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The polarization and regulation of type 2 innate immune responses

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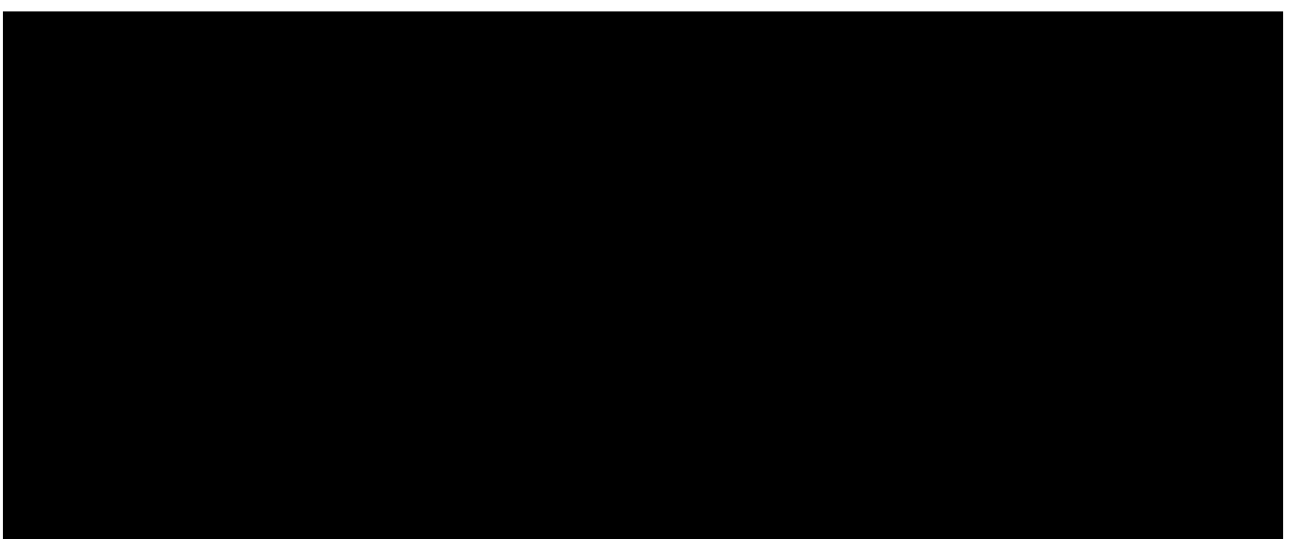

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Abstract

THE POLARIZATION AND REGULATION OF TYPE 2 INNATE IMMUNE RESPONSES

Kanade Shinkai

Recognition of pathogen-derived antigens induces rapid activation of innate immune cells, followed by differentiation of T helper lymphocytes to a subset appropriate for a given pathogen challenge. The development and regulation of type 2 innate and adaptive immune responses were studied using cytokine reporter mice in a murine model of helminthic infection, *Nippostrongylus brasiliensis.* Type 2 innate immunity was initiated but attenuated in the absence of adaptive immunity, reflecting the important role of adaptive immune cells in the normal orchestration of innate immune responses. In the absence of B and T cells, type 2 cytokine production by eosinophils remained intact but eosinophil degranulation was abrogated. Adoptive transfer of CD4+ T cells was sufficient to restore eosinophil degranulation *in vivo*, but only in the presence of cognate antigen. These data define differential requirements for activating pathways for cytokine production versus degranulation in eosinophils, and reveal a modulatory role for helper T cells in full activation of innate immunity. Helper T cells confer antigen-specificity as a requirement for degranulation of eosinophils, akin to the regulation of mast cell degranulation by IgE, which may be an important regulatory checkpoint for preventing tissue injury resulting from the release of

inflammatory mediators from innate immune cells. Consistent with this model, both gross pathologic and histologic signs of lung injury correlated with the presence of both eosinophils and helper T cells during helminth infection *in vivo,* and depletion of either cell subset was sufficient to abrogate tissue damage.

While these studies highlight the finding that components of adaptive immunity regulate innate immune cell effector function, cytokine production by the innate compartment may in turn play a critical role in differentiation of T helper subsets. In the absence of T cell-extrinsic type 2 cytokines, naïve T helper cells failed to polarize to the Th2 subset *in vivo.* The failure of Th2 development correlated with the inability to clear gut worm burden during *N.brasiliensis* infection. Taken together, these results reveal a highly interdependent relationship between the innate and adaptive arms of the immune responses *in vivo.*

Richard A. Corbett
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Chapter I:

The polarization of innate immune responses

Abstract

Pathogen encounter results in the rapid activation of innate immune cells, leading to a diverse spectrum of effector responses. However, in contrast to the prior belief that innate immune cells produce universal, stereotyped responses to pathogens, recent evidence has demonstrated that these responses can be highly specific and appropriate for a given pathogen. Evidence demonstrating the role of pattern recognition in the immune responses of *Drosophila melanogaster,* linking pathogen identity with selective induction of effector responses, has provided an attractive model whereby innate immune responses can be tailored to the nature of pathogen threat. The subsequent discovery and characterization of Toll like receptors (TLRs) in the mammalian immune system has broadened this concept to include both innate and adaptive immune responses, where there is a wealth of evidence demonstrating that TLRs play a role in establishing polarized, multicellular immune responses. However, this evidence almost exclusively supports such a mechanism for type 1 immunity; receptors that mediate type 2 immunity remain unknown.

The notion of type 2 innate immunity has been controversial and thus far, unsupported. This work is dedicated to the description of the discovery and characterization of type 2 innate immunity in host defense to helminths. A mouse model of a helminthic parasite of the gut, *Nippostrongylus brasiliensis,* was utilized to study the kinetics and regulation of type 2 innate immune responses *in vivo.* This introduction summates the state of the field at the time when this project was initiated, and introduces the key players in the experimental system described herein.

Pathogen encounter initiates innate immune responses

Successful immunity *in vivo* comprises the clearance or control of infectious agents and the capacity to more rapidly mount responses during subsequent pathogen challenges. One of the first, and perhaps most important, events during the immune response is the initial encounter of the immune system with pathogens. The significance of this event has been long appreciated, first described in a written publication by Elie Metchnikoff in 1883, which characterized the cellular process by which microorganisms could be phagocytosed by macrophages. Pathogen encounter results in the rapid activation of innate immune cells, leading to a diverse spectrum of effector responses that are sustained and elaborated by ensuing multi-cellular adaptive immune responses. Although this encounter involves highly stereotyped responses, these are by no means universal "danger" signals(Vance, 2000). Recent evidence has demonstrated that these responses can be highly specific and appropriate for a given pathogen.

The ability to generate specific immune responses towards pathogens is exemplified by T helper cell differentiation. Naïve CD4 T cells have the potential to differentiate into discrete subsets of effector T cells in response to infectious challenges *in vivo.* This differentiation generates highly polarized subsets of effector T helper cells – Th1 and Th2 cells – which are defined on the basis of distinct, heritable profiles of immunomodulatory cytokines(Grogan and Locksley, 2002). While the generation of an appropriate T helper subset to a given infectious challenge is critical to successful immunity *in vivo,* factors influencing the decision remain poorly defined. One possible

candidate for such a factor is the innate immune system, which may instruct or reaffirm expansion of a particular effector subset through selective establishment of a polarizing cytokine milieu in response to a given pathogen. Thus, a unifying hypothesis is that the capacity to selectively induce immune responses is most likely achieved at the level of pathogen recognition. This, in turn, leads to the establishment of a polarizing cytokine milieu that reinforces differentiation of an effector T helper subset to carry out an appropriate immune response to the given pathogen at hand. Finally, the establishment of immunological memory by specialized memory T helper cells ensures the expeditious induction of similar immune responses upon rechallenge. Research into how this selectivity is achieved is evolving into one of the most interesting and yet perplexing issues in all of immunology.

The concept of pathogen recognition

The innate immune system remains the prevailing candidate for achieving such selectivity due to its unique ability to discern between pathogens and non-pathogens. This discrimination is achieved by germ line-encoded proteins that recognize highly conserved pattern motifs unique to pathogens, and includes a diverse spectrum of recognition mechanisms. A classic example of such a recognition system is the Toll-like receptor (TLR) family. Recognition of pathogen-derived components by TLRs leads to induction of NFκB and elaboration of inflammatory cytokines such as TNFα, IL-1, and IL-6(Bauer et al., 2001). There are ten published murine TLRs, TLRs 1-10. Of these, six have known specificities: TLR2 (which pairs with TLR1, TLR6, or unknown TLRs to recognize lipoprotein), TLR3 (dsRNA), TLR4 (lipopolysaccharide),

TLR5 (flagellin), TLR7/8 (required for action of anti-viral compounds), and TLR9 (CpG DNA)(Aderem and Hume, 2000; Alexopoulou et al., 2001; Bauer et al., 2001; Beutler, 2000; Hacker et al., 2000; Hayashi et al., 2001; Hemmi et al., 2002; Hoshino et al., 1999; Schnare et al., 2000; Yang et al., 1998). All of these ligands bear structural motifs uniquely found in microbes but not mammalian cells. This recognition is presumably a direct one, although the subcellular location of recognition and processing requirements are controversial. The prevailing hypothesis is that TLRs are recruited to phagosomes, where they may survey internalized contents for the presence of pathogens(Underhill et al., 1999). The extent of processing prior to recognition is not known, and matching the relative ligand specificities with alternative downstream adapter molecules, such as MyD88, TIRAP, and Tollip remains to be determined(Burns et al., 2000; Horng et al., 2001; Zhang and Ghosh, 2002).

Other recognition receptors work upon a similar principle of recognizing pathogens. Fc receptors provide an indirect recognition pathway for microbe recognition, in which pathogens decorated by opsonizing antibodies are bound and internalized by phagocytes, leading to respiratory burst and microbe killing(Ravetch and Bolland, 2001). Other examples of activating pathways are exemplified by circulating effector proteins, such as complement, collectins, C-reactive proteins and coagulation factors. The mannan-binding lectin, an example of the collectin family, pathway exploits subtle differences in the glycochemistry of cell wall sugars between mammals and microbes(Turner, 1998). Binding of microbial sugars by the mannan-binding lectin complex, which consists of the binding domain in concert with two

protease zymogens, activates its proteolytic activities and results in cleavage of C4 and C2 to generate the C3 convertase, thus leading to partial activation of the complement cascade. C-reactive protein (CRP), which recognizes the phosphorylcholine portion of certain bacterial lipopolysaccharides and fungal cell wall mannans, similarly acts to activate the complement cascade. Like the collectins, CRP specifically exploits subtle differences in the chemical nature of phospholipids found in bacterial pathogens that differentiates them from phospholipids found on viable mammalian cells. But this story has an interesting twist. Pathogens may, in turn, exploit self ligand-driven pathways for their own benefit. CRP-binding, together with classical complement components, plays a critical homeostatic role, promoting opsonization and phagocytosis of apoptotic cells without activation of terminal complement casade, thus enabling removal of dying cells without inflammation(Gershov et al., 2000). This capacity may be important for handling apoptotic cells to prevent a loss of tolerance for self-antigens, a protective mechanism to prevent systemic autoimmunity. The CRP-binding by pathogens would thus enable them to infect in a stealth manner, promoting their entry into phagocytes but also suppressing inflammation, and may represent an important pathway for immune evasion.

This subversion is also exemplified by other pentraxin plasma proteins, such as serum amyloid P component (SAP). SAP sequesters extracellular/ exposed DNA in chromatin to prevent its degradation and subsequent inflammatory response, thus preventing loss of tolerance. The importance of this homeostatic function is highlighted by autoimmunity in SAP-deficient mice, which have enhanced antibody responses to

chromatin. However, SAP plays a second role in host defense, in which it selectively binds bacteria. SAP-deficient mice reveal the pleiotropic functions of SAP *in vivo,* in which they are more susceptible to certain SAP-binding bacteria, such as rough variants of *Escherichia coli.* In this case, SAP-binding by certain bacteria allows them to evade phagocytosis by neutrophils, and thus enhances their virulence(Noursadeghi et al., 2000). Bacteria exploit this normal pathway to suppress the immune responses directed towards them; SAP-deficient mice are more successful in controlling these binding strains of bacteria. However, this story is complicated by the finding that SAP-deficient mice display increased susceptibility to other non-binding variants of bacteria, such as smooth strains of *E. coli;* thus, SAP may have a second, distinct role in host defense. In these ways, SAP highlights a beautiful example a situation in which a molecule can play an equally important role in host defense to a role in virulence, and yet be exploited by pathogens. Further work will be required to unravel this paradoxical function of SAP *in vivo.*

A second mechanism for pathogen recognition is the upregulation of host-derived molecules, which display only after pathogen encounter. This system is typified by MIC-A/B, nonclassical class I MHC homologs, which are upregulated in response to viral infections such as cytomegalovirus(Groh et al., 1996). These molecules serve as ligands for NKG2D, an activating family of receptors found on NK, CD8+ T, and γδ T cells(Bauer et al., 1999). Cytokine elaboration and cytotoxic effector functions are driven upon receptor ligand engagement. Thus, pathogen recognition or encounter can lead to a diverse armamentarium of effector functions, ranging from

cytokine induction to phagocytosis, all intended for the purpose of microbe killing or destruction of microbe-harboring cells.

The specificity of innate immune responses: Type 1 and Type 2 innate immunity

The development of polarized cytokine production by innate immune effectors suggests a mechanism by which these cells recognize the origin of antigens and mount appropriate, polarized Type 1/2 responses to pathogens. Previous studies have revealed early, polarized cytokine production by innate immune cells to a variety of environmental stimuli (discussed in Chapter IV). The development of polarized cytokine production in the absence of an adaptive immune compartment has also strengthened the argument for pathogen recognition as a means for initiating both type 1 and 2 innate immune responses. This latter point has been studied in a number of systems. Infection of $Rag1^{-/-}$ mice with the nematode *Nippostrongylus brasiliensis* results in the development of IL-4 producing cells in the lung (discussed in Chapter III). IP injection of *Listeria monocytogenes* or *Aspergillus fumigatus*- derived antigens into $Rag1^{-/-}$ mice generates IFNγ and IL-4 producing populations, respectively (Wakil and Corry, unpublished data). Finally, infection of both $Rag1^{-/-}$ and TCR $C\alpha^{-/-}$ mice with *Leishmania major* demonstrates strain-dependent kinetics of footpad lesion growth, suggesting differences in immunity to *L.major* that is B and T cell independent (Kelly, Fowell, Shinkai, and Locksley, unpublished data). Infected TCR $C\alpha^{-/-}$ mice on a Balb/c background display identical parasite burden and footpad lesion growth to wild type Balb/c mice. In contrast, TCR $C\alpha^{-/-}$ mice on a B10.D2 background consistently demonstrate an intermediate phenotype to that seen in wild type Balb/c mice

(susceptibility) and wild type B10.D2 mice (resistance). This phenotype is recapitulated in Rag1$^{-/-}$ mice, involving both intermediate growth of the footpad lesion without necrosis (unlike TCR C$\alpha^{-/-}$ Balb/c) and intermediate parasite burden, suggesting that in the absence of T cells, there are differences in innate immunity to *L.major* that are genetically-encoded and strain-dependent.

One way that selectivity in the innate immune system may be achieved is by hard-wired pathways linking specific pathogen recognition receptors on the cell surface to distinct programs of cytokine gene expression. Selective activation of cytokine gene transcription could thus be regulated at the level of pathogen recognition by pattern recognition receptors (PRRs). This notion, most recently revived by Charlie Janeway and Ruslan Medzhitov, maintains that surface receptors are activated by pathogen associated molecular patterns (PAMPs) either directly or by products generated by PAMP recognition (i.e., complement receptors and Toll in *Drosophila melanogaster*)(Janeway and Medzhitov, 2002; Medzhitov and Janeway, 1997; Medzhitov and Janeway, 2002). The repertoire of PRRs on a given cell may confer upon it the ability to discriminate "type 1-" versus "type 2-" antigens. In such a system, engagement of a type 1 PRR might result in transcriptional activation of type 1 cytokines, such as IL-12 and IFNγ, whereas engagement of a type 2 PRR would activate type 2 cytokines, such as IL-4, IL-5, IL-13, and IL-17.

Investigations of *Drosophila melanogaster* suggest that this may be an evolutionarily conserved system: that even seemingly simple, stereotyped immune responses maintain a potential diversity of signaling pathways to induce distinct effector

mechanisms. Here, specificity at the level of pathogen recognition enables selective activation of pathways resulting in the production of either certain anti-bacterial peptides or anti-fungal agents. With increasing multicellular complexity of organisms throughout evolution, pathways downstream of pathogen-specific receptors may have been co-opted and linked to a diverse repertoire of antigen receptors of random specificities, clonally distributed on B and T lymphocytes. While this new system enables practically limitless pathogen recognition, it sacrifices the capacity to determine the origin or biological context of their ligands, no longer able to distinguish between deleterious pathogens and innocuous self-antigens. In vertebrates, these ancient principles of insect immunity may instead be maintained in the innate immune compartment, where the type of threat or pathogen encountered determines the induction of distinct effector functions by cytokines. As such, the initial hypothesis is that engagement of pathogen-specific recognition receptors on innate immune effectors activates hard wired signaling pathways that lead to distinct programs of cytokine gene expression, enabling the generation of polarized type 1/type 2 responses to pathogens.

The Toll and IMD pathways in flies enable selective induction of immune responses

The notion that specificity of immune responses is regulated at the level of pathogen recognition was first demonstrated in the invertebrate immune system by genetic studies in *Drosophila melanogaster*(Hoffmann et al., 1999; Hoffmann and Reichhart, 2002). The initial observation was that certain pathogen challenges induced selective activation of a spectrum of antimicrobial peptides, whereas other pathogen

challenges led to the activation of a distinct set of effector molecules. Around the same time, Bruno Lemaitre and Jules Hoffmann were studying the genetic regulation of rapid synthesis of antimicrobial peptides in response to injury(Lemaitre et al., 1996). They described the first recessive mutation, *immune deficiency (imd),* in flies that impairs the induction of many antibacterial peptides during pathogen challenge. However, during their analysis of the *imd* mutants, they made the observation that the induction of antifungal peptide drosomycin remained fully intact and thus revealed the existence of pathways leading to selective activation of antimicrobial peptides(Lemaitre et al., 1995). They subsequently identified the dorsoventral pathway, including *spatzle/ toll/ cactus*, as upstream regulatory molecules of the antifungal response. This observation was groundbreaking for many reasons; first, because it highlighted the pleiotropism of a signaling cascade at distinct developmental stages (important for body patterning during ontogeny, important for immune responses in the adult), and second, because they were the first to provide evidence for this pathway in some, but not all, antimicrobial responses. Furthermore, through analysis of a series of fly mutants, they were able to define and distinguish pathways leading to drosomycin (*spatzle/ toll/ cactus* pathway) and those leading to diptericin and drosocin (*imd* pathway). Thus, in demonstrating selective expression of genes encoding antimicrobial peptides that were regulated by distinct genetic pathways, they were the first to propose the model of how immune responses can be regulated at the level of pathogen recognition. Since 1996, a number of groups have identified the remaining pieces of this pathway, including the differential usage of NFkB family members to selectively target distinct groups of

antimicrobial peptide-encoding genes(Lemaitre et al., 1997; Rutschmann et al., 2000), and the recent discovery of peptidoglycan recognition protein (PGRP) within the Toll and *imd* pathways. PGRP, which was first described as a pathogen recognition molecule upstream of the prophenoloxidase pathway (phenoloxidase activation leads to the generation of melanin granules to sequester invading pathogens, i.e., the invertebrate granuloma), has now been described as an upstream regulator of both the Toll and *imd* pathways (Choe et al., 2002; Gottar et al., 2002; Kang et al., 1998). PGRP-SA (short form) has been shown to mediate recognition of Gram-positive bacteria through the Toll pathway, and epistasis experiments have placed PGRP-LC upstream of the *imd* pathway, mediating recognition of Gram-negative bacteria (Gottar et al., 2002). Thus, discrimination of distinct classes of pathogens is mediated by differential use of putative recognition proteins to drive selective activation of each pathway. Intriguingly, mammalian PGRP has been identified as a gene that is upregulated in two different models of helminthic infection, and is expressed on murine lung eosinophils activated by *Nippostrongylus brasiliensis*(Kang et al., 1998; Liu et al., 2000).

Mammalian Toll-like receptors

It was not long after the initial studies of TLRs in *Drosophila* that the mammalian Toll homolog was identified. The stretch was only a short one, as there were already many intriguing parallels between Toll-mediated induction of NFkB, leading to antimicrobial peptide expression, and NFkB-mediated induction of cytokine genes in the LPS response. In a paper that Charlie Janeway self-indulgently refers to as

"the most important paper of modern biology," Ruslan Medzhitov – whilst training in Charlie's lab – made the first description of a mammalian Toll homolog(Medzhitov et al., 1997). They pointed out the similarity in structural homology between the fly and human Toll, including a type I transmembrane protein with leucine-rich repeat extracellular domain and a cytoplasmic domain homologous to that of the IL-1 receptor. The demonstration that this pathway in mammals was upstream of proinflammatory cytokines such as IL-1, IL-6, and IL-8 clearly reiterated its role in invertebrate immunity, and thus this pivotal paper highlighted the structural and functional evolutionary conservation that suggested the importance of this recognition system within biology.

The TLR field rapidly expanded to encompass a diverse and competitive host of investigators formerly from the LPS-signalling, cytokine regulation, *Drosophila* geneticists, NFkB signaling, and sepsis fields. While many new players in the TLR-signaling pathway have been identified, the most important contributions have been made by cellular immunologists – in which the role of TLRs in the induction of immune responses has most clearly been demonstrated. Fernando Bazan described the structures of the human TLRs (Rock et al., 1998), and much research focused on the identification of ligand specificities for each of the known receptors.

The striking role of TLRs was demonstrated by studies *in vivo,* which addressed the relationship between specific TLR function and susceptibility to sepsis. The TLR2-/- and TLR4-/- mice fail to develop a septic response to lipoproteins and lipopolysaccharide, respectively, and the MyD88-/- mice also fail to respond to

lipopolysaccharide(Adachi et al., 1998; Hoshino et al., 1999; Kawai et al., 1999; Takeuchi et al., 1999). Likewise, TLR5-/- mice fail to produce systemic IL-6 in response to flagellin(Hayashi et al., 2001), and so repeatedly, the critical role of TLRs in pattern recognition *in vivo* have been recapitulated in numerous pathogen challenge studies. Most importantly, the absence of TLRs increases susceptibility to infection due to an inability to limit pathogen outgrowth, a testament to their role in immunity to infection. However, the emerging story is not as straightforward as previously thought. Although some of the cellular responses are abolished in the absence of MyD88, TLR4 – but not TLR9 – can still induce downstream signaling leading to activation of NFkB and MAPKs. The recent discovery of alternative adapter molecules for TLR4, such as TIRAP, in part may explain this inconsistency; however, what is still unclear is how these signaling pathways are differentially induced if they require the same upstream receptor(Horng et al., 2001). Already, differential signaling pathways through utilization of alternative adapter proteins has been demonstrated to induce distinct cytokines – for example, TLR4, through TIRAP, lies upstream of IRF3 and IFNβ, leading to Stat1α/β-dependent gene expression; this pathway is distinct from TLR2 signaling(Toshchakov et al., 2002). What remains to be seen is the mechanism by which specificity can be maintained – for certainly high dose, single pathogen challenges are less common in real life. This will probably involve pathways at the level of dendritic cell antigen processing, at the level of TLR-mediated signal transduction and NFkB activation, and at the level of proinflammatory cytokine responses. At first glance, this scenario suggests that specificity may be maintained

even more upstream of the TLRs, perhaps PGRPs, and this idea is consistent with the tight regulation of Toll and IMD signaling pathways by upstream protease cascades and protease inhibitors (serpins) in *Drosophila.*

Perhaps the most important mechanistic description of TLR function has been made for its role in dendritic cell maturation. Two papers, published back to back in Nature in the summer of 1997, together elucidated the pathways by which inflammatory stimuli – such as LPS – led to the dramatic alterations in dendritic cell biology, or dendritic cell maturation(Cella et al., 1997; Pierre et al., 1997). Although the recognition of immature dendritic cell (i.e., highly endocytic, low surface levels of Class II MHC) and mature dendritic cell (i.e., low endocytic, high surface levels of Class II MHC-peptide complexes) phenotypes was long appreciated, the role of inflammatory stimuli had not been causally linked to these developmental states. This observation in part correlated with a complex, inflammation-induced developmental program of dendritic cells, including upregulation of costimulatory molecules (i.e., CD80 and CD86), changes in endocytosis rate, and alterations in intracellular trafficking of Class II molecules. Kadowaki and colleagues later explicated the role of TLRs in this process, and numerous studies have supported the role of TLRs in dendritic cell maturation(Kadowaki et al., 2001).

The idea that TLRs (and other receptors) on DCs can decipher environmental signals, allowing DCs to mature and polarize (or tolerize) T cell responses according to the nature of the inflammatory stimulus, provides an attractive mechanistic model for linking pathogen recognition with appropriate ensuing immune responses. The ability

to discriminate between different pathogens was clearly linked to TLR repertoire in the aforementioned study by Kadowaki and colleagues, in which differential expression of TLRs by distinct DC subsets conferred the ability to sense the identity of pathogens. Medzhitov and colleagues have provided evidence that this may play a role in T helper polarization, showing that Th1 polarization is abrogated in the absence of MyD88(Schnare et al., 2001). These initial studies investigated antigen-specific Th1 responses to systemic immunization with OVA peptide (in complete Freund's adjuvant, CFA). Here, T cells from immunized MyD88-/- mice failed to proliferate and produce IFNγ in response to rechallenge *ex vivo.* Interestingly, these mice instead showed a type 2 bias, with intact IL-13 production to rechallenge *ex vivo,* in addition to high baseline serum IgE. The authors account for this phenotype by citing differences in the regulation of accessory signals by TLRs, which in turn is regulated by pathogen recognition. This notion was, in part, supported by the demonstration in the same study that dendritic cell maturation to mycobacterial challenge (components of CFA) requires MyD88; both IL-12 production and costimulatory molecule upregulation are dependent on the function of this adapter. Still, the phenotypes observed may be attributed to much more complex physiological defects *in vivo,* such as the inability of dendritic cells to properly mature and migrate from the periphery to nodes. This may only be an unlikely possibility, as type 2 responses are intact in these mice. The remaining explanation is that the receptors that mediate type 1 immunity are distinct from those that mediate type 2 immunity. Whether there are single or multiple signaling pathways mediating type 1 immunity remains unclear, as distinct pathways may be suggested by

the phenotype of Rip-2 (RICK/CARDIAK)-/- mice, which have deficient Th1 responses but intact Th2 responses(Allan et al., 1999; Kobayashi et al., 2002). The absence of default type 2 immune responses in these mice is corroborated by other work by Dragana Jankovic and colleagues, who have shown that CD4 T cells in IL-12-/- mice do not default to a Th2 pattern(Jankovic et al., 2002). In this work, they also show that T cells in MyD88-/- mice default to a Th2 response even when challenged with a type 1 stimulus; this may reflect a role for pathogen recognition in coordinating signals to silence opposing cytokine gene loci. Thus, the evidence – albeit preliminary – are beginning to point at a mechanism by which pathogen recognition differentially induces polarizing immunity. The precise nature of these initial signals, and whether they are carried out by polarizing cytokines or novel effector molecules, remains to be seen.

The notion of type 2 innate immunity

Despite the evidence supporting a role for TLRs in the initiation of type 1 immune responses, receptors or signaling pathways mediating type 2 immunity have not yet been described. My efforts were directed at establishing a mouse model system to characterize the existence and regulation of type 2 innate immunity, and after 3 years, we have finally arrived at a tractable system with which to pursue the receptors inducing type 2 innate immunity. The following thesis details the system I established, discusses my findings, and considers the strategies and caveats that should be taken to interrogate the molecular basis for type 2 innate immunity.

Mouse model of Type 2 immunity: *Nippostrongylus brasiliensis*

Numerous animal models have enabled the study of type 2 immune responses *in vivo.* Among them, perhaps the best studied are antigen- or fungal-induced airway hyperreactivity, contact sensitivity, and parasite infection models. These studies have highlighted many common principles between parasitic infection and allergic disease, and provided systems to ask focused questions into the pathogenesis of type 2 immunity *in vivo.* The well-studied nematode parasite of the gut, *Nippostrongylus brasiliensis,* is a potent inducer of type 2 immune responses in rodent hosts(Finkelman et al., 1997). Infection with this helminth, which occurs through skin during natural infections, is initiated in the lab by subcutaneous injection of 500 third-stage infective larvae (L_3). The worms invade the dermal blood vessels, through which they migrate to the lungs upon embolizing in the narrow pulmonary vasculature at 24-48 hours post-infection. There, they break through the pulmonary vessels into the alveolar air spaces, crawl up the trachea, and are subsequently swallowed into the gut at 48-72 hours post-infection, where they complete sexual maturation in the jejunum, mate, and lay eggs. Eggs and adult worms are passed out of the gut in immunocompetent hosts in less than 2 weeks post-inoculation, and the eggs give rise to infective larvae to reinitiate the infection cycle. Several differences exist between the suitability of hosts; rats are competent hosts for the complete life cycle, whereas mice are not – they fail to give rise to infectious larvae. However, mice display a vigorous type 2 immune response to *N.brasiliensis* infection, and have provided a genetic system in which to study the specific mechanisms of the immune response that are required for host defense.

Several components of the type 2 immune response are required for clearance of worm burden in infected murine hosts. Helper T cells are required for clearance of worms from the gut. Specifically, Th2 cells may be the critical subset that mediates this effect; anti-CD4 mAb treatment, but not anti-CD8 mAb, abrogates worm expulsion(Katona et al., 1988). Th2 cytokines, such as IL-13, but not IL-4 or IL-5, are required for host protection, and there is a wealth of evidence to suggest that this cytokine exerts its effect through IL-4R signaling, as both the IL-4R and its signaling component Stat6 are necessary(Urban et al., 1998). IL-4, although required for clearance of other intestinal helminthic parasites, is not required but is sufficient to clear chronic infections of *N. brasiliensis* in immunocompromised hosts, such as $Rag1^{-/-}$ or SCID mice, as demonstrated by treatment with IL-4 or reagents that stabilize IL-4 half-life in the serum(Urban et al., 1995). This treatment rescues via a mechanism that is B, T, and mast cell- and leukotriene-independent (as demonstrated by treatment in SCID or 5-lipoxygenase$^{-/-}$ mice)(Finkelman et al., 1997), and the data that hosts with defects in IL-4 signal transduction fail to clear worms suggests that this cytokine acts on the host and not the worm. Continuing studies are further examining the precise mechanisms of cytokine action. Urban and colleagues have demonstrated through the generation of bone marrow chimeric mice that the requirement for IL-4 signaling resides in the non-hematopoietic compartment. Here, IL-4 signaling may act to induce alterations in a wide range of gut epithelial physiology, such as mucus production, smooth muscle contractility, and enhancement of chemokine receptors or other trafficking addressins such as VCAM-1(Urban et al., 2001). Finally, in an immunocompetent host, B cells are

probably not required, as IgM-suppressed mice, which produce little antibody, expel *N.brasiliensis* normally(Jacobson et al., 1977).

