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Chitin in Allergic Immunity

by

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Abstract

CHITIN IN ALLERGIC IMMUNITY

Tiffany A. Reese

Allergic and parasitic helminth immunity is characterized by infiltration of tissues with IL-4- and IL-13-expressing cells, including Th2 cells, eosinophils and basophils. Additionally, tissue macrophages assume a distinct phenotype, designated alternatively activated macrophages. Relatively little is known regarding the early factors that trigger these host responses, however we do know that these host responses depend on Stat6 signaling downstream of IL-4 and IL-13 receptors.

We identified two mammalian chitinase-like proteins, AMCase and Ym2 as being Stat6-dependent and upregulated during an immune response to the helminth, *Nippostrongylus brasiliensis*. AMCase is an enzymatically active chitinase that degrades chitin, while Ym2 is a chitinase-like protein that has lost enzymatic activity. In experiments using transgenic mice that overexpressed AMCase or Ym2, as well as antibody blockade experiments we concluded that neither AMCase nor Ym2 were proinflammatory. Thus, we took the approach of exploring the effects of chitin, a carbohydrate ligand of AMCase and Ym2 to ascertain their functions in immunity to chitin-containing pathogens and allergens.

Chitin is the second most abundant polysaccharide in nature, and it confers structural rigidity to fungi, crustaceans, helminths and insects. We found that chitin induced the tissue accumulation of IL-4-expressing innate immune cells, including eosinophils and basophils, when given to mice. Moreover, chitin mediated alternative macrophage activation *in vivo* and induced leukotriene B4 production, which was required for optimal immune cell

recruitment. Importantly, we found that tissue infiltration of effector cells and alternative macrophage activation was abolished when chitin was treated with AMCase, but not Ym2. Therefore, we conclude that chitin is a recognition element for tissue infiltration by innate cells implicated in allergic and helminth immunity and this process is negatively regulated by a vertebrate chitinase.

This work implicates chitin, a common environmental biopolymer and component of aerosolized antigens as an pathogen associated molecular pattern that can trigger early inflammatory responses associated with allergic and parasitic immunity. In our model chitin induces alternative macrophage activation and recruitment of cells that can produce IL-4 and IL-13. IL-4/IL-13 expression in tissues leads to activation of Stat6-dependent genes, including AMCase and Ym2. While the function of Ym2 remains unclear at this time, AMCase appears to act in a negative feedback fashion to degrade chitin and dampen chitin-induced inflammation.

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Chapter I:

Initiation of Type-2 Immunity

The ability to distinguish self from non-self is an essential feature of all organisms, ranging from plants and insects to mammals. Intriguingly, many of the underlying mechanisms of self/non-self recognition are highly conserved across kingdoms. Plants have an immune system, even though they do not have mobile defense cells with the ability to neutralize pathogens or pathogen-infected cells. Plants depend on individual cells to recognize pathogens, to eliminate them, and to send warning signals to neighboring cells (Jones and Dangl, 2006; Nurnberger et al., 2004). Insects, such as *Drosophila melanogaster*, rely on both antimicrobial peptides secreted into the hemolymph, as well as hemocytes that phagocytose and encapsulate pathogens in the hemolymph (Lemaitre and Hoffmann, 2007). Importantly, a common feature of plants, insects, lower vertebrates and mammals is that they all rely on pattern recognition receptors (PRRs) that recognize pathogen associated molecular patterns (PAMPs). Because PAMPs are common microbial components that are often essential for the microorganism, they are consequently difficult for the microorganism to alter and likely provide common components that can be recognized by plants, insects and mammals.

Chitin, a polymer of N-acetyl-β-D-glucosamine is the second most abundant polysaccharide in nature after cellulose, and is a structural component of fungi, crustaceans and insects. Consequently, chitin recognition is an ancient and highly conserved mechanism for regulating diverse biologic pathways. In *Vibrio cholerae*, chitin-sensing is linked with a complex gene expression pattern that regulates attachment and degradation of chitin on zooplankton, development of transformation competence and attachment to human intestinal epithelia (Kirn et al., 2005; Meibom et al., 2005). Chitin provides tensile strength and osmotic stability to fungal cell walls (Bowman and

Free, 2006). In insects, chitin is used as a scaffold, functioning in the rigid exoskeleton and in tubular morphogenesis, and regulated expression of enzymes called chitinases that degrade chitin is required during molting and development (Devine et al., 2005; Merzendorfer and Zimoch, 2003). Nematode chitins are important for eggshell integrity and for structure of the rigid pharynx, including the buccal cavity and grinder, a specialized cuticle that is shed and resynthesized during molting (Zhang et al., 2005). The use of chitin by this wide range of organisms may have provided evolutionary pressure to maintain chitin recognition elements in mammals similar to those found in plants and protochordates.

Paradigms of Pattern Recognition

Chitin Recognition in Plants

The plant immune system recognizes PAMPs by utilizing both transmembrane receptors, as well as intracellular nucleotide-binding leucine rich repeat (NB-LRR) proteins similar to the NOD-family of proteins in mammals. Upon recognition of an invader, plant defense includes transcriptional activation of defense genes, such as lytic enzymes and anti-microbial proteins, and production of reactive oxygen intermediates (ROIs). Plants also commonly induce hypersensitive cell death in a highly localized fashion potentially to contain infection (Jones and Dangl, 2006; Nurnberger et al., 2004).

