely blocked in CD19-deficient B cells. In tolerized B cells, PIP3(3,4,5) depletion should be more severe than a CD19 deficiency. Therefore, CD19-deficient B cells are expected to be more sensitive to antigen induced tolerance signals due to the already lower levels of PIP3(3,4,5) in the B cells prior to BCR induced depletion. As expected from this model, CD19-deficient B cells flux calcium more poorly and activate NFKB and JNK less efficiently. CD19-deficient B cells appear to be more anergic like, and this may be due to less efficient generation of PIP3(3,4,5).

If contextual signals derived from signal 2 components are capable of subverting tolerogenic outcomes, then contextual signal 2 events must be capable of increasing the production of the PIP3(3,4,5) substrate. This in fact holds true for both non-classical and classical signal 2 sources. Signal 2 derived from complement tagged antigens that co-ligate the BCR with the

complement receptor 2 complex (CR2) leads to the enhanced recruitment and activation of CD19 which then recruits PI3K to promote the production of PIP3(3,4,5).[9, 23] Toll receptors capable of generating non-classical signal 2 can also directly promote PI3K activity and therefore combat the depletion of PIP3(3,4,5) by the BCR.[24, 25] Classical signal 2 derived from CD40 dependent T cell help also stimulates the activation of PI3K on B cells.[26]

The strength and duration of BCR signal models for the induction of tolerance implicate that BCR signaling itself must consume PIP3(3,4,5) in opposition to contextual enhancement of PIP3(3,4,5) generation. If true, then both rapid but strong BCR signaling and slow but sustained BCR signaling could both result in the depletion of PIP3(3,4,5) and the generation of a tolerogenic outcome however by different kinetics. To account for the temporal model, it may be possible that either the rate of PIP3(3,4,5) generation is substantially decreased in newly generated B cells or that BCR induced consumption of PIP3(3,4,5) is higher in tolerance-sensitive immature B cells neither of which has been tested.

How could BCR ligation induce the consumption of PIP3(3,4,5)? Although PTEN is capable of reducing the levels of PIP3(3,4,5) production, it is constitutively active and its activity is not believed to be induced by BCR signaling nor is its activity believed to be increased in anergic B cells. The

phosphatidly-inositol-5-phosphatase SHIP is also capable of reducing the levels of PIP3(3,4,5) by dephosphorylating the 5' phosphate of the inositol ring.[27] The SHIP phosphatase is activated by the specific co-ligation of Fcγ receptors by IgG immune complexed antigens, providing for feedback inhibition once antigen specific antibody has been generated.[28] Additionally, SHIP may be recruited by the inhibitory B cell receptor CD22, which binds to sialic acid containing antigens.[29] Both mechanisms function to induce the inactivation of B cells, however the Fcγ receptors mechanism probably has little to do with self versus non-self recognition. CD22, on the other hand, may distinguish self from non-self, as it binds sialic acids that are usually found on most secreted and surface self-antigens, promoting self/non-self discrimination based up sialic acid modification. Importantly, sialation is usually a late step in golgi transcytosis, and most intracellular antigens should not be sialated. As such, intracellular HEL expression has been found to promote auto-immune responses rather than tolerance.[30] However, the degree to which SHIP is specifically activated by BCR signaling alone is not understood. It could be recruited through binding to SHC/Grb2 associated with Igα, Syk and even the p85 subunit of PI3K.[31-34] CD22 deficiency should pre-dispose an animal to auto-immunity based upon the ability to inhibit BCR signaling, however evidence that SHIP activity is employed to tolerize B cells is lacking as of yet.[35]

PLCγ activity could be responsible for both cellular activation and the onset of tolerance. As tolerance should occur if the PIP3(3,4,5) consumption rate outpaces the generation of PIP3(3,4,5) by the activation of PI3K through contextual signaling receptors, PLCγ may represent a third enzyme that can reduce PIP3(3,4,5) levels by competing with PI3K for the substrate PIP2(4,5) that is necessary for generating PIP3(3,4,5). Importantly, consistent with the BCR induced model of tolerance, PLCγ is activated by BCR signaling, and its activity is probably maintained although decreased in anergic B cells, as discussed earlier. Although it has not as of yet been excluded as a possibility, PLCγ might also be able to decrease PIP3(3,4,5) availability by competing for it's binding or possibly by directly cleaving it into IP4 and DAG like it does for PIP2(4,5), producing IP3 and DAG. If this were true, then PIP3(3,4,5) depletion would occur more rapidly than the depletion of PIP2(4,5) during BCR induced activation of PLCγ, as PIP3(3,4,5) is already at scarce levels in B cells.

We have shown that an increase in PIP3(3,4,5) levels resulting from a deficiency in PTEN phosphatase is sufficient to overcome the induction of anergy. As it is believed that a recovery of both Ca2+ flux levels and PKC dependent activation of JNK and NFKB are necessary for breaking anergy, these pathways must be PIP3(3,4,5) dependent. Mechanistically, there is a need for a unifying theory on how PIP3(3,4,5) levels would regulate the

activation of both of these pathways. A depletion of PIP3(3,4,5) should generate a tolerogenic state where Ca2+ flux and PKC activation is lost, while the generation of PIP3(3,4,5) should promote Ca2+ flux and PKC activation.

One potential explanation for how a loss of PIP3(3,4,5) could result in the reduced Ca2+ levels in anergic B cells, is that PLCγ activity is regulated by PIP3(3,4,5) levels, and once PIP3(3,4,5) is depleted, then PLCγ activity should be decreased as well. PLCγ has been shown to bind to PIP3(3,4,5), however BCR dependent PLCγ recruitment appears to rely more on B cell linker BLNK (SLP-65) dependent recruitment and not PIP3(3,4,5).[36] Importantly, the tec kinase Btk enhances the activation of PLCγ, and its function is dependent on binding to PIP3(3,4,5).[37, 38]

A second possibility is that PIP3(3,4,5) may be capable of being converted to Inositol-4-Phosphate (IP4) by PLC activity, or alternatively that IP3 may be phosphorylated to IP4 by PI3K activity. IP4 is a second type of Ca2+ regulating second messenger known to be capable of inducing Ca2+ flux by binding to an as yet undefined IP4 Receptor (IP-4R).[39, 40] PTEN deficiency may promote the generation of IP4 in addition to IP3, and the subsequent opening of IP4 gated Ca2+ channels may give rise to a higher degree of Ca2+ flux than would normally be produced in tolerized cells by IP3 alone. IP4-R activation may be necessary for reaching and maintaining the

500ηM concentration, while the production of only the second messenger IP3 would result in the opening of only a subset of the potential Ca2+ channels and thus a reduced Ca2+ release in anergic cells. As CD19-deficient B cells are known to flux Ca2+ poorly in comparison to WT B cells, this may be due to a decreased ability to generate IP4.

Interestingly, the activity of some PKC family members has been shown to be PIP3(3,4,5) dependent.[41] B cells express one such PIP3(3,4,5) dependent PKC family member, PKCδ.[42] PIP3(3,4,5) regulation of PKCδ is not due to PIP3(3,4,5)-dependent membrane recruitment, as PKCδ does not contain a PH domain nor does it bind to PIP3(3,4,5) directly. The reason that PKCδ is PIP3(3,4,5) dependent is that in order to be activated PKCδ requires a phosphorylation in the activation loop specifically induced by the PIP3(3,4,5) recruited kinase PDK-1.[43] PDK-1 binding to the membrane is dependent on PIP3(3,4,5) association, making its activation of PKC PIP3(3,4,5) dependent. Reduced PIP3(3,4,5) levels in anergic B cells would result in the loss of PDK-1-dependent activation of this PKC family member. The loss of PKC activation would result in the subsequent loss of NFKB and JNK activation. A PTEN deficiency may subvert this regulatory pathway by providing for the constitutive activation of PIP3(3,4,5)-dependent PKC family members in tolerized B cells.

How PMA treatment leads to the PKC dependent activation of NFKB and JNK in anergized cells remains elusive since PMA does not instigate an increase in PIP3(3,4,5) generation, nor does it directly activate PDK-1. Instead, it is possible that PMA recruits to the plasma membrane PKC family members, which are normally not recruited in anergized B cells. These PKC family members, unlike PKCδ, may be of the PDK-1 independent PKC subtypes such as PKCβ and therefore would supplant the need for PIP3(3,4,5)-dependent activation of PKC through PDK-1 recruitment. This inappropriate recruitment of PKC activity to the membrane could result in the activation of JNK and NFKB in a tolerized B cell.

Normally in non-anergized B cells the PKCβ family is available for signal transduction, however in anergic B cells the PIP3(3,4,5) dependent PKCδ family member is highly expressed and as could inhibit the activation of other PIP3(3,4,5) independent PKC family members by competing for DAG availability. As expected by this model, the loss of PKCδ in mice results in a break in B cell tolerance and the activation of auto-specific B cells.[44] Without PKCδ, DAG may be freely accessibility to other PIP3(3,4,5)/PDK-1 independent PKC family members, leading to a PMA like outcome that is independent of PIP3(3,4,5) and PDK-1.

In summation, it appears that some signaling pathways remain intact in tolerized B cells, such as Ca2+/NFAT and ERK/RSK/egr-1, while others are not activated, such as PKC, NFKB, and JNK. The provision of increased PIP3(3,4,5) levels by the selective deletion of PTEN results in a break in tolerance, while a decrease in PIP3(3,4,5) generation due to the loss of PI3K recruitment by CD19 results in a state that promotes tolerance induction. Tolerance establishes at least two selective differences in signaling potential that can logically be attributed to the failed generation of PIP3(3,4,5); Ca2+ flux through a potential decrease in PLCγ activity or a decrease in IP-4 generation, and PKC activation of NFKB and JNK due to the possible failed recruitment of PDK-1. From this reasoning, it is evident that differential signaling in anergic versus activated naïve B cells is may be a product of differential levels in the abundance of the phosphatidyl-inositol product PIP3(3,4,5).