The role of innate immunity in host protection is complex, as effectors such as mast cells may play a dualistic role in promoting inflammation (hence a dramatic mastocytosis in the gut) yet promoting fecundity; treatment of mice with anti-stem cell factor anti-serum decreases both mast cell number and parasite egg burden, whereas treatment with stem cell factor promotes an increase in both mastocytosis and fecundity(Newlands et al., 1995). Eosinophils are dispensable for worm clearance, as neutralization of IL-5 has no effect on worm expulsion of *N.brasiliensis* or many other gut helminths(Coffman et al., 1989). The role of eosinophils, however, may be to kill *N.brasiliensis* larvae within the lung, thus reducing worm burden reaching the gut. The notion that eosinophils serve to reduce numbers of infective larvae as they migrate through the lung is supported by data that increased amounts of IL-5 – such as in the transgenic IL-5 over expressing mice – have lower worm burden compared to wild type mice(Dent et al., 1999). However, the conclusion that no single immune component is required may only serve to highlight the highly complex and functionally reiterative response – requiring synergy between type 2 cytokines and other innate immune effector functions – to induce host protection.

The *N. brasiliensis* model, unlike other models of type 2 immune responses such as Leishmaniasis, has no known strain differences. It was this aspect of the model that makes it an extremely attractive candidate for the study of type 2 innate and adaptive immune responses – as information gained from these studies should be generalizable to

all murine hosts. Thus, the ability to highlight common principles of helminthic disease, the short infection cycle, and the "relative" ease of maintaining parasites made this the ideal system in which to study the evolution and regulation of innate immune responses *in vivo*. This system also ultimately highlighted the reciprocal influences between the innate and adaptive arms of the immune response, an opportunity afforded only by interrogating the mouse model of infection. It takes a long time, but it's well worth the wait.

Conclusion

This thesis is dedicated to the analysis of the evolution and regulation of type 2 innate immune responses in host defense to helminths. It will begin with the report of the generation of a novel IL-4 bicistronic cytokine reporter mouse in the Locksley lab by Markus and Katja Mohrs, and the characterization of this reporter mouse - both *in vitro* and *in vivo* - by Markus Mohrs and myself. This novel technology enabled the experimental interrogation of the origins and evolution of Type 2 immunity *in vivo;* this work demonstrated that Type 2 innate immune responses are initiated independently of adaptive immunity but also revealed the dynamic interplay between the innate and adaptive arms of the immune response to infection. Most importantly, this research defined distinct pathways of activation in eosinophils, distinguishing differential requirements for cytokine production versus degranulation. Eosinophil degranulation is regulated *in vivo* by helper T cells; this may represent an important and previously unappreciated regulatory checkpoint during immune responses. Full activation of innate immune effectors may be tightly controlled by antigen-specific T cells for the

purposes of focusing tissue injury at sites of high antigen load. Preliminary work addressing the contribution of Type 2 innate immunity towards T helper effector differentiation is presented in chapter IV. Chapter V addresses the attempts to establish an *in vitro* experimental system to study eosinophil degranulation and thoughts for future directions of the project. Chapter VI is a methods section, detailing the experimental and support protocols used for the work described. In the final chapter, chapter VII, a previously published review of the structural and functional analyses of nonpolymorphic Major Histocompatibility Complex (MHC) molecules speculates the origins of molecular determinants of immune recognition in essential homeostatic processes required for tissue maintenance. This last chapter was added mostly for sentimental reasons, but also because it addresses the origins of immunity itself, introducing the idea that the immune system may have evolved from much more basic cellular processes.

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Chapter II:

Analysis of Type 2 immunity in vivo using a bicistronic IL-4 reporter

Abstract

Effector T cells mediate adaptive immunity and immunopathology, but methods for tracking such cells in vivo are limited. We engineered knock-in mice expressing IL-4 linked bicistronically via a viral IRES element with enhanced green fluorescent protein (EGFP). Reporter T cells primed under Th2 conditions showed sensitive and faithful EGFP expression and maintained endogenous IL-4 production. After Nippostrongylus infection, reporter expression demonstrated evolution of type 2 immunity from tissue lymphocytes and thence to lymph node CD4+ T cells, which subsequently migrated into tissue. Reporter expression of tissue, but not lymph node, lymphocytes was Stat6-dependent. Transferred EGFP-positive CD4+ T cells from infected animals conferred protection against Nippostrongylus to immunodeficient mice. These mice will provide a valuable reagent for assessing immunity in vivo.

Introduction

Host immunity and immunopathology are mediated largely by cytokines, short-lived effector molecules rapidly secreted by different cell types in many different tissue compartments. Analysis of cytokine activities in vivo has been extrapolated from in vitro studies using isolated cells and through generation of cytokine knock-out and transgenic over-expressing mice. Although many insights have been gained by these approaches, disadvantages are also apparent. First, analysis of cells in vitro removes them from their in situ environment and from potentially critical signals present in tissues. Second, exogenous cytokines are often provided using amounts that might not be physiologic. Third, knock-out or reporter knock-in mice preclude the ability to judge the contributions of the cytokine that has been deleted, since it can no longer contribute to the immune response. Finally, over-expression and transgenic reporter systems frequently result in loss of key regulatory elements that may be required to modulate expression when the cytokine is in the genome in its appropriate DNA context.

The need for cytokine reporters that can be introduced without deleting the cytokine itself led us to explore viral internal ribosomal entry site (IRES) elements as a mechanism for faithfully marking cells transcribing cytokines. This approach was applied successfully in mice to map projections of olfactory sensory neurons using an IRES-β-galactosidase reporter (Mombaerts et al. 1996, Wang et al., 1998). Using enhanced green fluorescent protein (EGFP), we elected to mark the endogenous IL-4 gene in mice in an attempt to visualize the host immune response in vivo. Produced by lymphocytes and non-lymphoid cells, IL-4 remains the canonical marker for Th2 cells,

which have been implicated in mucosal immunity against parasites and in allergic and atopic responses, including asthma (Finkelman et al., 1997; Wills-Karp, 1999). The ability of IL-4 itself to direct the differentiation of IL-4-producing CD4+ and CD8+ T cells has suggested an autocrine positive feedback mechanism for the generation of a type 2 immune response in vivo. However, the initial source of IL-4 necessary for Th2 development remains elusive - CD4+ T cells themselves are one candidate population. As such, a functional endogenous IL-4 gene might be critical in attempting to assess the biology in the intact animal. We characterize the generation of mice containing an endogenous reporter for IL-4-expressing cells using both in vitro assays and in vivo during infection with *Nippostrongylus brasiliensis* (Urban et al., 1998). The ability to identify IL-4-producing cells in vivo without the need for restimulation or cloning should provide a powerful reagent for assessing protective and pathologic correlates of immunity.

Results

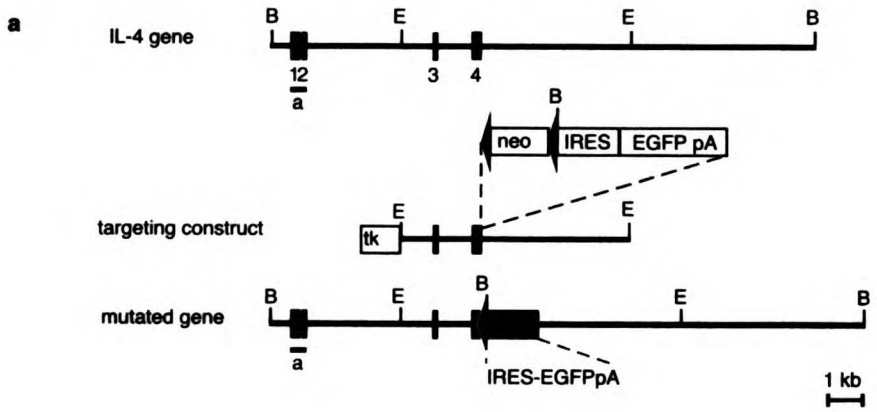
Generation of 4get mice. The targeting vector consisted of an IL-4 genomic EcoRI fragment comprising exons 3 and 4 with approximately 5 kb of 3' untranslated sequence (Figure 1a). A loxP-flanked neomycin expression cassette linked to the IRES-EGFP construct with the polyadenylation signal from bovine growth hormone was introduced just downstream of the translational stop and upstream of the endogenous polyadenylation signal in the 3' untranslated region of exon 4. A herpes simplex thymidine kinase expression cassette was cloned to one flank of the targeting construct for use as a counter-selectable marker for transgene integration. The construct was electroporated into 129/SvJ-derived PrmCre ES cells which express the Cre recombinase under control of the germline-specific protamine promoter, thus allowing Cre-mediated deletion of the neomycin gene from the male germline (O'Gorman et al., 1997). G418- and gancyclovir-resistant ES cell clones were screened for correct integration by Southern blot and targeted clones were injected into C57BL/6 blastocysts. Chimeric males were bred to wild-type BALB/c mice and offspring were screened by Southern blot for the mutated allele and for deletion of the neomycin selection cassette. Heterozygous animals were bred to BALB/c mice and offspring were screened for the presence of the reporter and absence of the Cre transgene. These mice were interbred to obtain homozygous animals (Figure 1b). The targeted mice, which were healthy and exhibited no obvious phenotype, were designated IL-4/GFP-enhanced transcript, or 4get, mice.

Figure 1. Targeting of the IRES-EGFP reporter into the mouse IL-4 locus.

a, Map of the mouse IL-4 locus, the reporter targeting construct and the mutated gene. A genomic 5 kb EcoRI fragment from the wild-type locus (top panel) was mutated by introduction of a loxP-flanked (filled triangles) drug-selectable neomycin cassette (neo), followed by an internal ribosomal entry site (IRES) element, enhanced green fluorescent protein (EGFP) and a polyadenylation signal (pA) (middle panel). After successful targeting in PrmCre embryonic stem cells, the neo cassette was deleted by cre-mediated recombination in the male germline of chimeric males, creating the final arrangement depicted at the bottom of the panel. Numbered filled boxes indicate exons. tk: thymidine kinase cassette; B: BamHI; E: EcoRI.

b, Southern blot analysis of DNA from tail biopsies from offspring of heterozygous breeding. DNA was digested with BamHI and hybridized with probe **a** (dark line, top panel, Fig. 1A above). A new BamHI site introduced by the targeting construct results in a 7 kb fragment in place of the 17 kb BamHI fragment in the wild-type locus. Genotypes are indicated as wild-type (+/+), heterozygous knock-in (+/4get) and homozygous knock-in (4get/4get).

Figure 1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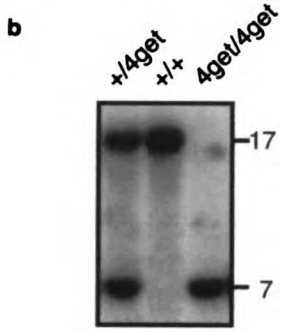
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Faithful IL-4 production by 4get naive CD4+ T cells in vitro. Naive (small, resting, EGFP-, CD62L^{hi}) CD4+ and CD8+ T cells were sorted from 4get mice to >99% purity. To assess fidelity of the reporter, naive T cells were stimulated with anti-TCR/anti-CD28 antibodies and irradiated APC under neutral (IL-2), Th1 (IL-2 with IL-12 plus anti-IL-4) or Th2 (IL-2 with IL-4 plus anti-IFN-γ) conditions, and analyzed for EGFP expression at designated times (Figure 2a, 2b). Under Th1 or neutral conditions, no stable EGFP expression was observed (Grogan et al., 2001). In contrast, under Th2 conditions, EGFP could be detected as early as 36 hours, prior to the onset of cell division (40-45 hrs, data not shown), as previously demonstrated using different methods (Richter et al., 1999; Laouar and Crispe, 2000; Grogan et al., 2001). The proportion of CD4+ T cells expressing EGFP peaked at days 4-5 in vitro, with 72-92% of cells positive in various experiments (Figure 2a and data not shown). Expression of EGFP at the peak of the response on days 3.5-4.5 after activation was significantly attenuated in the absence of Stat6 (Figure 2c).

In order to analyze cells in relation to cell division, we labeled the membranes of naïve CD4+ T cells with the red fluor PKH26 prior to cell stimulation and polarization. These experiments demonstrated that essentially all cells that undergo cell division express EGFP and are CD62L^{lo}. The EGFP-negative cells remaining in the culture consisted primarily of a population of undivided cells that maintained their CD62L^{hi} phenotype, possibly indicating incomplete activation (Figure 2d and data not shown). Of note, the EGFP mean fluorescence intensity (MFI) was not related to the numbers of cell divisions, consistent with the early induction and constant level of IL-4 transcripts

as assessed by sensitive RT-PCR methods (Grogan et al., 2001). By comparison, standard methods for intracellular IL-4 trapping after PMA/ionomycin and brefeldin A treatment of 5-day, Th2-primed naive CD4+ T cells revealed 15-28% IL-4-positive cells (data not shown), consistent with published studies (Openshaw et al., 1995; Noben-Trauth et al., 2000; Ouyang et al., 2000).

Polarization of CD4+ T cells from heterozygous 4get mice resulted in the same percentages of EGFP+ cells, but the MFI was only half that from homozygous 4get cells (Figure 2a and Table 1). Naive 4get CD8+ T cells displayed essentially the same activation kinetics under Th2 priming conditions, but the proportion of EGFP-expressing cells and the MFI per cell were less as compared to CD4+ T cells. Of note, the proportional numbers and the MFI of EGFP+ CD8+ T cells was the same using cells from either heterozygous or homozygous 4get mice, consistent with monoallelic IL-4 expression (Figure 2b).

To test the fidelity of the endogenous IL-4 genes, CD8-depleted spleen cells from wild-type and 4get heterozygous and homozygous mice were primed with TCR/CD28 antibodies under Th1, neutral, or Th2 conditions for 5 days, re-stimulated for 48 hr, and the supernatants were analyzed for cytokines by ELISA (Figure 3a). In multiple experiments, cells from these different mice produced comparable amounts of IL-4 and IFN-γ under the various priming conditions, indicating that the endogenous IL-4 gene remained intact and functional in 4get mice. Similarly, when assessed for numbers of IL-4-producing cells using ELISPOT assays, CD4+ T cells from wild-type and 4get mice primed under Th2 conditions generated comparable numbers of IL-4-

producing cells (Figure 3b); priming of Stat6-deficient 4get cells diminished the numbers of IL-4-producing cells, in accordance with the flow cytometric analysis and published observations (Kaplan et al., 1996).

Figure 2. EGFP expression of 4get T cells in vitro.

a, FACS histograms of wild-type (gray fill), heterozygous 4get (thin line), homozygous 4get (bold line) CD4+ T cells. Naïve CD4+, EGFP- T cells were purified by cell sorting and cultured under Th2 conditions in the presence of antigen presenting cells. After days indicated, EGFP expression was analyzed after gating on CD4+ cells.

b, As in **A**, but naïve CD8+ T cells were purified and primed instead of CD4+ T cells.

c, As in **A**, but $Stat6^{-/-}$/4get (dotted line) CD4+ T cells were compared to wild-type (gray fill) and homozygous 4get cells (bold line).

d, Purified, naïve CD4+, EGFP- homozygous 4get T cells were labeled with the red fluor PKH26. Cells were cultured under Th1, neutral or Th2 conditions in the presence of antigen presenting cells and analyzed on day 4 after gating on CD4+ cells. Wild-type cells and unstimulated cells were used to define quadrant gates.

Figure 2

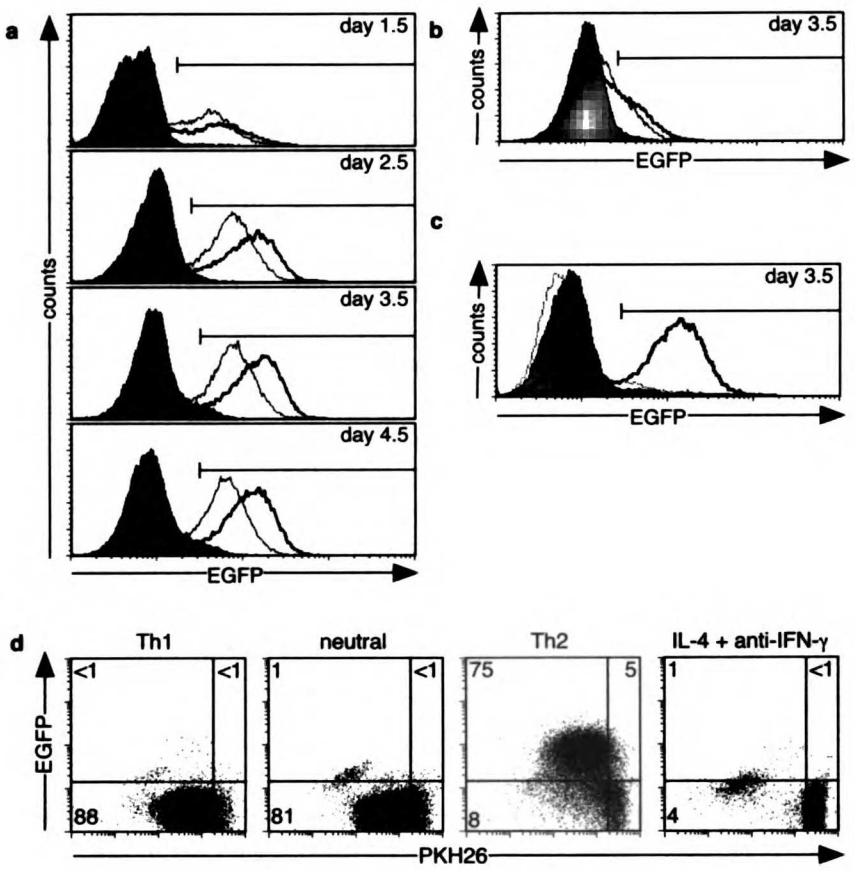

a
day 1.5
day 2.5
day 3.5
day 4.5
counts
EGFP
b
day 3.5
counts
EGFP
c
day 3.5
counts
EGFP
d
Th1
neutral
Th2
IL-4 + anti-IFN-γ
EGFP
PKH26
<1
<1
88
1
<1
81
75
5
8
1
<1
4

Table 1. Expression of EGFP in CD4 cells under Th2 conditions

Genotype	**Day 1.5**		**Day 2.5**		**Day 3.5**		**Day 4.5**	
	%	MFI	%	MFI	%	MFI	%	MFI
+/+	< 1	n.a.	< 1	n.a.	< 1	n.a.	< 1	n.a.
+/4get	34	48	63	87	70	88	66	75
4get/4get	30	68	57	140	73	157	73	136

n.a. not applicable

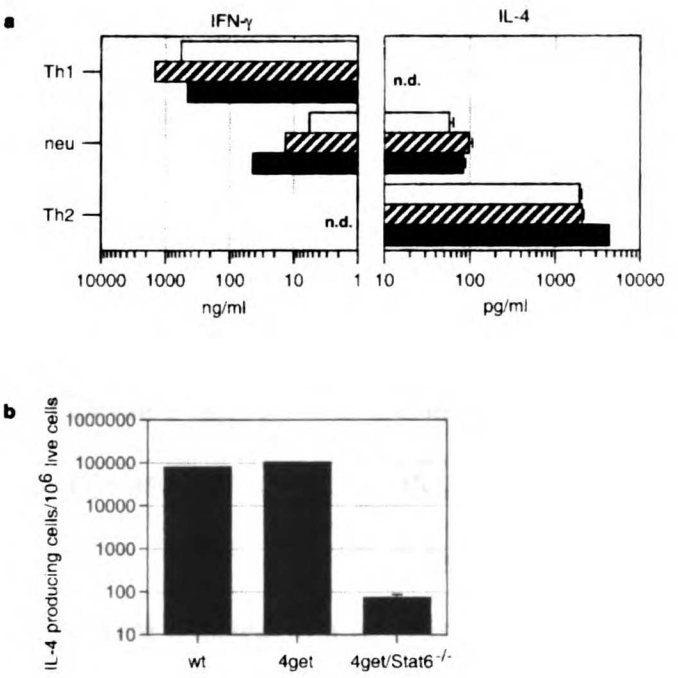
Purified naive CD4+ T cells from wild-type (+/+), heterozygous 4get (+/4get) and homozygous 4get (4get/4get) mice were incubated with anti-TCR/CD28 mAbs under Th2 conditions. On indicated days, samples of cells were analyzed for the percentage of cells that expressed EGFP (%) and for the mean fluorescent intensity (MFI). Data are representative of three comparable experiments.

Figure 3. Cytokine secretion by 4get T cells.

a, Splenocytes from wild-type (+/+, open bars) and heterozygous (+/4get, hatched bars) or homozygous (4get/4get, filled bars) 4get mice were depleted of $CD8^+$ cells by complement lysis and stimulated under Th1, neutral or Th2 conditions for 5 days. Cells were washed and re-stimulated, and supernatants were collected after 48 hr and analyzed for IL-4 and IFN-γ by ELISA. Mean and standard deviation of triplicate cultures are shown.

b, Purified, naïve CD4+, EGFP- T cells from wild-type, 4get and 4get/Stat6-/- were cultured under Th2 conditions with antigen presenting cells. After 5 days cells were re-stimulated with plate-bound anti-CD3 mAb for 6 hr and serial dilutions of viable cells were analyzed by ELISPOT. Mean and standard deviation of triplicate cultures are shown.

Figure 3


a
IFN-γ
IL-4
Th1
neu
Th2
n.d.
n.d.
10000 1000 100 10 1
10 100 1000 10000
ng/ml
pg/ml
b
IL-4 producing cells/10^6 live cells
1000000
100000
10000
1000
100
10
wt
4get
4get/Stat6-/-

Identification of IL-4-producing cells in vivo. Infection of mice with the helminth, *N. brasiliensis,* induces strong type 2 immunity. Subcutaneously inoculated larvae invade venules, embolize to the lungs after 1-2 days, escape into the alveolae, ascend the trachea, descend the esophagus and mature into egg-laying adults after arriving in the small bowel after 5-6 days (Finkelman et al., 1997). Infection is characterized by massive T cell and eosinophil infiltration into the lung during larval migration, along with mesenteric lymphadenopathy and intestinal mast cell hyperplasia, mucus production and elevated IgE levels as worms mature in the gut. Expulsion of adult worms after 10 days and immunity to re-infection is dependent on Th2 cells (Finkelman et al., 1997).

4get mice were infected with *N. brasiliensis* and examined after 10 days. No adult worms could be detected in the intestine at this time. To assess whether the reporter had been appropriately activated in vivo, cell suspensions were created from the lung and mesenteric lymph nodes, as well as from spleen and peripheral lymph nodes that did not drain the site of inoculation of the parasites. Non-infected littermates were examined concurrently.

Approximately 40% of the CD4+ T cells in the lungs of infected 4get mice spontaneously expressed EGFP without the need for re-stimulation (Figure 4a). Expression in the spleen and mesenteric lymph node CD4+ T cells was comparable in various experiments (approximately 15%), and consistently greater than in non-draining peripheral lymph nodes (approximately 5%). CD8+ T cells, although capable of EGFP expression when primed under Th2 conditions in vitro (Figure 2b), were not activated in

vivo, consistent with experimental evidence that these cells are not required in immunity against Nippostrongylus (Brown et al., 1996). In comparison, uninfected mice demonstrated baseline EGFP fluorescence in approximately 1% of spleen, lung, mesenteric or peripheral lymph node CD4+ T cells (or CD8+ T cells - data not shown) (Figure 4a). IgE levels in infected 4get mice were comparable to wild-type littermates (1040 +/- 90 ng/ml vs. 960 +/- 105 ng/ml, respectively), confirming that endogenous IL-4/IL-13-mediated Ig class-switching remained functional in these mice.

To examine the kinetics and cell composition of the EGFP-expressing cells, 4get mice were infected with *N. brasiliensis* and examined at various days after infection in conjunction with cell surface markers (Figure 4b). By 3 days after infection, when larvae are migrating through the lung, CD4+ T cells in pulmonary, but not lymphoid tissues, were already expressing EGFP above the background from uninfected mice. Analysis of these cells revealed a predominant DX5+, CD4+, CD3+ phenotype consistent with NK T cells (Bendelac et al., 1997). CD4+, DX5-, TCR αβ+ T cells appeared between days 5 and 8 in lung tissue and continued to increase in numbers through day 13. In the mesenteric lymph nodes, over 80% of the EGFP+ cells were conventional CD4+ T cells, although EGFP-expressing DX5- and DX5+ CD4+, TCR αβ+ T cells also appeared in spleen and peripheral lymph node compartments during this period. Mediastinal lymph nodes could not be reliably identified early in infection, but by day 13 the EGFP+ cells in these nodes were essentially all DX5- CD4+ T cells (data not shown). Finally, non-T cells, identified as predominantly eosinophils in the lung based on forward scatter profile and morphologic staining characteristics (data not

shown), were identified by EGFP fluorescence as a population that appeared in the lungs beginning on day 8.

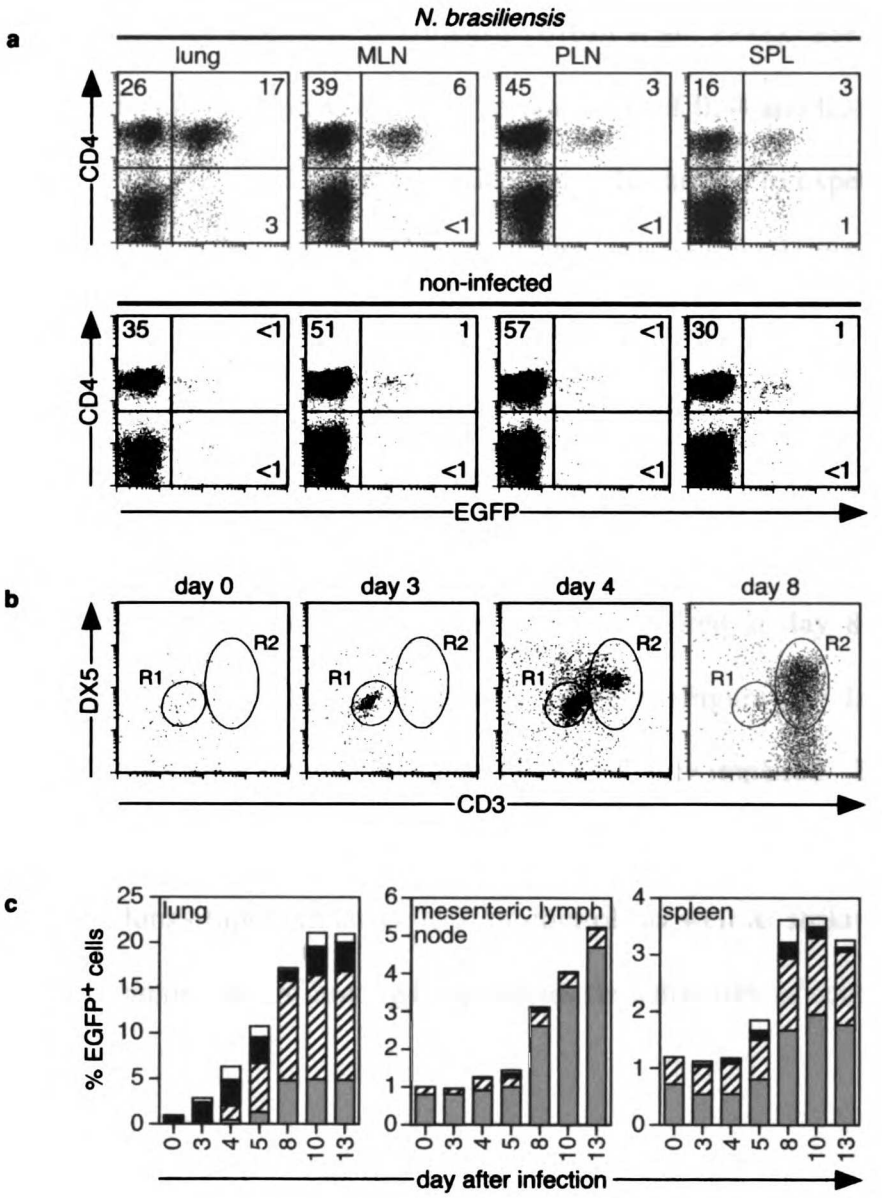
Figure 4. Spontaneous EGFP expression in 4get mice infected with *N. brasiliensis*.

a, Homozygous 4get mice were infected subcutaneously with *N. brasiliensis.* FACS analysis of lung, mesenteric lymph nodes (MLN), peripheral lymph nodes (PLN) and spleen (SPL) was performed after 10 days. Cells from the forward- and side-scatter lymphocyte gate were quantitated for expression of EGFP, CD4 and CD8. Non-infected control mice were housed in the same facility as infected mice. Cells from 3 mice were pooled for each analysis. Numbers indicate percentages per quadrant.

b, As in **A**, but the analysis was performed by gating on EGFP+ lung lymphocytes to analyze the expression of DX5 and CD3 on indicated days after infection. R1 = $DX5^{lo}$ $CD3^{lo}$ cells, R2 = DX5+ $CD3^{hi}$ cells.

c, As in **A,** but mice were analyzed on indicated days after infection for the percent of EGFP cells in designated tissues. Dark fill indicates CD4+ $CD3^{lo}$ DX5+ cells, hatched fill indicates CD4+ $CD3^{hi}$ DX5+ cells, gray fill indicates CD4+ $CD3^{hi}$ DX5- cells, and open fill indicates CD4- CD3- DX5- cells.