Chitin recognition by plant cells has been observed for some time, and is thought to be a mechanism of defense against fungal invaders. When tomato cells are exposed to chitin fragments in culture they induce transient alkalinization of the growth medium and protein phosphorylation, and both tomato cells and rice cells bind chitin oligosaccharides

(Baureithel et al., 1994; Shibuya et al., 1993). Receptors in rice and soybean plasma membranes have been isolated (Day et al., 2001; Ito et al., 1997), and in tobacco a chitinase-related receptor-like kinase (CHRK1) found in the plasma membrane undergoes autophosphorylation, although the chitinase domain is inactive due to a mutation in the catalytic center (Kim et al., 2000). Despite the observed up-regulation of CHRK1 in tobacco cells upon fungal infections, direct binding of chitin oligosaccharides to this receptor was not reported. However, it is an interesting candidate for a receptor that can sense chitin and induce a signal in the plant cell.

Recently, functional receptors for chitin have been identified in plant cells that are important in both symbiotic and potentially pathogenic interactions with bacteria and fungi. Legumes establish a symbiotic relationship with both mycorrhizal fungi for phosphorus fixing, and rhizobial bacteria for nitrogen fixing. While the fungal factors involved in symbiosis are unknown, the rhizobial factors essential for symbiosis, called Nodulation factors (NFs), are lipochito-oligosaccharides (Lerouge et al., 1990). Root nodules for nitrogen fixing are initiated when NFs secreted by rhizobia induce root hairs to undergo deformation and curling around the bacteria, followed by infection thread growth. In two different legume species, potential NF receptors were defined, although their direct binding to chito-oligosaccharides has not been shown. Mutants lacking either NFR1 or NFR5 of *Lotus japonicus* do not induce root hair responses to rhizobia (Madsen et al., 2003; Radutoiu et al., 2003). Mutants missing LYK3 and LYK4 of *Medicago truncatula* are able to entrap bacteria, but do not undergo infection thread growth (Limpens et al., 2003). Interestingly, all of these receptors are LysM domain receptor kinases. The extracellular region contains 2-3 LysM domains, while the intracellular

region contains the kinase domain. In rice cells another LysM-domain containing protein, CEBiP directly binds to chitin oligosaccharides, and knock-down of CEBiP by RNA interference leads to a reduction in reactive oxygen species and gene induction following treatment with chitin (Kaku et al., 2006).

LysM domains were initially identified in bacteria lysins and other enzymes that degrade bacterial cell walls. Interestingly, they are conserved in mammals. Three LysM domains can bind to peptidoglycan, specifically *N*-acetyl-glucosamine-*N*-acetyl-mureine (Steen et al., 2003). LysM domains are often found with amidase, protease, or chitinase domains, and may represent a conserved mechanism for chitin recognition in higher organisms.

Pattern Recognition in Drosophila

Secretion of antimicrobial peptides is a hallmark of *Drosophila* immunity, and their production is regulated through two distinct signaling pathways, Toll and Imd. These pathways are triggered by recognition of PAMPs, either through secreted receptors or transmembrane receptors. The Toll-signaling pathway is important in fly development, but also plays an essential role in fly immunity (Lemaitre and Hoffmann, 2007). Importantly, this signaling pathway is highly conserved in vertebrates, and individual Toll-like receptors (TLRs) in vertebrates recognize different PAMPs. In contrast to vertebrates, *Drosophila* toll receptors do not recognize pathogens themselves, but instead are activated by a cytokine, Spatzle (Lemaitre et al., 1996). Spatzle, normal present in an inactive pro-Spatzle form, is activated through proteolytic cascades induced by secreted recognition molecules that sense gram-positive bacteria (Hu et al., 2004;

Weber et al., 2003). These secreted recognition molecules fall into two categories, either peptidoglycan recognition proteins (PGRPs) or gram-negative binding proteins (GNBPs) (a misnomer since GNBPs recognize gram-positive bacteria) (Lemaitre and Hoffmann, 2007). PGRPs recognize peptidoglycan, an essential polymer in the cell wall of bacteria consisting of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid residues linked by short peptide chains (Mengin-Lecreulx and Lemaitre, 2005). Fungi and yeast also activate the Toll pathway by activation of different secreted PRRs. Ultimately, each pathway activates expression of different sets of antimicrobial peptides that have activity against different classes of pathogens, although the precise mechanism of their action is still unclear. Importantly, the toll signaling pathway is highly conserved and an essential part of vertebrate innate immunity to bacteria and viruses, and will be discussed again later.

Chitin-binding proteins in protochordates

Comparative studies of the immune systems of protochordates, such as amphioxus, provide researchers with clues to how immune recognition, as well as linkages between innate immunity and adaptive immunity have evolved. Although conventional adaptive immunity associated with recombination activating gene (RAG) expression does not appear in evolution until the jawed vertebrates, it is now recognized that some jawless vertebrates and protochordates have anticipatory immune systems with large multigene families of highly variable receptors (Litman et al., 2005). Interestingly, amphioxus has a multigene family that encodes an immunoglobulin-type molecule called V-region-containing chitin-binding protein (VCBP) that contains two N-terminal V-

domains and one C-terminal chitin-binding domain (Cannon et al., 2002). The V-domains of VCBP2 and VCBP5 have regions of extensive diversity, suggesting a role in immune function and pathogen recognition (Cannon et al., 2002; Cannon et al., 2004; Hernandez Prada et al., 2006). The association of a diversified V region with a chitin-binding domain represents a bifunctional molecule with a region that can recognize a range of pathogens combined with a domain that recognizes *N*-acetylglucosamine found in many microbes, similar to domains of innate immune receptors in plants. VCBPs are a potential example of how evolutionary pressure for sensing chitin, a common microbial component, has maintained chitin-binding domain elements and adapted them for immune recognition in highly diverse organisms.