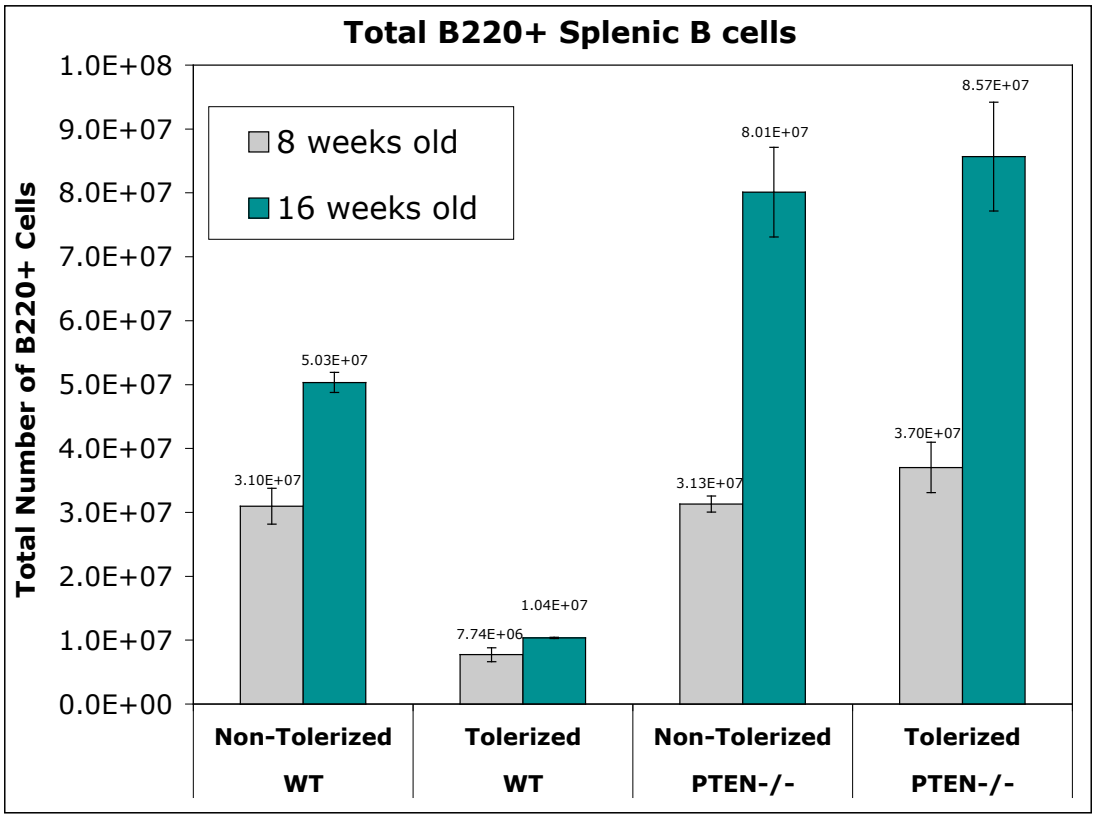


Figure 1a Total Splenic B220+ B cell Numbers

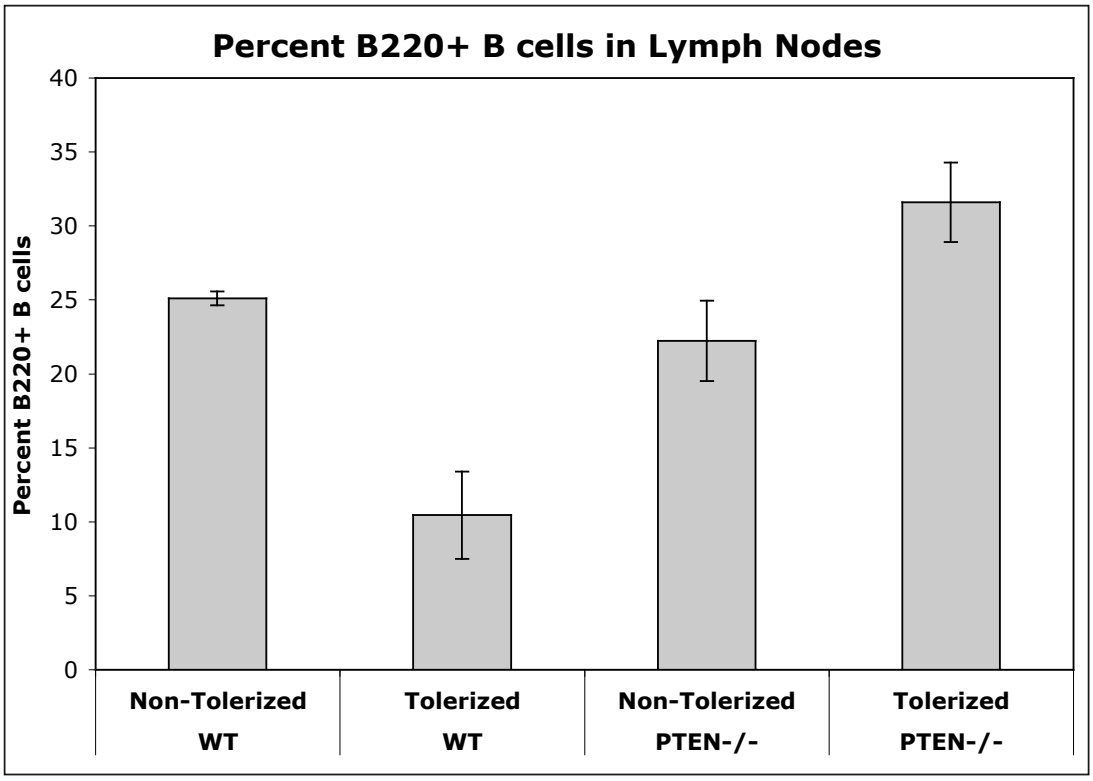


Figure 1b Enumeration of Lymph Node B cell Numbers

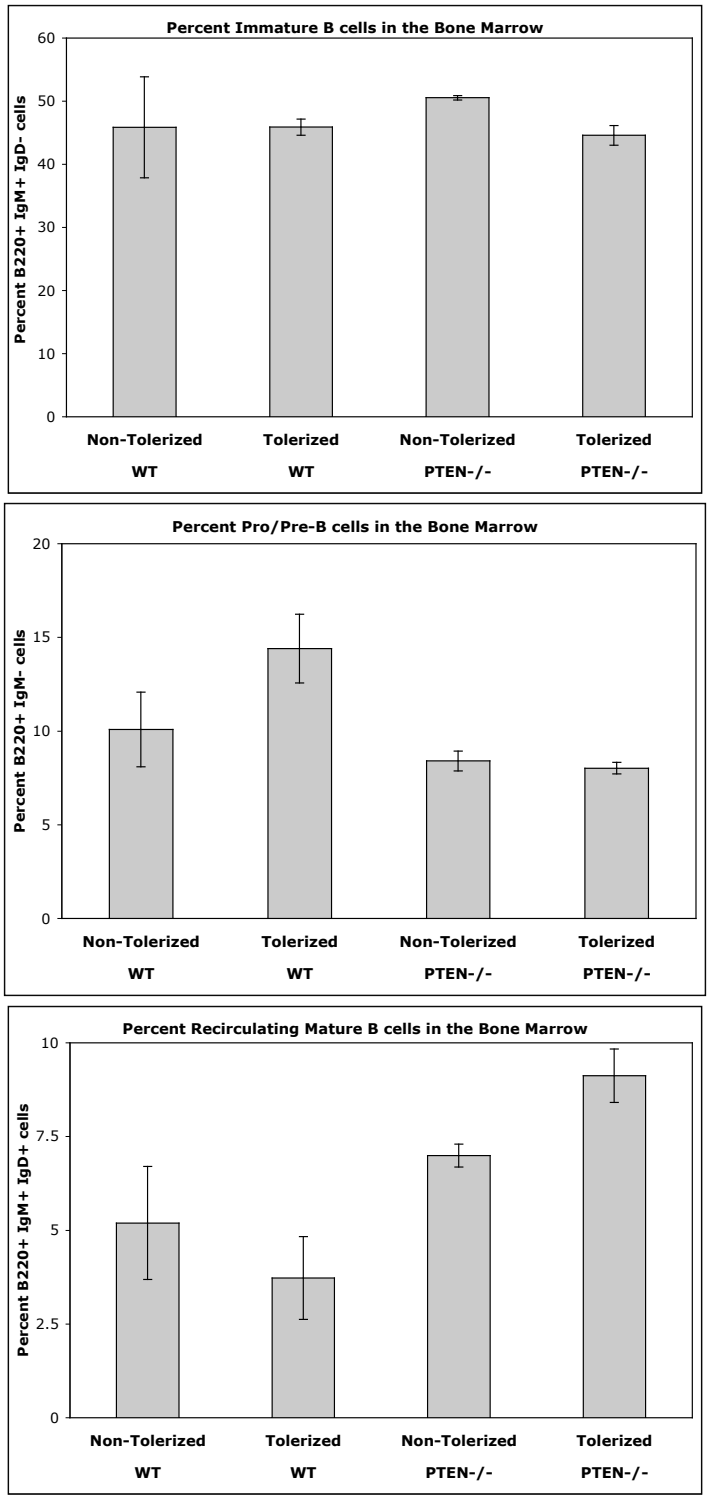


Figure 1c Enumeration of Bone Marrow B cell Populations

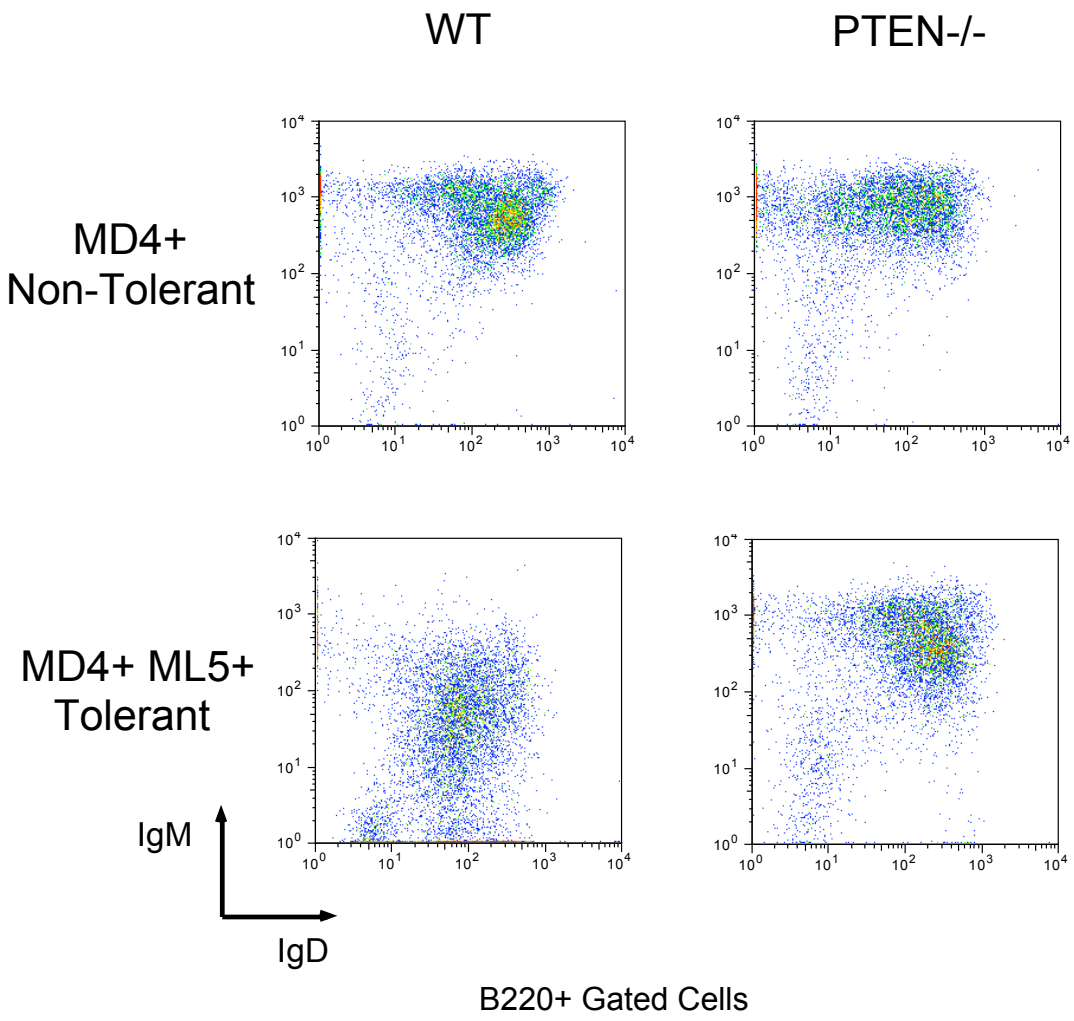
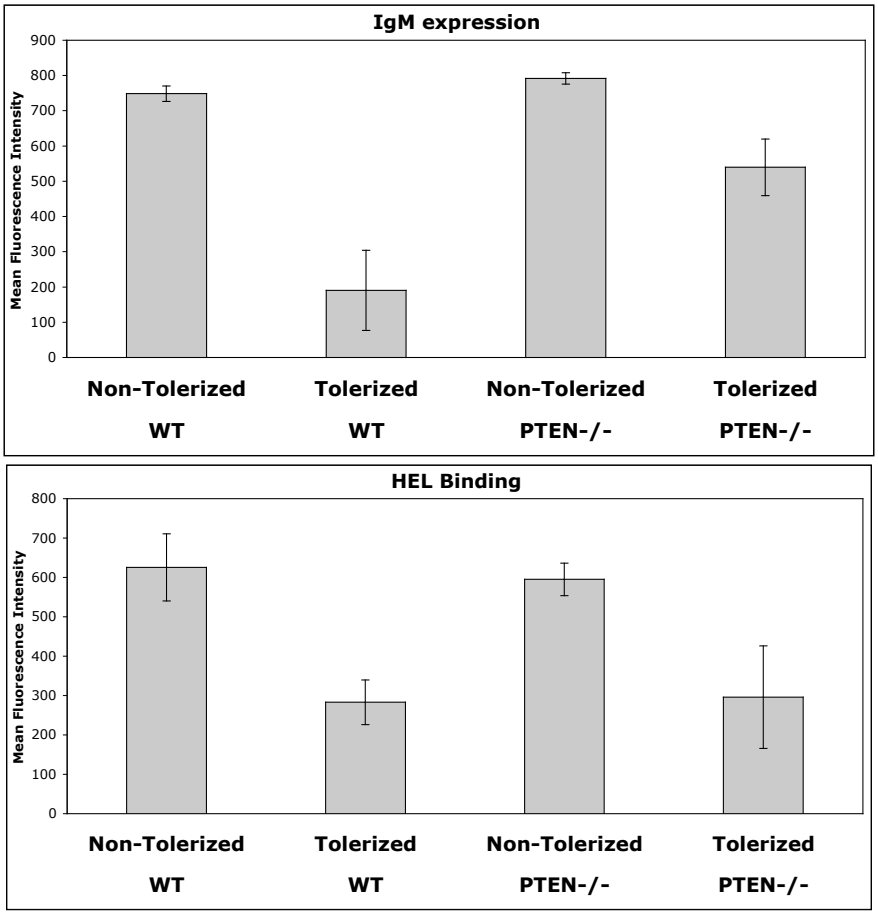