Figure 4

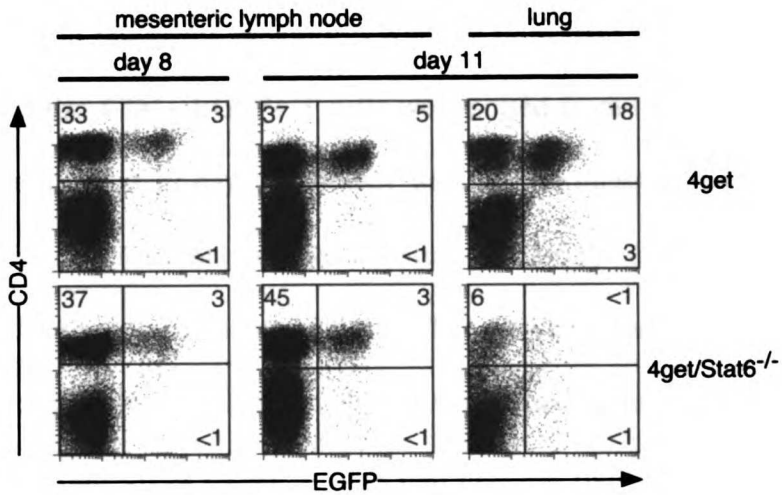

a
N. brasiliensis
lung
MLN
PLN
SPL
26
17
3
39
6
<1
45
3
<1
16
3
1
CD4
non-infected
35
<1
<1
51
1
<1
57
<1
<1
30
1
<1
EGFP
b
day 0
day 3
day 4
day 8
R1
R2
DX5
CD3
c
lung
mesenteric lymph node
spleen
% EGFP+ cells
day after infection
0
3
4
5
8
10
13

Activation requirements for tissue and lymph node lymphocyte EGFP expression. Prior studies have implicated signals through IL-4Rα and Stat6 as essential in mediating protective type 2 immunity against *N. brasiliensis* (Urban et al., 1998). Paradoxically, infected Stat6-deficient mice generated normal serum levels of IL-4 and IL-13, despite the finding that IL-4/IL-13 doubly-deficient mice were also unable to expel intestinal worms (Finkelman et al., 2000; McKenzie et al., 1999). To investigate these findings further, 4get mice were crossed to Stat6-deficient mice and infected with the parasite. After 11 days, by which time 4get littermates had expelled all intestinal worms, 4get/Stat6-deficient mice had numerous worms remaining in the intestine, consistent with prior studies (Urban et al., 1998; data not shown). Unexpectedly, activation of CD4+ T cells in the mesenteric lymph nodes was unimpaired at day 8 and only modestly compromised at day 11 by the absence of Stat6 (Figure 5). In contrast, expression of EGFP in CD4+ T cells in the lungs was strikingly impaired. The loss of IL-4-producing tissue lymphocytes was due to a reduction in the numbers of lymphocytes in the lungs (approximately 15% of control) as well as strikingly fewer EGFP-positive cells among the infiltrating lymphocytes (less than10% of control).

Figure 5. EGFP expression in 4get/Stat6$^{-/-}$ mice infected with *N. brasiliensis.*

Homozygous 4get and 4get/Stat6$^{-/-}$ mice were infected subcutaneously with *N. brasiliensis.* FACS analysis of lung and mesenteric lymph nodes was performed on day 8 and day 11 after infection. Cells from the forward- and side-scatter lymphocyte gate were quantitated for expression of EGFP and CD4. Cells from 3 mice were pooled prior to analysis. Numbers indicate percentages per quadrant.

Figure 5

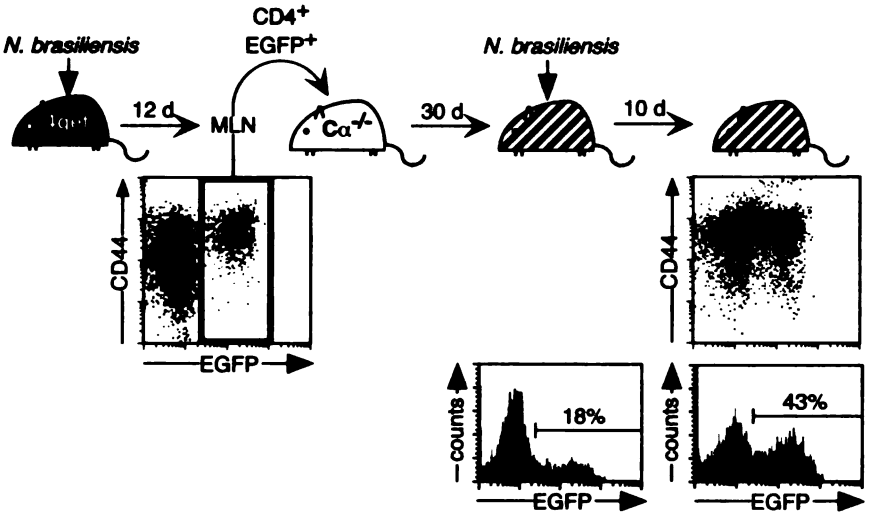

mesenteric lymph node
lung
day 8
day 11
33
3
<1
37
5
<1
20
18
3
4get
37
3
<1
45
3
<1
6
<1
<1
4get/Stat6-/-
CD4
EGFP

CD4+ EGFP+ effector cells mediate protective immunity. To confirm that activated, EGFP+ CD4+ T cells remained viable and functional, we used adoptive transfer to immunodeficient recipients to confirm whether protective memory could be conferred by effector cells. CD4+ EGFP+ T cells were sorted from the mesenteric lymph nodes of 4get mice infected 12 days previously with *N. brasiliensis.* Additional phenotyping of these cells confirmed that they were uniformly $CD62L^{lo}$ and $CD44^{hi}$. After washing, 7 x 10^5 cells were used to reconstitute TCR Cα-deficient mice, and after 30 days, reconstituted mice were infected with *N. brasiliensis.* Prior to infection, the majority (82%) of the transferred CD4+ T cells had become EGFP-negative. Ten days after infection, all reconstituted mice had expelled worms from the gut and the percentage of EGFP+ cells among the CD4+ T cell population had increased to 43% (Figure 6). CD4+ EGFP+ cells were readily isolated from both lung and lymphoid tissues. In contrast, non-reconstituted TCR Cα-deficient mice were unable to expel worms. Reconstituted infected mice generated significantly greater levels of IgE in serum as compared to non-reconstituted infected mice (205 + 10 μg/ml versus 0.06 + 0.02 μg/ml, respectively), confirming that B cell isotype switching to IgE could be mediated by EGFP+ 4get T cells. Thus, EGFP+ CD4+ T cells collected during the resolution of acute infection were capable of mediating protective immunity to Nippostrongylus in otherwise immunodeficient mice.

Figure 6. Adoptive transfer of EGFP+ effector T cells into TCR $C\alpha^{-/-}$ mice.

Homozygous 4get mice (depicted in black) were infected subcutaneously with *N. brasiliensis.* After 12 days, mesenteric lymph nodes (MLN) were analyzed for CD44 and EGFP expression in $CD4^+$ T cells and 7 x 10^5 purified EGFP+ T cells were transferred intravenously into TCR $C\alpha^{-/-}$ mice (depicted in white). 30 days later MLN of recipient mice (hatched) were analyzed for EGFP expression on gated $CD4^+$ cells. Remaining recipient mice were infected subcutaneously with *N. brasiliensis* and MLN cells were analyzed for CD44 and EGFP expression on gated $CD4^+$ T cells 10 days later.

Figure 6


N. brasiliensis
CD4+
EGFP+
12 d
MLN
Cα-/-
30 d
N. brasiliensis
10 d
CD44
EGFP
counts
18%
43%

Discussion

Cytokines mediate immunoprotective and immunopathologic responses. Despite their obvious importance, it is difficult to track cytokine-producing cells in vivo due to the rapid secretion of cytokines and the short half-lives of the proteins and mRNA transcripts. In an attempt to follow the development of effector immunity *in vivo,* we have engineered mice containing a bicistronic mRNA linking a readily identifiable reporter, EGFP, to IL-4 gene expression. Cells from these mice revealed that the majority of naive CD4+ T cells express IL-4 upon activation under Th2 priming conditions, a result substantially greater than previously appreciated using standard methods. Indeed, fully 30% of CD4+ T cells activated under Th2 conditions expressed EGFP prior to the onset of cell division. Using a prototypic type 2 immune stimulus - infection with the helminth *N. brasilensis* - we demonstrate several novel insights previously unappreciated using these reporter mice. First, IL-4 effector cells appear rapidly in lung tissues in response to parasite migration, suggesting initial cytokine activation can occur in situ prior to antigen processing and trafficking to the lymph nodes by dendritic cells. Second, Stat6-dependent activation requirements for IL-4 expression, as identified using the EGFP reporter, were different in comparing CD4+ T cells in draining lymph nodes to CD4+ T cells in tissue sites. Lastly, we confirm that antigen-experienced (memory) helper T cells capable of mediating protective immunity likely derive from effector T cell populations, as demonstrated previously for cytotoxic CD8+ T cells (Jacob and Baltimore, 1999; Opferman et al., 1999). Taken together,

these mice will provide valuable insights for understanding the development and maintenance of effector immunity *in vivo*.

A number of the problems inherent with existing methods for the detection of cytokine-producing cells can be overcome using this system. Conventional methods for identifying cytokine-producing cells require re-stimulation of cells *ex vivo* in order to allow detection of effector function (Openshaw et al., 1995; Richter et al., 1999). This typically involves activation using non-specific stimuli in order to overcome the need to identify relevant antigens presented in vivo. Further, some methods require intracellular trapping of cytokine in the endoplasmic reticulum and cell permeabilization, thus abrogating the ability to study these cells further (Openshaw et al., 1995). As shown here, the 4get cells are substantially more sensitive for identifying IL-4-expressing cells than currently standardized methods. Comparison of mean fluorescence intensity of cells from heterozygous and homozygous 4get mice demonstrated the capacity to quantitate two-fold differences in expression, suggesting that expression can be detected from individual alleles using these cells. Under the conditions used - optimal concentrations of anti-CD3 and -CD28 antibodies - the percentages of EGFP+ cells were comparable using cells from either heterozygous or homozygous 4get mice (Figure 2a, Table 1). These data suggest that monoallelic expression of the IL-4 gene occurs infrequently under these in vitro conditions, in contrast to the ease with which monoallelic expression of IL-4 has been documented using different systems (Bix and Locksley, 1998a; Hu-Li et al., 2001). Although further studies are needed, initial analysis suggests that naive CD8+ T cells, in contrast to naive

CD4+ T cells, may be incapable of expressing IL-4 from both alleles (Bix and Locksley, 1998a; Hu-Li et al., 2001), at least under these conditions.

After helminth infection, cells from 4get mice were activated in situ within relevant organs and required no further priming or stimulation for their identification. These mice create the opportunity to identify not only T cells, but potentially any immune cells that express IL-4. Viable cells can be phenotyped, sorted to high purity, and used in additional studies. As demonstrated here, sorted T cells remained fully capable of mediating protection after transfer into otherwise immunodeficient animals. This latter capacity likely reflects preservation of a functional IL-4 gene in 4get mice, in contrast to prior IL-4 knock-in models that result in deletion (Riviere et al., 1998; Hu-Li et al., 2001) or substantive modification (Ho et al., 1998) of the endogenous gene.

Current models for Th development envision instruction from dendritic cells that traffic from their tissue sites of activation over 1-3 days, during which time these cells mature from an antigen-capturing to an antigen-presenting phenotype (Banchereau and Steinman, 1998; Lanzavecchia and Sallusto, 2000). Such a process would be consistent with our observation that mesenteric lymph node CD4+ T cells began to express the EGFP reporter above background levels on day 8, approximately 2-3 days after arrival of worms in the intestine on days 5-6 (Figure 4b). In contrast, lung DX5+, CD4+ T cells expressed EGFP at the earliest time point analyzed, day 3, suggesting rapid site-specific response to migrating worms that reach the lungs between 1 and 2 days after infection. The DX5+CD4+CD3+ phenotype of these cells suggests they are NK T cells, although we have demonstrated appearance of the DX5 antigen on naive CD4+ T cells

after in vitro priming under either Th1 or Th2 conditions after 5 days (M. Mohrs; data not shown). Further studies will be required to define fully the lineage of these cells, particularly at later time points as DX5+ cells accumulate in tissues. In experiments not shown, these cells did not stain with α-GalCer tetramer reagents capable of binding to canonical CD1-restricted NK T cells (M. Mohrs, data not shown)(Benlagha et al., 2000; Matsuda et al., 2000). Indeed, prior studies have demonstrated that canonical NK T cells are unlikely to be required for host immunity against Nippostrongylus (Brown et al., 1996).

The amounts of IL-4 produced by naive αβ T cells - estimated to be 50 pg/10^6 cells in the initial 48 hr after activation - remain inefficient at Th2 priming in vitro (Noben-Trauth et al., 2000). The data here suggest that terminal Th2 differentiation may occur in the tissues in response to IL-4 produced by resident or recruited NK T cells activated by migrating parasites. Prior studies in Stat6-deficient mice infected with N. brasiliensis demonstrated essentially wild-type levels of IL-4 and IL-13 in serum despite the inability to expel intestinal worms (Finkelman et al., 2000). As shown here, Stat6-deficient T cells efficiently activated EGFP expression in mesenteric lymph nodes, but were unable to sustain stable IL-4 expression in tissues (Fig. 5). Thus, these mice demonstrate that cytokine responses can be anatomically compartmentalized, likely explaining the failure of worm expulsion in Stat6-deficient mice. Recent data suggest that initial activation of the IL-4 gene can be Stat6-independent, although subsequent stabilization required Stat6-mediated expression of GATA3 (Grogan et al., 2001); the latter transcription factor is necessary and sufficient

for Th2 cell differentiation (Zheng and Flavell, 1997). Together with the data presented here, we favor a model whereby naive T cells become activated to express cytokines by Stat-independent pathways in lymph nodes but become instructed to canonical committed Th subsets in response to cytokines elaborated by resident, specialized, cell populations within inflammatory foci. Further, signals mediated by Stat6 are required to express surface markers required for migration into infected tissues. By either or both mechanisms, in the absence of Stat6, IL-4/IL-13 expression cannot be sustained at tissue sites where these cytokines are required to mediate worm expulsion (McKenzie et al., 1999). These mice illustrate the value of the reporter in evaluation of tissue-specific cytokine expression *in vivo.*

The capacity to recover effector cells from tissues should provide important reagents for studies of homeostasis and memory, not only of canonical T cells, but also NK T cells or other effector cells that express IL-4. Current methods often rely on adoptive transfer of antigen-specific TCR transgenic T cells, which requires knowledge of relevant antigens prior to the analysis (Reinhardt et al., 2001). As shown here, transfer of EGFP+ T cells to immunodeficient mice was followed by loss of fluorescence as cells resided for 30 days in recipient mice. Although we have not thoroughly evaluated the half-life of EGFP in these cells, the disappearance of EGFP that appears early after activation of cells under Th1 conditions (Grogan et al., 2001) and during homeostatic re-population of TCR-Cα-deficient mice, as shown here, demonstrates that the protein does not persist indefinitely. These cells were capable of re-expressing EGFP after infection, of conferring host protection and of providing

cytokine signals necessary for production of IgE. As such, T cell lines generated from these effector cells might prove useful for identifying protective worm antigens using various expression systems. Indeed, CD4+ effector T cells as defined by EGFP expression were fully capable of conferring immunoprotection, extending to CD4+ T cells similar observations demonstrating that CD8+ T effector cells give rise to CD8+ memory T cells (Jacob and Baltimore, 1999; Opferman et al., 1999). The ability to mark the IL-4 gene should be readily applicable to other cytokine loci, and the simultaneous use of different fluorescent markers for multiple genes should allow an unprecedented look at the evolving immune response in the whole animal. Together with the capacity to obtain these effector cells in high purity for further studies, such engineered mice will prove valuable reagents in models of immunoprotection, immunopathology and autoimmunity.

Experimental Methods

Mice. BALB/c mice (Jackson Laboratories, Bar Harbor, ME), TCR Cα- (Philpott et al., 1992), and Stat6-deficient (Kaplan et al., 1996) mice were kept under specific pathogen-free conditions in the animal care facility at the University of California San Francisco. Mice were backcrossed at least 10 generations onto BALB/c prior to further breeding where designated.

Generation of IL-4-reporter mice. A 6 kb EcoRI fragment derived from 129/SvJ genomic IL-4 DNA was cloned into pgkTK (Tybulewicz et al., 1991). A SrfI site was introduced into the 3' untranslated region upstream of the endogenous polyadenylation signal in exon 4 by site-directed mutagenesis using the following oligonucleotides: IL-4 Srf sense 5'-CGT AGT ACT GAG CCC GGG CCA TGC TTT AAC-3', IL-4 Srf anti-sense 5'-GTT AAA GCA TGG CCC GGG CTC AGT ACT ACG-3'. The reporter cassette was derived from pIRES-EGFP (Clontech, Palo Alto, CA). A Kozak translation initiation sequence (Kozak, 1992) was introduced upstream of the 11th ATG of the EMCV IRES element (Jackson et al., 1990) by PCR-mediated mutagenesis using the following oligonucleotides: IRES-3' Nco 5'-CCA TGG TAT CAT CGT GTT TTT CAA AGG-3', IRES-5' 5'-CTG CAG GTC GAG CAT GCA TCT AGG G-3'. The modified IRES-EGFP cassette, including the bovine growth hormone polyadenylation signal, was ligated to the loxP-flanked neo-cassette, pL2neo2 (Gu et al., 1993), to generate the reporter/selection cassette. This cassette was cloned into the SrfI site of the modified genomic DNA to generate the final targeting construct.

PrmCre ES cells, which express the Cre recombinase under the protamine promoter (O'Gorman et al., 1997), were electroporated with the NotI-linearized targeting construct and selected in the presence of 400 μg/ml G418 and 2 μM gancyclovir. Resistant ES cell clones were screened for homologous integration by Southern blot. Targeted clones were injected into C57BL/6 blastocysts to create chimeric mice. The neomycin resistance cassette was deleted in the male germline by Cre-mediated recombination after breeding chimeric mice to wild-type BALB/c females. Heterozygous animals were bred to BALB/c mice and offspring were screened and selected for the presence of the reporter and the absence of the Cre transgene. Finally, heterozygous animals were interbred to obtain homozygous mice.

Cell purification. Naive CD4+ T cells were sorted from lymph nodes and spleen after labeling with anti-CD4-PE (YTS191.1, Caltag Laboratories, San Francisco, CA) and anti-CD62L-APC (MEL-14, PharMingen, San Diego, CA) using flow cytometry (MoFlo Multi-Laser Flow Cytometer, Cytomation, Ft. Collins, CO). Sorted cells were small, EGFP-negative lymphocytes on forward-side scatter analysis and in independent analysis were >99% CD4+, $CD62L^{hi}$. Naive CD8+ T cells were sorted for the same parameters using anti-CD8-PE (Caltag Laboratories). Where designated, naive T cells were pre-incubated with the vital fluorescent dye, PKH26 (2 μM; Sigma Chemical Co., St. Louis, MO), as described (Zhang et al., 1997). Where noted, enriched CD4+ T cells were prepared from spleen cells by complement-mediated lysis of CD8+ T cells as described (Bix et al., 1998b).

Antigen presenting cells (APC) were prepared from the spleens of TCR-Cα-deficient mice after lysis of red cells and γ-irradiation (2500 rad), as described (Fowell et al., 1999).

In vitro T cell priming. Purified naive CD4+ or CD8+T cells in media (RPMI 1640 with 10% heat-inactivated fetal calf serum, 50 μM β2-mercaptoethanol, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin) were stimulated in 24-well plates at 10^6 cells/ml with mAbs to TCRβ (H57.597; 1 μg/ml) and CD28 (37N51.1; 5 μg/ml) and 50 U/ml recombinant human IL-2 (neutral priming) in the presence of 5 x 10^6 irradiated APC. Under Th1 priming conditions, murine recombinant IL-12 (5 ng/ml) and 20 μg/ml anti-IL-4 mAb (11B11), and, under Th2 priming conditions, murine recombinant IL-4 (50 ng/ml) and 50 μg/ml anti-IFN-γ mAb (XMG1.2), were added to the respective cultures. Where indicated, cells were washed after 5 days and re-stimulated using anti-TCRβ mAb and fresh irradiated APC. Supernatants were collected after 2 days and analyzed for cytokines by ELISA (Fowell et al., 1999).

Parasites and infection. Third-stage larvae (L3) of *N. brasiliensis* were isolated from feces of infected rats, washed extensively, and 750 organisms were injected subcutaneously in 0.2 ml PBS at the base of the tail as described (Fowell et al., 1999). Mice were killed at designated times and the presence of adult worms in the intestines was assessed using inverted microscopy. The lungs, spleens, mesenteric and peripheral (axillary and inguinal) lymph nodes were excised and dispersed into single-cell suspensions. Where designated, lung suspensions were purified further by

centrifugation over Ficoll. Serum was collected for determination of total serum IgE by ELISA as described (Brown et al., 1996).

Flow cytometric analysis. Designated single cell populations isolated from tissues, lymphoid organs or in vitro cultures were labeled with the indicated conjugated mAb to cell surface markers (PharMingen and Santa Cruz Biotechnologies, Santa Cruz, CA). Lung and spleen cells were pre-incubated with mAb to CD16/CD32 (Fc block; PharMingen) to block non-specific binding. Analysis was acquired on a FACScalibur flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA) after setting parameters using isotype matched control mAb and non-fluorescent cells from wild-type mice.

Cytokine assays. Mouse IL-4 and IFN-γ were determined by sandwich ELISA with detection limits of 50 pg/ml for IL-4 and 1 ng/ml for IFN-γ, as described (Fowell et al., 1999). ELISPOT assays were performed after priming T cells for 5 days, purifying cells over Ficoll and re-stimulating with plate-bound anti-CD3 mAb for 6 hr as described (Brown et al., 1996).

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Chapter III:

Regulation of Type 2 innate immune responses by adaptive immunity

Abstract

Type 2 immunity requires orchestration of innate and adaptive immune responses to protect mucosal sites from pathogens. Dysregulated type 2 responses result in allergy or asthma (Will-Karp, 1999). T helper 2 (Th2) cells elaborate cytokines, such as interleukin (IL)-4, IL-5, IL-9 and IL-13, that work in concert with toxic mediators of innate immune cells to establish environments inhospitable to helminth or arthropod invaders (Finkelman, 1997). The importance of Th2 cells in coordinating innate immune cells at sites of inflammation is not known. Here, we show that polarized type 2 immune responses are initiated independently of adaptive immunity. In the absence of B and T cells, IL-4-expressing eosinophils were recruited to tissues of mice infected with the helminth, *Nippostrongylus brasiliensis*. Unexpectedly, eosinophils failed to degranulate in the absence of adaptive immunity. Reconstitution with CD4 T cells promoted degranulation, but only if T cells were stimulated with cognate antigen. Degranulation correlated with tissue destruction that was attenuated if eosinophils were depleted. Helper T cells confer antigen specificity on eosinophil cytotoxicity, but not cytokine responses, defining a novel mechanism that focuses tissue injury at sites of immune challenge.

Helper T cells regulate eosinophil degranulation *in vivo*

Many of the polarized cytokines produced by Th1 and Th2 cells play an important role in coordinating innate immune responses at sites of inflammation. However, recent evidence suggests that innate immune cells are sufficient to induce type 1 immunity through Toll-like receptor (TLR)-mediated, pathogen-driven activation (Akira, 2001; Schnare, 2001; Chin, 2002; Kobayashi, 2002; Suzuki, 2002). To determine whether the innate arm of type 2 immunity can be initiated in the absence of an adaptive immune compartment, we used Rag-$1^{-/-}$ mice, which lack T and B cells, to examine the capacity of the helminth, *Nippostrongylus brasiliensis*, to activate IL-4 expression from innate immune cells. *N. brasiliensis* powerfully induces a type 2 immune response during larval migration through the lung (Finkelman, 1997). IL-4-secreting cells appeared in the lungs of *N. brasiliensis*-infected Rag-$1^{-/-}$ animals, peaking at day 8 post-infection, consistent with the peak of IL-4 production in infected wild-type mice (Figure 1a). As compared to wild-type mice, the response was four-fold less abundant and failed to be maintained after 12 days, presumably reflecting the absence of a reinforcing adaptive immune response. No IFNγ-producing cells were detected in infected wild-type or Rag-$1^{-/-}$ mice, consistent with the expected polarized type 2 response (data not shown). Thus, innate cells are capable of expressing a polarized type 2 cytokine response in the absence of an adaptive immune compartment.

To track cells involved in the type 2 response, we used IL-4 reporter mice, designated 4get mice (IL-4 green fluorescent protein-enhanced transcript), which

contain a knockin IL-4 gene modified to express a bicistronic message linking IL-4 via an internal ribosomal entry site (IRES) element with enhanced green fluorescent protein (eGFP) (Mohrs, 2001). Cells from 4get mice faithfully report IL-4 expression without the need for restimulation while leaving endogenous IL-4 intact. 4get and 4get x Rag-1$^{-/-}$ mice were infected with *N. brasiliensis* and kinetic analysis of eGFP fluorescence was determined by flow cytometry. In wild-type mice, eGFP+ cells accumulated first in lungs, and later in mesenteric lymph nodes and spleen, reflecting the migratory path of the worms as they travel from subcutaneous sites of inoculation through the lungs, up the trachea, down the esophagus and finally to the intestines (Mohrs, 2001). In 4get x Rag-1$^{-/-}$ mice, the magnitude of the innate cytokine response was attenuated in the absence of adaptive immunity. Analysis of the numbers of non-B, non-T eGFP+ cells per lung or spleen of age- and size-matched, infected 4get or 4get x Rag-1$^{-/-}$ mice revealed a defect in the later phase of recruitment and/or activation of IL-4-expressing innate immune cells in the absence of B and T cells (Figure 1b). Although quantitatively similar at early time points, IL-4-expressing innate cells were six-fold reduced on day six and forty-fold reduced on day twelve in lungs in 4get x Rag-1$^{-/-}$ mice; a similar defect was observed in the spleen. However, as assessed by comparable mean fluorescence intensity (MFI) of eGFP, innate IL-4-expressing cells in lungs of both wild-type and Rag-1$^{-/-}$ mice demonstrated a robust cytokine-activating pathway independent of adaptive immunity (Figure 1c).

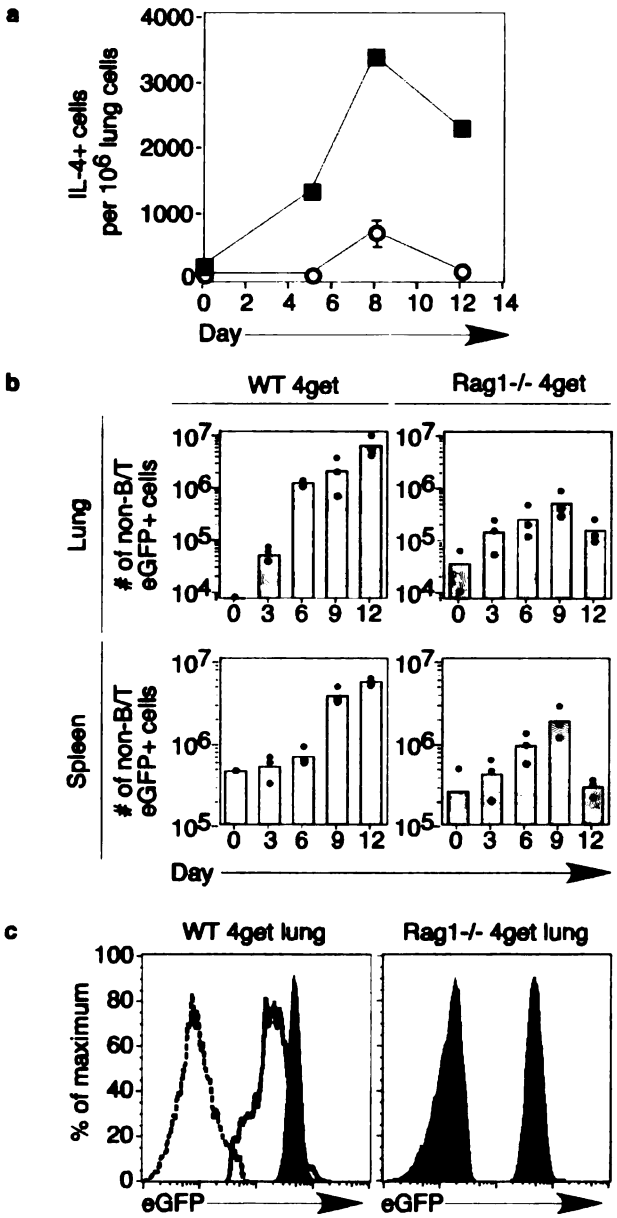
Figure 1. Activation of IL-4 expressing cells in the absence of adaptive immunity.

a, Wild-type (filled squares) and Rag-$1^{-/-}$ (circles) mice were infected with *N. brasiliensis*. On designated days, lung cells were analyzed for spontaneous IL-4 secretion by ELISPOT.

b, 4get and 4get x Rag-$1^{-/-}$ mice were infected with *N. brasiliensis*. On designated days, non-B, non-T cells from lung and spleen were analyzed for spontaneous eGFP fluorescence by flow cytometry. Circles depict values in individual mice, bars depict group mean values.

c, On day 9 post-infection, lung cells were analyzed for mean fluorescence intensity of eGFP in CD4+ T cells (in 4get mice) and non-B, non-T cells (in 4get and 4get x Rag-$1^{-/-}$ mice). CD4+, eGFP+ (tinted with solid black line); CD4+, eGFP- (black dashed line); non-B/T, eGFP+ (solid black); non-B/T, eGFP- (solid gray). Data are representative of three comparable experiments.

Figure 1


a
IL-4+ cells per 10^6 lung cells
4000
3000
2000
1000
0
0 2 4 6 8 10 12 14
Day
b
WT 4get
Rag1-/- 4get
Lung
Spleen
# of non-B/T eGFP+ cells
10^7
10^6
10^5
10^4
0 3 6 9 12
Day
c
WT 4get lung
Rag1-/- 4get lung
% of maximum
100
80
60
40
20
0
eGFP
eGFP

Non-B, non-T eGFP+ cells were isolated from lungs of infected wild-type 4get mice by flow cytometry for histologic examination. Cells in the high side-scatter gate, which did not stain with the neutrophil-specific monoclonal antibody 7/4, were highly enriched for eosinophils (>90%), as characterized by lobulated nuclei and many dense, eosin-staining granules (Figure 2a, 2b). The eGFP+ cells in the low side-scatter gate were also 7/4-negative and enriched for cells with morphologically similar nuclei, but contained only a perinuclear blush of lightly eosin-positive granules, and no dense granules. Both populations were viable, as determined by trypan blue and 7-AAD exclusion, and did not stain with annexin V (data not shown). RT-PCR analysis of mRNA from each purified population confirmed comparable expression of eosinophil-specific genes, including major basic protein-1 (MBP-1) and eosinophil peroxidase (EPO), but not neutrophil-specific elastase, suggesting that both were eosinophils (Figure 2c). Eosinophil-specific genes were not expressed by 7/4+ neutrophils. The hypothesis that the low side-scatter eosinophils might appear after degranulation of the granule-rich high side-scatter eosinophils was supported by the finding that treatment of the latter population with phorbol myristate acetate and ionomycin, agents that provoke eosinophil degranulation, resulted in the appearance of cells with the same side-scatter profile and morphologic appearance as those in the low side-scatter gate (Figure 2d, and data not shown). The appearance of degranulated eosinophils in the low side-scatter gate was concomitant with the reduction of eosinophils in the high side-scatter gate. Transmission electron microscopy of the eGFP+ high side-scatter cells isolated from

lungs of infected 4get mice confirmed the typical lobulated nuclei and crystalloid core-containing specific granules of eosinophils (Figure 2e). Ultrastructural study of the eGFP+ low side-scatter cells revealed intact cells with ruffled membranes containing similar lobulated nuclei but altered granules, including loss of the crystalloid core from specific granules and the accumulation of cytoplasmic vacuoles.