Initiation of Immunity in Mammals

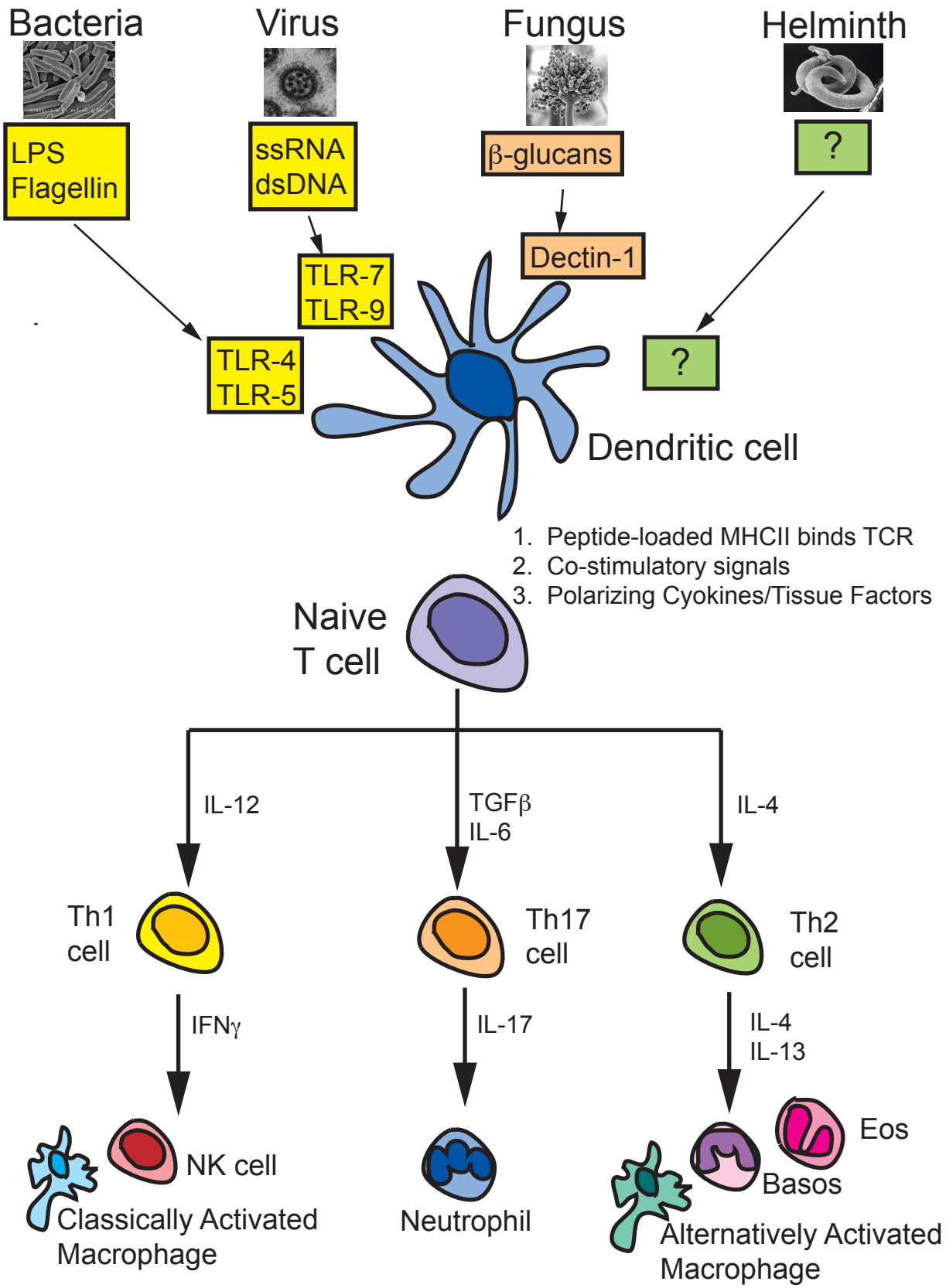
In mammals, defense against pathogenic microorganisms is critically dependent on the coordination of pathogen non-specific innate cells and pathogen specific adaptive immune cells. Dendritic cells (DCs) are specialized antigen presenting cells (APCs) that are critical for coordinating innate recognition of pathogens with generation of appropriate adaptive immune responses. DCs are sentinel cells positioned at sites throughout the body, particularly at mucosal surfaces and the skin where pathogens are most often encountered. When DCs encounter pathogen they undergo a transformation process. First, they phagocytose the pathogen and process it into peptides for presentation on major histocompatibility complex (MHC) class I and II. During this process DCs also up-regulate chemokine receptors such as CCR7 and downregulate other receptors such as CCR6 (Dieu et al., 1998; Sallusto et al., 1998), thus facilitating their

movement from peripheral sites to lymph nodes. During migration to the lymph node, DCs undergo maturation, and up-regulate costimulatory molecules and cytokines that are important for T cell activation. Once in the lymph node, DCs present MHC class II loaded with pathogen-derived peptides to naïve $CD4^+$ T cells and induce their differentiation into effector cells.

As already alluded to, naïve T cell fate is determined by the signals that DCs primed by a pathogen are thought to provide. Signal 1 is generated through the engagement of the T-cell receptor by peptide bound by MHC class II on the DC. This determines the antigen-specificity of the immune response. Signal 2 is achieved through costimulation, whereby CD28 on the T cell interacts with B7-1/B7-2 on the mature dendritic cell, and CD40 ligand (CD40L) on the T cell binds to CD40 on the dendritic cell. Without signal 2 T cells become anergic and fail to proliferate in response to antigenic stimulation. While there are data that implicate the strength of signal 1 (Constant et al., 1995; Hosken et al., 1995; Tao et al., 1997) or the nature of signal 2 (Corry et al., 1994; Kuchroo et al., 1995; Lenschow et al., 1995) to the ultimate polarization of T cells to acquire different effector fates, evidence suggests the need for a third signal derived from the dendritic cell that favors differentiation of naïve T cells into specific effector cells (Figure 1) (Kalinski et al., 1999).