Figure 2a Ig Profiles of Splenic B cells



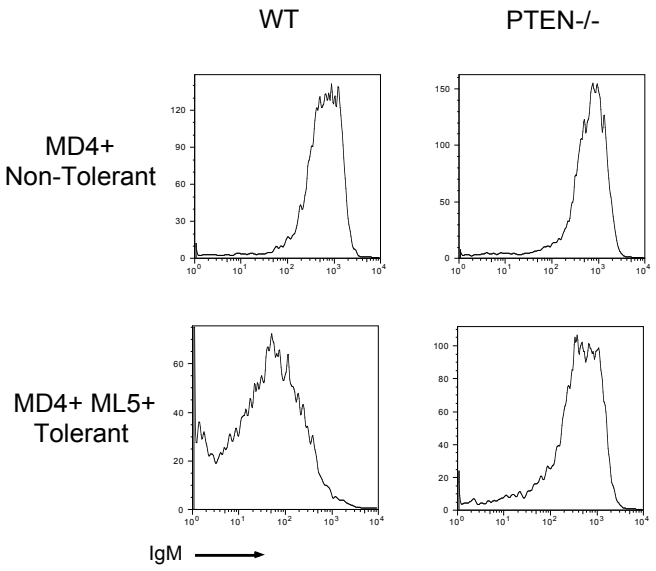


Figure 2b Ig Surface levels and HEL Binding of Splenic B cells

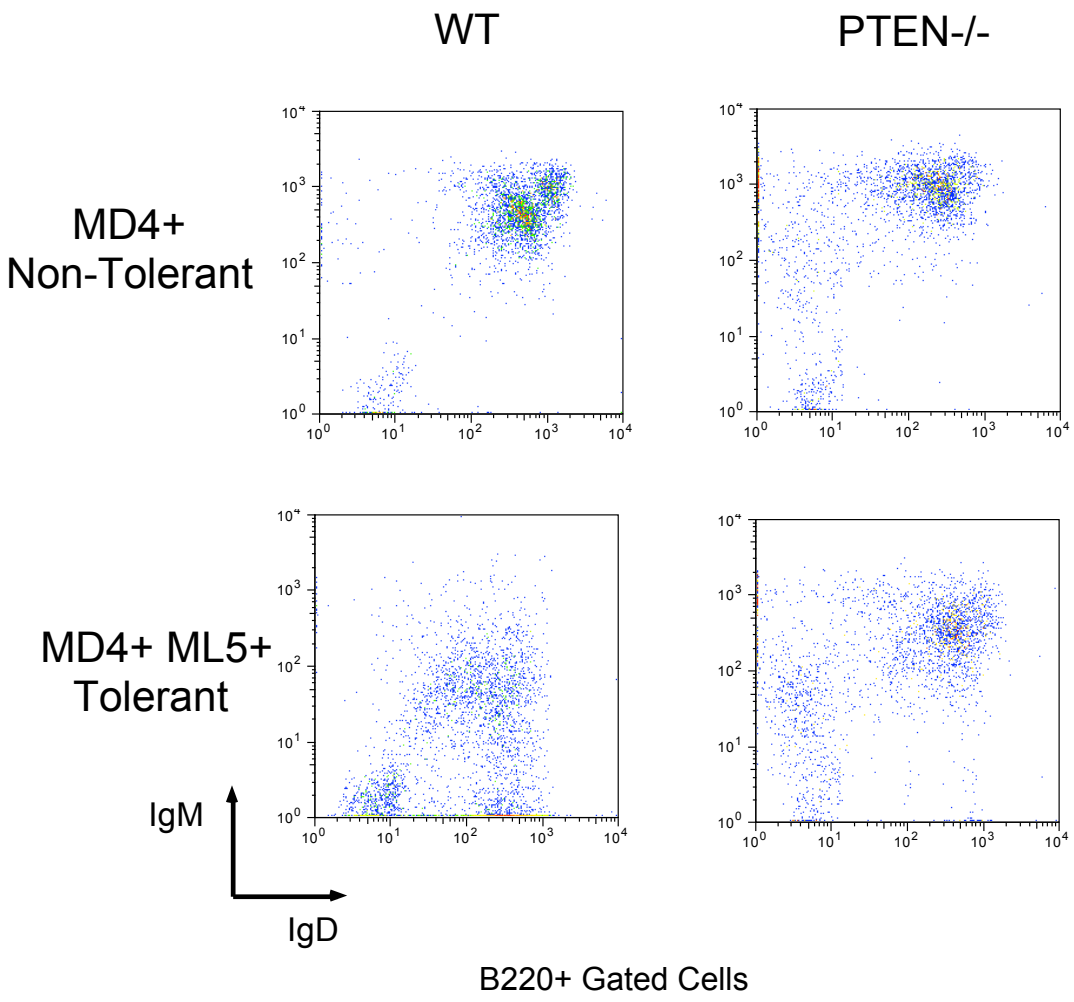


Figure 2c Ig Profiles of Lymph Node B cells

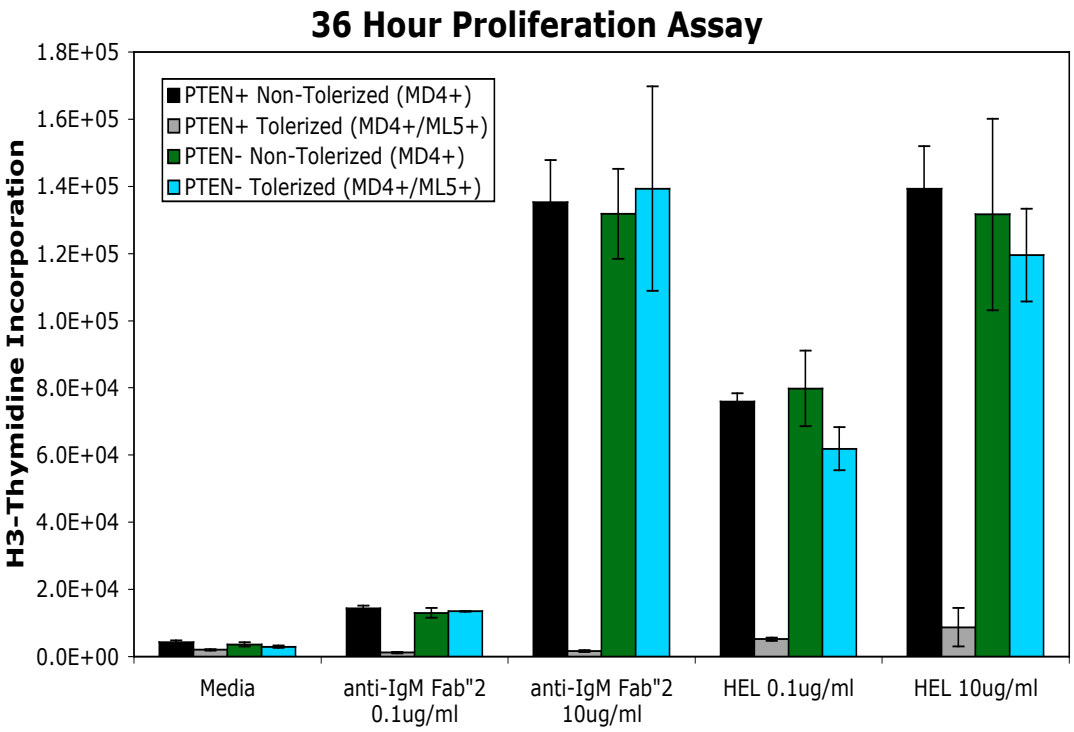


Figure 3a Failed Antigen Specific Proliferative Anergy

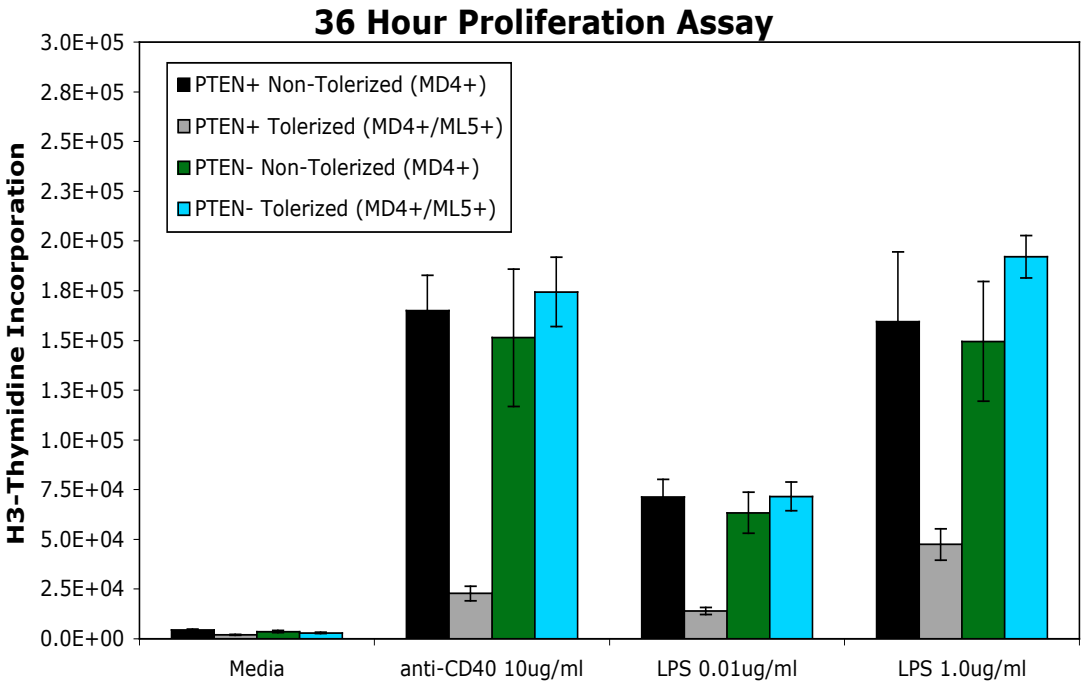


Figure 3b Failed Proliferative Anergy to Mitogens

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Chapter 3 CD21 and CD19 in the initiation of auto-immune arthritis

Introduction:

Complement C3 cleavage products mediate the recognition and clearance of toxic or infectious agents. In addition, the C3d fragment promotes B lymphocyte activation by binding to antigen and allowing for the coengagment of complement receptor 2 (CD21) and the B cell antigen receptor (BCR). Here we show, using the DBA/1 collagen-induced arthritis (CIA) model, that conjugation of C3d to heterologous type II collagen (CII) is sufficient to cause disease in the absence of the mycobacterial components of complete Freund's adjuvant (CFA). Consistent with this observation, transient depletion of C3 during the inductive phase of CIA delayed and lessened the severity of disease. DBA/1 mice deficient in either *Cd21* or *Cd19* do not develop disease. Moreover, adoptive transfer experiments showed that CD21 expression on B cells or FDCs is sufficient to confer susceptibility to disease. Although $CD19^{-/-}$ and $CD21^{-/-}$ mice produced primary antibody responses to heterologous and autologous CII, they are impaired in the ability to prime T cells, form germinal centers and produce secondary autoantibody responses. We show that disease is associated with an increase in titers to autologous type II collagen and an acquired specificity for type I collagen. These findings identify inappropriate C3d deposition as a potential causative mechanism of B cell-dependent autoimmune disease.

Infection and inflammation are important contributors to autoimmunity. Introduction of foreign antigens crossreactive with self-constituents, or the sustained activation of innate immunity can both lead to self-reactivity. Accordingly, many of the induced animal models (eg. experimental autoimmune encephalomyelitis (EAE), collagen induced arthritis (CIA) and adjuvant arthritis) of autoimmunity require mycobacterial components of CFA, which causes local inflammatory reactions, enhances antigen uptake and promotes Toll-like receptor (TLR)-dependent cytokine release. Mycobacteria can activate the complement cascade either directly via microbial cell wall components (alternate pathway) or indirectly via bound antibody (classical pathway) [1, 2]. Early steps in complement activation are distinct for each pathway of induction, but unite in the cleavage of complement C3 into active fragments that function as important chemoattractants (C3a), opsonins (C3b, iC3b, C3d,g), and precursors to the generation of the chemoattractant C5a and the formation of the membrane-attack-complex (C5b-9) [3]. Although complement plays an essential role in the recognition and clearance of extracellular pathogens and toxins, deregulated complement activation during the acute inflammatory response can promote autoimmune disease brought on by the binding of antibodies to self tissues, or deposition of immune complexes in glomeruli and small vessels [4].

In addition to the roles of antibody and complement C3 as soluble effector molecules, the cleavage product C3d acts during the inductive phase of the antibody response by binding to CD21 on B cells and FDCs [5]. Specific recognition of C3d-bound antigen results in coclustering of the BCR and CD21, as well as CD21-associated CD19. CD19 functions as a transmembrane adaptor protein for both CD21 and the BCR, and is thought to be responsible for the noted adjuvant effect of C3d on antigen-specific B cells [6, 7]. Mice deficient for *Cd19*, *Cr2* (encoding CD21 and CD35) or *C3* all exhibit impairments in the production of natural antibody and induced responses to T cell-dependent (TD) antigens and some T cell-independent (TI) antigens [8-14]. In the case of $CD19^{-/-}$ mice, impaired responses to some TI antigens may be explained by the absence of the marginal zone (MZ) and B-1 populations, combined with attenuated BCR signaling by follicular B cells. $Cr2^{-/-}$ mice retain the MZ and B-1 cell compartments and show quantitative defects in antibody responses to TI antigens [11, 12, 14]. CD21 deficiency is less severe than CD19 since CD21 only participates in B cell responses to opsonized antigens. However, CD21 exerts an additional CD19-independent role on FDCs to capture and retain opsonized antigens for B cell selection and propagation [15, 16]. Together, these findings suggest that C3d deposition on foreign antigen and subsequent recognition by CD19/CD21 is a crucial parameter of the antibody response.

Complement activation needs to be carefully regulated to prevent autodestructive effects such as that which occurs during chronic inflammation. Rheumatoid arthritis (RA) is a common chronic inflammatory disease affecting the joints, and is correlated with local complement activation [17, 18]. In RA, release of collagen types I (CI) and II (CII) serve as biomarkers for damaged bone and cartilage, respectively [19]. In mice, collagen-induced arthritis (CIA) displays similar pathology and inflammation-associated sequelae with RA [20]. Immunization of susceptible strains of mice with heterologous “foreign” type II collagen leads to the production of autoantibodies and the subsequent initiation of inflammation and destruction of cartilage and subchondral bone [21]. DBA/1 mice (H-2^q) show a high penetrance of disease following immunization with heterologous CII [22]. Autoimmune disease does not develop when mice are depleted of $CD4^+$ T-cells [23]. Additionally, B cells are required for the production of arthritogenic anti-CII antibodies [24, 25], and increasing evidence suggests that they play an important role in the recognition, processing and presentation of native CII [26, 27].

Disease progression in CIA can be divided into the early lymphocyte-dependent inductive phase, and the antibody dependent effector phase where it has been shown that transfer of arthritogenic anti-CII antibodies is sufficient to cause disease [25, 28, 29]. Articular cartilage bound by CII-specific antibodies as well as immune complex formation activate complement and Fc

receptor-bearing cells which combine to promote inflammation and joint destruction. Indeed, the pathology associated with CIA requires the complement components C3 and C5, and cells bearing the C5a and/or FcγR receptors [30-35]. C3 cleavage products function as opsonins, chemoattractants and precursors to C5a formation in the effector phase, and are also required for optimal anti-CII titers [31]. Several reports have now shown that C3d(g)-binding promotes antibody responses to foreign antigens [7, 36, 37], however the consequences of C3d deposition and CD21-dependent adjuvant effects have not been addressed in CIA or other models of autoimmune disease.