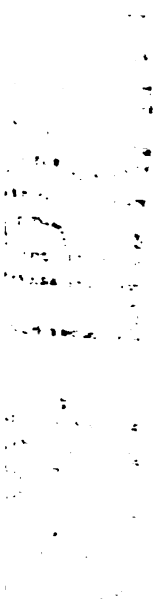


Figure 2. Subsets of eosinophils identified by flow cytometry.

a, 4get mice were infected with *N. brasiliensis.* eGFP+, non-B, non-T lung cells were analyzed by flow cytometry (day 7 post-infection). Cells from indicated gates were sorted onto slides and stained with hematoxylin and eosin (right panels).

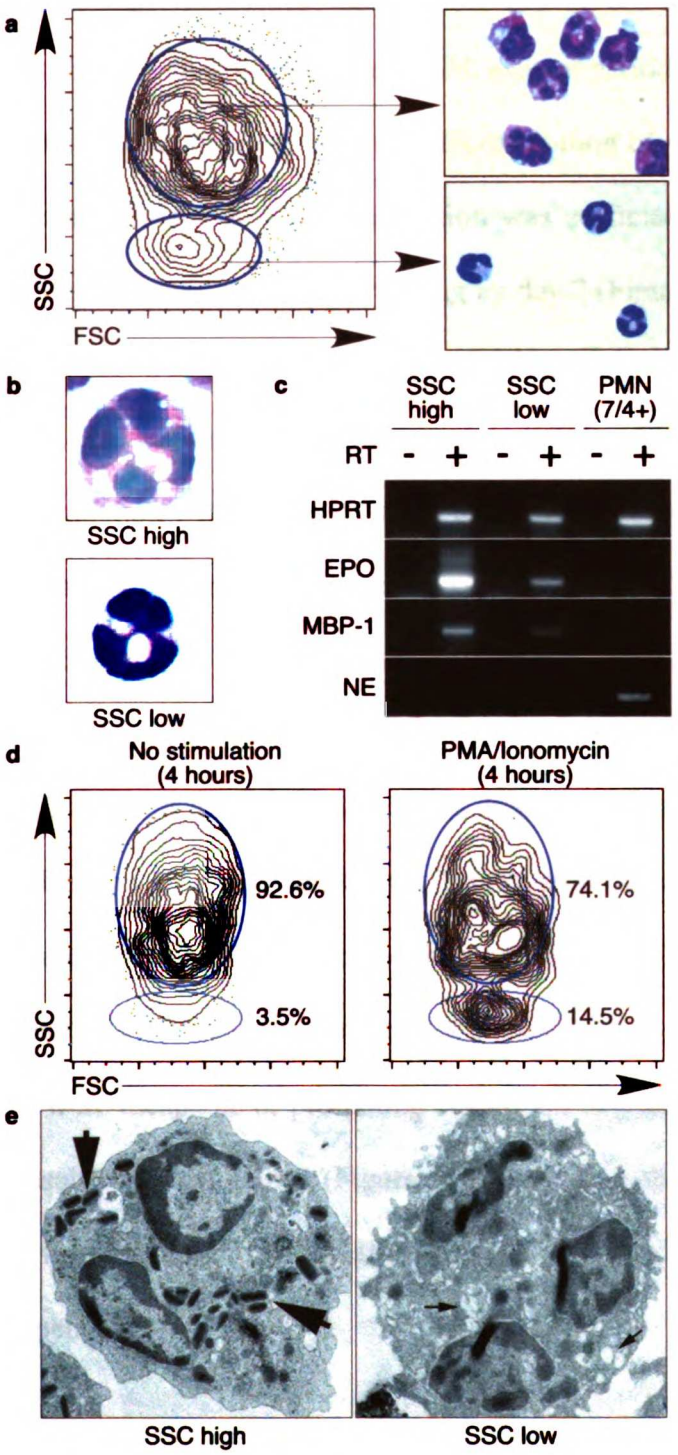
b, Detail of cells sorted from SSC (side-scatter) high and low gates.

c, eGFP+ SSC-high cells, eGFP+ SSC-low cells, and eGFP- 7/4+ neutrophils (PMN) were sorted from lungs of infected 4get mice (day 7 post-infection) and analyzed by reverse transcriptase-PCR analysis for expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT), eosinophil peroxidase (EPO), major basic protein (MBP-1) and neutrophil elastase (NE).

d, 4get x Rag-$1^{-/-}$ mice were infected with *N. brasiliensis.* On day 7, lung cells were maintained in media alone (left panel) or stimulated with PMA and ionomycin (right panel). After 4 hrs, eGFP+ cells were analyzed by flow cytometry.

e, Transmission electron microscopy of side-scatter high (left panel) and side-scatter low (right panel) non-B, non-T eGFP+ cells sorted from the lungs of infected 4get mice. Crystalloid-containing specific granules and cytoplasmic vacuoles indicated by thick and thin arrows, respectively. Data are representative of three comparable experiments.

Figure 2


a
SSC
FSC
b
SSC high
SSC low
c
SSC high
SSC low
PMN (7/4+)
RT - + - + - +
HPRT
EPO
MBP-1
NE
d
No stimulation (4 hours)
PMA/Ionomycin (4 hours)
92.6%
3.5%
74.1%
14.5%
SSC
FSC
e
SSC high
SSC low

In contrast to infected wild-type mice, in which up to 30% of the lung eGFP+ eosinophils were degranulated by day 7, none (<1%) of the eGFP+ eosinophils in infected 4get x Rag-$1^{-/-}$ mice were degranulated, even at periods as late as 14 days post-infection (Figure 3a, and data not shown). Reconstitution of 4get x Rag-$1^{-/-}$ mice with wild-type spleen cells at the time of infection was sufficient to restore the normal appearance of degranulated eosinophils in lung by day 7 (Figure 3a). Spleen cells from TCR-Cα-deficient mice did not restore eosinophil degranulation, whereas purified wild-type CD4 T cells reconstituted the process, suggesting that CD4 T cells were sufficient to confer degranulation without the need for antibody. Although signals from CD4 T cells are presumably imparted by Th2 cells, spleen cells purified from IL-4-deficient, IL-4/IL-13-deficient and Stat6-deficient mice restored eosinophil degranulation comparably to wild-type T cells (Figure 3b). Thus, despite defects in Th2 development and, in IL-4/IL-13- and Stat6-deficient mice, inability to expel *N. brasiliensis* after infection (McKenzie, 1999; Urban, 1998), T cells from these mice are competent to mediate degranulation of activated eosinophils. Although DO11.10 CD4 T cells from ovalbumin-specific TCR transgenic mice on a TCR-$C\alpha^{-/-}$ background were recruited to the lung, they were incapable of promoting eosinophil degranulation unless mice were treated intranasally with ovalbumin (Figure 3c, and data not shown). Thus, CD4 T cells are sufficient for eosinophil degranulation by a process that is antigen-dependent but antibody-independent.

Degranulation of eosinophils discharges toxic granule constituents, including MBP-1, EPO, eosinophil-derived neurotoxin (EDNT), eosinophil cationic protein (ECP) and other inflammatory mediators (Walsh, 2001). During infection with *N. brasiliensis,* the capacity of eosinophils to degranulate correlated with tissue injury. Lungs from infected wild-type mice were edematous and visibly inflamed, in contrast to lungs from infected Rag-$1^{-/-}$ mice (Figure 4a, 4b). Reconstitution of Rag-$1^{-/-}$ mice with CD4 T cells resulted in increased whole lung weight and inflammation comparable to wild-type mice. Histologic examination revealed inflammation spilling into alveoli, epithelial injury and goblet cell mucus production in wild-type mice that was minimal in Rag-$1^{-/-}$ mice, although peribronchial infiltrates were unaffected (Figure 4c, 4d, 4e, and 4f). Depletion of either helper T cells using CD4 antibody or recruited eosinophils using IL-5 neutralizing antibody resulted in reduced inflammation and attenuation of lung weight in infected mice (Figure 4a, 4b, 4g, 4h, and data not shown).

These studies reveal novel interactions between innate and adaptive effector cells during the evolution of type 2 immune responses. First, eosinophils can activate IL-4 expression in the absence of adaptive immune cells *in vivo*. Thus, in agreement with prior observations in systems using Schistosoma egg and various environmental allergens, eosinophils must express receptors capable of transducing signals from the environment to the IL-4 gene (Rumbley, 1999; Justice, 2002). Whether these receptors are pattern recognition receptors that interact with specific pathogen moieties, or alternatively, that interact with ligands, cytokines, or chemokines induced in normal

tissue in response to specific types of injury will require further study (Foster, 2001). The type 2 bias induced in mice in response to certain cytokines, like IL-25 (Fort, 2001), or in the absence of defined receptors, like DR6 (Liu, 2001), suggests evidence for such potential signals. The preservation or default appearance of type 2 responses in MyD88-, Rip2- and IRAK4-deficient mice, however, indicates that these signals are distinct from those important in type 1 immunity (Schnare, 2001; Chin, 2002; Kobayashi, 2002; Suzuki, 2002).

Figure 3. T helper cells regulate eosinophil degranulation *in vivo*.

a, Designated recipient mice were reconstituted with cells from the indicated donors and infected with *N. brasiliensis*. After 7 days, eGFP+, non-B, non-T lung cells were sorted onto slides, stained and analyzed for eosinophil degranulation by histologic examination. Values are given relative to the percentage of eosinophil degranulation in 4get mice. Circles represent values in individual mice, bars represent group mean values. Approximately 30% of eosinophils were degranulated in 4get mice.

b, As in **a**, with source of donor cells as indicated.

c, As in **a**, with source of donor cells as indicated. Where noted, cohorts of mice were treated additionally with intranasal ovalbumin twice prior to isolation of lung cells. Data are representative of three comparable experiments.

Figure 3

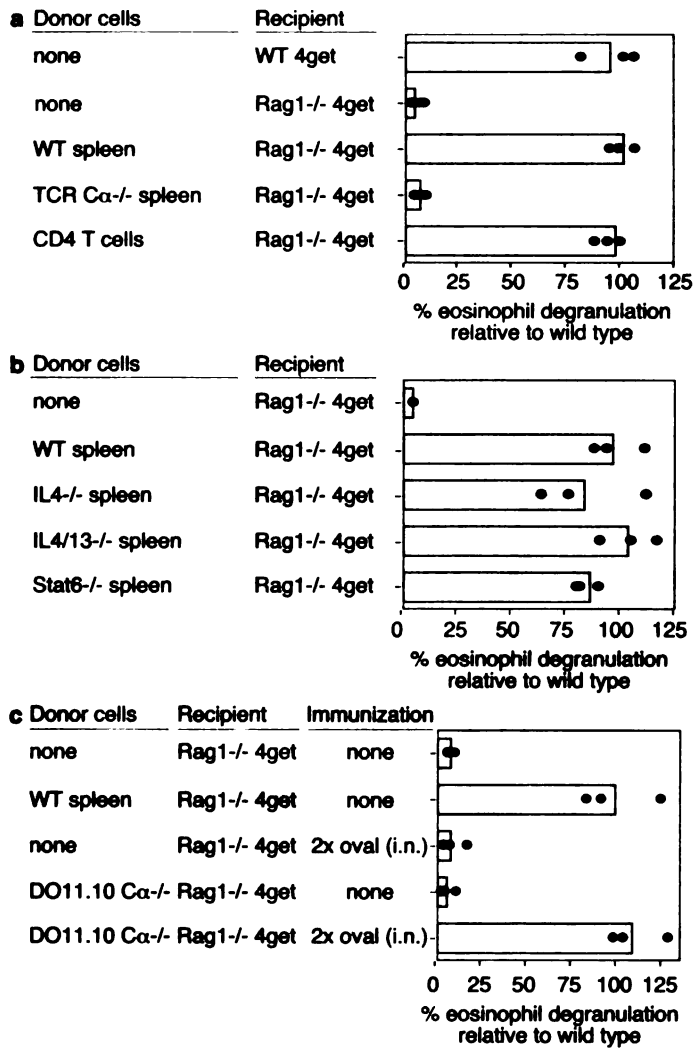

a
Donor cells
Recipient
none
WT 4get
none
Rag1-/- 4get
WT spleen
Rag1-/- 4get
TCR Cα-/- spleen
Rag1-/- 4get
CD4 T cells
Rag1-/- 4get
0 25 50 75 100 125
% eosinophil degranulation relative to wild type
b
Donor cells
Recipient
none
Rag1-/- 4get
WT spleen
Rag1-/- 4get
IL4-/- spleen
Rag1-/- 4get
IL4/13-/- spleen
Rag1-/- 4get
Stat6-/- spleen
Rag1-/- 4get
0 25 50 75 100 125
% eosinophil degranulation relative to wild type
c
Donor cells
Recipient
Immunization
none
Rag1-/- 4get
none
WT spleen
Rag1-/- 4get
none
none
Rag1-/- 4get
2x oval (i.n.)
DO11.10 Cα-/-
Rag1-/- 4get
none
DO11.10 Cα-/-
Rag1-/- 4get
2x oval (i.n.)
0 25 50 75 100 125
% eosinophil degranulation relative to wild type

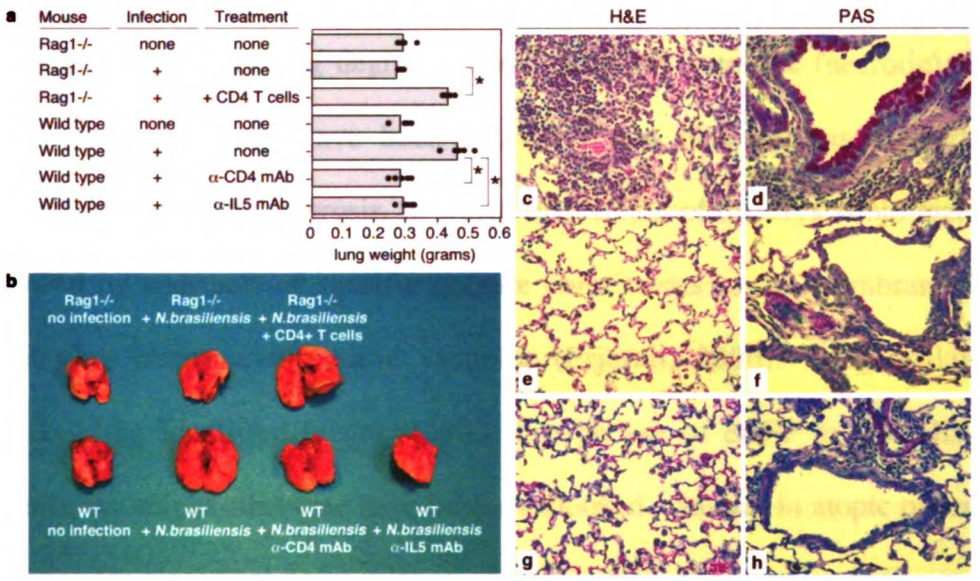
Figure 4. Tissue injury correlates with presence of both CD4 T cells and eosinophils.

a, Designated mice were infected with *N. brasiliensis* after receiving adoptively transferred CD4 T cells or depleting antibodies to CD4 or IL-5. After 7 days, lungs were excised without perfusion and weighed. Circles depict values from individual mice, bars depict group mean values. Significant differences denoted by asterisks ($p < 0.005$).

b, Appearance of excised lungs from representative mice from each group.

c-g, Hematoxylin/eosin (left, H&E) and periodic acid Schiff (right, PAS) stains of lung tissues on day 7 after infection in wild-type mice (**c**, **d**), Rag-$1^{-/-}$ mice (**e**, **f**), and wild-type mice treated with anti-IL-5 antibody (**g**, **h**). Magnification 400X. Data are representative of 5 mice per group.

Figure 4


a
Mouse
Infection
Treatment
Rag1-/- none none
Rag1-/- + none
Rag1-/- + + CD4 T cells
Wild type none none
Wild type + none
Wild type + α-CD4 mAb
Wild type + α-IL5 mAb
0 0.1 0.2 0.3 0.4 0.5 0.6
lung weight (grams)
b
Rag1-/- no infection
Rag1-/- + N.brasiliensis
Rag1-/- + N.brasiliensis + CD4+ T cells
WT no infection
WT + N.brasiliensis
WT + N.brasiliensis α-CD4 mAb
WT + N.brasiliensis α-IL5 mAb
H&E
PAS
c
d
e
f
g
h

Eosinophil degranulation has been variably reported in models of allergic lung disease (Mould, 2000; Denzler, 2001). In part, this may reflect histologic uncertainty over the mechanism(s) underlying degranulation. Although cytolytic (necrotic) and classical exocytosis pathways have been described, our observations support a mechanism termed 'piecemeal' necrosis, in which dissolution of the crystalloid matrix core is followed by transport of specific granule constituents to the membrane for secretion, leaving empty cytoplasmic vacuoles (Erjefalt, 2000). Degranulated eosinophils could be identified in our studies through use of eGFP to label IL-4-expressing cells, but might otherwise have been overlooked. Studies in atopic patients support evidence for piecemeal degranulation of eosinophils after allergen exposure *in vivo* (Karawajczyk, 2000). Unexpectedly, our quantitative approach revealed a profound role for antigen-activated CD4 T cells in mediating this process independent of the capacity for eosinophils to activate IL-4 expression. Intriguingly, CD4 T cells lacking components critical to Th2 development, including IL-4, IL-13 and the signal transducing element, Stat6, were competent to mediate degranulation. Prior evidence has suggested that IL-5 might mediate eosinophil degranulation and/or IL-4 expression (Mould, 2000; Sabin, 1996), although these activities were completely dissociated in our experiments. IL-5 critically affects bone marrow generation of eosinophils and eosinophil survival, but transgenic mice over-expressing IL-5 do not demonstrate overt tissue cytotoxicity despite massive blood eosinophilia (Dent, 1990; Lee, 1997).

CD4 T cell regulation of eosinophil degranulation may represent an important checkpoint spatially restricting the release of tissue-destructive inflammatory mediators contained in eosinophil granules. Eosinophil cationic proteins can generate transmembrane pores *in vitro*, and ECP and EPO catalyze formation of toxic intermediates from halides and oxygen, creating the potential for tissue cytotoxicity. Although these proteins are host protective in the setting of parasitic infection, the correlation between increased concentrations of MBP and EPO in bronchial lavage fluid and clinical exacerbations in asthma patients supports a cytotoxic role for eosinophil granular contents in disease (Walsh, 2001). Although initial studies using neutralizing IL-5 antibody in asthma patients failed to demonstrate an impact of eosinophil depletion on bronchial hyperreactivity (Leckie, 2000), confounding effects including incomplete tissue eosinophil depletion and IL-5-independent pathways for eosinophil activation preclude definitive conclusions (Foster, 2002).

The concept that effector T cells regulate the discharge of eosinophil granular contents, although unexpected, is conceptually similar to the role of IgE in mediating the discharge of mast cell contents. In both cell types, antigen-specific components of adaptive immunity regulate the ultimate decision mediating the discharge of toxic contents. The capacity to ameliorate asthma by targeting IgE (Milgrom, 1999) suggests that interventions abrogating the pathway by which antigen-specific T cells mediate eosinophil degranulation may prove fruitful therapeutic targets. Indeed, targeting helper

T cells might attack dual components of the pathologic response by both removing allergen-specific adaptive immunity and interfering with eosinophil cytotoxicity.

Experimental Methods

Mice. Female BALB/c mice (Charles River Laboratories) were maintained in the UCSF specific pathogen-free facility and used at 6-8 weeks of age. Interleukin-4 GFP enhanced transcript (4get) mice (Mohrs, 2001) were backcrossed three generations to BALB/c prior to use. 4get x Rag-$1^{-/-}$ mice were generated by crossing N3 BALB/c 4get mice onto N10 BALB/c Rag-$1^{-/-}$ mice. TCR-C$\alpha^{-/-}$ mice were backcrossed ten generations onto BALB/c. N10 BALB/c TCR-C$\alpha^{-/-}$ mice were crossed to DO11.10 TCR transgenic mice (Murphy, 1990). IL4-, IL-4/IL-$13^{-/-}$ (McKenzie, 1999) and $Stat6^{-/-}$ (Urban, 1998) mice were on the BALB/c background.

Parasites and infection. Infective third-stage *N. brasiliensis* larvae were isolated from the feces of infected rats by modified Baermann technique. After washing, 500 larvae in 0.2 ml saline were injected subcutaneously at the base of tail. Infected mice were maintained on antibiotic-containing water.

Cell purification. On specified days, mice were killed and the lungs perfused with sterile phosphate buffered saline (PBS) via the right cardiac ventricle. Lungs and spleen were excised and mechanically dispersed into single-cell suspensions. Non-B, non-T cells were purified after labeling cells with antibodies to CD4 (YTS191.1, Caltag Laboratories, S. San Francisco, CA), CD8 (5H10, Caltag), and CD19 (1D3, PharMingen, San Diego, CA) using flow cytometry (MoFlo Multi-laser Cytometer, Cytomation, Ft. Collins, CO). Recovered cells were >98% CD4/CD8/CD19-negative.

Spleen and T cell reconstitutions and depletions. 8 x 10^7 spleen cells were injected intravenously in 0.2 ml PBS into Rag-$1^{-/-}$ mice. Where indicated, CD4 T lymphocytes were enriched to 90-99% purity by either cell sorting or antibody- and complement-mediated lysis of CD8, class II MHC and heat stable antigen-bearing cells as described (Fowell, 1997). Cell depletions *in vivo* were performed by single intraperitoneal injection of 2 mg GK1.5 mAb (for CD4 T cells) or TRFK5 mAb (anti-IL-5, for eosinophils) concomitant with infection.

Cytokine analysis. Lung cells were washed and incubated at 10^7 live cells/ml in culture medium (RPMI 1640 with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin) overnight at 37°C in 5% CO_2 on anti-IL-4 (11B11)- or anti-IFNγ (R46A2)-coated, 96-well ELISPOT plates without restimulation. After 16 hrs, plates were washed and developed to detect the frequency of cytokine-expressing cells as described (Brown, 1996).

Histology. Cells purified by flow cytometry were centrifuged onto slides, fixed in methanol and stained using modified Wright-Giemsa. Eosinophil degranulation was assessed morphologically by enumerating at least 3000 granulated or degranulated cells per individual mouse by light microscropy. For tissue histology, lungs were surgically excised without cardiac perfusion, inflated and fixed in formalin in PBS for 48 hrs, paraffin embedded and cut into 3 μm sections prior to staining with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) stains. For ultrastructural microscopy, sorted cells were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide,

dehydrated stepwise in ethanol and propylene oxide, and embedded in epon araldite. After sectioning, samples were mounted on grids and analyzed on a Tecnai20 electron microscope.

Reverse transcriptase-PCR analysis. Activated granulocytes were sorted from single-cell lung suspensions from infected mice (day 7 post-infection) based on forward- and side-scatter profiles and eGFP fluorescence. Sorted cells were >98% pure by histologic examination. Total RNA was purified (Biotecx Laboratories, Houston, TX), templated with oligo(dT) and random hexamer primers to make cDNA (Clontech Laboratories, Palo Alto, CA) and used for PCR analysis with the following primers: hypoxanthine-guanine phosphoribosyltransferase (HPRT): sense 5'-CCT GCT GGA TTA CAT CAA AGC ACT G - 3', anti-sense 5'-TCC AAC ACT TCG TGG GGT CCT-3'; mouse MBP-1: sense 5'-TCT ACT TCT GGC TCT TCT AGT CGGG-3', anti-sense 5'-GAC ACA GTG AGA TAG ACG CCA GTG; mouse EPO: sense 5'-ACT GTT TCC TGC TAG AGC TTT TGC-3', anti-sense 5'-AGA GTG CTG CTG TTC CTT CAG G-3'; mouse NE: sense 5'-GGA ACT GAA CGT CAC GGT GGT C-3', anti-sense 5'-GTT TTG AAT CCA GTC CAC ATA C-3'.

Intranasal immunizations. Individual 4get x Rag-$1^{-/-}$ recipients of wild-type, DO11.10 x TCR-Cα-/-, or no spleen cells were immunized on days 3 and 6 post-infection. Chicken ovalbumin (Sigma Chemical Company, St. Louis, MO) was administered to anesthetized mice via the intranasal route using 2.5 mg each immunization delivered in 50 μL sterile PBS. PBS alone was used as a control.

In vitro degranulation of eosinophils. Lung cells were isolated as described above, washed and incubated at 10^7 live cells/ml in culture medium at 37°C in 5% CO_2 for four hrs in the presence or absence of phorbol 12-myristate 13-acetate (PMA, 40 pg/ml) and ionomycin (2 μg/ml) prior to analysis of forward- and side-scatter characteristics by flow cytometry.

Statistics. Analysis of lung weight differences was performed by matched pairs comparison (t test with four degrees of freedom).

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Chapter IV:

T cell-extrinsic type 2 cytokines influence T helper 2 effector development

Abstract

The factors influencing differentiation of naïve T helper cells are critical to the expansion of an effector subset appropriate for a given infectious challenge. The nature of cytokine milieus experienced during T helper priming and activation has been postulated as an important factor influencing T helper differentiation. Here, we report that T cell extrinsic type 2 cytokines play an important role in instructing the differentiation of naïve T helper cells into Th2 cells. Although adoptive transfer of CD4+ T cells into *Nippostrongylus brasiliensis*- infected TCR $C\alpha^{-/-}$ mice resulted in Th2 development analogous to priming in wild type mice, CD4+ T cells transferred into IL4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients failed to activate the IL-4 gene, and Th2 differentiation was abrogated. Inability to activate the IL-4 gene corresponded with failure to clear worms from the gut. T cell-extrinsic type 2 cytokines may serve to relay critical information regarding the identity of pathogens to T cells, thus influencing the selective expansion of a polarized helper T subset best suited for a type 2 pathogen.

Introduction

Naïve CD4+ T cells have the potential to differentiate into discrete subsets of effector T helper cells in response to infectious challenges *in vivo.* This differentiation process requires extensive programming of gene expression and generates highly polarized populations of effector T helper cells – Th1 and Th2 cells – which are defined on the basis of distinct, heritable profiles of immunomodulatory cytokines. Th1 cells characteristically produce IFNγ and lymphotoxin (TNFβ), which promote inflammatory reactions through the activation of phagocytes and cytotoxic lymphocytes. Th2 cells typically secrete IL-4, IL-5 and IL-13, which mediate mucosal immunity and the expulsion of intestinal helminths. The factors influencing this polarization are of great interest because successful immunity *in vivo* depends on expansion of appropriate subsets in response to a given infectious challenge(Grogan and Locksley, 2002).

Several models have been put forth to explicate the mechanism by which naïve T helper cells are polarized. A stochastic model of T helper differentiation subsumes the idea that naïve T helper cells encounter their fates in a random manner, with equal probability of becoming a Th1 or Th2 cell regardless of the nature of the pathogen. In contrast, an instructive model holds that naïve T cells are influenced by the context of their environment, such that signals from the environment selectively drive programs of cytokine gene expression suitable for the given pathogen. An attractive candidate for how pathogen identity is encoded within the environment is through innate immune cells, which bear receptors for pathogen recognition, and in turn, selectively elaborate polarizing cytokines. Thus, a polarizing cytokine milieu established by innate immune

cells instructs the expansion of a particular T helper subset appropriate for the given pathogen(Fearon and Locksley, 1996).

Although the identity of cell types that establish the early cytokine milieus instructive for differentiating T helper cells are not well characterized, there have been several reports suggesting that a range of cell types – dendritic cells and macrophages – produce IL-12 in response to pathogens such as *Listeria monocytogenes* and influence Th1 development(Hsieh et al., 1993). An analogous definitive role of non-T cell-derived type 2 cytokines in Th2 differentiation has been suggested(Sabin and Pearce, 1995). Here, we report that T cell extrinsic type 2 cytokines play an important role in instructing the differentiation of naïve T helper cells into Th2 cells. Although adoptive transfer of CD4+ T cells into TCR $C\alpha^{-/-}$ resulted in Th2 development analogous to priming in wild type mice, CD4+ T cells transferred into IL4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients failed to activate the IL-4 gene, and thus Th2 differentiation was abrogated. Inability to activate IL-4 gene transcription correlated with a failure to clear worms from the gut. T cell-extrinsic type 2 cytokines thus play an important role in polarizing naïve T helper cells, and may serve to relay critical information regarding the identity of pathogens to T cells in order to induce expansion of a subset appropriate for a given pathogen challenge.

Results

Naïve CD4+ T cells fail to activate IL-4 gene expression in the absence of T cell-extrinsic type II cytokines. The contribution of T cell-extrinsic type 2 cytokines, IL-4 and IL-13, on Th2 differentiation was assessed by an adoptive transfer approach. CD4+ T cells were isolated from a previously described bicistronic cytokine transcription reporter mouse, the 4get mouse, and intravenously transferred into TCR $C\alpha^{-/-}$ recipients(Mohrs et al., 2001). Subsequent infection with the helminthic parasite of the gut, *Nippostrongylus brasiliensis,* resulted in Th2 development analogous to wild type mice. Between 20-35% of CD4+ T cells in lung and 10-25% of CD4+ T cells in mesenteric lymph nodes activated the IL-4 gene by day 9 post-infection, as determined by eGFP fluorescence without restimulation *ex vivo* (Figure 1a and b). In contrast, 4get CD4+ T cells transferred into IL-4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients failed to activate IL-4 gene expression; less than 3% of CD4+ T cells were eGFP+. Consistent with the finding that Th2 cells are required for clearance of adult worms from the gastrointestinal tract, 4get CD4+ T cell -> IL-4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients also failed to clear worms from the gut in contrast to their wild type counterparts (Figure 1c).

Previous work has demonstrated a requirement for IL-4 receptor signaling for the recruitment of immune effector cells into sites of inflammation. Recruitment of both helper T cells and eosinophils is dependent on IL-4 receptor signaling component, Stat6; Stat6$^{-/-}$ mice fail to recruit these inflammatory cell types into the airways in an antigen-induced model of airway hyperreactivity(Mathew et al., 2001).