Biased differentiation of naïve T cells into different effector fates is a hallmark of adaptive immunity, and is essential for orchestrating an appropriate adaptive immune response that can eliminate a pathogen. Coffman and Mosmann first proposed the hypothesis that CD4 T cells adopt different fates leading to different immunological outcomes in the mid 1980s (Cher and Mosmann, 1987; Mosmann et al., 1986). We now

Figure 1 | Dendritic cells promote polarized immune responses associated with Th1, Th2, and Th17 development by integrating pathogen-associated signals. Th1 polarization occurs when DCs recognized bacteria and viruses through pattern recognition receptors. For example, bacteria can activate TLR4 or TLR5 with LPS or flagellin, respectively. Th1 polarization can also occur when viruses activate TLR7 or TLR9 with ssRNA or dsDNA, respectively. Additionally, there are other receptors that bacteria and viruses can trigger that are not mentioned here. On the other hand, fungal β-glucans bind dectin-1, leading to Th17 responses. When these pathogens are phagocytosed, DCs present pathogen-associated peptides on MHC class II and up-regulate costimulatory molecules for activation of naïve T cells. DCs also secrete polarizing cytokines depending on the particular PRR that is activated by PAMPs. These polarizing cytokines promote development of different effector $CD4^+$ T cells that secrete IFNγ, IL-17, or IL-4/IL-13. Associated populations of myeloid cells are also activated. While much is known about the effector cell responses associated with Th2 cell development and type-2 immune responses, a PAMP from a helminth that binds a PRR on DCs, leading to DC-derived signals specific for a type-2 immune response is unknown.


Bacteria
Virus
Fungus
Helminth
LPS
Flagellin
ssRNA
dsDNA
β-glucans
?
TLR-7
TLR-9
Dectin-1
TLR-4
TLR-5
?
Dendritic cell
1. Peptide-loaded MHCII binds TCR
2. Co-stimulatory signals
3. Polarizing Cyokines/Tissue Factors
Naive
T cell
IL-12
TGFβ
IL-6
IL-4
Th1
cell
Th17
cell
Th2
cell
IFNγ
IL-17
IL-4
IL-13
NK cell
Classically Activated
Macrophage
Neutrophil
Eos
Basos
Alternatively Activated
Macrophage

recognize that these effector CD4+ T cell lineages include, but are not limited to, T helper 1 (Th1), Th2, and the recently identified subset Th17. Coffman and Mosmann's observations established a paradigm that would remain largely unchanged until recently whereby Th1 and Th2 helper T cell subsets stimulate different sets of effector activities, promote the development of more T helper cells of that same subset, and inhibit the development of the reciprocal T helper cell subset. It is now recognized that there are other important T helper cell subsets, including T regulatory cells (Sakaguchi et al., 2006) and Th17 cells (Harrington et al., 2005; Park et al., 2005), which are important in different infectious models and immune pathologies. However, even with the addition of these new T helper subsets, the ideas that specialized subsets of T cells for different types of pathogens exist, and that these T cell subsets have reciprocal interactions and cross-regulation remain defining principals in immunology. Appropriate differentiation and regulation of these helper T cell subsets is critical for clearance of microorganisms, as well as prevention of immune mediated pathology associated with autoimmunity and allergy. Moreover, adding to the complexity of T helper cell subsets, is the broader concept of "Type-1" and Type-2" immunity that includes non-T cells. It is now accepted that along with the differentiation of T cell subsets goes the activation of distinct populations of dendritic cells, macrophages, and other myeloid cell types (Figure 1).

Differentiation of Th1, Th17 and Th2 cells

For Th1 cell differentiation interleukin-12 (IL-12) from dendritic cells and macrophages signals through signal transducer and activator of transcription 4 (Stat4) and drives interferon-γ (IFNγ) production. IFNγ acts in an autocrine fashion to up-regulate

the IL-12 receptor on T cells and expression of Tbet, a member of the T-box family of transcription factors that is Th1-specific and is thought to be the "master switch" that controls Th1 differentiation. Tbet drives expression of more IFNγ, creating a positive feedback loop, and it suppresses expression of IL-4. Th1 cell differentiation and type-1 immunity is also associated with activation of natural killer (NK) cells and classically activated macrophages that eliminate pathogen-infected cells (Murphy and Reiner, 2002).

Th17 cells are relative newcomers to the field of T cell subsets. IL-6 and TGF-β are essential cytokines for Th17 differentiation (Stockinger and Veldhoen, 2007), and signaling through the IL-6 receptor and Stat3 leads to upregulation of the lineage determining transcription factor RORγt, an orphan nuclear receptor (Ivanov et al., 2006). Th17 cells are associated with autoimmunity (Weaver et al., 2007), and are important for defense against some extracellular bacteria such as *Klebsiella pneumoniae* (Happel et al., 2005) or *Bacteroides fragilis* (Chung et al., 2003), and against fungi such as *Candida albicans* (Huang et al., 2004).