Here we show that C3d-bound CII is sufficient to cause disease independently of the broad immunostimulatory effects of CFA that are otherwise required. Consistent with this finding, transient depletion of C3 at the time of immunization with CII in CFA lessens the incidence and severity of CIA. Expression of CD19 and CD21 on B cells as well as CD21 on FDCs are required for CIA, indicating that C3d augments B cell activation both directly and through increased antigen retention. In contrast to other protein antigens, $CD21^{-/-}$ mice do not efficiently prime CII-specific T cells, indicating that coreceptor function is required for the role of B cells as the primary antigen presenting cell type in this disease. These findings demonstrate that the natural adjuvancy of C3d can induce production of pathogenic autoantibodies,

and thus is of relevance to other B cell-dependent autoimmune diseases that are initiated in the context of inflammation. Importantly, we report for the first time that immunization with heterologous CII induces a crossreactive response to autologous CI that bears a stronger correlation with disease than autologous CII. We explain these findings in the context of hierarchical recognition of T and B cell epitopes during the immune response to heterologous and autologous collagen types.

Results

Immunization with a C3d$_3$-CII conjugate is sufficient to cause CIA.

During immunization, complement C3d is rapidly generated and acts as a potent opsonin to promote B cell activation upon antigen co-recognition by CD21 and the BCR [5, 7, 36, 37]. We conjugated C3d to bovine type II collagen (BCII) to determine whether C3d deposition on a crossreactive self-antigen can induce autoimmune disease. The native conformation of CII is a triple-helical structure which needs to be retained to cause disease [20, 38]. Therefore, instead of preparing a BCII-C3d$_3$ fusion protein, murine C3d was produced from a bacterial expression vector that encoded an N-terminal biotin tag fused to 3 copies of C3d (C3d$_3$-bio). The C3d$_3$-BCII conjugate was generated by admixing C3d$_3$-bio and *in vitro* biotinylated BCII in the presence of avidin (see Materials and Methods).

To test the pathogenicity of $C3d_3$-BCII, DBA/1 mice were immunized intradermally with $C3d_3$-BCII in incomplete Freund's adjuvant (IFA), which lacks the mycobacterial membrane component of CFA. These animals were directly compared with a group of DBA/1 mice that received an equivalent amount of unconjugated BCII-bio in CFA. All mice received a second intraperitoneal injection of BCII in PBS at day 21. Disease was monitored over a 60-day period as per standard protocols [39]. Strikingly, we found that immunization with $C3d_3$-BCII induced disease of similar clinical severity relative to that observed in BCII/CFA-treated mice (Fig. 1a). Importantly, multimerized BCII or unconjugated $C3d_3$-bio/BCII-bio were non-immunogenic (Fig. 1a), indicating that linkage of C3d to BCII is required for its adjuvancy. Consistent with the clinical scoring, only immunization with $C3d_3$-BCII/IFA or BCII/CFA induced BCII-specific antibody (Fig. 1b). Of note, BCII in CFA induced a more robust IgG2a response (Fig. 1b), which is likely attributed to the known T_H1 bias induced by CFA immunization. These findings demonstrate that C3d fixation to BCII is sufficient to cause autoimmune disease in the absence of co-administered mycobacteria.

Conjugation of C3d to BCII obviates the need for proteolytic processing of C3.

To determine if complement C3-derived opsonins promote CIA when present during the early inductive phase of the disease, DBA/1 mice were

transiently depleted of C3 by treatment with Cobra Venom Factor (CVF) one day prior to primary and secondary immunization with BCII. Effective depletion of serum C3 was verified by ELISA at the time of primary immunization with BCII in CFA and secondary immunization with BCII in PBS; C3 levels returned to normal within 10 days (data not shown). We found that C3 depletion at the time of immunization delayed the onset and reduced the clinical severity of CIA relative to immunized mice that did not receive CVF pretreatment (Fig. 1c). Thus, C3 is important during the inductive phase of CIA, in addition to its other known effector functions during the inflammatory phase of the disease [31].

CD21$^{-/-}$ and CD19$^{-/-}$ mice are protected from CIA.

C3d binds CD21 expressed on B cells and follicular dendritic cells (FDCs). On B cells, CD21 is thought to promote activation via recruitment of CD19, whereas on FDCs CD21 is thought to act by passively retaining C3d-bearing antigens and immune complexes [5]. To determine the cellular and molecular basis of C3d-dependent autoimmune disease induced by BCII, we examined disease progression in CD21$^{-/-}$ and CD19$^{-/-}$ mice backcrossed to DBA/1 7 or 10 generations, respectively. Groups of age- and sex-matched mice were immunized intradermally with BCII/CFA or C3d$_3$-BCII/IFA, and boosted with BCII in PBS on day 21. Therefore, differences in disease are attributable to the initial immunization. CD21$^{-/-}$ mice were found to be resistant

to development of CIA induced by BCII/CFA or $C3d_3$-BCII/IFA (Fig. 2a). The failure to develop disease could be due to impaired FDC and/or B cell function. Therefore, reciprocal bone marrow chimeric animals were generated, immunized with BCII or $C3d_3$-BCII and evaluated by clinical score. Interestingly, expression of CD21 either on B cells or radioresistant FDCs was sufficient to cause disease (Fig. 2b). As CD19 is thought to be the primary signal transducing component for CD21 on B cells, we examined disease progression in $CD19^{-/-}$ DBA/1 mice. No clinical signs of CIA were observed in BCII-immunized $CD19^{-/-}$ DBA/1 mice, which were indistinguishable from mice immunized with CFA alone (Fig. 2c). Thus, both CD19 and CD21 are essential for CIA. CD21 exerts an important role on FDCs as well as B cells, whereas CD19 appears to have both CD21-dependent and –independent functions on B cells responding to BCII.

$CD19^{-/-}$ and $CD21^{-/-}$ mice produce altered and distinct antibody responses to BCII.

Since passive transfer of CII-specific antibody is sufficient to cause arthritis [25, 28, 29], we reasoned that $CD19^{-/-}$ and $CD21^{-/-}$ DBA/1 mice may be nonsusceptible to CIA because of an impaired humoral response to BCII. Therefore, during the course of clinical evaluation, blood sera was collected at weekly intervals from immunized mice and BCII-specific antibody titers were measured. Both $CD19^{-/-}$ and $CD21^{-/-}$ DBA/1 animals displayed reduced IgM

and IgG responses to BCII following primary immunization with BCII/CFA, and failed to mount an elevated IgG response upon secondary immunization with BCII (Fig. 3ab). Disease incidence was 100% in DBA/1 mice and 0% in DBA/1-backcrossed (F10) $CD19^{-/-}$ mice (Fig. 2 and data not shown). $CD21^{+/+}$ mice derived from an F5 or F7 intercross of $CD21^{+/-}$ mice on the DBA/1 background presented disease at an incidence of 55% or 85%, respectively, consistent with the required coinheritance of multiple DBA/1-derived susceptibility genes (data not shown). Interestingly, $CD21^{+/+}$ mice derived from these crosses and found to be arthritic generated high titers of BCII-specific IgG, while anti-BCII IgG titers from non-arthritic wild type animals approximated those of $CD21^{-/-}$ animals (Fig. 3b). BCII-specific antibody responses elicited by $C3d_3$-BCII/IFA immunization of $CD19^{-/-}$, $CD21^{-/-}$ and DBA/1 mice were of similar kinetics and magnitude to that of BCII/CFA-immunized animals (Fig. 3cd).

Although both $CD19^{-/-}$ and $CD21^{-/-}$ mice were protected from developing CIA and exhibited similar impairments in total anti-BCII IgG titers, they differ significantly in the contribution of particular isotypes (Fig 3e,f). $CD19^{-/-}$ mice mounted a reduced primary IgM response, a gradual increase in IgG2a/IgG2b titers and failed to produce any measurable BCII-specific IgG1 antibody (Fig 3e). $CD21^{-/-}$ mice also exhibited an impaired primary IgM response, and produced reduced but measurable titers of IgG2a, IgG2b and

IgG1 to BCII (Fig 3e). Since IgG1 does not efficiently activate complement, shared reductions in BCII-specific IgG2a/b in $CD19^{-/-}$ and $CD21^{-/-}$ mice are likely of greater relevance to the disease. Secondary immunization at day 21 failed to induce elevated levels of BCII-specific antibody in $CD19^{-/-}$ mice, and only induced a modest response in $CD21^{-/-}$ mice (Fig 3e,f). Thus, the humoral response to BCII immunization is present but reduced in DBA/1 mice lacking CD19 or CD21, with a more marked impairment in the secondary response.

$CD21^{-/-}$ and $CD19^{-/-}$ mice exhibited quantitative reductions in BCII-specific antibody, but no evidence of arthritis as measured by joint swelling. Therefore, to ensure that our clinical scoring was consistent with joint pathology, sections of rear ankle tissue of experimental mice were examined 60 days after immunization for histological signs of arthritic lesions as revealed by hematoxylin and eosin staining (Fig. 4). Bone and cartilage erosion, synovial hyperplasia, pannus tissue formation and extra-articular inflammation were evident in BCII-immunized $CD19^{+/+}$ DBA/1 mice (Fig. 4, left panels). However, joint tissue sections from $CD19^{-/-}$ DBA/1 mice immunized with BCII appeared normal (Fig. 4, right panels). These findings indicate that the modest levels of anti-BCII IgG induced in $CD19^{-/-}$ animals are not arthritogenic.

Reduced autoantibody production to mouse collagen type II (MCII) in CD19$^{-/-}$ and CD21$^{-/-}$ mice.

In the experiments described above, a heterologous source of CII induces autoreactivity and the onset of disease. However, since chronic disease and autodestruction of joint tissues is due to the propagation, activation and differentiation of B cells reactive to autologous collagen, we assessed autoantibody titers to cartilage-derived MCII. Immunization with BCII/CFA (Fig. 5a,b) or C3d$_3$-BCII/IFA (Fig. 5cd) induced production of MCII-specific IgG in DBA/1 mice. As was the case with antibody titers to BCII, both CD19$^{-/-}$ (Fig. 5a,c) and CD21$^{-/-}$ (Fig. 5b,d) mice produced significant but reduced titers of anti MC-II antibodies. Wild type mice that developed arthritis produced elevated and sustained levels of MCII-specific antibodies while non-arthritic wild type animals produced titers similar to CD19$^{-/-}$ or CD21$^{-/-}$ animals. These reduced titers may account for the reduced susceptibility to CIA observed in CD19$^{-/-}$ and CD21$^{-/-}$ animals.

CD19$^{-/-}$ and CD21$^{-/-}$ mice do not produce germinal centers in response to BCII immunization.

We have shown that immunization with either C3d$_3$-BCII/IFA or BCII/CFA induces the production of pathogenic antibodies to MCII. The generation of these antibodies may arise from the induced differentiation of pre-existing self-reactive B cells. Alternatively, the process of somatic

hypermutation, which drives affinity maturation in the GC, may also allow for the generation of B cells that have acquired auto-specificities. Provision of T cell help allows for further B cell differentiation and antigen-driven selection in the GC. In $CD19^{-/-}$ mice, GCs are generally absent in the spleen and lymph nodes [8, 9], but can be found in the Peyer's patches [40]. Germinal centers are reduced in size and frequency in $CD21^{-/-}$ mice [11, 12, 41], but the defect is less severe than in $CD19^{-/-}$ animals. For these reasons, it was important to evaluate GC formation in $CD19^{-/-}$, $CD21^{-/-}$ and DBA/1 mice following CII injection. Cells were isolated from the draining lymph node of naïve or BCII-immunized (day 11) mice and analyzed by flow cytometry to enumerate B cells ($B220^+$), GC B cells ($B220^+GL7^+$) and T_H cells ($CD4^+$) (Fig. 6). No significant change in $CD4^+$ T_H cells numbers was observed in any of the mice following immunization (data not shown), but the percentage of B cells in the draining lymph nodes of all immunized mice was approximately 2 fold higher following immunization (Fig. 6). Interestingly, only DBA/1 mice generated $GL7^+$ GC B cells (Fig. 6). The GC phenotype was confirmed by histologic staining of lymph node sections with PNA lectin (data not shown). The observed failure to generate GCs is consistent with the weak secondary response to BCII and MCII.

Reduced priming of BCII-specific T cells in $CD19^{-/-}$ and $CD21^{-/-}$ mice.

Generation of pathogenic IgG antibodies requires the provision of T cell-derived stimuli, which largely explains the contributions of B and T cells to CIA. However, since the native conformation of CII is required for immunogenicity, it is also apparent that B cells function as key antigen presenting cells in this disease. Therefore, to determine the contributions of CD19 and CD21 to T cell activation, lymph node cells were isolated from immunized $CD19^{-/-}$, $CD21^{-/-}$ and DBA/1 mice and assayed for T and B cell proliferation following antigenic challenge *in vitro*. Draining lymph node cells were isolated 11 days post-immunization with 100 μg BCII in CFA, or from non-immunized mice. Cells were cultured with or without 50 μg/ml of native or heat denatured BCII for 96 hours, and proliferation measured by BrdU incorporation. Naive lymph node cells from either non-immunized mice, $CD19^{-/-}$ or $CD21^{-/-}$ mice did not proliferate in response to BCII challenge *in vitro* (Fig. 7a,b). Lymph node B and T cells from immunized DBA/1 proliferated in response to antigen re-challenge. However, lymph node cells from immunized $CD19^{-/-}$ and $CD21^{-/-}$ mice could not be induced to proliferate in response to BCII, but did respond to B and T cell mitogens (Fig. 7a,b).