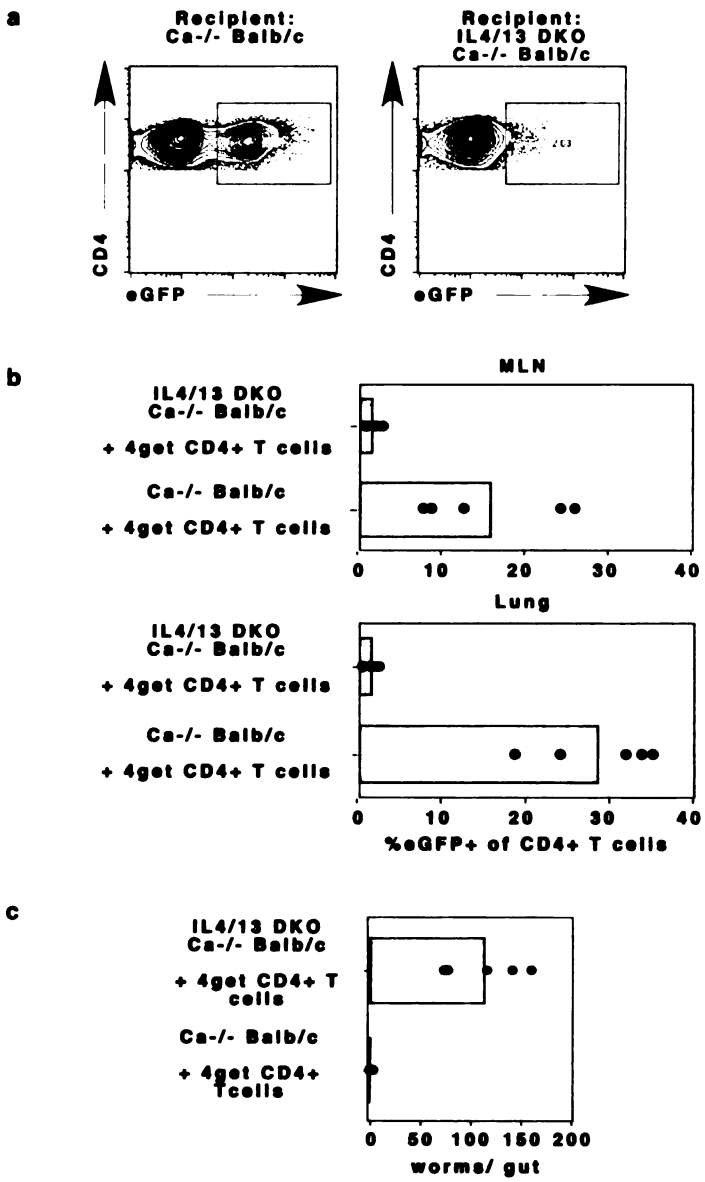
Figure 1. Th2 development is abrogated in the absence of T cell-extrinsic type 2 cytokines.

a, CD4+ T cells from 4get mice were transferred into TCR $C\alpha^{-/-}$ or IL-4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients and infected with *N. brasiliensis.* On day 10 post-infection, lungs were harvested and analyzed for eGFP fluorescence by flow cytometry.

b, As in **A**, showing the percentage of eGFP+ cells within the CD4+ T cell compartment in lungs and mesenteric lymph nodes (MLN) was determined in groups of mice (n=5).

c, As in **A**, showing the intestinal worm burden on day 10 post-infection. Data are representative of two comparable experiments.

Figure 1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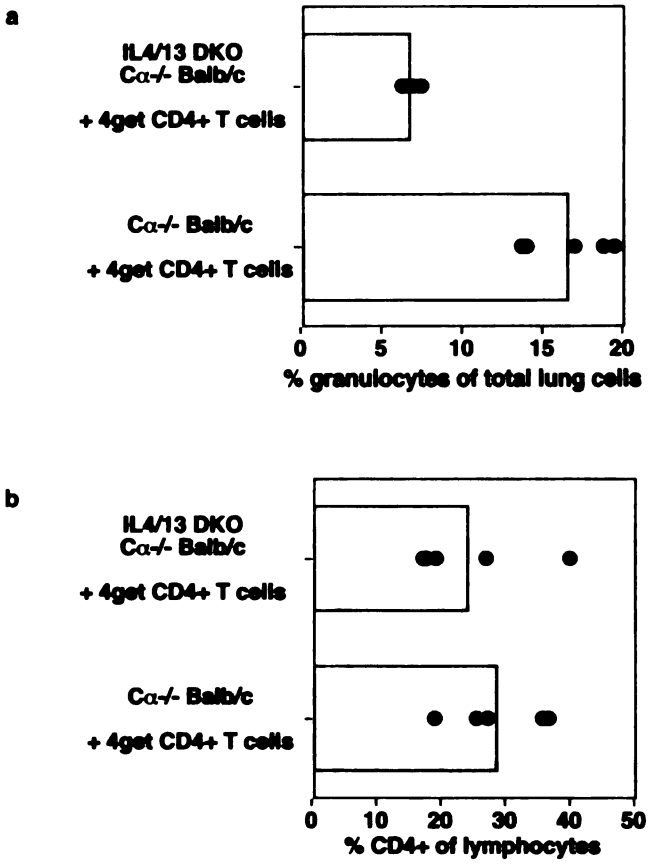
worms/ gut

Figure 2. Granulocyte recruitment, but not CD4+ T cell recruitment, is affected by the absence of T cell-extrinsic type 2 cytokines.

a, CD4+ T cells from 4get mice were transferred into TCR $C\alpha^{-/-}$ or IL-4/13$^{-/-}$ x TCR $C\alpha^{-/-}$ recipients and were infected with *N. brasiliensis.* On day 10 post-infection, lungs were assessed for granulocyte recruitment as a percentage of total lung cell number.

b, As in **A**, showing CD4+ T cell recruitment into the lung on day 10 post-infection. Data are representative of two comparable experiments.

Figure 2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Granulocyte recruitment was reduced almost two-fold in IL-4/13$^{-/-}$ x TCR C$\alpha^{-/-}$ recipients (Figure 2a). However, CD4+ T cell recruitment into the lungs was not statistically different between the two groups (Figure 2b). Thus, failure of Th2 priming may be attributed to requirements for signals provided by type 2 cytokines in the milieu but not failure of recruitment of these cells to the airways.

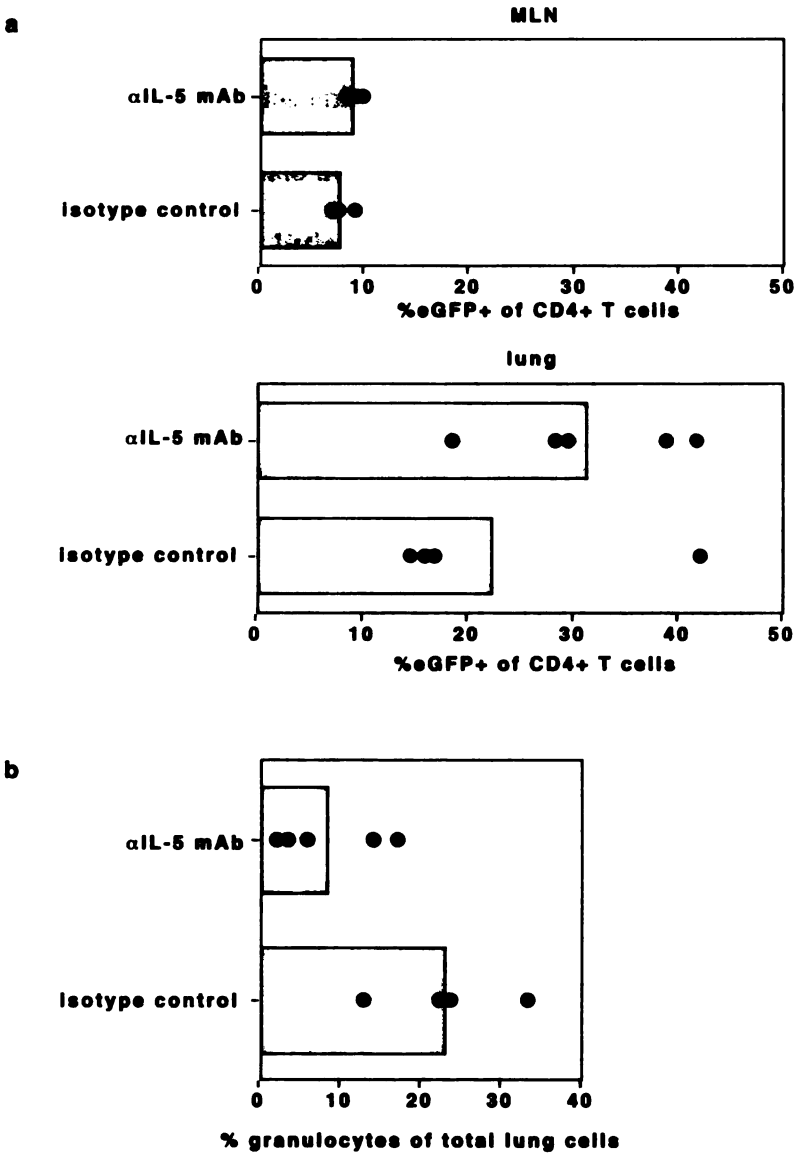
Blockade of eosinophil recruitment during *N. brasiliensis* infection results in normal Th2 development. *N. brasiliensis* is a powerful inducing stimulus of type 2 cytokine responses *in vivo*(Finkelman et al., 1997). This cytokine response is comprised of both innate and adaptive immune effector cells. While a wide spectrum of innate immune cell types produce type 2 cytokines during infection, eosinophils comprise over 90% of this cellular response (discussed in Chapter III). In order to test the role of eosinophils in Th2 development, *N. brasiliensis*-infected 4get reporter mice were treated with IL-5 neutralizing monoclonal antibody, TRFK5, which inhibits the terminal differentiation of eosinophils in the bone marrow, thus blocking eosinophil recruitment. Blockade of blood and tissue eosinophilia had no influence on the outcome of Th2 development, as determined on day 10 post-infection by eGFP fluorescence in CD4+ T cells by flow cytometry (Figure 3a). Induction of IL-4 gene expression in CD4+ T cells was not significantly different between TRFK5-treated mice or mice treated with an isotype control. Both groups of mice were successful in clearing worm burden from the gut by day 10 post-infection (data not shown). However, eosinophils were still present in the TRFK5-treated mice, as the antibody does not deplete eosinophils within peripheral tissues (Figure 3b). The level of eosinophils in TRFK5-treated mice was comparable to

levels of eosinophils in uninfected cohorts (data not shown), presumably reflecting the population of tissue resident eosinophils in lung parenchyma. Thus, although the dramatic blood and tissue eosinophilia was blocked, Th2 development was little affected, and antibody-treated mice went on to clear worm burden.

Figure 3. Blockade of eosinophil recruitment during *N. brasiliensis* infection does not interfere with Th2 development.

a, 4get mice were treated with either IL-5 neutralizing monoclonal antibody or isotype control and infected with *N. brasiliensis.* On day 10 post-infection, eGFP fluorescence in CD4+ T cells was assessed in lungs and mesenteric lymph nodes by flow cytometry. **b,** As in **A**, showing granulocyte recruitment into the lungs in IL-5 neutralizing or isotype matched monoclonal antibody-treated mice. Data are representative of two comparable experiments.

Figure 3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Discussion

This study suggests a critical role for T cell-extrinsic type 2 cytokines in development of Th2 effectors during *N. brasiliensis*-infection *in vivo.* However, the source of these cytokines remains unknown. Although eosinophils constitute over 90% of the recruited cells of the innate immune system during helminth infection, blockade of recruited eosinophils had little effect on the outcome of Th2 differentiation. However, other innate immune effector cells, as well as innate-like adaptive immune cells such as natural killer T cells, have been shown to be an early source of cytokines in many infectious disease and atopy models. Eosinophils, mast cells, macrophages, and natural killer (NK) cells have all been shown to activate the IL-4 gene during helminthic infection (discussed in Chapter III). The relative contribution of each of these cell types is currently under investigation. Differential production of IFNγ and IL-4 by γδ T cells has been observed in response to Type 1- or 2-stimulating pathogens in an intraperitoneal infection model(Ferrick et al., 1995). However, beta-2-microglobulin-dependent NKT cells - an early source of both IL-4 and IFNγ - have been shown to be dispensable for Th2 development in a number of *in vivo* models: protein immunization, antigen-induced airway hyperreactivity, and infection with *Nippostrongylus brasiliensis, Schistosoma mansoni,* and *Leishmania major*(Brown et al., 1996). It has also been previously reported that non-T cell-derived IL-4 may not be sufficient to drive physiologic actions of IL-4; B cell class switching to IgE in IL-4 deficient mice is only supported by transfer of IL-4 sufficient CD4+ T cells, but not IL-4 sufficient non-T cells(Schmitz et al., 1994).

The development of Th1-polarized cytokine production by innate immune effectors has been observed in antigen-presenting cells in several murine models of infection. Macrophages and NK cells activated by *Listeria monocytogenes* produce IL-12 and IFNγ, respectively, and can drive the development of Th1 cells. A report has also identified CD8α+ lymphoid-derived dendritic cells as a source for early IFNγ in response to the same pathogen(Ohteki et al., 1999). Thus, antigen-presenting cells may play an important role not only by initiating immune responses through antigen presentation but also in directing subsequent adaptive responses by establishing early, polarized cytokine milieus. This notion is consistent with recent work that has reported the role of different DC subsets in directing the development of distinct T helper effector subsets. Protein extracts derived from the helminth *Schistosoma mansoni* was reported to prime development of a Th2-promoting dendritic cell subset via an OX40 ligand-dependent mechanism(Whelan et al., 2000). However, other reports have shown that dendritic cell-derived IL-4 was not essential for Th2 development(MacDonald and Pearce, 2002).

The mechanism by which either naïve T helper cells or innate immune cells produce polarized responses remains unknown. Given the essential differences in antigen recognition between the innate and adaptive arms of immunity, one appealing hypothesis that must be highlighted is the molecular basis for making type 1/2 decisions in either system. For example, while T helper cells primarily recognize peptides derived from protein antigens in the context of class II MHC, they have no inherent ability to discriminate pathogens from innocuous substances on the basis of seeing only

the peptide-MHC complex, and thus rely on exogenous cues to direct or select the outgrowth of appropriate effector subsets. Differentiation of CD4+ T cells may thus be modulated by route and dose of antigen administration, activation of costimulation, TCR affinity for peptide-MHC complexes, genetic background, and cytokine milieu during T cell priming(Constant and Bottomly, 1997; Hosken et al., 1995; O'Garra, 1998). In contrast, a cardinal property of innate immunity is its ability to divide the universe of potential antigens into pathogens and non-pathogens on the basis of germline encoded receptors that recognize highly conserved pattern motifs unique to pathogens, or ligands upregulated during infection and inflammation(Medzhitov and Janeway, 1997). Whereas a role of Toll-like receptors (TLRs) in initiating Th1 responses has been demonstrated, it is appealing to speculate that an analogous pattern recognition receptor system functions during the evolution of type 2 immune responses(Kobayashi et al., 2002; Schnare et al., 2001). Receptors mediating type 2 immunity have yet to be identified, but are a topic of great interest.

A critical caveat to the interpretation of our data has been the consideration of the precise role of the type 2 cytokines. As the addition of type 2 cytokines to stimulatory cultures of purified, naïve T cells *in vitro* is sufficient to drive Th2 differentiation, it is tempting to speculate that the role of these cytokines *in vivo* is to directly stimulate naïve T helper cells to differentiate into Th2 cells. However, the mechanism by which these cytokines exert their effects may be more complex than previously thought. A potential role for type 2 cytokines in the upregulation of chemokine production at sites of inflammation has been revealed by studies in Stat6-/-

mice. Adoptive transfer of Stat6-sufficient T cells into Stat6-/- recipients has elucidated the contribution of Stat6-mediated signaling in the T cell- and non-T cell-compartments; absence of Stat6-/- in recipients completely abrogates Th2 immune responses within an allergic pulmonary inflammation model(Mathew et al., 2001). Most importantly, the lack of Stat6-/- in the recipient, but not the T cells, resulted in a dramatic loss of Th2 and eosinophil-active chemokine production. Thus the role of type 2 cytokine signaling may be to upregulate inflammatory signals in the non-T cell compartment – possibly on lung epithelium or pulmonary vascular endothelium – to promote recruitment of T cells and other immune effector cells, such as eosinophils, to the site of inflammation. Type 2 cytokines may also contribute to type 2 immune responses in entirely different ways. Urban and colleagues have shown that IL-4 responsiveness is required in non-bone marrow-derived cells to expel gastrointestinal helminths(Urban et al., 2001). In this case, type 2 cytokines may be critical to induce physiologic changes upon non-hematoipoietic cells, such as gut epithelium, goblet cells, and smooth muscle, necessary to mediate worm expulsion. Whether the role of IL-4 responsiveness is solely in non-hematopoietic cells, or whether these complement other hematopoietic cells, remains to be seen. Mast cells and macrophages represent potential candidates for early type 2 cytokine production. The relative contribution of each of these populations can be tested. Purging macrophages by toxin-loaded vesicles or use of a CD11a or c-promoter driven toxin receptor transgenic mice (Littman group, unpublished) would provide useful tools for testing the role of macrophages and dendritic cells, respectively, in this process *in vivo*. Finding requirements for Th

priming, such as whether IL-4-sufficient mast cells or eosinophils are sufficient to drive Th2-promoting effects within an IL-4/13-/- recipient can also be tested by adoptive transfer. However, a direct instructive role of these cytokines cannot be established at this time, and future studies will consider the requirements for physiologic action of early type 2 cytokines during Th2 differentiation. Indeed, elucidating the pathways by which early cytokine milieus impact development of Th2 effector subsets may suggest important therapeutic avenues for allergy and atopic disease.

Experimental Methods

Mice. All mice were maintained in the UCSF specific pathogen-free facility and used at 6-8 weeks of age. Interleukin-4 GFP enhanced transcript (4get) mice were previously generated, and backcrossed ten generations to BALB/c prior to use. TCR $C\alpha^{-/-}$ and IL-$4/13^{-/-}$ mice were previously generated (Murphy, 1990; McKenzie, 1999), and were backcrossed 10 or 5, respectively, generations onto BALB/c. These mice were then intercrossed to generate IL-$4/13^{-/-}$ x TCR $C\alpha^{-/-}$ mice.

Parasites and infection. Infective third-stage *Nippostrongylus brasiliensis* larvae (L3) were isolated from the feces of infected rats by modified Baermann technique. After washing, 500 larvae in 0.2 mL saline were injected subcutaneously at the base of tail. Infected mice were maintained on antibiotic-containing water.

T cell reconstitutions. CD4+ T lymphocytes from 4get mice were enriched to 90-99% purity by antibody- and complement-mediated lysis of CD8, class II MHC, and heat stable antigen (HSA)-bearing cells as described. 5 x 10^6 4get CD4+ T cells were injected intravenously in 0.2 mL phosphate-buffered saline (PBS) into indicated recipients.

Cell purification and analysis of cytokine expression. On specified days, mice were killed and the lungs perfused with sterile PBS via right cardiac ventricle. Lungs and mesenteric lymph nodes were excised and mechanically dispersed into single-cell suspensions. After red blood cell lysis, cell suspensions were labeled with antibodies to CD4 (YTS191.1, Caltag) and CD19 (1D3, Pharmingen) in the presence of Fc Block

(CD16/32, Pharmingen) and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

Determination of worm burden. Intestines, from gastroduodenal sphincter to the ileocecal junction, were surgically excised from *N. brasiliensis*- infected mice, cut longitudinally to expose the lumen and incubated in sterile Hanks buffer at 37°C for two hours. After incubation, adult worms were enumerated by light microscopy under a dissecting microscope.

In vivo depletion of eosinophils. Designated mice were treated with intraperitoneal injection of 2 mg monoclonal antibodies concomitant to time of infection. Eosinophil recruitment from the bone marrow was blocked with the IL-5 neutralizing monoclonal antibody, TRFK5, and control mice were treated with an isotype-matched monoclonal antibody, Y13-259 (anti-ras).

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Chapter V:

Future directions and conclusions

Abstract

The studies presented here reveal a novel and unexpected regulation of eosinophil degranulation by antigen-specific helper T cells. Subsequent studies were aimed at dissecting the molecular mechanism of this process by attempting to establish an *in vitro* culture system.. Stimulation of purified eosinophils with pharmacologic agents was not sufficient to induce degranulation *in vitro.* Culture of purified eosinophils with CD4 T cells or CD4 T cell culture supernatants also failed to provoke degranulation. Stimulation of dispersed lung cultures with a combination of a phorbol ester and ionomycin was the only stimulus capable of recapitulating degranulation in the tissue culture system. Here, preliminary studies towards the establishment of an *in vitro* system are analyzed, and strategies for defining the molecular basis of eosinophil degranulation are discussed.

In vitro assays for eosinophil degranulation

The regulation of eosinophil degranulation by antigen-specific helper T cells defines a novel aspect of the dynamic interplay between innate and adaptive arms of immune responses *in vivo.* This study progressed to investigate the cellular and molecular mechanisms by which T cells regulate eosinophil degranulation. Although important for stable Th2 development, signal transduction factors such as Stat6 and Type 2 cytokines such as IL-4 and IL-13 have already been found to be dispensable *in vivo.* Finding a single experimental approach to identifying degranulation-activating ligands subsumes the idea that the mechanism itself is clear; more preliminary work to define the basic outline of the mechanism may be required in order to rationally pursue target molecules. For example, two prominent outstanding questions are first, determining whether the regulation occurs via soluble factors or cell-cell contact, and second, determining whether the two cell types – T cells and eosinophils – are sufficient to recapitulate this phenomenon *in vitro.* The identification of intermediate cellular players in this process would greatly complicate the discovery of the molecular machinery involved.

As discussed in Chapter III, the mechanisms by which eosinophils degranulate have been an extremely contentious field of research. Most importantly, the relative contributions of several identified processes are not known. Transmission electron microscopy is currently considered the gold standard in the determination of degranulation states, as well as in the determination of eosinophils *in situ.* Three processes have been most widely reported. The first is the classic exocytotic pathway

by which intracellular vesicles are selectively transported to the cell membrane by a SNARE-mediated process(Egesten et al., 2001). This may occur at the surface membrane, but also through targeting to the phagolysosomal compartment – which may be important for eradication of internalized microbes. Other non-classical pathways include piecemeal degranulation and cytolytic degranulation (i.e., necrosis). Piecemeal degranulation involves a regulated redistribution of granules within activated eosinophils, whereby small granule-containing vesicles bud off of larger specific granules (Karawajczyk et al., 2000). How these smaller vesicles are transported to the membrane is not known, but the intracellular redistribution of granules has been observed in a number of studies. The IFNγ-induced release of the chemokine RANTES is believed to occur through piecemeal degranulation, but the pathways downstream of IFN-receptors leading to degranulation are not fully known(Lacy et al., 1999). The final pathway is cytolytic degranulation. This process is also termed necrosis, defined by morphological evidence of membrane disruption at the cell surface and nucleus, and correlated with other features of necrosis – such as chromatolysis and cytoplasmic degradation(Erjefalt et al., 1999). This may involve a general "accidental" spilling of granule contents secondary to cell death, occurring in a fashion to induce local inflammatory responses. Although observed in several disease states, the relative contribution of this and the other pathways in disease are not known(Erjefalt et al., 2001). Transmission electron microscopic analysis of eosinophil degranulation during *N. brasiliensis*- infection revealed evidence of piecemeal degranulation, as discussed in Chapter III.

Experimental *in vitro* studies into these questions were initiated but, unfortunately, have not yet yielded definitive insights into the general mechanism by which helper T cells facilitate eosinophil degranulation. In fact, most of the studies to date have failed to recapitulate the degranulation process *in vitro.* This includes studies treating highly purified SSC high eosinophils (thus, presumably non-degranulated) with various pharmacological agents: phorbol esters (PMA), ionophores (ionomycin), and interleukin-5 (IL-5), either alone or in combination. In the first series of studies, eGFP+, SSC high eosinophils were isolated on day 7 post-infection from lungs of *N. brasiliensis*-infected 4get mice by flow cytometric cell sorting and cultured *in vitro* in culture medium with ionomycin only. Degranulation was assessed by FACS analysis of forward- and side-scatter profiles (FSC versus SSC) of cells over a time course post-stimulation. At two different concentrations of ionomycin, 1 μg/mL or 5 μg/mL, eosinophils failed to degranulate at 0.5, 1.5, 4 and up to 44 hours post-stimulation (Figure 1a). By 44 hours post-stimulation, cells were no longer viable – as assessed by both FACS analysis and by histology (Figure 1b and c). Untreated cells were also not viable at 44 hours (data not shown). Treatment of sorted eGFP+, SSC high eosinophils with IL-5 (0.15 ng/mL) in addition to ionomycin (0.1 μg/mL), or IL-5 alone, also failed to sustain cell viability in culture, and the cells did not degranulate after 18 hours (Figure 2a). Treatment of these cells with PMA (41.6 pg/mL), ionomycin (2 μg/mL), and IL-5 (0.15 ng/mL) still failed to degranulate eosinophils *in vitro* at 18 hours (Figure 2b). Similarly, treatment of eGFP+, SSC high eosinophils with *N. brasiliensis* antigen extract, prepared from detergent-solubilized, sonicated infective larvae, failed to trigger

degranulation after 18 hours, even in the presence of IL-5 (0.15 ng/mL) (Figure 2c). Treatment of SSC low eosinophils with IL-5 (0.15 ng/mL) failed to result in the generation of SSC high eosinophils after 18 hours, casting doubt on the null hypothesis that the SSC low eosinophils were developmental precursors to the SSC high eosinophils (Figure 2D).

The basic requirements for the interaction between CD4 T cells and eosinophils were interrogated in a series of *in vitro* experiments. CD4 T cells were isolated from lungs or mesenteric lymph nodes of *N. brasiliensis*-infected 4get mice on day 7 post-infection and co-cultured with eGFP+, SSC high eosinophils; these conditions were not sufficient to drive degranulation after 1.5, 3.0, or 16 hours of co-culture even if T cells were pre-activated *in vitro* with PMA/ionomycin (42 pg/mL and 2 μg/mL) for 2 hours prior to co-culture (Figure 3a). As expected, similar results were obtained when T cells were cultured with eosinophils in a transwell culture system, where the cells were separated by a physical barrier preventing cell-cell contact (data not shown).

The idea that T cell-derived soluble factors would be sufficient to induce eosinophil degranulation was tested by incubating culture supernatants of CD4 T cells isolated from lungs of *N. brasiliensis*-infected 4get mice with dispersed lung cultures of infected Rag1-/- 4get mice. Specifically, CD4 T cells were purified from lungs of infected 4get mice on day 7 post-infection, stimulated *in vitro* by TCR (H57, anti-TCR beta chain ligation) and CD28 ligation in IL-2 (50 U/mL), and culture supernatants were collected at 48 hours. Incubation of eosinophils with either the pre-activated CD4 T cells or their culture supernatants failed to induce degranulation (Figure 3b and c,

respectively). Thus, attempts to establish a simple *in vitro* model of eosinophil degranulation were not successful.

The experiments that did successfully demonstrate this process *in vitro* were those in which SSC-high eosinophils could be degranulated with phorbol esters and ionophores (PMA & ionomycin) within the context of lung cultures. These lung cultures consist of mechanically dispersed lungs from *N. brasiliensis*-infected mice. This result was true in both lung cultures isolated from infected wild type and Rag1-deficient mice, presumably through a direct effect on the eosinophils. The ability of PMA/ionomycin to induce degranulation in Rag1-deficient mice suggests that the pharmacologic effect does not require T cells. The cellular context of the eosinophils may be critical, as suggested by the failure of highly purified SSC high eosinophils, isolated by cell sorting, to degranulate under similar stimulatory conditions. Factors contributing to the proper context are not known; they could involve issues of cellular density or adhesion to other cells within the culture (i.e., epithelial cells, macrophages), or the involvement of a third cellular participant. Several ways to dissect out the components involved may include the use of coated tissue culture plates, such as fibronectin- or collagen-matrix coated plates, or plating eosinophils on a "feeder" layer of cells, such as monolayers of epithelial cells or macrophages.

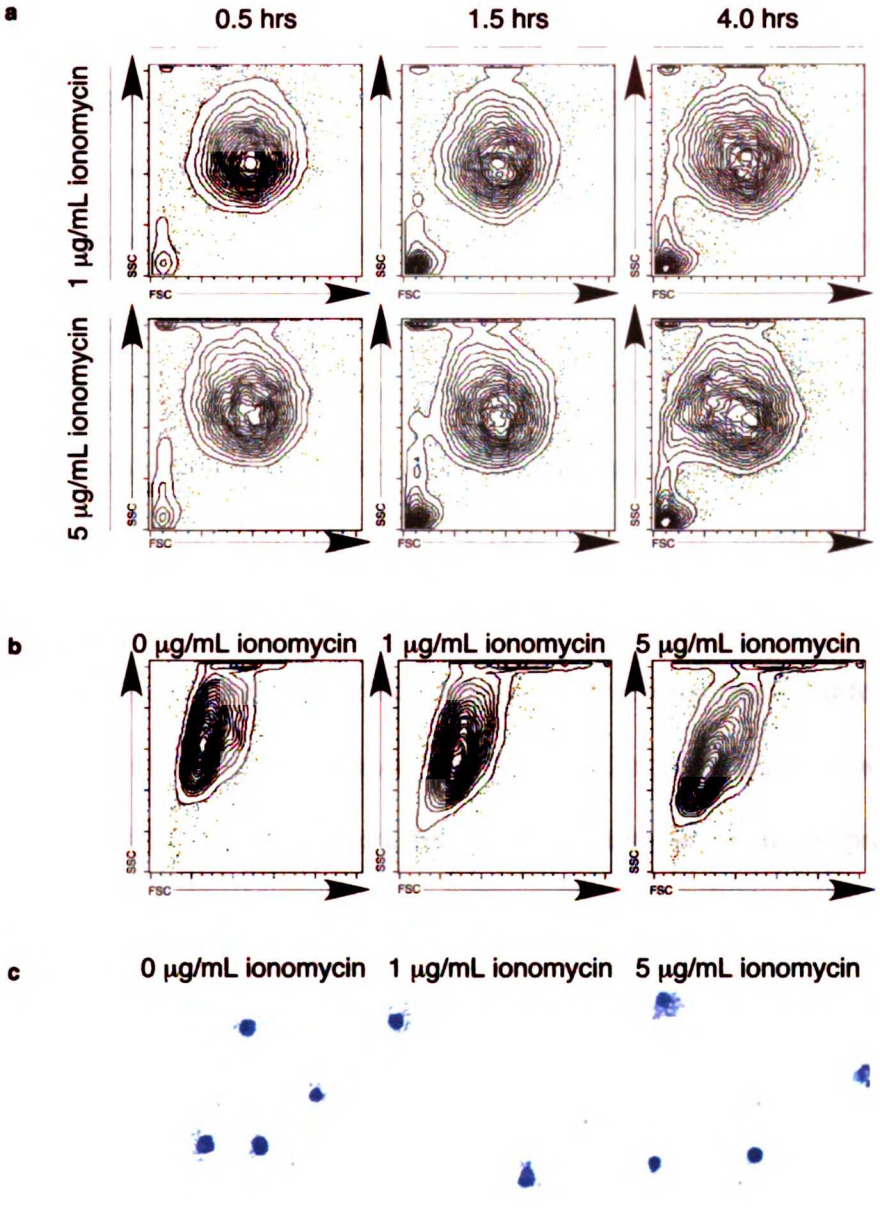
Figure 1. Treatment of eosinophils with ionomycin, PMA, and/or IL-5 fails to induce degranulation *in vitro*.

a, eGFP+, SSC high eosinophils were isolated on day 7 post-infection from lungs of *N. brasiliensis*-infected 4get mice by flow cytometric cell sorting and cultured *in vitro* in culture medium with ionomycin only. Degranulation was assessed by FACS analysis of forward- and side-scatter profiles (FSC versus SSC) of cells over a time course post-stimulation. At two different concentrations of ionomycin, 1 μg/mL or 5 μg/mL, eosinophils failed to degranulate at 0.5, 1.5, 4 and up to 44 hours post-stimulation.

b, Assessment of eosinophil viability 44 hours post-treatment with ionomycin by FACS analysis of FSC and SSC profiles.

c, Assessment of eosinophil viability 44 hours post-treatment by histologic analysis.