For Th2 cell differentiation IL-4 signals through Stat6 to promote T cell polarization. IL-4/Stat6 signals induce expression of GATA3, a zinc-finger transcription factor that is essential for Th2 cell polarization and maintenance. GATA3 regulates a broad array of Th2 cytokines in T cells. GATA3 is also thought to suppress IL-12 signaling through Stat4, thus inhibiting Th1 differentiation (Murphy and Reiner, 2002). Importantly, although IL-4 is important in T cell differentiation, DCs are not thought to make IL-4. The initial source of IL-4 could be from a low level expression by T cells themselves from the time they get activated (Schmitz et al., 1994) that gets amplified in the absence of IL-12, or from the other cells types, such as NK T cells (Yoshimoto and

Paul, 1994) or basophils (Gessner et al., 2005; Voehringer et al., 2004). However, this raises the questions of how these accessory cell types happen to be at the right place at the right time, and if there could be other as yet unappreciated signals provided by DCs that promote Th2 cell differentiation. It is also important to note that Stat6-deficient mice can still make Th2 cells, even though the T cells cannot receive IL-4 signals through the IL-4R (Finkelman et al., 2000; McKenzie et al., 1999; Mohrs et al., 2001). Although the initial source of IL-4 and the identity of other polarizing factors are unknown, it is clear that type-2 immunity is highly associated with the recruitment and activation of cells that can make IL-4 (Voehringer et al., 2004), including eosinophils and basophils, along with differentiation of alternatively activated macrophages that promote tissue remodeling (Gordon, 2003).

Innate immune signals instruct T cell fate

While the differentiation of $CD4^+$ helper T cells has interested immunologists for decades, and we recognize that DCs are important for driving T cell differentiation, there is still relatively little known about the earliest signals that promote the differentiation of one population or another. Janeway and colleagues proposed the pioneering concept that DCs recognize classes of pathogens through PRRs similar to receptors described earlier in *Drosophila*, and subsequently direct pathogen-specific T cells to acquire a protective effector phenotype (Janeway, 1989). Subsequent to this seminal proposal, significant progress has been made in identifying the signals provided by bacteria and viruses that drive Th1 differentiation and a type-1 immune response, including the identification of multiple Toll-like receptors (TLRs) in mouse and human. However, comparatively little

is know about the factors that drive Th2 differentiation and a type-2 immune response associated with parasitic helminthes (Figure 1).

How is a DC conditioned to promote Th2 cell differentiation?

Pattern Recognition Receptors

If a DC ultimately instructs or promotes naïve T cells to differentiate into Th1, Th2, or Th17 cells then an important question is how a DC discriminates one type of pathogen from another. As mentioned earlier, Janeway proposed the idea that there are conserved receptors that distinguish types of pathogens by recognizing evolutionary conserved patterns (Janeway, 1989). The validity of this hypothesis was first recognized with the identification of TLRs. There are twelve members identified in mammals, so far, and the TLRs are highly conserved from *Caenorhabiditis elegans* to mammals. TLRs are type I transmembrane glycoproteins containing multiple leucine rich repeats (LRRs) in the extracellular domain, and a cytoplasmic tail containing a Toll/interleukin 1 receptor (IL-1R) homology (TIR) domain. Some TLRs recognize lipid PAMPs, such as TLR1, TLR2, and TLR6, which can all heterodimerize with each other to recognize components of bacterial cell walls. Other TLRs, such as TLR7, TLR8, and TLR9 recognize nucleic acid motifs most often presented by viruses. TLRs are most highly expressed on antigen-presenting cells (APCs), including DCs and macrophages. All the TLRs activate components of the IL-1R signaling pathway leading to inflammatory cytokine production, such as IL-12 and IL-6, and some induce type-I interferon production. Thus, stimulation of most TLRs appears to lead to Th1 differentiation, and not Th2 differentiation (Figure 1) (Akira et al., 2006).

Other types of PRRs include C-type lectin receptors (CLRs) that have the capacity to regulate gene expression. CLRs are receptors with C-type lectin domains that can bind to carbohydrate motifs. Many CLRs can bind to PAMPs, although not all are cell associated (Robinson et al., 2006). For example, the collectins, including the mannose binding lectin, are found in serum and can bind to sugar motifs found on bacteria, viruses, fungi and protozoans leading to complement activation (Takahashi et al., 2006). Many other CLRs are found on myeloid cells, and most are considered to be phagocytic receptors, but dectin-1 has been shown to signal through Syk and to mediate recognition of the fungal component, zymosan, leading to IL-10 and IL-2 production by DCs (Rogers et al., 2005). Specifically, it was recently demonstrated that dectin-1 recognizes β-glucan motifs in zymosan, and recognition of these motifs by DCs leads to Th17 differentiation of naïve T cells (LeibundGut-Landmann et al., 2007). Importantly, mice lacking dectin-1 do not control fungal infection (Saijo et al., 2007; Taylor et al., 2007).

Despite the abundance of research in the area of innate immune recognition, a PAMP associated with a helminth parasite that binds a PRR leading, ultimately, to Th2 cell differentiation remains illusive. There are reports of helminth-derived products able to induce Th2 cell differentiation (Whelan et al., 2000). One glycoprotein from the filarial nematode, *Acanthocheilonema viteae* called ES-62 induces DCs with the capacity to drive Th2 differentiation *in vitro* and *in vivo*. These DCs, however, do not up-regulate of CD80/CD86 or CD40, therefore the status or mechanism of their maturation is unclear. Additionally, in these experiment the authors found that treatment of DCs with ES-62 induces small amounts of IL-12, and they do not rule out that their preparations of ES-62 could be contaminated with TLR ligands. They also provide no data identifying a

receptor recognizing ES-62, or confirming a product of DCs that promotes Th2 differentiation in the presence of ES-62.