To directly evaluate T_H cell differentiation in terms of cytokine production, in vitro stimulated lymph node cells were surface stained for CD4 followed by intracellular staining for IL-2, IFNγ, and IL-4 production. The percentage of IL-2, IFNγ, or IL-4 producing cells was enumerated by flow

cytometry (Fig. 7c-e). Only lymph node $CD4^+$ T-cells from BCII-primed DBA/1 mice were activated in an antigen specific manner to become BCII-specific T_H effectors, as measured by IL-2, IFNγ, and IL-4 production upon re-encounter with native BCII. The native conformation of BCII was important for the immunogenicity of BCII, as heat denatured BCII did not induce cytokine production. Additionally, the stimulation of wild type T cells with the antigen non-specific mitogen ConA led to the production of all three cytokines (Fig. 7c-e). By contrast, $CD19^{-/-}$ and $CD21^{-/-}$ T cells did not respond in an antigen specific manner to BCII re-encounter, consistent with the proliferation data. These findings suggest that the reduced GC and secondary antibody responses observed in BCII-immunized $CD19^{-/-}$ mice may be attributed at least in part to inefficient antigen specific T-cell activation. Moreover, this work underscores the importance of B cells and, specifically, the B cell coreceptor complex in the presentation of CII and elicitation of T cell help as a prerequisite for the development of disease.

Autoantibody production against mouse collagen type I (MCI).

It was surprising that given the qualitative differences in disease susceptibility in $CD19^{-/-}$ or $CD21^{-/-}$ versus wild type mice there were only quantitative differences in BCII and MCII-specific antibody production. Since chronic disease and autodestruction of joint tissues results in the release of collagen types I (CI) in addition to type II (CII) as a biomarker of disease

progression, and because type I but not type II collagen is expressed in the bone that is targeted for immunological degradation, we assessed autoantibody titers to bone-derived MCI. As shown in figure 5, Immunization with BCII/CFA (Fig. 8ab) or $C3d_3$-BCII/IFA (Fig. 8cd) induced production of MCII-specific IgG in DBA/1 mice. As was the case with antibody titers to BCII, both $CD19^{-/-}$ (Fig. 8ac) and $CD21^{-/-}$ (Fig. 8bd) mice produced significant but reduced titers of anti MC-II antibodies. Wild type mice that developed arthritis produced elevated and sustained levels of MCII-specific antibodies while non-arthritic wild type animals produced titers similar to $CD19^{-/-}$ or $CD21^{-/-}$ animals. By contrast, only arthritic wild type mice generated measurable titers of MCI-specific antibodies. The anti-MCI response exhibited a delayed onset relative to the anti-MCII or anti-BCII response. To associate anti-collagen specificity with arthritogenic potential, we compared the relative reactivity of immune sera from arthritic animals with a cocktail of CII-specific monoclonal antibodies that are known to be sufficient to cause reactive inflammatory arthritis in naïve recipient mice [28]. In addition to binding CII, the antibody cocktail exhibited strong reactivity with MCI (Fig. 8e), suggesting that crossreactive antibodies to anti-MCII and –MCI can cause disease.

Discussion

In the current work, we demonstrate that mice immunized with C3d-bound BCII in IFA develop an autoimmune condition similar to that observed

in mice treated with BCII in CFA. Susceptibility to CIA requires CD19/CD21 on B cells as well as CD21 on FDCs to promote B cell activation and propagation. Impaired priming of CII-specific T cells and a failure to form germinal centers in $CD19^{-/-}/CD21^{-/-}$ DBA/1 mice culminate in the inability to produce elevated and sustained levels of BCII- and MCII-specific IgG. Hence, C3d-binding and coreceptor-dependent B cell activation are important determinants for developing CIA.

The enzymatic cleavage of C3 is a highly amplified step in the complement cascade. To avoid autodestruction of host constituents, most C3 products are short-lived or rapidly inactivated. C3d may be an exception to this regulation, since it is a terminal cleavage product. Of relevance, C3d accumulation and excretion is of diagnostic value for autoimmune diseases involving impaired clearance of immune complexes such as RA or lupus [42, 43]. To date, the adjuvant activity of C3d has only been studied in the context of antibody responses to foreign antigens. Here, we utilized the CIA model to demonstrate that inappropriate deposition of C3d is not only correlative but can be causative of B cell-dependent autoimmune disease.

Similar to other models of induced autoimmune disease, the DBA/1 model of inflammatory arthritis requires the use of CFA to activate innate immunity [44]. One of the immunostimulatory effects of CFA is complement

activation [2], resulting in C5a-dependent recruitment and activation of effector cells that precipitate joint destruction in CIA [30-32, 35]. $C3^{-/-}$ mice and mice with impaired C3 convertase activity also show reduced susceptibility to CIA [31, 45, 46]. However, these studies cannot assign the relative importance of C3 in inducing lymphocyte activation in the early asymptomatic stages of the disease, versus recruitment of myeloid effector cells during the late symptomatic stages. We found that transient depletion of C3 by pretreatment of mice with CVF significantly decreased the onset and severity of CIA (Fig 1C). Since serum C3 levels return to normal long before any clinical symptoms of disease are noted, this finding supports an early role for C3 in the inductive phase of the disease as well as serving as an essential precursor for the biosynthesis of C5a during the effector phase.

Cleavage of C3 generates a soluble C3a fragment and a bound C3b fragment that is further cleaved into the opsonins iC3b and C3d,g. The fact that C3d fixation to CII was sufficient to cause disease without the overt activation of complement and other inflammatory mediators elicited by CFA indicates a direct role for C3d in the activation of CII-specific B cells and production of arthritogenic antibodies. Indeed, we showed that expression of the C3d receptor CD21 was required for disease susceptibility, but bone marrow chimera analysis revealed that expression of CD21 either on B cells or FDCs was sufficient to allow disease following immunization with either

BCII/CFA or $C3d_3$-BCII/IFA. The additive yet distinct contributions of CD21 expressed on FDCs and B cells to the antibody response to BCII is in agreement with that described for the response to other T cell-dependent antigens [16, 47]. CD21 is not thought to have a signal transducing ability for FDCs, but acts to retain opsonized antigens and immune complexes. By contrast, CD21 acts in concert with the BCR to mediate co-recognition of C3d(g)-bearing antigens [5]. In this context, CD21 is thought to augment B cell activation through CD19 recruitment. CD19 is also a proximal substrate for signal transduction induced by BCR engagement alone. We found that $CD19^{-/-}$ mice are not susceptible to CIA following immunization with BCII/CFA or $C3d_3$-BCII/IFA. This finding underscores a prominent CD21-independent role for CD19 in B cell function, since chimeric mice lacking CD21 expression on B cells remain susceptible to CIA.

Previous studies have shown that B cells are required for susceptibility to CIA [24], and that a selected cocktail of CII-specific monoclonal antibodies is sufficient to induce an acute arthritic condition upon transfer into both susceptible and non-susceptible mouse strains [28]. However, in addition to secreting antibodies, B cells can serve a crucial function as antigen presenting cells (APCs), which has been noted in the etiology of autoimmune diseases such as type I diabetes, lupus and RA [48-50]. Since the onset of CIA is critically dependent upon immunization with CII in its native conformation [20,

38], and CII-specific antibodies that recognize articular cartilage also recognize the intact triple helical structure of recombinant CII epitopes [38], the unique ability of B cells to recognize conformational epitopes of CII strongly implicates their importance as antigen-presenting cells in CIA [26, 27]. We found that lymph node cells from immunized $CD19^{-/-}$ or $CD21^{-/-}$ mice do not promote $CD4^{+}$ T cell proliferation nor IL-2, IFNγ, and IL-4 production upon *in vitro* re-challenge with native or denatured BCII. Since the loss of CD21 function does not affect T cell responses to other protein antigens [51, 52], impaired T cell priming in $CD21^{-/-}$ mice may be specific to CII. Several reports have shown that CD21-bound antigens can be directed to appropriate vesicular pathways for presentation by MHC class II [53-57], although it is unclear whether this process requires CD19. Moreover, CD19/CD21 engagement can promote T cell costimulation by inducing upregulation of CD80/CD86 [58]. These collective findings further implicate B cells as the primary APC type in this disease.

While the production of CII-specific antibody is clearly required for CIA, parameters of the anti-CII antibody response relative to the onset and severity of disease are not well characterized. We found a strong correlation of clinical disease and the amount of anti-MCII/BCII IgG titers. Both $CD19^{-/-}$ and $CD21^{-/-}$ mice exhibited reduced anti-CII IgG and were resistant to disease. Anti-CII titers and disease susceptibility were restored in chimeric mice expressing

CD21 on B cells or FDCs. Passive immunization with anti-CII antibodies induces a severe yet transient arthritic condition [25, 28, 29], indicating that continued autoantibody production and persistence are required for chronic disease. In addition to quantitative differences in anti-CII IgG, qualitative differences in antibody specificity, affinity and isotype are likely key determinants in the generation of arthritogenic antibodies. Notably, these refinements in antibody binding and effector function may be dependent upon further B cell differentiation in the germinal center, which is halted in CII-immunized $CD19^{-/-}/CD21^{-/-}$ mice.

Consistent with this interpretation, $CD19^{-/-}/CD21^{-/-}$ mice do not mount a strong secondary response to CII. It has been found that in CIA, gross titers of anti-CII antibody is not a good correlate for the development of arthritis, indicating that qualitative differences in antibody production determine the outcome of disease [35, 44, 59, 60]. Based upon these observations and our current findings, we postulate that MCI/MCII-specific B cells may be generated as an outcome of BCR diversification and intraclonal expansion in the GC. Analysis of immunoglobulin V gene usage during the primary and secondary immune responses to heterologous collagen in DBA/1 mice suggests that a shift in epitope specificity of anti-collagen antibodies occurs that may define the onset of CIA. As the acquisition of altered BCR specificities can arise from V gene hypermutation in the GC, this mechanism

may result in the recognition of autologous mouse collagen derived from an initial response to heterologous bovine collagen. Hybridomas isolated from the primary response most commonly express germline sequences encoding antibodies against the major C1 epitope in the cyanogen bromide (CB) cleaved CB11 fragment of heterologous CII (including BCII), while antibodies of the secondary response show evidence of somatic hypermutation and frequently display a shift and expansion in epitope specificity towards the recognition of new epitopes located in the CB10 fragment [61]. A shift in epitope specificity may be crucial for the generation of arthritogenic antibodies, including those specific for MCI which we have shown arise later in the response to BCII. Since $CD19^{-/-}$ and $CD21^{-/-}$ mice fail to form GCs, this shift in epitope specificity and affinity maturation may not occur, thus preventing the development of CIA. In this respect, $CD19^{-/-}$ and $CD21^{-/-}$ mice on the DBA/1 background bear resemblance to non-susceptible mouse strains which respond to heterologous CII, but fail to recruit T cell help and do not produce pathogenic antibodies reactive to autologous CII..

The significance of these cumulative findings can be integrated into a model for CIA whereby CD21 facilitates recognition of C3d-opsonized CII produced during primary immunization. B cell activation and proliferation is promoted by CD19 acting downstream of the BCR and CD21. In addition, the CD19/CD21 complex may promote the uptake and presentation of CII peptide

determinants to T_H cells, and induce the upregulation of costimulatory molecules. Activation of CII-specific T_H cells and the subsequent production of cytokines promotes B cell growth and differentiation into IgG-secreting cells. In parallel, some B cells responding to CII seed the germinal center to undergo further differentiation, and engender the abundant production of arthritogenic antibodies to both MCII and MCI. Future studies of spontaneous or inflammation-induced binding of C3d to self-antigens will likely reveal the importance of this innate mechanism in the etiology of other B cell-dependent autoimmune diseases.

Materials and Methods:

Mice. $CD19^{-/-}$ mice were generated previously [9]. CD21-/- mice were generously provided by V. Michael Holers, (Departments of Medicine and Immunology, University of Colorado Health Sciences Center, Denver, Colorado). $CD19^{-/-}$ and $CD21^{-/-}$ mice were back-crossed to DBA/1J wild type mice (Jackson Labs, Bar Harbor, Maine) for 7 generations or more. All experiments were performed on back-crossed, age matched, sex matched, $CD19^{-/-}$ and $CD19^{+/+}$ or $CD21^{-/-}$ and $CD21^{+/+}$ mice. $CD19^{+}$, $CD19^{-}$, $CD21^{+}$, $CD21^{-}$ genotyping was performed by PCR and CD19 or CD21 deficiency was confirmed by flow cytometry on peripheral blood leukocytes using either anti-mouse CD19 PE (phycoerythrin), or anti-mouse CD21/CD35 FITC (fluoresein isothiocyanate) (Pharmingen, San Diego, California). CD21 chimeric mice

were generated by the reconstitution of DBA/1J back-crossed $CD21^{-/-}$ and $CD21^{+/+}$ animals after lethal irradiation (1000 rads). Chimeric reconstitutions were performed by intra-venous (IV) injection of bone marrow isolated from either back-crossed $CD21^{-/-}$ DBA/1J or $CD21^{+/+}$ DBA/1J animals. To assure effective reconstitution, chimeric mice were screened after 8 weeks for the absence or presence of CD21 expressing cells in peripheral blood by flow cytometry. All animals were maintained under pathogen-free conditions and handled in accordance with the guidelines set forth by the Animal Subjects Program at the University of California, San Diego.

Serum C3 depletion in mice. Mice were given 18 units of CVF (Quidel, San Diego, California) 24 hours prior to primary or secondary immunization with BCII. Serum levels of C3 at the time of immunization and 10 days following immunization were measured by ELISA on serum samples collected by retro-orbital bleed. ELISAs for C3 serum levels were performed by coating 96 well plates (Becton Dickinson, Mansfield, Massachusetts) with serial dilutions of serum, and C3 levels detected by means of a polyclonal goat anti-mouse C3 IgG antisera (ICN Biochemicals, Aurora, Ohio), followed by a secondary rabbit anti-goat IgG Alkaline Phosphatase (AP)-labeled reagent (Southern Biotech, Birmingham, Alabama), and PNPP (Southern Biotech, Birmingham, Alabama) substrate for visualization at 405nm using a Versamax microplate reader (Molecular Devices, Sunnyvale, California).