Figure 1


a
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1.5 hrs
4.0 hrs
1 µg/mL ionomycin
5 µg/mL ionomycin
SSC
FSC
b
0 µg/mL ionomycin
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5 µg/mL ionomycin
SSC
FSC
c
0 µg/mL ionomycin
1 µg/mL ionomycin
5 µg/mL ionomycin

Figure 2. Treatment of eosinophils with IL-5, ionomycin, PMA, and/or *N. brasiliensis* extract.

a, eGFP+, SSC high eosinophils (eos) purified from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection were treated with IL-5 (0.15 ng/mL) in the presence or absence of ionomycin (0.1 μg/mL) and analyzed by FACS (FSC versus SSC profile) at 18 hours post-stimulation.

b, eGFP+, SSC high eosinophils purified from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection were treated with PMA (42 pg/mL), ionomycin (2 μg/mL), and IL-5 (0.15 ng/mL) and analyzed by FACS (FSC versus SSC profile) at 18 hours post-stimulation.

c, eGFP+, SSC high eosinophils purified from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection were treated with *N. brasiliensis* antigen extract (Nippo Ag), prepared from detergent-solubilized, sonicated infective larvae, in the presence of IL-5 (0.15 ng/mL) and analyzed by FACS (FSC versus SSC profile) at 18 hours post-stimulation.

d, eGFP+, SSC low eosinophils (eos) purified from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection were treated with IL-5 (0.15 ng/mL) and analyzed by FACS (FSC versus SSC profile) at 18 hours post-stimulation.

Figure 2

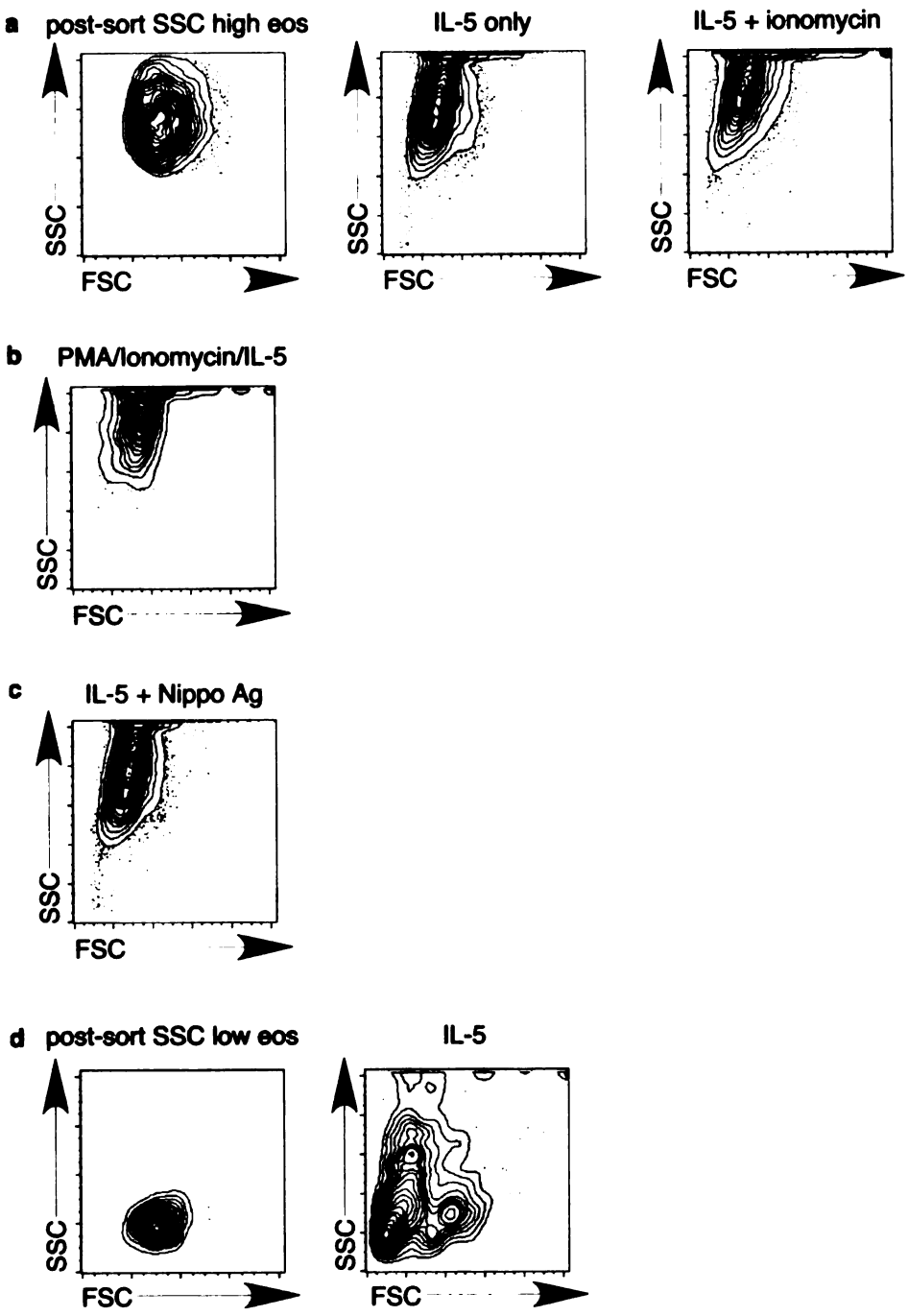

a
post-sort SSC high eos
IL-5 only
IL-5 + ionomycin
SSC
FSC
b
PMA/Ionomycin/IL-5
c
IL-5 + Nippo Ag
d
post-sort SSC low eos
IL-5

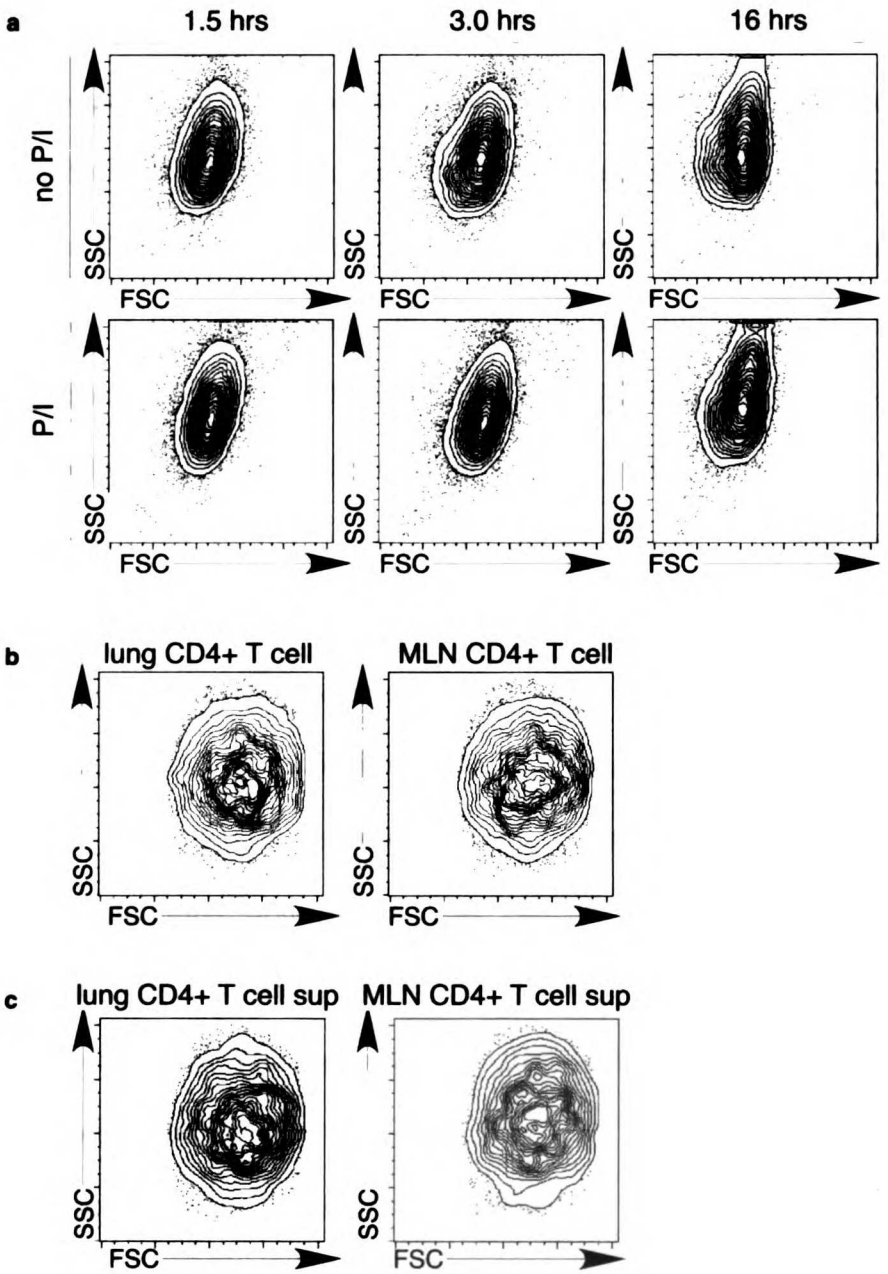
Figure 3. Co-culture and transwell cultures of eosinophils and CD4 T cells.

a, Co-culture of eosinophils and CD4 T cells purified by cell sorting from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection were analyzed by FACS (FSC versus SSC profiles). CD4 T cells were isolated by cell sorting, pre-activated for 2 hours with PMA (41.6 μg/mL) and ionomycin (2 μg/mL), washed extensively, and then co-cultured with eGFP+, SSC high eosinophils for 1.5, 3, or 16 hours.

b, eGFP+, SSC high eosinophils were co-cultured with pre-activated CD4 T cells. CD4 T cells were sorted from lungs or MLN of infected 4get mice on day 7 post-infection that were stimulated for 48 hours *ex vivo* by TCR/CD28 ligation. The cells were washed extensively, and then cultured for 6 hours with eGFP+, SSC high eosinophils sorted from lungs of *N. brasiliensis*- infected 4get mice on day 7 post-infection.

c, Lung cultures derived from *N. brasiliensis*- infected Rag1-deficient mice on day 7 post-infection were cultured *in vitro* for 24 hours with CD4 T cell culture supernatants. Supernatants were derived from CD4 T cells sorted from lungs or mesenteric lymph nodes of infected 4get mice on day 7 post-infection that were stimulated for 48 hours *ex vivo* by TCR/CD28 ligation.

Figure 3


a
1.5 hrs
3.0 hrs
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b
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MLN CD4+ T cell
c
lung CD4+ T cell sup
MLN CD4+ T cell sup

Dissecting the mechanism(s) of eosinophil degranulation

From a more theoretical standpoint, the regulation of eosinophil degranulation may proceed through numerous hypothetical mechanisms. The simplest would be a single receptor-ligand pair between helper T cell and eosinophil; this would be analogous to the CD40-CD40L system in T cell: dendritic cell / macrophage interactions. The Tumor Necrosis Factor (TNF) receptor superfamily may represent a family of molecules that are induced in a manner both temporally and spatially appropriate for such regulation: CD40-CD40L, OX40-OX40L, and RANK-RANKL pairs represent potential candidates(Locksley et al., 2001). Such interactions could be investigated by candidate approach, using blocking antibodies or soluble antagonist competitors; however, such a candidate screen would most certainly be predicated on an established *in vitro* culture system, although *in vivo* screening would be a possibility if a wealth of blocking reagents were readily available. A genetic approach could also be used to identify pathways involved; for example, using eosinophils or T cells derived from genetically mutated donors, such as the Src family member-deficient mice(Stafford et al., 2002), Itk-deficient (Tec family kinase)(Fowell et al., 1999), or the aforementioned candidate TNFRs or their signaling intermediates (i.e., TRAF-deficient)(Chung et al., 2002).

Alternative pathways for such regulation may include a more complex, multicellular program (i.e., T cells activate an intermediate cell, such as epithelium, to display a receptor that physically interacts with eosinophils), a pathway of neglect (i.e., down regulation of inhibitory receptors which hold degranulation in check), or even

interaction of T cell-derived factors with soluble intermediates that only together are sufficient to activate eosinophils. These alternate pathways would pose great experimental difficulty in defining a single assay for clarifying the molecular components involved; multiple assays of disparate approaches may be required to narrow the search for ligands. One way to ask which pathways are involved in a more open-ended manner is to perform careful comparative studies on the two target populations – for example, comparing granulated versus degranulated eosinophil subsets, or IL-4 expressing versus non-IL-4-expressing eosinophils. Such studies could interrogate at the level of expression, translational or post-translational differences. The comparison of transcriptional differences could be done by differential mRNA display, subtractive hybridization, or even gene chip approaches. At the translational or post-translational modification level, a comparative proteomics approach could be undertaken, but one could also attempt a more simplified approach, exploring differences in protein expression by 2-D gel analysis or differences in protein modification – for example, comparing patterns of tyrosine phosphorylation between the different groups. Clearly, identifying readily-available sources of large numbers of cells would facilitate this process, and utilizing cell lines or eosinophils from IL-5 transgenic overexpressing mice would be useful reagents(Lee et al., 1997).

Highlighting the involved pathways would open the door towards more rigorous testing of the involvement of individual players in the process. Such screens could be utilized to also pursue the T cell derived factors required for eosinophil activation; again, a comparative study of transcriptional or translational programs could be

performed in antigen-specific versus non-specifically activated T cells, or in *Nippostrongylus*- activated cells from disparate tissue sites – for example, from lung versus draining lymph nodes.

If the discovery of even a single member of a receptor-ligand pair could be made on the basis of preliminary work, then it would be fruitful to consider expression cloning as a methodology for identifying the cognate ligand. Fc-fusion proteins have been used as "bait" to screen for expression of binding partners induced by a retroviral expression library. For example, this approach has been successfully employed by Cerwenka and colleagues, who generated an Fc-fusion protein of murine NKG2D to identify its ligands, members of the *h60* and *rae-1* gene families(Cerwenka et al., 2000). Thus, generation of an Fc-fusion protein of the known receptor could be used to screen an otherwise non-binding cell line that has been retrovirally transduced with a cDNA expression library derived from eosinophils (or T cells).

In the absence of a single identified binding ligand, a more general approach could be attempted, such as the generation of hybridoma panels that could be screened for degranulation-stimulatory (or, theoretically, inhibitory) activity. For example, the generation of hybridoma panels from rats or rabbits immunized with mouse eosinophils could provide ample starting material to then screen panels of antibodies for their ability to drive degranulation of eosinophils or eosinophil cell lines *in vitro*. Identification of an antibody panel would then require further subdivision of the panel to identify the single hybridoma clone(s) with the stimulatory property. One way to optimize this assay for a more high-throughput approach is to utilize eosinophil cell lines that express

known granule contents, and then to screen the supernatants for granule contents either by ELISA or by colorimetric assay (i.e., an enzyme substrate that changes color upon cleavage). Simultaneous generation of reagents for screening degranulation, such as antibodies to MBP-1, ECP, and other granule contents, would clearly be worthwhile to facilitate such *in vitro* and future *in vivo* studies(Mould et al., 2000).

Eosinophil degranulation as a therapeutic target

Defining the pathways by which eosinophil degranulation is regulated by helper T cells may ultimately identify fruitful targets for therapeutic intervention. Such specific targets have long been elusive, and have resulted in the generation of less efficacious therapies or worse yet, the need for total immunosuppression in relatively mild instances of atopy. If our hypothesis that eosinophil degranulation results in tissue damage is true, then identification of such targets would allow the development of drugs to inhibit specific aspects of activated T cells without the need for total T cell suppression. Identifying specific therapeutic targets would most definitely reduce the side effects and health risks associated with long-term immunocompromised status. The efficacy of treatments targeting IgE on mast cells suggest that an analogous treatment blocking degranulation in eosinophils would provide a conceptual breakthrough to the current armamentarium of allergy and asthma medications: anti-histamines, mast cell stabilizers, steroidal anti-inflammatories, inhibitors of leukotriene synthesis, "immunotherapy" such as allergy shots, and epinephrine rescue(Kay, 2001). In addition, this pathway may be exploited for not only blockade of allergic disease, but also for harnessing eosinophils for tumor killing; recent evidence in the literature

suggests an important role for eosinophils for immune-mediated destruction of tumors(Hung et al., 1998).

Conclusion: the molecular basis of type 2 immunity

One remaining issue, last but certainly not least in importance, is the molecular mechanism by which the IL-4 gene is activated in non-T cells, and specifically, in cells of the innate immune system. The system described here clearly demonstrates the existence of type 2 innate immunity, suggesting a recognition event by which signals from the external environment – presumably staged by *Nippostrongylus brasiliensis* itself – are transduced to the IL-4 gene. The molecular basis of this recognition event underlies important pathways leading to the initiation of type 2 immune responses, and may represent the highly sought "type 2 pattern recognition receptor." Although analogous pathways have been described for type 1 immunity, such as the TLRs, the pathways leading to type 2 pattern recognition have remained far more elusive.

Preliminary studies into the nature of this ligand have been initiated by Ruslan Medzhitov and colleagues, who set out to address this question by immunizing rabbits with a mouse mastocytoma cell line, generating hybridoma panels, and screening for stimulatory antibodies that drive IL-4 production. So far, these studies have only pulled out weak agonists of the pathway. These are relayed independently of MyD88, suggesting a non-TLR-mediated system of pattern recognition, or at least it may not involved none of the known TLRs that signal through MyD88 (Medzhitov, 2002). A similar approach could be attempted with eosinophils, or eosinophil cell lines, although the realistic prediction would be that one would obtain similarly inconclusive results.

One improvement to this system would be to utilize a reporter system to facilitate the screening of the IL-4 phenotype. A cell line such as mastocytomas, EL4, or even Jurkat could be stably transfected with the bicistronic IL-4 reporter construct and then used to screen stimulatory antibodies(Mohrs et al., 2001). An alternative approach would be to generate eosinophil cell lines from 4get mice by transformation to screen candidate antibodies. Finally, expression cloning would be a distant possibility, using retroviral transduction of a cDNA library generated from IL-4+ eosinophils into a non-IL-4 expressing eosinophil or mast cell line and using IL-4 as a reporter read-out. The potential caveats of such an experiment are numerous, and broadening the source of cDNA may eliminate general assumptions inherent to the experimental strategy – such as using cDNA from lungs of *Nippostrongylus brasiliensis* –infected mice and transducing a more nonspecific, even non-hematopoietic, cell line to make IL-4. Again, the caveat here is that the remainder of the signaling components required to access the IL-4 gene will be intact, and thus using a recipient cell line that is closest to a type 2 innate immune effector cell, such as a eosinophil or mast cell, may best approximate the signaling pathways required for IL-4 gene expression.

A recent report by Ogawa and colleagues has demonstrated findings for a role for TLR2 in mast cell degranulation in response to peptidoglycan(Supajatura et al., 2002). In this report, *in vitro* stimulation of bone marrow-derived mast cells revealed differential roles for TLR2 and TLR4 in mediating recognition of LPS versus peptidoglycan. Whereas peptidoglycan recognition leading to degranulation was dependent on TLR2, but not TLR4, and resulted in nuclear translocation of NFκB, LPS

recognition was solely mediated by TLR4. TLR2-dependent recognition of peptidoglycan resulted in mast cell production of many type 2 cytokines, including IL-4, IL-5, and IL-13; this was in contrast to TLR4-dependent recognition of LPS, which stimulated TNF-a, IL-1B, IL-6, IL-13, but not IL-4 nor IL-5. TLR2 was required in the mast cell compartment for inflammation in response to intradermal injection of peptidoglycan. While this study implicates a role for TLR2 and TLR4 and mast cell effector function, it is still not yet clear what this role is – for example, TLR 4, but not TLR2, was required in the mast cell compartment for full expression of innate immunity in a mast cell-dependent model of sepsis (cecal ligation and puncture model); this effect may be downstream of defective neutrophil recruitment and reduced inflammatory cytokine production. An important piece of the puzzle that remains unanswered is identifying the signaling pathways by which TLR2 signals. Although it does not signal on its own or even when homodimerized, there is a widely accepted speculation that TLR2 signals in the context of a heterodimer. This notion, in the context of the previously discussed results by Ogawa and colleagues, may highlight the existence of other yet identified mast cell-specific TLR family members or adapter. Whether the TLRs play an analogous role in eosinophil degranulation remains to be seen; while Medzhitov's data renders evidence that type 2 cytokine release is independent of MyD88, it remains possible that TLRs could signal through an alternative coreceptor (i.e., PGRP) or adapter protein.

The biggest caveat of all is that the type 2 pattern recognition is not an active process, as we have interpreted from our data, but instead represents a default pathway.

While there is a vast body of evidence against this hypothesis, the possibility of this alternative mechanism should at least be discussed here. Recent work has led to the interesting suggestion that in contrast to the clear role of TLRs in the activation of Th1 immunity, innate immune recognition may not be required for the initiation of type 2 adaptive immune responses(Schnare et al., 2001). This suggestion arises from the interpretation of phenotypes of mice deficient for signaling components of the TLR pathway, such as the MyD88 knockout, that display increased Th2 bias in the absence of Th1 polarization(Kobayashi et al., 2002). Inherent to this hypothesis is the idea that type 2 responses are initiated in a manner fundamentally distinct from type 1 responses, foremost that type 2 responses represent a "default" pathway that occurs in the absence of signals driving type 1 responses. By this alternative explanation, Th2 cells become IL-4 producers by default, without instruction from the innate compartment. This suggestion rests on the speculation that the nature of signals driving type 2 adaptive responses – namely allergens and multicellular, eukaryotic parasites, as opposed to less molecularly-complex PAMPS such as lipopolysaccharide, CpG DNA, and peptidoglycan – utilize mechanisms distinct from those used to activate the type 1 pathway. Indeed, preliminary results by Medzhitov and colleagues indicate that type 2 immune responses to *Schistosoma mansoni* are not dependent on MyD88. However, the possibility of existing but yet unknown pattern recognition receptors precludes a definitive refute of a pathway for innate induction of type 2 immunity analogous to the TLRs for type 1 immunity. One possibility is the existence of yet undefined receptors, with a second hypothesis that type 2 immune recognition occurs through alternative

downstream signaling of known Toll-like receptors. The existence of such alternative signaling cascades, for example, the recent identification of novel adapters of the Toll pathway – Tollip and TIRAP – by Medzhitov, Volpe, and Ghosh laboratories provides appealing candidates for study of such a mechanism(Burns et al., 2000; Horng et al., 2001; Zhang and Ghosh, 2002). In particular, alternative signaling cascades may represent an important mechanism because they may act as negative regulators of the activating pathways downstream of TLRs – as has been demonstrated for Tollip, which negatively regulates TLR2 and TLR4. Such a mechanism may work to suppress type 1 responses through an active mechanism, and thus, in their absence, type 2 pathways prevail. Rather, there is growing evidence that the two pathways reciprocally balance one another, through active suppression and not by default(Salkowski et al., 1999; Toshchakov et al., 2002). This mechanism, where the ultimate phenotype of the immune response may rest on a balance of integrated signals, is akin to the system of activating and inhibitory receptors on natural killer (NK) cells as determinants for NK cell effector function.

Investigations of the molecular basis of immune responses in *Drosophila melanogaster* suggest that selective activation of pathways resulting in the production of discrete anti-microbial effector molecules is regulated at the level of pathogen recognition(Hoffmann and Reichhart, 2002). Beyond such candidates also lies the possibility that receptors for the initiation of type 2 immunity are not driven by pattern recognition at all, but rather through recognition of self-ligands, such as ligands upregulated by tissue injury or proteolytic modification of constitutively-expressed self

ligands. One favorite unifying candidate is proteases, a common allergen (i.e., feline salivary proteases, cockroach fecal antigens, house dustmite antigens such as Derp1 and Derp2, subtilisin, papain, fungal-derived proteases) and effector molecules of helminths (i.e., utilized to access tissue compartments). Simultaneously exploring the common features of helminthic infection and atopic disease may be the only true path to understanding the pathologic basis for allergic disease; there is no doubt that the parallel investigations in either field will ultimately reveal common principles of the etiology of type 2 immune responses.

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Chapter VI:

Experimental Methods

Reverse Transcription Reaction: RNA --> cDNA Preparation

Cell suspensions, 4 x 10^6 cells per sample, were harvested by centrifugation into a 15 mL Falcon tube. The supernatant was removed, and cell pellet mixed in 1 mL RNAzol, and incubated on ice for 5 minutes. Samples were quick frozen in liquid nitrogen, and stored at -70°C until extraction of RNA. Extraction was performed by adding 100 μL $CHCl_3$ to 1 mL homogenate, covered, and shaken for 15 seconds. After incubation on ice for 5 minutes, the sample was centrifuged for 15 minutes at 4°C. The aqueous phase was extracted and added to 300 μL isopropanol, incubated at 4°C for 15 minutes, and then centrifuged for 15 minutes at 4°C. After removal of the supernatant, the pellet was washed in 1mL 70% EtOH at room temperature, and spun at 4°C for 8 minutes. Upon removal of the supernatant, the pellet was dried under vacuum, and dissolved in 20μL 1mM EDTA, pH 7.0. RNA was stored at -70°C prior to reverse transcription.

Reverse transcription reaction:

10 μL of each RNA sample was mixed with 2.5μL DEPC-H_2O to yield a total volume of 12.5 μL. 1 μL of random hexamer primers, or a 1:1 mix random hexamers/ oligo dT 12-18 primers, heated to 70°C for 2 minutes, and then quenched on ice. The reverse transcription reaction consisted of: 5x reaction buffer (4 μL) dNTP (1 μL), Rnase inhibitor (0.5 μL) RT enzyme (1 μL) for a total volume of 6.5 μL. This 6.5 μL reaction was added to the 12.5 μL RNA sample to yield a total volume of 20 μL, mixed, and incubated at 42°C for 60 minutes. The samples were then incubated at 94°C for 5 minutes, spun briefly, and then diluted to a final volume of 100 μL with DEPC-H_2O.

The samples were vortexed, briefly spun, and then stored at -70°C prior to DNase treatment.

DNAse treatment of RNA

5 μg RNA in 29 μL DEPC-H_20 was used as starting material for DNase treatment. If the sample was 1 μg or less, 1.25 - 5.0 μg acetylated BSA (Gibco BRL 15561-012) was added to the RNA sample. The DNAse reaction was as follows: 5x First Strand buffer (Gibco-BRL)(8 μL), RNAsin (BM)(1 μL), and RNase-free DNAseI (BM)(2 μL), incubated at 37°C for 20 minutes, incubated 70°C for 10 minutes, and then stored at 4°C. Oligo d(T)12-18 primers (BM)(5 μL) and random hexamers 500 ng/μL (BM)(0.5 μL) were heated to 70°C for 10 minutes, then quenched on ice. Contents of tube were spun down, and the remainder of the reverse transcription reaction (49.5 μL) was added: 5x First Strand buffer (Gibco-BRL)(10 μL), 0.1M DTT (Gibco-BRL)(12 μL), 10 mM dNTP mix (5 μL), RNAsin (2 μL), and ddH_20 (20.5 μL). This reaction was incubated at 42°C for 2 minutes, and then 5 μL of Superscript II (Gibco-BRL) RT was added. The reaction was then incubated at 42°C for 50 minutes, then incubated at 70°C for 15 minutes. The final reaction is 5 μg RNA/ 100 μL, of which 10 - 50 ng cDNA was used per PCR/ Taqman reaction.

Polymerase Chain Reaction (PCR)

100 μL reactions were set up on ice as follows: 10x PCR reaction buffer (10 μL), dNTP (10mM stock)(1 μL), and 5' and 3' primers (20 mM stock)(2 μL), ddH_20 (81 μL), cDNA (5 μL), and Taq polymerase (1 μL). The reaction conditions were 94°C x 5 minutes, followed by 36 cycles of 94°C x 30 seconds, 60°C x 20 seconds, 72°C x 40

seconds, followed by 72°C x 7 minutes, and a 4°C hold. For screening IL4/13-/- mice, a combination of three primers were used: (IL13 exon 3, 5') CCT GGA TTC CCT GAC CAA CAT C, (IL13 exon 3, 3') GGC CTT GCG GTT ACA GAG GCC, (neomycin cassette) ACC ACA CTG CTC GAC ATT GGG TG. This results in the amplification of an 80 bp amplicon in wild type mice, and a 100 bp amplicon in IL4/13-/- mice. For screening Rag1-/- mice, a combination of four primers were used in the same reaction: (Rag1 5') GAT CGA CGT GAA GGC AGA TG, (Rag1 3') GTC TCT TCC TCT TGA GTC CC, (Neomycin, 5') CTT GGG TGG AGA GGC TAT TC, (Neomycin, 3') AGG TGA GAT GAC AGG AGA TC. This results in the amplification of two bands: neomycin (200bp) and Rag1-/- (<100bp).