Schistosoma mansoni, another helminth parasite, induces a strong Th2 response. Specifically the Schistosome soluble egg antigens (SEA) potently induces Th2 responses, including increased serum IgE and eosinophilia in the lung following repetitive intranasal doses (Okano et al., 1999a). Using periodate-treated SEA to disrupt specifically the carbohydrate component of SEA reveals that the carbohydrate component of SEA is necessary to induce SEA-specific IgE and IgG1, as well as the production of IL-4, IL-5 and IL-10 from nasal lymphocytes. Importantly, they show that although the carbohydrates on SEA are important for immunogenicity, they are not the actual targets of the SEA-specific IgE (Okano et al., 1999b). Subsequently, the same group found that conjugating protein antigen to lacto-*N*-fucopentaose III, the predominant carbohydrate component of SEA, induces Th2 differentiation *in vivo*, including antigen specific IgE, IgG1 and cytokine production in nasal lymphocytes (Okano et al., 2001). Other groups demonstrated that SEA-treated DCs promote Th2 responses *in vivo* (de Jong et al., 2002; MacDonald et al., 2001). Despite their ability to induce Th2 cytokine production *in vivo*, these DCs exhibit no conventional signs of maturation, with no up-regulation of CD80/86, CD40 or OX40L (MacDonald et al., 2001). Another group did see up-regulation on SEA-primed DCs of OX40L, a costimulatory molecule on DCs thought to be involved in Th2 cell differentiation (Flynn et al., 1998), thus providing a possible mechanism for how the SEA-primed DCs could induce Th2 differentiation. However, treatment of DCs with other Th2-inducing factors, such as *Vibrio cholerae* toxin, does

not induce OX40L (de Jong et al., 2002). Therefore, even if OX40L plays a role in SEA-primed DCs, there must be other unidentified mechanisms for different Th2 stimuli.

Tissue Factors

Many allergic or type-2 immune responses occur at mucosal surfaces, suggesting that mucosal surfaces or mucosal DCs could be predisposed to promote type-2 inflammation. Indeed, transfer of antigen-pulsed DCs into the airways of naïve mice, followed by aerosol antigen challenge leads to recruitment of eosinophils into the bronchoalveolar lavage, IL-4 and IL-5 production by $CD4^+$ T cells, and perivascular and peribronchial eosinophilic infiltrates (Lambrecht et al., 2000). In the gut, either myeloid DCs from the Peyer's patch (Iwasaki and Kelsall, 2001) or mesenteric lymph node DCs (Alpan et al., 2001) preferentially prime naïve T cells to produce higher levels of IL-4 and IL-10 compared with splenic DCs or peripheral DCs from other lymph nodes. These experiments imply that DCs populations in the lung or in the gut are influenced by their microenvironment to preferentially induce Th2 cell polarization.

One possibility for how DCs are primed to make Th2 cells in certain tissues is that the epithelial cells of some tissues preferentially produce tissue factors that favor Th2 cell differentiation. For example, Prostaglandin E2 (PGE_2), primarily produced by tissue macrophages, selectively inhibits IFNγ production from mouse and human T cell clones, promotes IL-4 and IL-5 production (Betz and Fox, 1991; Snijdewint et al., 1993), and inhibits IL-12 receptor expression (Wu et al., 1998). Naïve T cells exposed to PGE_2 during the priming phase develop selectively into Th2 cells, as determined by their secretion of IL-4, IL-5, and IL-13 and a lack of IFNγ production (Demeure et al., 1997).

Importantly, PGE_2 also acts on the level of the APC, by inhibiting production of IL-12p70, a heterodimer of IL-12p40 and IL-12p35 (Kalinski et al., 2001; van der Pouw Kraan et al., 1995). Interestingly, PGE_2 selectively induces the IL-12p40 subunit, but not the IL-12p35 subunit in DCs. IL-12p40 can form homodimers, and the homodimers are thought to be antagonists of the IL-12R, thus providing a potential mechanism for how PGE_2 not only promotes Th2 cell development, but also actively inhibits Th1 cell development.

Similarly, other factors like PGE_2 that induce increased cAMP also prevent Th1 development and promote Th2 development. Histamine inhibits IFNγ production by T cells, and TNFα and IL-1 production by monocytes (Dohlsten et al., 1988; Dohlsten et al., 1987; Vannier et al., 1991). Histamine also blocks production of IL-12p40 and IL-12p70 (van der Pouw Kraan et al., 1998), even in the presence of LPS (Caron 2001). DCs matured in the presence of histamine promote Th2 differentiation, even when IFNγ is added to the culture media (Caron et al., 2001). Moreover, β2-adrenergic receptor agonists selectively inhibit IL-12 production from macrophages and DCs stimulated with LPS, and accordingly priming of naïve T cells in the presence of β2-adrenergic receptor agonists inhibits Th1 cell development and enhances Th2 cell development (Panina-Bordignon et al., 1997). Taken together, these data indicate that certain tissue-derived factors with the ability to increase intracellular cAMP can modulate Th1/Th2 cell development. However, there is little *in vivo* data to support these *in vitro* differentiation assays, and mechanisms by which these factors are induced to promote Th2 cell differentiation is unclear. We know that histamine is released from mast cells and basophils through IgE receptor crosslinking, but that implies that the antigen specific IgE

already exists to promote histamine release and subsequent Th2 polarization. Perhaps these factors play more of an amplification or positive-feedback role in the process of Th2 differentiation.

Tissue-derived cytokines

Thymic stromal lymphopoietin (TSLP), an IL-7-like cytokine, is a potential epithelial cell-derived cytokine that can instruct DCs to prime Th2 cells. TSLP potently activates human DCs to up-regulate MHC class II and costimulatory markers, without inducing the up-regulation of the Th1 polarizing cytokine, IL-12p70 or the proinflammatory cytokines IL-6, IL-1β and TNF-α (Soumelis et al., 2002). Additionally, TSLP-treated DCs up-regulate the chemokines TARC and MDC that attract Th2 cells (Soumelis et al., 2002). TSLP-activated DCs also polarize naïve T cells to become Th2 cells that secrete IL-4, IL-5, and IL-13 (Soumelis et al., 2002). TSLP-activated DCs induce expression of OX-40L, and blockade of OX-40L with a neutralizing antibody inhibits Th2 cell development (Ito et al., 2005).