Collagen induced arthritis. For $CD21^{-/-}$ or $CD19^{-/-}$ experiments, 6-8 week old mice were immunized by intra-dermal (ID) injection at the base of the tail with either 100 μg type II bovine collagen (50 μl), (Chondrex, Redmond, Washington), plus 50 μl of CFA (Sigma, St. Louis, Missouri), or with CFA plus Phosphate Buffered Saline (PBS) alone. A boost of either 100 μg BCII in PBS, or PBS alone, was injected intra-peritoneal (IP) on day 21 in experiments involving clinical, histopathological, and serological analysis. CD21 chimeric mice were allowed 8 weeks to reconstitute the peripheral leukocyte pool after lethal irradiation and prior to immunization as described above. $C3d_3$-bio was prepared by cloning 3 copies of murine C3d into the PinPoint™ vector (Promega Corporation, Madison, WI), which allows for *in vivo* biotinylation in *E. coli*. The recombinant protein bearing a His-tag sequence was prepared by batch purification on Co beads (Pierce), dialyzed extensively and found to be endotoxin-free. C3d-bio was coupled to *in vitro* biotinylated (EZ-Link Sulfo-NHS-Biotin, Pierce) BCII in the presence of avidin at 1:2:1 molar ratio (BCII-bio:avidin:C3d-bio). An amount of $C3d_3$-BCII equivalent to 100 μg of BCII was emulsified in IFA (Sigma-Aldrich) prior to injection.

Clinical scoring. A 0-16 clinical scoring method was utilized to evaluate the induction and severity of arthritis. Each foot was evaluated on a 0-4 severity scale, (“0” being no inflammation; “1” being swelling surrounding the ankle; “2” being swelling extending into the upper foot; “3” being swelling

extending into the lower foot; “4” being swelling extending into the toes). The summation of individual evaluation scores for all four feet per animal produced a 0-16 clinical score per mouse. All group scores were taken as the average clinical score for all mice in each group, and standard deviations were calculated. All clinical evaluations were performed blind to investigators. This scoring method was learned and performed under the guidance of Dr. Gary S. Firestein (University of California, San Diego).

Antibody titers. Detection of anti-collagen antibodies in the serum of mice was performed by standard ELISA. Peripheral blood serum was isolated from mice by retro-orbital bleed prior to immunization and at 7-10 day intervals following immunization. An empirically determined optimal dilution of 1/500 was chosen as it represented 50% maximum binding on the linear portion of the binding curve. Collagen-specific antibodies were captured from serum using either 10 μg/ml BCII (Chondrex, Redmond, Washington), 10 μg/ml MCII or 10 μg/ml MCI (Biotrend Chemicals, Destin, Florida) coated 96 well plates (Becton Dickinson, Mansfield, Massachusetts) and detected with alkaline phosphatase (AP)-conjugated rat anti-mouse IgG, IgM, IgG2a, IgG2b, IgG1 and IgG3 secondary antibodies (Southern Biotech, Birmingham, Alabama) applied at 1:2000 dilution. PNPP (Southern Biotech, Birmingham, Alabama) substrate was used for visualization at 405 nm using a Versamax microplate reader (Molecular Devices, Sunnyvale, California).

Cell culture. Popliteal and inguinal lymph nodes were isolated 11 days after immunization with BCII (immunizations were performed as described above) and single cell suspensions were prepared in Dulbecco's Modified Eagle Medium (Mediatech, Herndon, Virginia) plus 10% FBS. $1x10^6$ cells were plated in 96 well polystyrene round bottom plates (Corning, Costar, New York, New York).

Cell proliferation. Lymph node cells were incubated for 96 hours at 37 ^oC in 5% CO_2, stimulated in either medium alone, with 50 μg/ml BCII (Chondrex, Redmond, Washington) or with 50 μg/ml heat denatured BCII (hdBCII). To make hdBCII, native BCII was heated at 100 ^0C for 15 minutes prior to addition to cell cultures. Proliferation was measured in triplicates by adding 0.5 μCi/well of ^3H-thymidine (ICN Biochemicals, Aurora, Ohio) for the last 18 hours of culture, and measuring incorporation of radioactive label by means of a 96 well cell harvester and a scintillation counter.

Enumeration of cell populations. Popliteal and inguinal lymph nodes were isolated 11 days after immunization with BCII (immunizations were performed as described above) and single cell suspensions were prepared. $1x10^6$ cells were stained for fifteen minutes on ice with anti-GL7-FITC, anti-B220-APC (allophycocyanin), (both from Pharmingen, San Diego, California) and biotinylated anti-CD4 (Ebioscience, San Diego, California) diluted in PBS plus 1% Fetal Bovine Serum (FBS) (Omega Scientific, Tarzana, California). Cells were washed with PBS plus 1% FBS and incubated with streptavidin-PE

(phycoerythrin) (Pharmingen, San Diego, California) diluted in PBS plus 1% FBS. Flow cytometric analysis was performed using a dual laser FACSCalibur instrument (Becton Dickinson, Mansfield, Massachusetts).

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Figure legends

Figure 1. Immunization with a $C3d_3$-BCII conjugate is sufficient to cause CIA.

a, b. Groups (n=4) of wild type DBA/1 mice were immunized with either unconjugated BCII-bio in CFA (▲), avidin (Av)-crosslinked BCII-bio in IFA (○), conjugated $C3d_3$-BCII in IFA (●), unconjugated BCII-bio and $C3d_3$–bio (□) or CFA alone (◊). a. Mice were evaluated for clinical severity of CIA over time on a 0-16 severity scale. b. Production of BCII-specific serum antibodies were measured by ELISA for total IgG, IgM, IgG2a, IgG2b, and IgG1. Results are the mean ± s.d. of ELISAs performed in triplicates. c. Groups (n=5) of wild-type DBA/1 mice were immunized with either BCII in CFA (▲), CFA alone (◊), or temporarily depleted of complement C3 by CVF treatment just prior to primary and secondary BCII immunization (●), mice were evaluated for clinical severity of CIA over time on a 0-16 severity scale. Data shown are the representative of two experiments.

Figure 2. $CD21^{-/-}$ and $CD19^{-/-}$ mice are protected from CIA with BCII in CFA or $C3d_3$-BCII.

a. $CD21^{+/+}$ (▲), and $CD21^{-/-}$ (●) DBA/1 mice immunized with either BCII in CFA, CFA alone (◊) (n=9) or with conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊) (n=7), were evaluated for clinical signs of CIA as in 1a. b. $CD21^{-/-}/CD21^{+/+}$ chimeric mice derived from lethally irradiated recipients reconstituted with syngeneic bone marrow; WT reconstituted with WT bone marrow (▲), $CD21^{-/-}$ reconstituted with WT bone marrow (■), and WT reconstituted with $CD21^{-/-}$ bone marrow (●), were

immunized with either BCII in CFA (n=6) or with conjugated $C3d_3$-BCII in IFA (n=6), and evaluated for clinical signs of CIA as in 1a. c. $CD19^{+/+}$ (▲), and $CD19^{-/-}$ (●) DBA/1 mice immunized with either BCII in CFA, CFA alone (◊) (n=9) or with conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊) (n=7), were evaluated for clinical signs of CIA as in 1a. Data shown are the representative of multiple experiments.

Figure 3. $CD19^{-/-}$ and $CD21^{-/-}$ mice produce altered antibody responses to BCII.

a. c. The production of BCII-specific serum antibodies was measured by ELISA for total IgG in $CD19^{+/+}$ (▲), and $CD19^{-/-}$ (●) DBA/1 mice immunized with either (a) BCII in CFA or CFA alone (◊) (n=9) and with either (c) conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊) (n=7). b. d. The production of BCII-specific serum antibodies was measured by ELISA for total IgG in $CD21^{+/+}$ mice that developed arthritis (▲),$CD21^{+/+}$ mice that did not develop arthritis (■), or $CD21^{-/-}$ mice (●) DBA/1 mice immunized with either (b) BCII in CFA, CFA alone (◊) (n=9) or with (d) conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊) (n=7). e. f. BCII-specific serum antibody isotypes were measured by ELISA for IgM, IgG2a, IgG2b and IgG1 isotypes in both (e) $CD19^{+/+}$ (▲), and $CD19^{-/-}$ (●) DBA/1 mice after immunization with BCII in CFA (n=9), or (f) $CD21^{+/+}$ mice (▲) DBA/1 mice and $CD21^{-/-}$ mice (●) DBA/1 mice immunized with either BCII

in CFA or CFA alone (◊) (n=9). Results are the mean ± s.d. of ELISAs performed in triplicates. Data shown are the representative of multiple experiments.

Figure 4. $CD19^{-/-}$ mice do not develop histological signs of joint inflammation.

Histologic examination of rear hind leg ankle joint tissue (H&E staining) 60 days post primary immunization correlates evidence of; synovial hyperplasia (1), extra-articular inflammation (2), bone and cartilage erosion (3, 4 respectively), consistent with the clinical scoring data. 10X and 40X magnification shown. Images provided are representative of groups.

Figure 5. $CD21^{-/-}$ and $CD19^{-/-}$ mice show decreased production of auto-antibodies reactive to MCII and fail to mount MCII specific secondary antibody responses.

a. c. $CD19^{+/+}$ (▲) and $CD19^{-/-}$ (●) mice immunized with (a) heterologous BCII in CFA or CFA alone (◊) (n=9) and (c) conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊) (n=7), were analyzed for the production of total serum IgG, antibodies specific for autologous MCII by ELISA. b. d. $CD21^{+/+}$ mice that developed arthritis (▲), $CD21^{+/+}$ mice that did not develop arthritis (■), and $CD21^{-/-}$ mice (●) immunized with (b) heterologous BCII in CFA or CFA alone (◊) (n=9) and (d) conjugated $C3d_3$-BCII in IFA or unconjugated BCII-bio and $C3d_3$–bio in IFA (◊)

(n=7), were analyzed for the production of total serum IgG, antibodies specific for autologous MCII by ELISA. Results are the mean ± s.d. of ELISAs performed in triplicates. Data shown are the representative of multiple experiments.

Figure 6. $CD19^{-/-}$ and $CD21^{-/-}$ mice do not produce germinal centers in response to BCII immunization.

Lymph nodes from non-immunized and BCII in CFA immunized (a) $CD19^{+/+}$ and $CD19^{-/-}$ mice, and (b) $CD21^{+/+}$ and $CD21^{-/-}$ were examined for the percent of total lymph node cells that are B cells ($B220^{+}$), and germinal center B cells ($B220^{+}$ $GL7^{+}$), 11 days after immunization. Data shown are the representative of multiple experiments performed with multiple mice per group.

Figure 7. Ineffective generation of BCII-specific B and T cells in $CD19^{-/-}$ and $CD21^{-/-}$ mice.

a. b. Draining lymph node cells were isolated from WT, $CD19^{-/-}$ and $CD21^{-/-}$ mice 9 days after immunization with BCII in CFA or CFA alone. Lymph node cells were cultured with either medium alone, 50 μg/ml BCII, 50 μg/ml heat denatured BCII (hdBCII), or 1 μg/ml LPS or 1 μg/ml concanavalin A (ConA) for 48 hours in the presence of BrdU. The percent of proliferating (a.) $B220^{+}$ gated and (b.) CD4+ gated lymph node cells was determined by

intracellular flow cytometry staining with an anti-BrdU antibody. c. d. e. Draining lymph node cells were isolated from WT, $CD19^{-/-}$ and $CD21^{-/-}$ mice 5 days after immunization with BCII in CFA or CFA alone. Lymph node cells were cultured with either medium alone, 100 μg/ml BCII, 100 μg/ml heat denatured BCII (hdBCII), or 1 μg/ml concanavalin A (ConA) for 48 hours, then percent CD4+ lymph node cells producing cytokines were enumerated by intracellular flow cytometry staining for (c.) IFNγ, (d.) IL-2, or (e.) IL-4.

Figure 8. BCII immunized $CD21^{-/-}$ and $CD19^{-/-}$ mice produce modified auto-antibody responses to MCII and MCI.

Immunized mice were analyzed by standard ELISA for the production of crossreactive IgG antibodies specific for autologous MCII and MCI over time. a. Groups (n=7) of $CD19^{+/+}$ DBA/1 (▲) and $CD19^{-/-}$ DBA/1 (●) mice immunized with heterologous BCII in CFA or $CD19^{+/+}$ DBA/1 mice immunized with CFA alone (◊), b. Groups (n=7) of $CD21^{+/+}$ DBA/1 that developed arthritis (▲), $CD21^{+/+}$ DBA/1 that did not develop arthritis (■) and of $CD21^{-/-}$ DBA/1 (●) mice all immunized with heterologous BCII in CFA or $CD21^{+/+}$ DBA/1 mice immunized with CFA alone (◊), c. Groups (n=7) of $CD19^{+/+}$ DBA/1 (▲) and $CD19^{-/-}$ DBA/1 (●) mice all immunized with heterologous BCII linked to C3d with avidin in IFA or $CD19^{+/+}$ DBA/1 mice immunized with BCII and C3d unlinked (w/o avidin) in IFA (◊), d. Groups (n=7) of $CD21^{+/+}$ DBA/1 that developed arthritis (▲), $CD21^{+/+}$ DBA/1 that did not develop arthritis (■) and of

$CD21^{-/-}$ DBA/1 (●) mice all immunized with heterologous BCII linked to C3d with avidin in IFA or $CD21^{+/+}$ DBA/1 mice immunized with BCII and C3d unlinked (w/o avidin) in IFA (◊). All BCII immunized mice a-d. received BCII in PBS and CFA immunized mice received PBS alone on Day 21 (IP) as a boost. e. Monoclonal antibody cocktail capable of passively inducing arthritis (Chondrex Inc.) (●) like polyclonal mouse serum isolated from WT arthritic DBA/1 mice (▲) shows cross reactivity with BCII, MCII and MCI by standard IgG specific ELISA over a dilution curve, while polyclonal mouse serum from WT mice given CFA alone does not. Results are the mean ± s.d. of ELISAs performed in triplicates. Data shown are the representative of multiple experiments.