Immunoprecipitation from primary mouse CD4+ T cells

Lymph nodes and spleen were surgically isolated from mice, pushed through a 70 μm cell strainer placed on top of 50 mL Falcon tube using a 3 cc syringe plunger. After rinsing the cell strainer with 30 mL RPMI-10, cells were centrifuged at 1200 rpm for 5 minutes at 4°C, and washed three times. After enumerating cell counts, cell suspensions were resuspended at $3x10^7$ cells per mL complement lysis mix. The complement lysis mix was as follows: 3.155 (anti-Lyt2/CD8 antibody), BP107 (anti-Class II MHC (Ia^d), J11D (anti-Heat Stable Antigen), with guinea pig complement (GpC'), and rabbit complement (Rc'), in RPMI-10, filter-sterilized through a 0.22 μm filter. The complement lysis reaction was incubated at 45 min at 37°C, and viable cells isolated by centrifugation over a Ficoll gradient, spun at 2500 rpm for 15 minutes at room temperature (no brake), or a Histopaque gradient, spun at 1600 rpm for 25

minutes at room temperaure (no brake). Cells were counted, with an aliquot taken out for post-lysis analysis of purity by flow cytometry, washed three times in RPMI-10, rested at 37°C for 2 hours in serum-free RPMI prior to stimulation. Cells were resuspended in 120 μL RPMI-10, with 25 μg/mL biotinylated anti-CD3_ Ab (Pharmingen 01032D), and incubated on ice for 10 minutes. 80 μL streptavidin (at a final concentration of 50 μg/mL) was added, and samples were incubated at 37°C for the given length of a time point ranging between 30 seconds to 30 minutes. Activating reactions were terminated by addition of 5 mL ice-cold PBS + 20 mM NaF and 1 mM Na3VO4, and washed twice in the same termination buffer. The cells were then lysed into 350 μL cell lysis buffer + protease inhibitors by incubation on ice for 15 minutes in 4°C room. Lysis buffer consisted of 50 mM Hepes pH7.4, 150 mM NaCl, 1% NP-40 or Brij 97, 2 mM EDTA, 10% glycerol, with freshly added aprotinin (10 μg/mL), leupeptin (10 μg/mL), pepstatin A (1 μg/mL), 10 nM okadaic acid, 10 mM NaF, and 10 mM NaPP, and freshly-prepared 2mM Na_3VO_4 and 1mM PMSF. Lysates were immediately utilized for immunoprecipitations or stored at -80°C. Samples were thawed at room temperature, then spun at 4°C at 14,000 rpm for 10 minutes. 350 μL of each lysate was transferred to fresh microfuge tubes, and 10μL immunoprecipitating monoclonal antibody was added to each tube of 350 μL lysate, and incubated for 1 hour on ice at 4°C. 50μL supernatant was also removed to check depletion. Following incubation with the primary antibody, 100 μL (30 – 150 μL) of washed (in lysis buffer + inhibitors 3-4x @ 4°C) 50% protein G-sepharose/PBS slurry was added to each tube and incubated on a rotary wheel at 4°C for 30' - 1h (pipetting protein G-sepharose

through wide-bore pipet tips). Sepharose was pelleted by brief centrifugation, from which the supernatant was aspirated off, utilizing a gel-loading tip for aspiration. Sepharose beads were rapidly washed three times with 0.5 mL fresh, cold lysis buffer at 4°C: resuspending beads in wash buffer, spinning washes for 5s, aspirating wash buffer away. Proteins were eluted from sepharose in 100 μL of 2x sample buffer for 2 minutes at 100°C, spun for 5 seconds to pellet the beads, and supernatants loaded onto a 7% polyacrylamide gel, or snap-frozen in liquid nitrogen and stored at –80°C until loading onto gel. Gel was equilibrated in BSN transfer buffer for 15 minutes at room temperature, and then transferred onto nitrocellulose at 20V for 30 minutes. Following blocking at 1 hour in 5% milk in TBS/T at room temperature, blots were incubated in primary antibody for 1 hour at room temperature or 4°C overnight, washed four times for 10 minutes in TBS/T, and then incubated in secondary reagents such as goat anti-mouse IgG antibody (1:20, 000) in TBS/T, 0.25% milk for 1 hour at room temperature, followed by four, ten-minute washings in TBS/T. Blots were developed in ECL (mixed 1:1 to make a final 10 mL volume), wrapped in plastic wrap, and exposed to autoradiographic film.

Preparation of genomic DNA from mouse tails for genotyping

Tail clips were digested at 5 - 60°C in 0.5 mL tail digestion buffer with Proteinase K, 5 hours to overnight. Tail digestion buffer was prepared by (for 500 mL) 437.5 mL ddH_20, 10 mL 5M NaCl, 24 mL 1M Tris-Cl, pH8.0, 15 mL 0.25M EDTA, and 12.5 mL 20% SDS, a solution that is stable at room temperature, where proteinase K was added to a final concentration of 0.5 mg/mL just prior to use (proteinase K was

maintained as a stock solution at - 20°C). Digestion reactions were spun at 14, 000 rpm for 10 minutes at room temperature, and supernatants pipetted into 0.5 mL isopropanol. DNA was precipitated by gentle, repeated inversions for 15 seconds, and DNA pellet was transferred by a sterile pipet tip into 500 μL 1x TE and warmed at 55°C for 1 hour to facilitate dissolution into solution.

ELISA Protocol

ELISA assays were performed on supernatants of lymphocytes following restimulation ex vivo. At specified days, or under specified conditions, 40 μL of culture supernatants (which can be frozen and maintained at -20°C until analysis) were incubated for 2 hours at room temperature on anti-IL-4 (11b11)-, anti-IFNγ (R46A2)-, anti-IL5 (Trfk5) or anti-IL13 -coated, pre-blocked (30 minutes at 37°C in 10% FCS/PBS) 96-well Dynatech Immulon 4 flat-bottomed plates (coated overnight at 4°C in PBS, 40 μL / well), upon 1:2 serial dilutions of triplicate wells per sample. Standards were as follows: IL4 standard, starting at 100 ng/mL and diluting 1:2 serial dilutions, IFN-gamma standard, starting at 1 μg/mL and diluting 1:2 serial dilutions, and IL-13, starting at 80 ng/mL and diluting 1:2 serial dilutions. After 2 hours, plates were washed extensively with PBS/ 0.1% Triton-X/ 0.05% NaAzide (at least 5 rinses), and incubated for 1 hour at room temperature with biotinylated detection antibody (BVD6, XMG1.2, or Trfk4, respectively) in 5% BSA/PBS (100 μL/well). Plates were washed again, and incubated for 30 minutes at room temperature with streptavidin-linked alkaline phosphatase in 5% BSA/PBS (100 μL/well). Plates were washed with PBS before adding NPP substrate (0.65 mg/mL) in alkaline buffer solution (100 μL/well). Plates

were allowed to develop at room temperature for 3 hours, and development was terminated with 0.5M NaOH (add 50 μL/well after first row of standards develop).

ELISPOT Protocol

ELISPOT assays were performed on total lung, spleen, or mesenteric lymph node cells in the absence of restimulation ex vivo. At specified days, or under specified conditions, tissues were surgically removed from mice and dispersed into single-cell suspensions through a 70 μm cell strainer. Red blood cells were lysed in ACK lysis buffer, and the remaining cells washed, counted, and resuspended at 10^7 live cells/mL in RPMI-10 culture medium for cytokine ELISPOT assays and incubated overnight at 37°C in 5% CO_2 on anti-IL-4 (11b11)- or anti-IFNγ (R46A2)- or anti-IL5 (Trfk5) coated, pre-blocked (30 minutes at 37°C in 10% FCS/PBS) 96-well Dynatech Immulon 4 flat-bottomed plates (coated overnight at 4°C in PBS, 40 μL / well), upon serial dilutions of triplicate wells per sample (100 μL/well). After 16 hours, plates were washed extensively with PBS/ 0.1% Triton-X/ 0.05% NaAzide (at least 5 rinses), and incubated for 1 hour at room temperature with biotinylated detection antibody (BVD6, XMG1.2, or Trfk4, respectively) in 5% BSA/PBS (100 μL/well). Plates were washed again, and incubated for 30 minutes at room temperature with streptavidin-linked alkaline phosphatase in 5% BSA/PBS (100 μL/well). Plates were washed with PBS before adding BCIP substrate (1 mg/mL) in alkaline buffer solution with 0.6% agarose solution (100 μL/well). Plates were allowed to polymerize and develop at room temperature for 3 hours, and scored by inverted microscopy.

Complement lysis-mediated enrichment of T cell-depleted splenocytes (for APCs)

Spleen and lymph nodes (cervical, axillary, inguinal, peri-aortic, popliteal, and mesenteric) were surgically removed from mice and dispersed into single-cell suspensions through a 70 μm cell strainer. Red blood cells were lysed in ACK lysis buffer, and the remaining cells washed, counted, and resuspended at $3x10^7$ cells per mL complement lysis mix. Complement lysis mix consists of J1J (anti-Thy1.2 antibody), guinea pig complement (GpC'), rabbit complement (RC'), in RPMI-10, sterilized through a 0.22 μm filter. Complement lysis reaction was performed at 37°C for 45 minutes. Following incubation, the complement reaction was resuspended well with a pipet prior to addition of pre-warmed (37°C) Ficoll to the bottom of the tube (10 mL of Ficoll per 10 mL of complement lysis reaction in a 50 mL Falcon tube). Viable cells were collected from the Ficoll: media interface after centrifugation at 2500 rpm for 15 minutes (brake off) at room temperature, or 1600 rpm for 25 minutes at room temperature for Histopaque 1086. Isolated cells were washed extensively in RPMI-10, and counted to determine yield; a small aliquot also removed prior to culture in order to determine percentage purity by FACS analysis. If utilized for antigen-presentation cells, cells were irradiated prior to culture at 2500 rad, and then cultured at 5 to 10:1 ratio (APC: T cell).

Complement lysis-mediated enrichment of CD4+ T cells

Spleen and lymph nodes (cervical, axillary, inguinal, peri-aortic, popliteal, and mesenteric) were surgically removed from mice and dispersed into single-cell suspensions through a 70 μm cell strainer. Red blood cells were lysed in ACK lysis

buffer, and the remaining cells washed, counted, and resuspended at $3x10^7$ cells per mL complement lysis mix. Complement lysis mix consists of 3.155 (anti-CD8 alpha/ Lyt 2), J11D (anti-Heat Stable Antigen), BP107 (anti-Class II MHC), guinea pig complement (GpC'), rabbit complement (RC'), in RPMI-10, sterilized in a 0.22 μm filter. Complement lysis reaction was performed at 37°C for 45 minutes. Following incubation, the complement reaction was resuspended well with a pipet prior to addition of pre-warmed (37°C) Ficoll to the bottom of the tube (10 mL of Ficoll per 10 mL of complement lysis reaction in a 50 mL Falcon tube). Viable cells were collected from the Ficoll: media interface after centrifugation at 2500 rpm for 15 minutes (brake off) at room temperature, or 1600 rpm for 25 minutes at room temperature for Histopaque 1086. Isolated cells were washed extensively in RPMI-10, and counted to determine yield; a small aliquot also removed prior to culture in order to determine percentage purity by FACS analysis (CD4 versus CD8β, should be approximately 85 - 95%).

Growing and testing antibodies from hybridomas

For maintenance of hybridoma cell lines, frozen cell stocks were thawed, washed, and incubated in a six-well plate in RPMI-10. The cell culture was gradually expanded to 10^8 cells (approximately two T75 flasks) in RPMI-10 prior to seeding a CellMax cell growth apparatus and maintained according to manufacturer's protocol. Alternatively, three to four T 75 flasks were used to intraperitoneally seed fifteen mice for ascites at Harlan Biosciences. Ascites fluid harvests were precipitated by ammonium sulfate cut and dialyzed against PBS. Antibody preparations were titrated by two independent assays: a cell lysis analysis and by flow cytometry to determine

specificity of lysis; these were always tested in comparison to the previous antibody batch. Single-cell suspensions of splenocytes from wild-type mice were isolated and purified over Ficoll and resuspended in RPMI-10 to 6 x 10^7/ mL, thus at two fold the final concentration. In a 96-well plate, 50 μL of cells with guinea pig complement (GpC' at 1/6) and rabbit complement (RC' at 1/50) were incubated with 50 μL of antibody, serially diluted in RPMI-10 between 1/10 to 1/1000 dilution (i.e., 1/10, 1/30....1/810), including controls lacking either complement, antibody, or both. Titrations were performed in duplicate (one set for cell lysis analysis, one set for FACS), and antibodies were prepared at 2x and then added to the pre-aliquotted cells. Following 45 minutes of incubation at 37°C, the plate was immediately quenched on ice, and 100 μL trypan blue added to sets intended for cell analysis. Determination of a percentage lysis (# of lysed cells/ # total cells equates the percentage lysed) was performed by trypan blue exclusion on a hemocytometer – lysed cells appearing as large, faint membrane "ghosts," up to 30 μm in size). Determination of specificity was determined by flow cytometry, utilizing surface markers selected for by complement lysis to measure their efficacy. The controls were essential to determine that the ascites or CellMax preparation did not contain any inherent lysis capability. Antibody preparations were aliquoted and frozen for storage at – 80°C.

Antibody staining of cells for flow cytometry

Spleen and lymph nodes (cervical, axillary, inguinal, peri-aortic, popliteal, and mesenteric) were surgically removed from mice and dispersed into single-cell suspensions through a 70 um cell strainer. Red blood cells were lysed in ACK lysis

buffer, and the remaining cells washed, counted, and resuspended at 10^8 cells/mL in PBS with 5% FCS. Prior to staining, cells were pre-stained in FcγR Block: (CD16/32, at 1/10 dilution) for 10 minutes at room temperature. Cells were then immediately stained at10^8 cells/mL in PBS with 5% FCS for 30 minutes on ice at 1:200 - 1:1000 (depending on prior antibody titration). Cells were then extensively washed in PBS with 5% FCS, and maintained on ice until FACS analysis (< 3 hours), or washed in PBS only and fixed in 1% paraformaldehyde in PBS and maintained in the dark (wrapped in aluminum foil) at 4°C until analysis could be performed.

MACS isolation of cells

Solution for MACS was prepared (PBS without Ca2+ or Mg2+, 2 mM EDTA, 0.5% Bovine Serum Albumin), sterile-filtered, and de-gassed. Tissues were harvested and made into cell suspension; following lysis of red blood cells in ACK lysis buffer, filtered through a 70 μm cell strainer, and resuspended at 10^7 cells / 90 μL buffer/ 10 μL MACS beads, mixed well. Cells were incubated at 4°C (not ice) for 15 minutes to allow staining, washed in 30 mL MACS solution, counted, and resuspended at 10^8/ 500 μL. Isolation column (i.e., MACS RS+) was pre-equilibrated with 500 μL MACS solution, making certain that there were no air bubbles trapped within, and then cells laid onto column, allowing flow-through at an extremely slow rate (approximately 1 drop per 2 minutes). After three 500 μL washes, 1 mL MACS solution was added to top of column, and, using a plunger to push the cells through, cells harvested into a tube. Following extensive washing, cells were enumerated and a small aliquot removed to assess purity of yield by FACS analysis.

***in vitro* T cell activation and proliferation assays**

Stimulation of CD4 T cells by plate-bound antibody was initiated by first coating flat-bottomed 96-well plates with H57 (anti-TCR Beta chain antibody) and anti-CD28 antibody at 5 μg per mL in PBS, incubated at 37°C for 2 hours, followed by three washes of PBS. Stimulation by antigen-presenting cells (APCs) was initiated by incubating APCs with H57 at 10 μg/mL and CD28 at 5 μg/mL in RPMI-10 in a round-bottomed 96-well plate. CD4+ T cells, isolated by flow cytometric sorting or by complement lysis-mediated enrichment, were added to the wells at 20,000 per well (or 1 million cells per well for a 24-well plate). For neutral priming, cells were plated with only recombinant human IL-2 (50 U/mL). For Th2 conditions, cells were plated with IL-4 (50 ng/mL) in addition to IL-2. For Th1 conditions, cells were plated with IL-12 (5 ng/mL) and anti-IL-4 antibody (neutralizing antibody, 11b11 clone) (20 μg/mL) in addition to IL-2. Cultures were set up in triplicate, with serial dilutions of stimulating antibodies ranging from strong to suboptimal stimulation. Primary stimulations were carried out for 5 to 7 days before restimulation. Upon restimulation, T cells were activated by plate-bound antibody (anti-TCR only) or APCs (anti-TCR only), without polarizing cytokine conditions. After 24 hours, cell supernatants were analyzed for cytokine production, or RNA harvested. For measuring proliferative capacity, tritiated-thymidine (^{3}H) was added to cell cultures at 18 hours, and cells harvested at 24 hours and counted in a scintilation counter (supernatants tested at 1/5, 1/10, and 1/20 dilutions).

Baermann's *Nippostrongylus brasiliensis* culture

Infection of mice and rats. Infective third-stage *Nippostrongylus brasiliensis* larvae (L_3) were isolated by modified Baermann technique (Figure 1). Feces of *Nippostronglus*-infected rats were bathed in pre-warmed, filter-sterilized 0.9% NaCl saline for 3 minutes at 37°C administered to larval cultures in petri dishes using a pasteur pipet and then poured into a paper sieve stabilized within a clamped funnel. The sieve consists of a funnel with Nalgene tubing on the bottom - this is clamped off to cut off flow. The filter paper sits on top of the funnel, pre-wet in approximately 50 ml pre-warmed saline. The funnel itself is clamped onto a ring stand.

Incubation for one hour allows the live larvae to burrow through the sieve and accumulate at the bottom of the tubing. After one hour, 50 mL was drawn off the bottom into a centrifuge tube and allowed to settle for 20 minutes. The pellet of settled larvae was washed three times in 0.9% NaCl saline. Parasite number was enumerated by inverted microscopy by streaking 10 μL of elution across a glass microscopy slide, in triplicate, and the final concentration of larvae determined by back calculation. Larvae should be resuspended at 2500 to 3750 live larvae per milliliter. Mice were then infected by subcutaneous injection at the base of the tail with 500 to 750 infective third-stage larvae in 0.2 mL 0.9% NaCl solution through a 27.5G needle, and maintained on antibiotic-containing water for the duration of the infection: polymyxin B sulfate (0.1mg/ml), neomycin sulfate (2 mg/mL). Rats were injected with 3000 - 4000 L_3 per rat, whereas the infective dose was 500 - 1000 L_3 per mouse.

Determination of worm burden. Mice were dissected in order to expose the peritoneal cavity, followed by surgical removal of the intestinal tract, from duodenal sphincter of the stomach to the cecal pouch. Intestines were placed into a 10 cm petri dish in 15 mL Hanks buffer, and cut longitudinally to expose the lumen. Plates were incubated at 37°C for up to 3 hours in order to allow worms to detach from the lumenal epithelium, and enumerated by microscopy under a dissecting microscope.

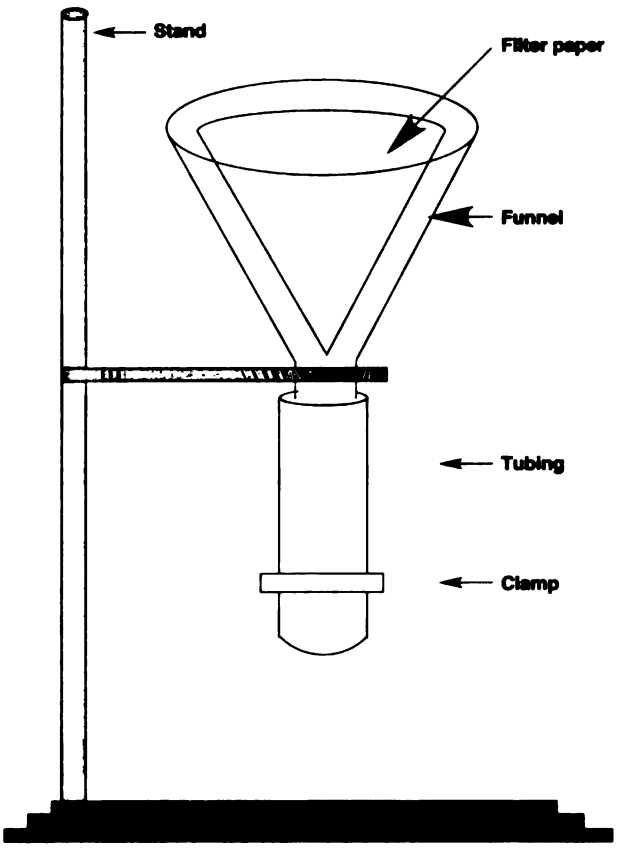
Preparation of larval cultures. Infective larvae can only be obtained from feces of infected rats, and not mice, as rats (such as Lewis strain) are permissive hosts for generating infective larvae in the feces. Six to eight days after infection, infected rats were placed overnight on grids, to allow the feces to fall through the grids. Bedding was replaced with wet paper towels, lining the bottom of cage in order to keep feces moist. Rat fecal pellets were collected on days 7, 8, and 9 of infection. An optional step is perform an egg count, performed by adding 3 g feces to 45 mL saturated NaCl solution, and mixing well prior to adding this suspension to six columns of a McMaster chamber. Eggs counted in six columns x 100 equates the number of eggs per gram, and a good infection will yield 50,000 eggs per gram of feces. Feces were mixed with distilled water to form a slurry at approximately a 1:1 ratio of water to feces. Charcoal (Sigma activated charcoal) was added, again at approximately equal volume to the fecal slurry to form a black, semi-moist consistency. A small, round, compact mound of this final slurry was placed onto the center of a round circle of pre-soaked (ddH20) filter paper (i.e., 3MM Whatman paper) within a deep 15 cm diameter petri dish. The diameter of the filter paper should be approximately 13.5 cm, to allow a border onto

which the larvae will migrate and are easily collected. Petri dishes were dated and placed in humidified tupperware (tupperware lined with wet paper towels). Larvae hatch within a few days and are viable for 4 to 8 weeks.

Figure 1. Baermann's isolation of *Nippostrongylus brasiliensis*

Infective larvae, with or without charcoal, were suspended in pre-warmed (37°C), sterile 0.9% NaCl saline and placed over a Baermann column. The column apparatus consists of a clamp stand suspending a funnel. The funnel bottom was attached to a 6 inch piece of Nalgene tubing, which is clamped off approximately 2 inches from the bottom. Within the funnel rests a cone of 2-ply blue Kimwipe paper, trimmed to fit the funnel opening. The live infective larvae burrow through the Kimwipe paper into the funnel tubing, and then precipitate towards the clamped off piece of tubing. After 45 minutes, the clamp can be released and larvae eluted into a 50 mL Falcon tube.

Figure 1



***In vivo* cell depletions**

Short-term (< 30 day) cell depletions in vivo were performed by intraperitoneal injection of 2 mg of depleting antibodies. For eosinophil depletion, TRFK5 mAb was used to neutralize IL-5, which resultingly blocks eosinophil terminal differentiation and release from the bone marrow. Y13-259 (anti-ras mAb) was used as an isotype control. GK1.5 mAb was used to deplete CD4+ T cells. Efficacy of cell depletions was determined by flow cytometry of samples of peripheral blood leukocytes, spleen, lymph node cells, or broncho-alveolar lavage. In infection experiments, depletions were performed by antibody injections simultaneous to initiation of infection.

Adoptive transfer of cells

Adoptive transfer of cells into mouse recipients was done by intravenous or intraperitoneal route. For intravenous route, donor cells were prepared using sterile technique and loaded into 1 cc syringes with 30G needle in 0.2 mL PBS. Recipient mice were pre-warmed under a red heating lamp for 5 minutes and placed into a restraint without anesthesia. Recipient tails were prepared by gentle stroking with warm sterile water with a sterilized cotton swab to enhance visibility of tail vein. Injections were placed at 1 inch from distal end of tail.

Formalin fixation for H&E

10% Formalin (PBS) solution was prepared by diluting 37% formaldehyde at 1:10 in PBS. This solution can be kept at room temperature as a stock solution. Dissection is dependent on the organs being harvested, but for lungs the procedure is initiated by careful cervical dislocation or by lethal dose injectable anesthesia via

intraperitoneal route. The sternum, antero-lateral portions of the ribcage, and submandibular "strap" muscles are removed to expose the trachea. The trachea was then pierced between cartilaginous intratracheal rings with a 26G needle on a 3 mL syringe filled with 10% formalin solution. The trachea was sealed through reinforcement with thread tie to firmly seal the trachea around the needle. Lungs were perfused with formalin solution until fully expanded; approximately 2 mL per lung. Lungs and heart were removed together, and incubated in 5 mL 10% formalin in 50 mL Falcon tube at RT for up to 48 hours. The heart and thymus were removed, and 10 mm pieces of the lung were placed into cassettes for paraffin embedding.

H&E Histological Staining Protocol (for paraffin sections)

Paraffin-embedded sections were fixed in acetone for five minutes at room temperature in in pre-chilled (4°C) acetone, and allowed to dry. The staining protocol was hematoxylin for 6 minutes (Harris' Hem/ Fisher #SH26-500D), rinse in ddH_20 (5 dips), then differentiated with 2 quick dips in acid EtOH (70% EtOH, 1% HCL of 10 mM solution), then blue with 5 dips in Li_2CO_3 (Lithium carbonate, 1% w/v ddH_20), rinse in ddH_20 (5 dips), then one dip in 80% EtOH, then eosin Y for 6 minutes (Labchem, Inc. 1% Alcohol #LC14030-1), followed by dehydration through graded alcohols, as quickly as possible- 80% 5 dips, 100% 5 dips, 100% 5 dips (less for darker eosin slide), cleared through 2 to 5 dips through 2 baths of xylene, cover-slipped out of Xylene in Permount. Reservoirs were exchanged weekly if used often.

Immunohistochemistry

Immunohistochemistry procedures were kindly provided by Mehrdad Matloubian and Jason Cyster of UCSF. Dissection was dependent on the organs being harvested, but for lungs the procedure is initiated by careful cervical dislocation or by lethal dose injectable anesthesia via intraperitoneal route. The sternum, antero-lateral portions of the ribcage, and submandibular "strap" muscles are removed to expose the trachea. The trachea was then pierced between cartilaginous intratracheal rings with a 26G needle on a 3 mL syringe filled with 50% OCT media in PBS. The trachea was sealed through reinforcement with thread tie to firmly seal the trachea around the needle. The animal was perfused via right ventricle with 10 mL 1%PFA in PBS, whereby lungs should expand, and then the lungs perfused with OCT media. Paraformaldehyde fixation buffer should be prepared as a 1% PFA/PBS solution for each experiment from a 5% PFA/PBS stock. (Stock solution: 5% w/v PFA (powder) in PBS. Heat to dissolve. After restoring to RT, pH7.2-7.4. Store @ -80°C.) A 20mm piece of lung tissue was placed in a Tissue Tek holder, covered with OTC, frozen on dry ice, and stored @ -80°C. For other tissues, such as spleen and lymph nodes, tissues were sliced into 3 mm pieces, incubated in 1% PFA/PBS in a microcentrifuge tube at 4°C for two hours prior to preparation in the Tissue Tek block and storage at –80°C. 7 to 10 μm tissue sections were cut on a Cryostat. Sections were pressed onto 4 well slides (C.A.Hendley, LTD, Oakwood Hill Industrial Estate, Loughton, Essex UK, Tel.#0181-502-1821, PH005), and then fixed in prechilled (4°C) acetone for 5 minutes at room temperature. Slides were rinsed in Tris-buffered saline (TBS, pH7.5) with

0.1% BSA, twice for 5 minutes. All steps were performed in a humidified chamber, with each well holding 70 μL volume, working quickly to avoid drying. Slides were blocked for 10 minutes in TBS with 0.1% BSA and 0.25% Normal mouse serum (Sigma M5905) and Normal Goat Serum (Jackson Immunoresearch 005000121, 10mL). Slides were immediately, without washing, placed into primary antibody, a rabbit anti-GFP (Molecular Probes), diluted 1:1000 in TBS with 0.1% BSA and 0.5% NGS and 0.25% NMS) for one hour at room temperature. Slides were washed one to two times for 5 minutes before addition of biotinylated goat anti-rabbit IgG (Pharmingen 550338), diluted at 1:100 in TBS with 0.1% BSA and 0.25% NMS and Rat anti-mouse B220 (unconjugated), diluted at 1:70 (Pharmingen 550286) with 0.5% Normal Donkey Serum (Jackson Immunoresearch 017-000-121) for 45 - 60 minutes at room temperature. Following incubation, the slides were washed one to two times for 5 minutes. Thirty minutes before needed, but no earlier, the streptavidin-AP was prepared in (ABC Kit from Vectastain, Vector Labs #AK5000) plain TBS, at a 1:100 dilution of A and 1:100 of B. Just prior to use, BSA was added to a final concentration of 0.1% BSA and anti-rat IgG HRP (i.e., for 1 mL: 10μL A + 10μL B + 6.7μL Donkey anti Rat IgG-HRP (Jax 712-035-153) at 1:150 and 10μL 10% BSA in TBS. This solution was added to the slides and incubated for forty minutes at room temperature. For developing, the AP should be developed first. Five milligrams of Fast Blue Salt (Sigma F0250) was added to five milliliters of thawed Fast Blue Buffer. 50 mL Fast Blue Buffer was first prepared by adding 40 mg Levamisole (Sigma L9756) to 1.5 mL dimethylformamide in a glass pipet to dissolve, then adding 18 mg Napthol AsMx Phosphate (Sigma N4875),

and 0.1M Tris buffer pH9.2, and stored at – 80°C). Following gentle shaking, this solution should be filtered through a 0.22 μm filter prior to application on the slide. Adding the AP developing buffer to the slide after washing, the slide should be developed for approximately 10 to 20 minutes, terminating the reaction in TBS. The HRP is developed next, by addition of 2 Sigma Fast DAB tablets (Sigma D4293, one is urea H_2O_2, one is DAB) to 5 mL of ddH_2O (filter 0.22 μm), and allowed to develop 2 to 3 minutes, terminating the reaction in TBS. The slides were then mounted in Crystal Mount (Fisher BM-M02) for 55 to 65°C for 30 minutes.

Cytocentrifugation and staining of cell suspensions

Cells were resuspended at 10^6 cells per mL in RPMI-10. 200 μL was carefully loaded onto a cytocentrifugation funnel (clamped together: cytocentrifugation funnel, filter paper, and slide, in that order), carefully to avoid air bubbles. Fisher Plus (positive charged) slides were utilized. Following cytocentrifugation at 500 rpm for 5 minutes (Cytospin II), the slides were released from the cytocentrifugation funnel and allowed to air-dry in a hood for 15 minutes. The Diffquick (VWR, modified Giemsa stain) staining protocol was 30 seconds in fixative, 40 seconds in red dye, 40 seconds in blue dye, followed by 6 to 10 washes in ddH_20, then again in a fresh bath of ddH_20. Slides were allowed to air dry in hood for 15 minutes to overnight prior to mounting of coverslips in Permount, adding several drops to slide, and placing coverslips - laying one edge down first, and gradually, diagonally placing it flush to slide to prevent air bubbles from becoming trapped in the Permount.

Chapter VII:

CD1, Tuberculosis, and the Evolution of MHC Molecules

Preface

This review is the result of an engaging series of conversations about the evolution of immune responses that originated in the Locksley lab. Although my advisor Rich held the primary vision of the review's thesis, through its writing, I learned the art of systematic, critical dissection of scientific literature and how to form opinions on research that is not one's own. While the notion that pathogenic organisms have co-opted basic homeostatic functions of cellular biology is not entirely new, countless examples of innate and adaptive immunity have illuminated the striking similarities of principles, and even mechanisms, used for the seemingly-disparate purposes of host defense and homeostasis. This review focuses on the roles of nonpolymorphic MHC (major histocompatibility complex) molecules in sampling distinct cellular components for tissue maintenance, and discusses the potential evolutionary roots of polymorphic MHC molecules, used in immunity, in essential, basic mechanisms of self-maintenance. This concept has been reiterated within the innate immunity literature now by several different gene families. For example, the dual role of the Toll signaling pathway in *Drosophila melanogaster,* first, in the developmental pathway of body patterning establishment, and second, in immune recognition of pathogens leading to activation of anti-microbial products in the adult organism, beautifully highlights the pleiotropic functions of a molecular family during distinct stages of development and for divergent purposes. It is this duality that we hypothesize is exploited by *Mycobacterial* pathogens - or perhaps instead by the host response - subverting basic cellular processes to initiate immune responses to pathogenic invaders. This notion may underlie the complex

exploitation of all pattern recognition pathways by pathogens in manipulating immunity for the purposes of self-preservation.