Significantly, high TSLP expression in keratinocytes is associated with patients with acute and chronic atopic dermatitis (Soumelis et al., 2002), and TSLP expression is increased in the airways of asthmatic patients (Ying et al., 2005). The link between TSLP and allergic inflammatory diseases is also observed in mouse models of asthma, with increased TSLP expression in the lungs of mice with OVA-induced airway inflammation. TSLP receptor-deficient mice have greatly attenuated disease in the OVA asthma model, and overexpression of TSLP specifically in the lung leads to airway inflammation, spontaneous airway hyperreactivity, increased serum IgE, and goblet cell

hyperplasia – all hallmarks of allergic airway disease (Zhou et al., 2005). Lastly, inducible TSLP overexpression in the skin of mice results in development of atopic dermatitis-like phenotype (Yoo et al., 2005). Taken together, these mouse models suggest that TSLP can initiate allergic inflammation by activating DCs to promote Th2 cell development. However, observations that transgenic mice crossed to T cell deficient mice still have skin inflammation imply that TSLP exerts many of its proinflammatory effects on myeloid cells and that disease initiation mediated by TSLP does not require Th2 cells (Yoo et al., 2005). Further clarification of the necessity for Th2 cells in TSLP-mediated pathologies needs investigation.

IL-25, also known as IL-17E, is part of the IL-17 family of cytokines, and may represent another tissue-derived factor important in type-2 biology. IL-25 was originally identified as a cytokine made by Th2 cells (Fort et al., 2001), although mast cells can also make it after IgE-receptor crosslinking (Ikeda et al., 2003). Treatment of mice with recombinant IL-25 (rIL-25) systemically induces eosinophilia, serum IgE, and IL-4, IL-5, and IL-13 from a non-B/non-T cell that is MHC class II^{hi} (Fort et al., 2001). Treatment intranasally induces eosinonophilia in the lung and BAL, airway hyperresponsiveness, and mucus hypersecretion (Hurst et al., 2002). IL-$25^{-/-}$ mice are more susceptible to infection with the intestinal helminth, *Trichuris muris* (Owyang et al., 2006), and have delayed worm clearance of *N. brasiliensis* (Fallon et al., 2006). Administration of rIL-25 in $Rag^{-/-}$ leads to rapid worm expulsion of *N. brasiliensis* (Fallon et al., 2006). One group reported spontaneous inflammation and allergic pathology in IL-25 transgenic mice that overexpressed IL-25 in the lung (Angkasekwinai et al., 2007). They also found that adding IL-25 to *in vitro* cultures for T cell differentiation enhances Th2 polarization in

the presence of anti-CD3/CD28 and IL-2 (Angkasekwinai et al., 2007). Taken together, these results suggest a role for IL-25 in the earliest events leading to induction of type-2 immunity and Th2 differentiation, possibly through effects on an innate cell type. However, the *in vivo* source of IL-25 and the cell type(s) that express the receptor remain controversial. Recently, it was found that IL-25 receptor in human is expressed mainly on memory Th2 cells and that IL-25 enhances cytokine production from memory Th2 cells upon restimulation (Wang et al., 2007). Until the cellular source and the stimuli that induce expression of IL-25 are identified, the importance of IL-25 in inducing type-2 immune responses will be difficult to ascertain.

IL-33 is an IL-1 family cytokine that signals via the IL-1 receptor-like protein ST2 expressed on Th2 cells and mast cells (Trajkovic et al., 2004). Blockade of the ST2 receptor in an asthma model attenuates eosinophil recruitment and IL-4 and IL-5 production (Coyle et al., 1999; Lohning et al., 1998). *In vitro* differentiation of Th2 cells is impaired when ST2 is blocked during priming (Coyle et al., 1999). One group found that mice deficient in ST2 have impaired pulmonary granuloma formation and defects in Th2 cytokine production following injection of *Schistosoma mansoni* eggs (Townsend et al., 2000), while $ST2^{-/-}$ mice made by a different group have no defect in response to *N. brasiliensis* (Hoshino et al., 1999).

The ligand for ST2 is IL-33, and subsequently it was found that administration of systemic IL-33 induces eosinophilia and increases in IL-4, IL-5 and IL-13 gene expression, similar to IL-25. Furthermore, mucosal tissues exhibit histological changes, including inflammatory infiltrates in the lung and digestive tract, and increased mucus production (Schmitz et al., 2005). The authors found a role for IL-33 in polarized Th2

cells restimulated in the presence of IL-33, however they could not find a role for IL-33 during the initial *in vitro* priming of Th2 cells. RT-PCR of both mouse and human cDNA libraries show IL-33 to be expressed broadly in various organs including lung, lymph node, stomach, skin, and brain, among others. Moreover, IL-33 transcript is found specifically in epithelial cells of the lung and skin, as well as low-level expression in resting dendritic cells and activated macrophages in the mouse (Schmitz et al., 2005). Similar to IL-1β, IL-33 is produced with a prodomain that must be cleaved to make biologically active IL-33. Thus identifying the cellular source of IL-33 and the enzyme that activates it will be essential to understanding the biology of IL-33 and clarifying the role for IL-33 in the induction of type-2 immune responses.

Importantly, the missing link in assessing the importance of tissue-derived cytokines in the initiation of type-2 immune responses is determining how TSLP, IL-25, and/or IL-33 are induced in tissues. Are receptors triggered by pathogens on epithelial cells, or is simple physical injury or epithelial barrier disruption enough? One report found that stimulation of primary epithelial cells with TLR ligands or proinflammatory cytokines induce TSLP. Additionally, physical trauma of the skin resulting from biopsy correlates with TSLP expression (Allakhverdi et al., 2007). It remains unclear how specificity is achieved for cytokine production by epithelial cells and subsequent Th2 cell polarization with these stimuli.