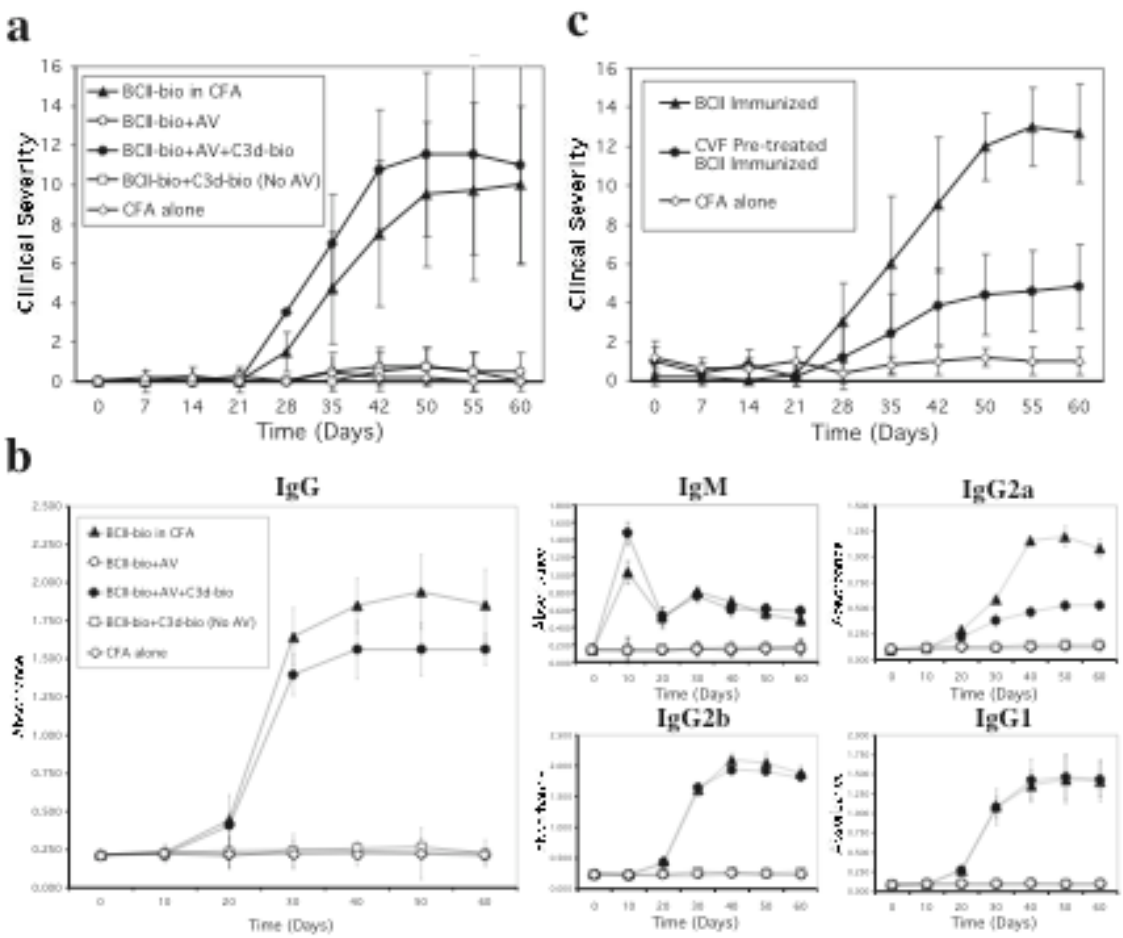

Figure 1 Immunization with a C3d3-BCII conjugate can cause CIA

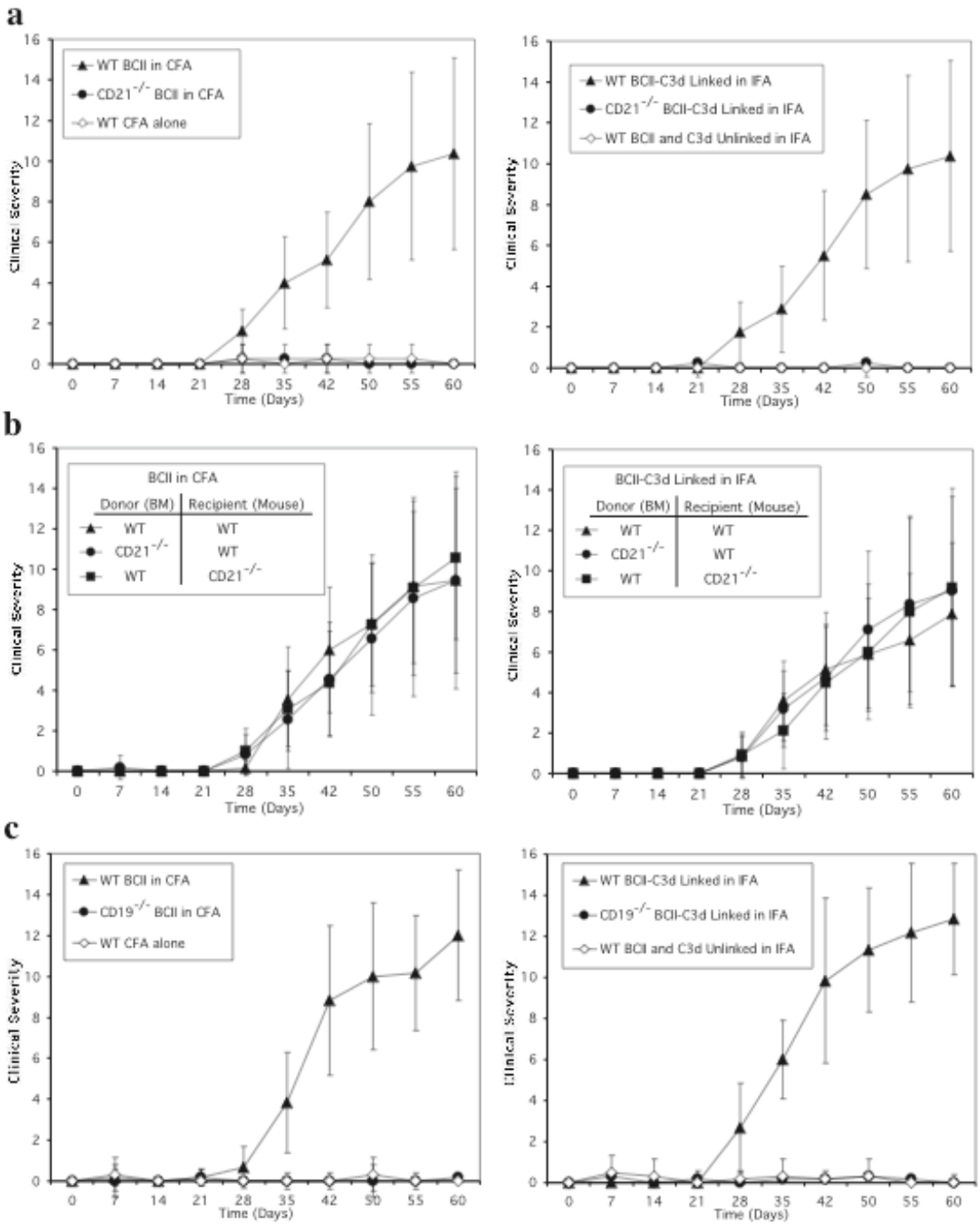

Figure 2 CD21-/- and CD19-/- mice are protected from CIA

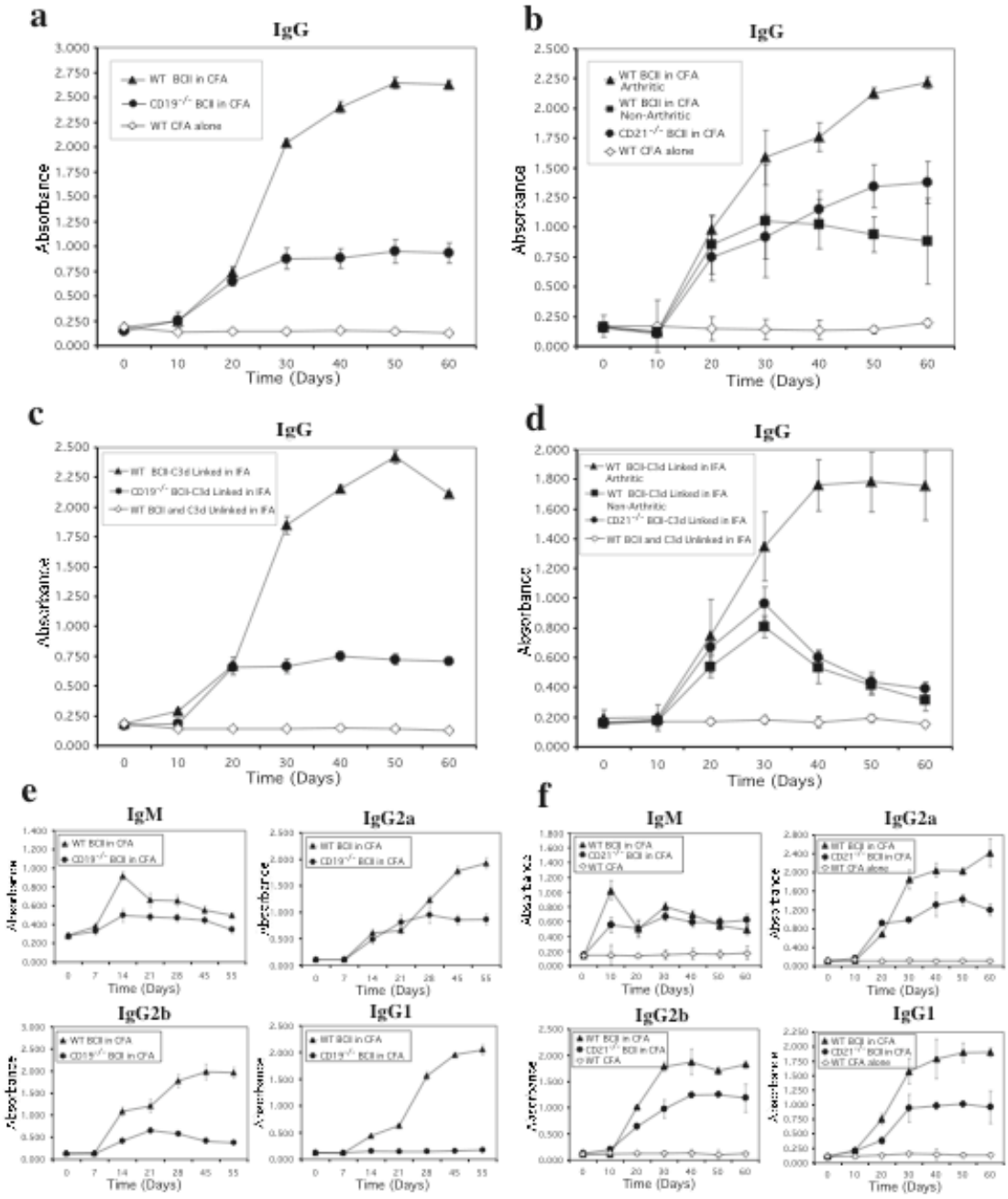

Figure 3 CD21-/- and CD19-/- mice have altered anti-BCII responses

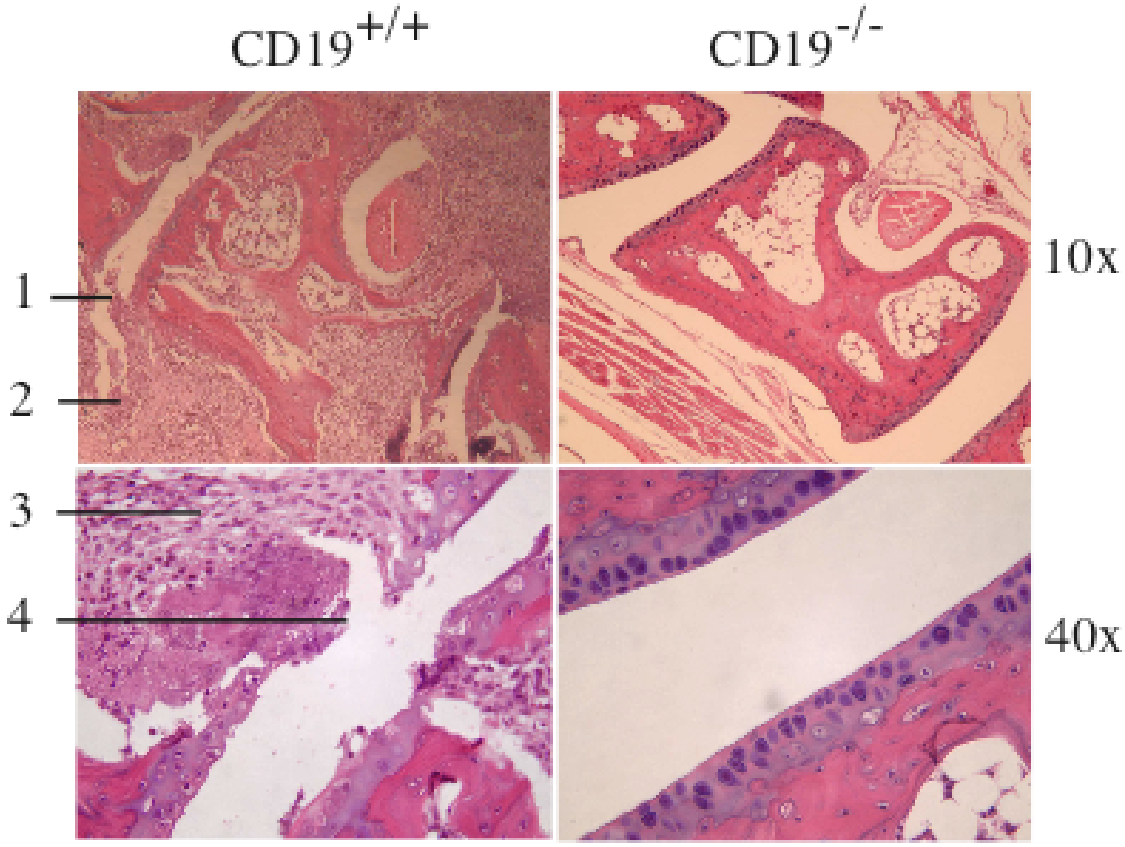

Figure 4 CD19-/- mice do not show histological signs of CIA

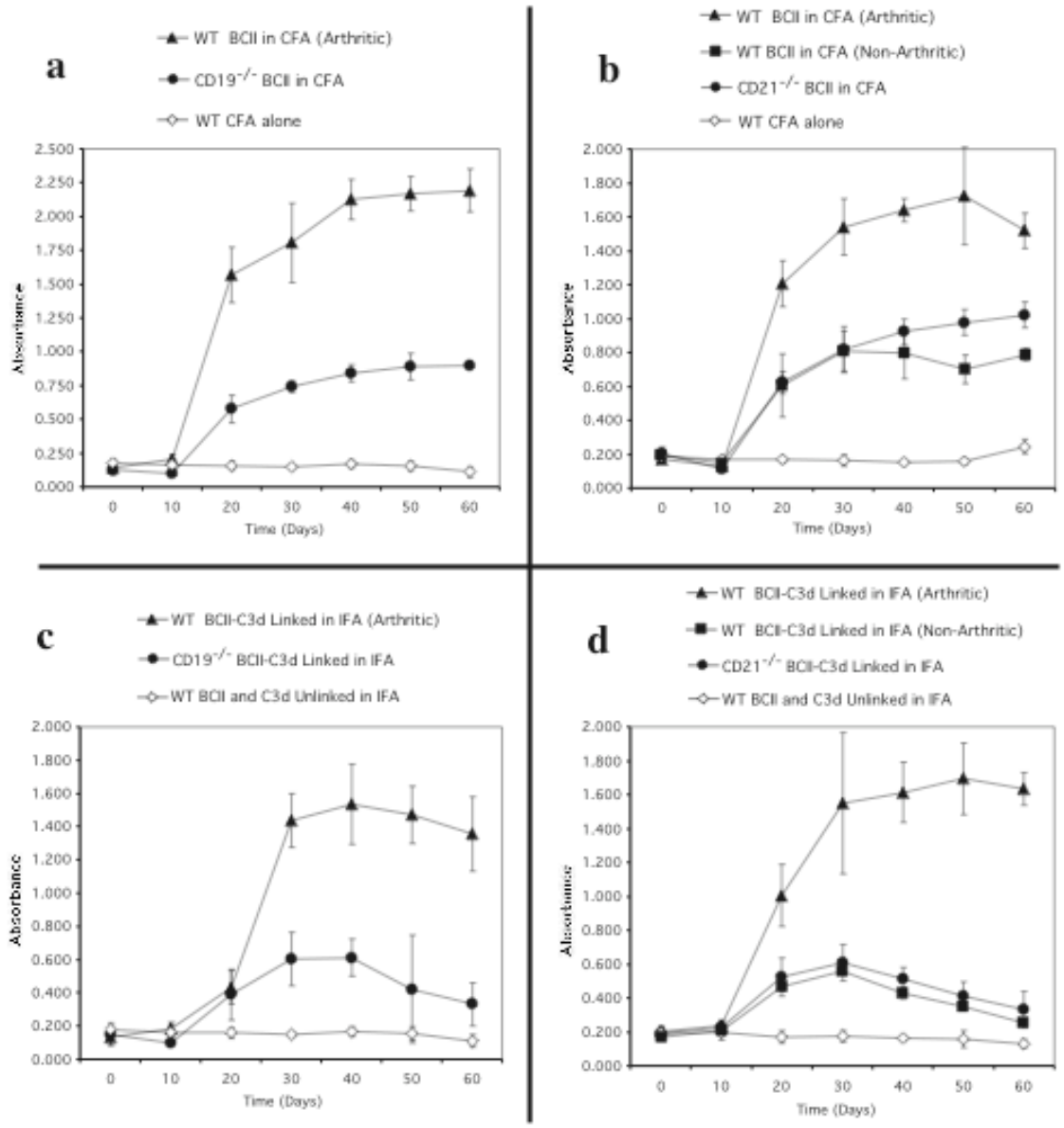