As an aside, when we first wrote the review, we received many inflammatory letters from people in the field, and worse yet, it went uncited for several years. Two years later, investigators in the field started to warm up to the idea. This was perhaps best exemplified by Mike Bevan (HHMI, University of Washington, Seattle), who wrote to us: "...when I first read your commentary in JEM 2000 containing the suggestion that H2-M3-restricted CD8 T cells may have a 'homeostatic' role in surveilling for damaged mitochondria...well, I thought it was CRAP!...[but] now we have shown that ALL class Ib-restricted T cells are strange in the sense that 1. they are all pre-activated in naïve mice and 2. they all have the ability to be positively selected in hematopoietic cells in the thymus rather than, or in addition to, non-hematopoietic cells (epithelial) cells. So they may resemble NKT cells. Does this mean you are smarter than me? Perhaps. We are coming around to the notion that the Class Ib cells are kept on alert by self-epitopes in the periphery, after being selected by high affinity interactions in thymus."

CD1, tuberculosis, and the evolution of MHC molecules

Although the role of class I and class II MHC in adaptive immunity against microbial pathogens is clear, the participation of nonpolymorphic MHC molecules in host defense remains less well defined. Roles for Qa-2 and H2-M3 have been suggested (Kurlander, 1992; Pamer, 1992; Rotzschke, 1993), and accumulating data have prompted speculation that CD1 family members may be important in immunity against pathogenic mycobacteria and parasites (Porcelli, 1999). Spada *et al.* recently report the restriction of at least some human Vγ2, Vδ1 T cells, the most common tissue γδ T cells, by CD1c (Spada, 1999). Like other populations of CD1-restricted T cells - as discussed further below - the CD1c-restricted cells were autoreactive in vitro. These cells produced IFN-γ, but not IL-4, and displayed cytotoxicity against CD1c+ targets, leading the authors to speculate that such cells might be involved in innate host defense against prevalent pathogens. In this way, γδ and other CD1-restricted T cells would represent unique small populations of lymphocytes that have been evolutionarily maintained due to their capacity to react rapidly to microbes.

Pathogens are clever, however, and an equally plausible hypothesis is that pathogens have exploited unusual T cell populations that exist for reasons different than immunity. Indeed, based on comparisons with other nonpolymorphic MHC molecules, we suspect that the primary role of such molecules may not entail immunity to infectious organisms, but may rather underlie a basic mechanism for the maintenance of cell and tissue homeostasis. The ancient process by which MHC molecules sample distinct cellular compartments may have been later co-opted by classical MHC molecules to

mediate protective immunity at the time of acquisition of bacteria-derived RAG transposases and the establishment of a system for adaptive immunity (Thompson, 1995). By this alternate hypothesis, mycobacteria uniquely exploit the underlying biologic processes mediated by CD1.

The CD1 family: genomic organization and structure

The CD1 family comprises a heterogeneous group of β2-microglobulin (β2m)-associated transmembrane proteins that bear strong structural resemblance to the classic MHC antigens (Porcelli, 1999). In contrast to the latter, however, CD1 genes are relatively nonpolymorphic and are encoded by genes distant from the classic MHC loci. In humans, five CD1 genes on chromosome 1 are known to encode four proteins, designated CD1a, b, c and d; CD1e may represent a pseudogene. The CD1 family is further subdivided into group 1, comprising CD1a, b and c, and group 2, comprising CD1d, based on sequence and functional homology. In the mouse, an ancient translocation likely resulted in the loss of the group 1 genes (Balk, 1995); only CD1d1 and a duplicated gene, CD1d2, remain, on the syntenic region of chromosome 3.

Crystallographic analysis of mouse CD1d1 confirmed the preservation of domain organization between this molecule and the structure of classic MHC (Zeng, 1997). As with class I MHC, an externally disposed binding cleft formed by an eight-stranded anti-parallel β-sheet floor bounded by the α1 and α2 helices provided evidence for a molecule involved in ligand display. In contrast to the sequential small binding pockets that accommodate individual amino acids of the peptide backbone in classic MHC, however, the CD1d1 cleft consisted of two large pockets lined with hydrophobic

residues. Although capable of binding long, highly hydrophobic, peptides (Castano, 1995), it is likely that the unique structure underlies the capacity of CD1 to present lipid ligands. The functionally interchangeable nature of mouse and human CD1d molecules, such that mouse CD1 can present to human CD1-restricted T cells and vice versa (Brossay, 1998), suggests that the human CD1d structure will be similar.

Recognition of CD1 by T cells bearing limited TCR diversity

The description of CD1-restricted, tumor cytotoxicity, by CD4-CD8- αβ or γδ human T cells provided the initial hypothesis that CD1 might subserve an immune function (Porcelli, 1989; Faure, 1990; Balk, 1991). Shortly thereafter, murine NK1.1+ T cells were demonstrated to be CD1-restricted (Bendelac, 1995).

The unusual nature of the TCRs that recognize these nonpolymorphic CD1 molecules suggested limited ligand diversity. Best characterized are CD1d-restricted TCRs expressed on NK1.1 T cells from mice and humans (Bendelac, 1997). In mice, these double-negative or CD4+ T cells express an invariant Vα14, Jα281 TCR paired with a highly restricted set of Vβ chains, usually Vβ8, Vβ7 or Vβ2, that may reflect tissue-specific expansion or homing. Strikingly, CD1d-restricted human T cells use essentially the same TCR, the homologous Vα24, JαQ/Vβ11 TCR. In mice, these cells co-express typical NK lineage markers and undergo thymic selection by CD1-expressing, bone marrow-derived cortical thymocytes (Bendelac, 1995). A second subset of CD1d-restricted cells do not express NK lineage markers or the invariant Vα14 TCR, although the expressed TCRs reveal highly restricted VαJα usage, frequently paired with Vβ8 (Chiu, 1999).

Although αβ TCR usage by T cells restricted by CD1 antigens other than CD1d remains less well studied, emerging evidence suggests that these, too, will show limited diversity. In humans, such cells are typically double-negative or CD8αα+. Expressed TCRs from such cells displayed limited numbers of TCR α chains - Vα4, Vα7, Vα19 and Vα24 - that were shared among individuals with no identity at classic MHC molecules (Han, 1999). The most prevalent - human Vα7S2, Jα33 and Vβ2S1 or Vβ13 - were restricted by CD1b, and strikingly, were completely homologous to TCRs on double-negative T cells from mice and cattle (Tilloy, 1999). In mice, such cells were present in CD1d-deficient, but not β2-microglobulin-deficient, mice, and the selecting ligand remains unknown. The report by Spada *et al.* extends the concept of limited diversity to human CD1c-restricted T cells. Although extrapolated from relatively few examples, these cells all expressed Vγ2, Vδ1 TCRs with or without CD8αα, and reacted to CD1c presented by a variety of cell types.

CD1-restricted αβ and γδ T cells are present in low numbers at birth and then rise progressively in blood and select tissues - liver, bone marrow, and spleen - to constitute from 1-20% of cells, a prevalence not unlike NK cells. In humans, but not mice or rats, they can express CD8αα, which may reflect their activation status (Paliard, 1988). These cells display a memory-effector antigen profile on their surface that is consistent with their capacity to secrete rapidly large amounts of cytokines or to generate cytotoxicity after TCR ligation (Porcelli, 1999; Bendelac, 1997). Such instantaneous effector capacity distinguishes these cells as compared to mainstream naive CD4 and CD8 T lymphocytes. Although the Vγ2, Vδ1 T cells described by

Spada *et al.* (Spada, 1999) demonstrated cytotoxicity and a type 1 cytokine profile, such findings need to be tempered by the observation that these cells were selected by repeated incubation with mycobacterial antigens that mediate IL-12 production from antigen presenting cells through Toll-like receptor interactions (Brightbill, 1999). It is likely that such cells, when derived under less biased conditions, might display a wide range of cytokine and effector potential (Ferrick, 1995).

Intracellular trafficking of CD1 molecules

An important property of CD1-restricted T cells is their inherent autoreactivity (Porcelli, 1999; Bendelac, 1997). Different types of CD1-restricted T cells can respond to CD1 molecules in distinct tissue compartments, suggesting that auto-antigens are expressed in different organs (Chiu, 1999; Eberl, 1999). More intriguingly, the same CD1 molecule, at least for the group II CD1d antigen, can activate distinct classes of CD1-restricted T cells depending on its pattern of intracellular trafficking. Normally, CD1d molecules traffic from their synthesis in the Golgi to the cell surface. There, after ligation or antigen loading, CD1 is internalized to endosomes that subsequently acidify and eventually co-localize in MIIC, the class II peptide-loading compartment (Jackman, 1998). Internalized CD1 is then returned to the cell surface. By mutating the tyrosine-based amino acid motif in the CD1 tail such that the molecule failed to be internalized to endosomes, Bendelac and colleagues could indirectly assess the effects of endolysosomal trafficking on the capacity of CD1 to activate NK T cells (Chiu, 1999). Unexpectedly, CD1 that had trafficked through MIIC activated NK1+, Vα14, Jα281 T cells, whereas CD1 that had trafficked to the cell membrane from the Golgi activated

only the non-NK, non-Vα14 CD1d-restricted T cells. Thus, these two sets of CD1d-restricted T cells respond to self-antigens differentially localized to cytosolic secretory or endosomal compartments in a manner highly reminiscent of the way in which CD8+ and CD4+ T cells respond to peptides presented by class I and class II antigens. With the exception of CD1a, which does not traffic to MIIC, the other CD1 family members contain similar targeting motifs embedded within their cytoplasmic tails.

Presentation of nonpeptide mycobacterial ligands by group 1 CD1 molecules and GPI's by CD1d

Substantial invigoration of the field occurred with the isolation of human T cell clones that reacted to antigens from *Mycobacterium tuberculosis* and *M. leprae* in a CD1b-restricted manner (Porcelli, 1992). Antigen processing required endosomal trafficking, but was TAP- and HLA-DM-independent. A number of human double-negative or CD8 lines and clones that reacted in a CD1a-, 1b- or 1c-restricted manner to various antigens derived from mycobacterial cell walls, including mycolic acid, glucose monomycolate (GMM) and lipoarabinomannan (LAM), have been described; most generated IFN-γ or demonstrated cytotoxicity upon activation (Beckman, 1994; Beckman, 1995; Dellabona, 1993; Sieling, 1995; Thomssen, 1995). Although most such lines expressed αβ TCR, γδ TCR were also seen. Analysis of the antigens revealed rigid requirements for carbohydrates and other polar moieties with the lipid requirements being less specific. Thus, in this model, lipid components of GMM or LAM would be accommodated in the large hydrophobic cleft in CD1 in order to

position hydrophilic structures outwards to enable recognition by the TCR (Moody, 1997).

The concept of presentation of lipid antigens via CD1 to T cells provoked a series of experiments to find the ligand for CD1d, the only known CD1 family member retained in the mouse. Despite the ease with which human group 1 CD1-restricted T cells reactive to mycobacterial antigens could be isolated in vitro, it seems clear that CD1d plays no role in immunity to *M. tuberculosis* in the mouse based on intact immunity in CD1d-deficient animals (Behar, 1999). Evidence has been reported supporting the role of endogenous glycosylphosphatidylinositol (GPI) anchors as a CD1d ligand (Joyce, 1998) and of parasite-derived GPI anchors in mediating CD1d-dependent NK T cell activation for B cell help in antibody production (Schofield, 1999). A striking observation remains the capacity of α-galactosylceramide, a glycolipid naturally present in sea sponges but undetectable in mammals, to activate Vα14- and Vα24-bearing T cells in mice and humans, respectively (Brossay, 1998;Kawano, 1997; Spada, 1998). Despite only 60% identity in the peptide-binding domains of mouse and human CD1d, either molecule could present this ligand and activate both mouse and human NK T cells (Brossay, 1998).

The host defense model of CD1 family members

The presence in mice and humans of nonpolymorphic MHC molecules that present nonpeptide antigens across species to T cells of limited diversity that circulate with a pre-programmed effector phenotype suggests strong evolutionary pressure for their maintenance. Although the CD1d-deficient mouse has a rather limited phenotype

(Smiley, 1997; Chen, 1997; Mendiratta, 1997), the persistence of similar types of T cells in these mice suggests that other nonpolymorphic MHC molecules might contribute to this pool of cells (Tilloy, 1999). The ability to demonstrate CD1 group 1- and group 2-restricted reactivity to mycobacterial and parasite-derived antigens, respectively, suggests the plausible hypothesis that these lipid- and glycolipid-presenting molecules have been maintained to confer the ability to respond to conserved cell wall determinants from prevalent pathogens of substantial morbidity and mortality. Indeed, the most prevalent γδ T cells in human blood - Vγ2, Vδ2 - also recognize conserved nonpeptide antigens from mycobacteria, e.g.; prenyl pyrophosphate (Morita, 1995) and phosphorylated thymidine nucleotides (41), as well as widely distributed alkylamine compounds (Constant, 1994). Together, these models envision marked evolutionary pressure from mycobacteria, and perhaps other organisms, such that substantial numbers of T cells with limited diversity exist to enhance early immune responses to these pathogens. Before considering some of the problems inherent in such a model, a review of the emerging functions of other nonpolymorphic MHC-like molecules will be considered.

Nonpolymorphic MHC molecules participate in diverse homeostatic functions

A wealth of recent data has cast new light on the role of nonpolymorphic MHC molecules (Table 1). The unexpected roles for several of these molecules were revealed in β2-microglobulin-deficient mice, which are not only deficient of class I MHC, but also of a number of nonpolymorphic MHC molecules that rely on β2-microglobulin for protein stability and expression. As reviewed below, a number of these molecules

demonstrate a chaperone function for ligated 'cargo', and, as with CD1, serve as ligands for receptors on distinct populations of effector T cells (Table 1). An emerging theme is the function of these molecules to sample cellular compartments, perhaps reflecting a role in monitoring ligands that serve as surrogate markers indicative of cellular health.

HFE is a nonpolymorphic MHC molecule involved in the regulation of iron metabolism. Mutation of the gene in humans is responsible for hereditary hemochromatosis, a disorder of iron storage that affects between 1 in 200 and 1 in 400 individuals of northern European ancestry (Bacon, 1999). Tissue iron overload can eventually lead to chronic liver failure, as well as dysfunction of other organs. The crystal structure of HFE revealed a classic MHC-like structure, although the putative peptide binding groove was narrowed by a translation of the α1 helix that resulted in burial of potential peptide-binding pockets in the floor of the cleft (Lebron, 1998). Biochemical and mutational studies have defined a pathway by which membrane HFE forms a ternary complex with iron-bound transferrin and the transferrin receptor that assists in the endosomal targeting of the complex (Figure 1). A histidine patch in HFE may serve to titrate a pH-dependent dissociation of HFE from the complex within endosomes, allowing the delivery of cellular iron. The most common abnormalities leading to hemachromatosis are missense mutations that abrogate the association of HFE with β2-microglobulin, thus destabilizing the protein.

FcRn, the neonatal Fc immunoglobulin receptor, facilitates the transport of IgG from the mother's serum across the placenta and from the mother's milk across the neonatal intestine. The receptor is widely expressed on vascular endothelium

throughout life, where it participates in maintaining normal levels of serum IgG; mice deficient in β2-microglobulin, and hence, deficient in FcRn, demonstrate markedly accelerated clearance of IgG (Junghans, 1996). The potential peptide-binding groove in FcRn is essentially closed, with a surface area too small, approximately 235 A^2, to accommodate peptides like MHC class I molecules, which have a pocket surface area of approximately 760 A^2 (Burmeister, 1994). Rather, like HFE, FcRn binds IgG along the side at the interface of the Fc C_H2 and C_H3 domains of immunoglobulin near a cluster of histidines that could facilitate the pH-dependent association of the molecules, analogous to the HFE pathway (Burmeister, 1994). In adults, FcRn may sequester endocytosed serum IgG, thus protecting it from lysosomal degradation (Figure 1); saturation of FcRn may underlie the therapeutic efficacy of high-dose immunoglobulin for autoimmune disease by accelerating the degradation of autoreactive antibodies (Yu, 1999).

CD1, in contrast to HFE and FcRn, has a large hydrophobic pocket that does bind ligands, and CD1 presents ligands to specialized types of lymphocytes. In fact, both of these properties are shared with other nonpolymorphic MHC molecules. Of these, the molecule with a binding cleft most similar to CD1 is ZAG, zinc-α2-globulin, that circulates as a soluble serum protein. ZAG, unlike CD1, has a network of hydrogen bonds that stabilize the molecule without a requirement for β2-microglobulin, but, like CD1, forms a large, central hydrophobic pocket which, although smaller than CD1, contained a nonpeptide ligand in the crystal structure (Sanchez, 1999). In vitro and in vivo, ZAG promotes lipolysis and fat depletion; it was isolated as a lipid-catalyzing moiety from the urine of cancer patients with wasting (Hirai, 1998). Although further

work is required, evidence is consistent with a role for ZAG in mediating lipid homeostasis in normal or pathologic conditions. Concise mechanisms for regulating cellular membrane lipids are beginning to be elucidated and are consistent with such homeostatic processes (Brown, 1997).

Whereas CD1 interacts with TCR via its externally disposed binding pocket, FcRn interacts with IgG, or soluble BCR, at a region distinct from its closed cleft. A number of other nonpolymorphic MHC molecules, including H2-M3, HLA-E (Qa-1 in mouse), HLA-G, MIC-A and MIC-B, can interact with immune receptors on lymphocytes (Table 1). H2-M3 is a murine class I homolog that also forms a large surface area pocket of neutral and hydrophobic residues capable of accommodating peptides with N-formylated methionine termini (Wang, 1995). H2-M3-restricted CTL that respond to formylated peptides from bacteria have been demonstrated (Pamer, 1992). The ability of H2-M3 to bind endogenous formylated peptides from mitochondria would be consistent with a primitive role in monitoring mitochondrial integrity, although no evidence exists for such a function. Selection of an H2-M3-restricted TCR by one of the endogenous 13 formylated proteins of mitochondria - NADH dehydrogenase subunit 1 - has been demonstrated (Berg, 1999). Recent evidence suggests that preformed H2-M3 exists intracellularly and can be rapidly mobilized to the surface when provided with an appropriate formylated peptide ligand (Chiu, 1999). After infection with Listeria, the rapid accumulation of H2-M3-restricted CD8+ T cells with specificity for Listeria-derived antigens could be detected using H2-M3 tetramer reagents (Kersiek, 1999). In contrast to conventional class I or II-restricted

T cells, such cells displayed no evidence for a memory response. Although the authors used one mitchondrial-derived control peptide to assess specificity, analysis with each of the self-peptide mitochondrial tetramers will be required to show definitively that these T cells are not reacting to self peptides that mediate stress-induced rescue of intracellular H2-M3.

HLA-E in humans and Qa-1 in mice are homologous MHC molecules that present leader sequences acquired in the endoplasmic reticulum from MHC class I molecules in a peptide-binding cleft at the cell surface (Lanier, 1998). Using tetramer technology, the ligand for HLA-E and its bound peptide was shown to be CD94/NKG2A, -B or -C, a heterodimeric, C-type lectin superfamily receptor on NK and some T cells that regulates NK cell activation for cytotoxicity through association with the adaptor protein, DAP12 (Braud, 1998; Lanier, 1998). In this way, HLA-E serves to monitor the homeostatic surface expression of cell class I MHC molecules for surveillance by effector lymphocytes, and thus constitutes a mechanism for rapidly removing cells pathologically altered by a number of conditions (Figure 1).

The intestinal epithelial MHC-like molecules, MIC-A and MIC-B, illustrate remarkably the preservation of this homeostatic function despite the substantial alteration of the underlying MHC domains. A disordering of the $\alpha 2$ helix results in complete occlusion of the putative binding cleft, and the molecules adopt an extended structure that is incompatible with binding by $\beta 2$-microglobulin (Li, 1999). MIC-A and MIC-B are induced on intestinal cells by stress-activatible promoters, where they are recognized by $V\delta 1$-expressing $\gamma\delta T$ cells that occupy subepithelial locations in the

intestine (Groh, 1998). Diverse nonhuman primate MIC molecules activate cytotoxicity by human Vδ1 T cell clones, consistent with recognition of a conserved surface on the side of MIC molecules near the footprint occupied by β2-microglobulin in class I MHC. Despite interaction at a domain distinct from the residual binding pocket, MIC molecules, like HLA-E, interact with C-type lectin NK receptors, NKG2D, expressed on Vδ1 T cells, NK cells and some CD8+ T cells (Bauer, 1999). Activation of the lymphocytes occurs through association of NKG2D with an activating adaptor protein, DAP10, resulting in cell death of the MIC-bearing target (Wu, 1999). Although its biologic function remains unknown, MIC molecules likely play a role in monitoring intestinal epithelial integrity. Indeed, the turnover of epithelial cells in intestinal villi is substantially reduced in γδ-deficient mice (Komano, 1995).

A reevaluation of CD1

As shown by the above examples, the capacity to bind ligands, including lipids, and to interact with relatively invariant lymphocyte receptors are properties of CD1 shared by other nonpolymorphic MHC molecules (Table 1). Frequently, T cells restricted by nonpolymorphic MHC are localized to distinct tissue compartments. Examples include NK T cells in the liver, and MICA- and MICB-restricted T cells and CD8αα intraepithelial T cells, in the intestine (Das, 1999; Park, 1999). The restricted expression of HLA-G in the placenta may represent yet another example suggesting a specialized homeostatic function for these invariant molecules in surveying placental integrity (Carosella, 1999). These considerations suggest that these various properties were evolutionarily endowed upon CD1 before the appearance of mycobacterial human

pathogens. The concept that CD1 remains 'hard-wired' in order to confront *M. tuberculosis* or *M. leprae* is difficult to conceptualize given the biology of these organisms. First, *M. tuberculosis* has probably existed as a human pathogen only since the domestication of cattle some 7500 years ago, presumably reflecting a trans-species adaptation by *M. bovis.* Second, both *M. tuberculosis* and *M. leprae,* although undeniably of immense public health concern, cause disease in only a minority of persons infected during their reproductive lifespans. Third, although the group 1 CD1 proteins can present antigens from *M. tuberculosis,* mice, which are inherently more resistant to tuberculosis than humans, have deleted the group 1 CD1 genes, whereas guinea pigs, which have expanded numbers of these genes, are extraordinarily susceptible to tuberculosis (Porcelli, 1999). Finally, the ubiquitous environmental mycobacteria are relatively nonpathogenic organisms that require marked deficiencies in the adaptive immune system - e.g., advanced HIV infection or genetic deletions of the IL-12, IL-12R or IFN-γR genes (Altare, 1998) - in order to cause disease. The likelihood that the evolutionary impetus for CD1 derived from these organisms seems remote.

The sequencing of the *M. tuberculosis* genome offers extraordinary insights regarding the biology of this organism (Cole, 1998). As compared to *E. coli,* which has only 50 genes devoted to fatty acid metabolism, *M. tuberculosis* has over 250 distinct enzymes involved in the synthesis and catabolism of a diverse array of lipophilic molecules. Mycobacteria contain enzymes representing all of the classes of lipid and polyketide biosynthesis normally found in mammals, plants and bacteria. It seems

likely that mycobacteria infection would result in substantial effects on endogenous lipid metabolism of the cell. If CD1 molecules were normally loaded with an endogenous cell-derived lipid, as suggested by the autoreactive nature of NK T cells (Porcelli, 1999; Bendelac, 1997) or the Vγ2, Vδ1 T cells isolated by Spada *et al.* (Spada, 1999), it is likely that disruption of normal lipid synthetic pathways could alter the distribution or amount of the endogenous CD1 ligand. As such, CD1 function would more closely resemble that of other nonpolymorphic MHC molecules in providing information regarding deviation from normal cellular biosynthetic pathways, perhaps relating to lipid membrane homeostasis (Table 1). In this model, mycobacteria have evolved to displace or induce the normal CD1-associated ligand. But why would *M. tuberculosis* have evolved to activate CD1-restricted T cells?

M. tuberculosis is unusual because of it airborne transmissibility. In contrast, most bacteria that cause pneumonia do so after establishing colonization in the pharynx, from which microaspiration can occur and cause disease in the appropriate host and clinical setting. Airborne transmission relies on the discharge of enormous numbers of bacilli, usually by coughing. The pathology of tuberculosis is marked by an inflammatory granulomatous response that results in sequestration of organisms within areas of liquefaction and tissue destruction, termed caseous necrosis. It is in these caseous areas that organisms remain viable for years, awaiting periods of diminished immunity that will allow reactivation of dormant bacilli. The capacity of mycobacteria to elicit strong inflammatory responses - the adjuvant in Freund's adjuvant - may be critical in inducing sufficient tissue destruction to facilitate passage of organisms to the

airspaces, where triggering of cough and transmission to the environment can occur. It is likely that mycobacteria have exploited the inherent effector capacity of NK and related T cells, and perhaps γδ T cells, to engender the granulomatous inflammatory response that both ensures longevity in the host and eventual transmission to others. Recent experiments suggest such contributions from NK T cells (Apostolou, 1999). By this hypothesis, *M. tuberculosis* exploits the effector potential and ligand recognition properties of CD1, which have evolved for unrelated housekeeping functions, to ensure its survival and transmission, consistent with the capacity of this organism to have infected up to one-third of the human population on earth.

On the origin of class I and class II MHC

Taken together, we would speculate that it is the ability to present, either through recognition of the molecule itself or of its chaperoned cargo, information regarding the general baseline health of the cell that unites the function of these diverse MHC molecules. Frequently, this involves trafficking through distinct cellular compartments that allow dynamic interactions with their various ligands. This, of course, is exactly the function of class I and class II MHC, which display peptide ligands acquired from different cell compartments for surveillance by lymphocytes. For these molecules, however, the capture of bacterial transposases allowed the unlimited rearrangements of the scanning receptors, establishing the capacity to monitor cellular homeostasis with unprecedented precision. Of importance is the presence of these surveillance detectors on lymphocytes, cells capable of clonal expansion, thus enabling the amplification of highly specific effector programs. Again, evolutionary precedents

established by the nonpolymorphic MHC molecules have identified a preexisting relationship whereby lymphocytes survey these types of molecules in order to unleash effector pathways involved in monitoring tissue health and integrity. It is intriguing to speculate that some primordial footprint of the evolutionary role of class I and class II MHC might remain that is independent of the widely accepted role of these molecules in adaptive immunity.

Table 1. Proposed homeostatic roles of mouse CD1 and other nonpolymorphic MHC-like molecules.

MHC or MHC-like Molecule	Functional Binding Groove	Cargo	Recognition by Lymphocyte Receptor	Interacting Ligand and/or Cell Types	Proposed Homeostatic Function
Classical					
Class I	+	peptide	+	CD8/TCR αβ T cells	host defense
Class II	+	peptide	+	CD4/TCR αβ T cells	host defense
Nonpolymorphic					
CD1	+	lipid	+	CD4+ or DN NK, CD8αα+, γδ T cells	lipid membrane integrity
H2-M3	+	formylated peptide	+	CD8/TCR αβT cells	mitochondrial integrity?
HLA-E (Qa-1)	+	MHC I leader peptide	+	CD94/NKG2A, B, C NK cells & CD8+ T cells	Class I MHC expression
HLA-G	+	peptide	+	ILT-2, p49 KIR NK & T cells	materno-fetal tolerance
MIC-A/MIC-B	-	unknown	+	CD94/NKG2D NK, γδ T cells, CD8+ T cells	gut epithelial integrity?
FcRn	-	IgG	+	IgG	serum immunoglobulin
ZAG	probable	lipid	unknown	unknown	lipid metabolism?
HFE	-	transferrin receptor	-	transferrin/iron/TfR	iron

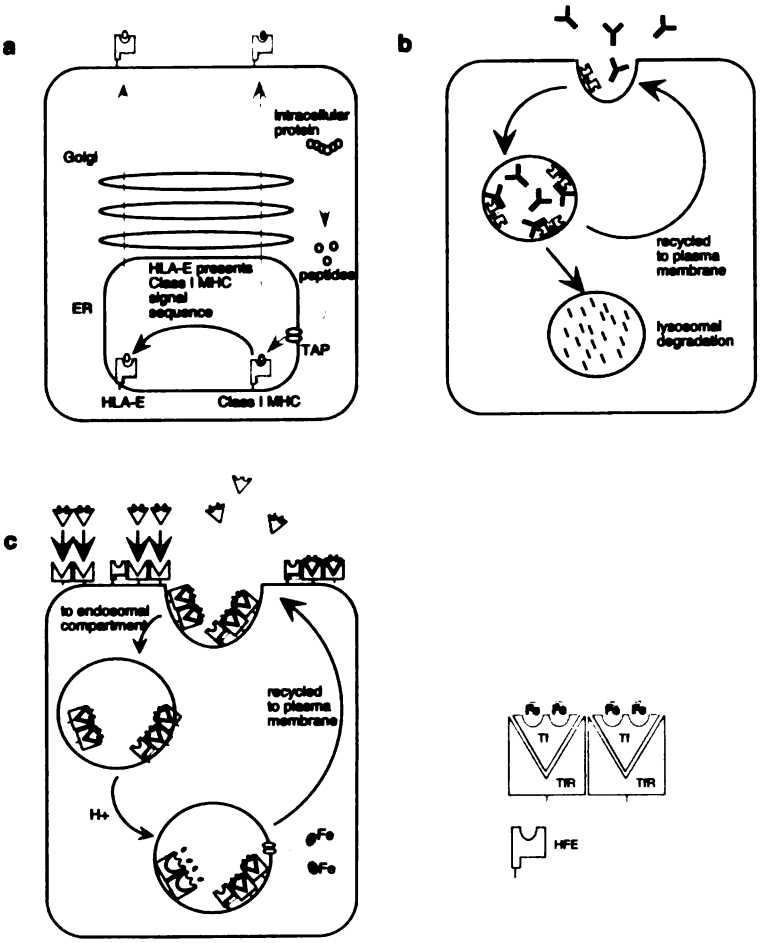
Figure 1. Nonpolymorphic MHC-related molecules are involved in homeostatic maintenance by surveying self-ligands from distinct intracellular compartments.

a, HLA-E presents peptides derived from the signal sequences of Class I MHC molecules that are loaded in the endoplasmic reticulum.

b, The neonatal Fc receptor transports IgG from neonatal gut epithelium to blood (not shown), and protects IgG catabolism in adult vascular endothelium, skin, muscle, and gut epithelium.

c, HFE binds iron-bound transferrin on the cell surface of gut epithelium, and is trafficked through the endosomal compartment. HFE acts as a negative modulator of transferrin receptor (TfR)-mediated uptake of transferrin (Tf)-bound iron, and thus plays an important role in iron homeostasis.

Figure 1


a
Intracellular protein
Golgi
peptides
HLA-E presents Class I MHC signal sequence
ER
TAP
HLA-E
Class I MHC
b
recycled to plasma membrane
lysosomal degradation
c
to endosomal compartment
recycled to plasma membrane
H+
Fe
Fe
TfR
TfR
HFE

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