Figure 5 Loss of secondary responses to MCII in CD21-/- and CD19-/- mice

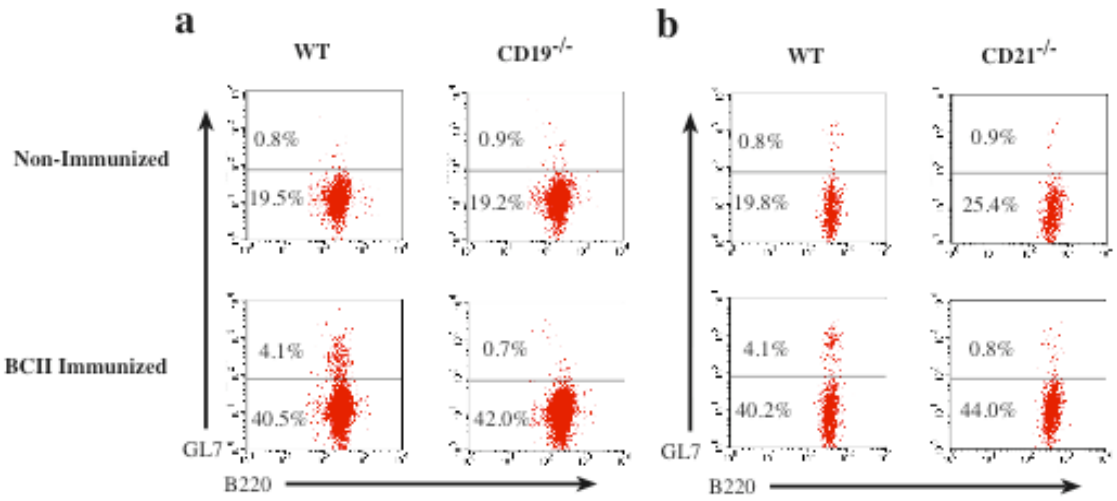

Figure 6 CD21-/- and CD19-/- mice do not produce germinal centers

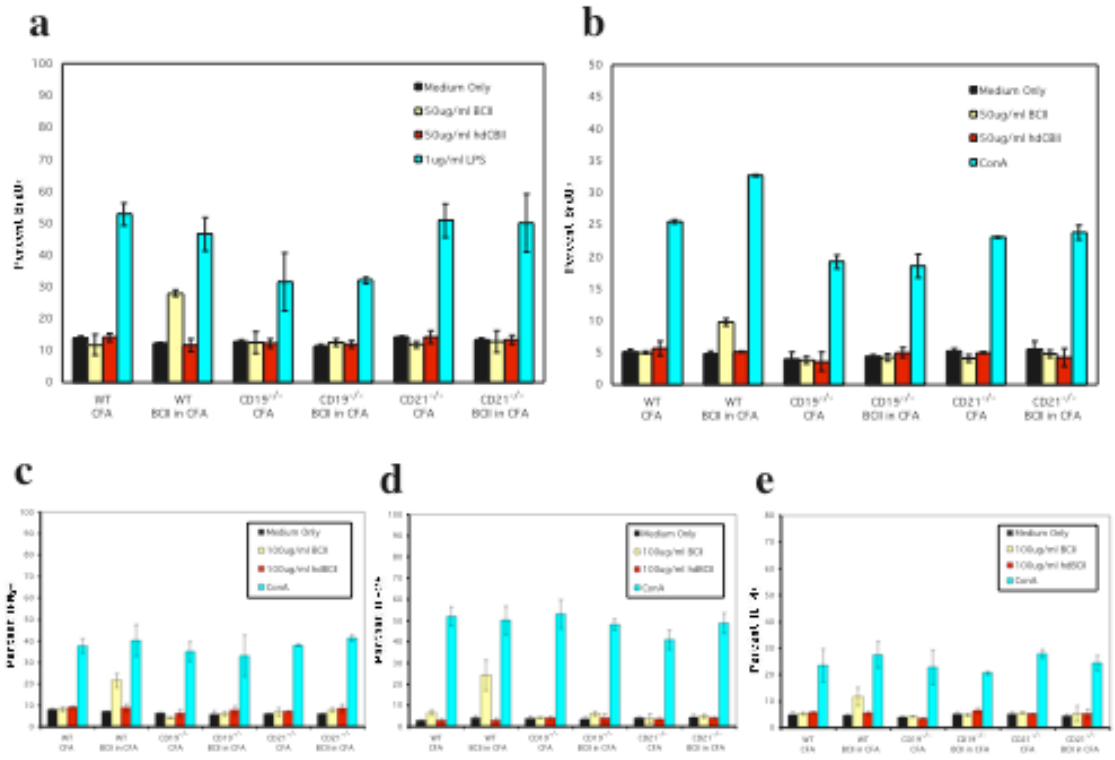

Figure 7 Ineffective generation of BCII-specific B and T cells

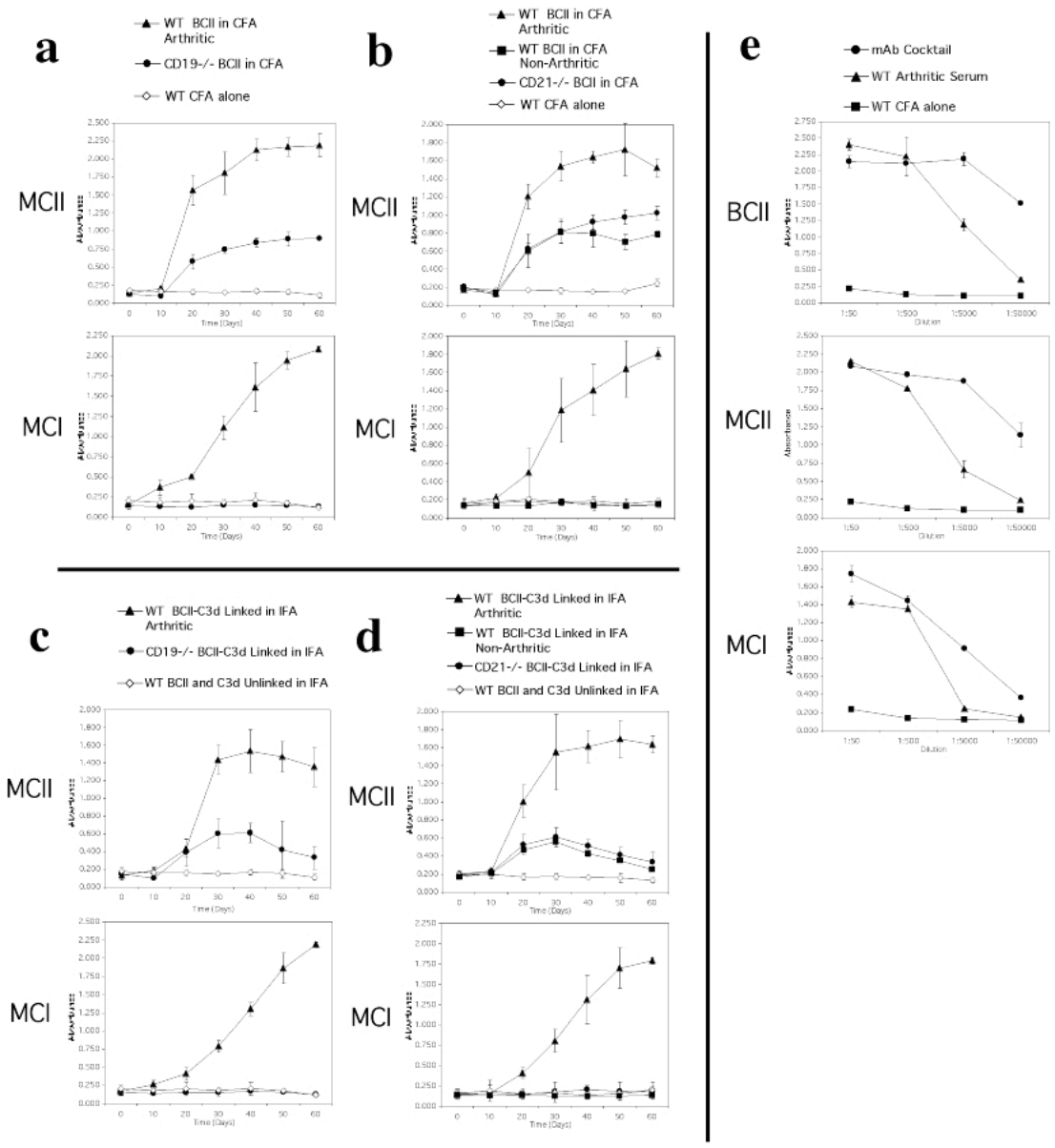

Figure 8 Disease correlates with MCI antibody production

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