Proteases

Many allergens are noted to have proteolytic activity, and this raises the possibility that proteases could be a common feature of substances that induce type-2

responses by affecting the epithelial barrier. Allergic reactions to the protease papain from *Carica papaya* found in meat tenderizers, contact lense solutions, digestive aids, lotions, and papaya fruit has been well documented. These reactions are associated with the development of allergic rhinitis, asthma, atopic dermatitis, and even hypersensitivity reactions due to oral exposure (Mansfield and Bowers, 1983; Novey et al., 1979; Soto-Mera et al., 2000). Other well studied allergens, including Der p 1, the major allergen of the house dust mite *Dermatophagoides pteronyssinus*, and allergens derived from *Aspergillus fumigatus* have protease activity. Immunization of mice with proteolytically active Der p 1, but not inactive Der p 1, promotes type-2 responses and induces Der p 1-specific IgE (Comoy et al., 1998; Gough et al., 1999). *A. fumigatus*, *A. oryzae*, and *Ambrosia artemisiifolia* (ragweed) allergens all induce airway hyperresponsiveness and eosinophil accumulation in the BAL that is dependent on their protease activity (Kheradmand et al., 2002). Additionally, combining OVA, which alone induces tolerance, with purified *A. fumigatus* protease leads to airway hyperresponsiveness and eosinophil accumulation in the BAL (Kheradmand et al., 2002). A purified protease from *Leishmania mexicana* is able to induce IL-4 and IgE production that is dependent on enzymatic activity (Pollock et al., 2003). Taken together, these observations suggest that exogenous proteases can induce type-2 immune responses, whether they are derived from allergens or parasites.

How do proteases promote type-2 immune responses? Some speculate that allergen proteases disrupt the epithelial barrier (Tomee et al., 1997) perhaps by disrupting tight junctions (Wan et al., 2001; Wan et al., 1999), thus increasing access of antigen presenting cells to the allergen (Herbert et al., 1995). Another possibility is that allergen

proteases activate specific receptors, such as protease-activated receptors (PARs) that lead to cytokine, chemokine and prostaglandin production. PARs are 7-transmembrane G protein coupled receptors activated by both endogenous and exogenous serine proteases (Reed and Kita, 2004). PAR-2 can be activated by allergen-derived serine proteases, Der p 3 and Der p 9 (King et al., 1998; Sun et al., 2001), as well as the cysteine protease Der p 1 (Asokananthan et al., 2002). In fact, it has been noted that PAR-2 expression is increased in patients with asthma (Knight et al., 2001; Roche et al., 2003). Moreover, researchers have observed that pulmonary epithelial cell lines treated with proteases release IL-8, IL-6, and monocyte chemotactic protein-1 (MCP-1) (Asokananthan et al., 2002; Tomee et al., 1997). Epithelial barrier disruption by proteases could also induce other signals, such as TSLP or synthesis of leukotrienes and prostaglandins that activate cells and recruit in myeloid cells.

Innate Cells Associated with Type-2 Immunity

Differential cell recruitment and alternative macrophage activation represent another mechanism for guiding the development of type-2 immunity and Th2 cell polarization. Importantly, type-2 immune responses are defined by the recruitment of effector cells to sites of inflammation that can produce IL-4, IL-5, and IL-13, including eosinophils, mast cells and basophils. Within tissue, resident macrophages acquire a distinct phenotype associated with type-2 immunity called alternative activation (Figure 1). These alternatively activated macrophages along with recruited cell types lead to further cell recruitment and tissue remodeling. Researchers are exploring the potential functions of innate cells and the significance of innate cell-derived cytokines in type-2

immune responses. It is increasingly clear that innate cell-derived IL-4/IL-13 is important in *Nippostrongylus brasiliensis,* a helminth model of type-2 immune responses.

A model type-2 pathogen: Nippostrongylus brasiliensis

To study type-2 immunity scientists experimentally adapted the rat helminth parasite, *Nippostrongylus brasiliensis* for use in the mouse. *N. brasiliensis* in a normal mouse induces a robust Th2 response, which leads to clearance of the parasite and protection against subsequent infections. Much has been learned about the requirements for induction of type-2 immunity using this model of acute infection.

N. brasiliensis L3 larvae, similar to a hookworm in humans, infect through the skin. The larvae penetrate the skin and enter the blood vessels, and are transported to the lungs via the circulation. Once trapped in the lungs, the worms undergo a molt. The L4 larvae are cough-up by the mouse and swallowed, which is how they make their way to the intestines. Once in the small intestines of the mouse the larvae mature to the adult form and begin to lay eggs. Due to a robust Th2 response and extensive mastocytosis in the intestines the adult worms are expelled from the mouse by day 10 (Ogilvie and Hockley, 1968). Subsequent attempts to secondarily infect a mouse with *N. brasiliensis* induce a rapid recall response, thus preventing secondary infection.

The immune response to N. brasiliensis

Expulsion of *N. brasiliensis* is critically dependent on $CD4^+$ T cells (Katona et al., 1988), but not on IL-4 (Kopf et al., 1993; Madden et al., 1991). Mice treated with a monoclonal antibody to IL-4 (Madden et al., 1991), or mice deficient in the IL-4 gene

(Kopf et al., 1993) expel worms normally. However, mice deficient in the IL-4 receptor α chain (IL-4Rα) (part of both the IL-4 and IL-13 receptor), or signal transducer and activator of transcription 6 (Stat6) fail to expel *N. brasiliensis* (Urban et al., 1998). The transcription factor Stat6 regulates the expression of several genes downstream of the IL-4/IL-13 receptor and thereby mediates effector cell recruitment and immunoglobulin isotype switching.

While IL-4 and IL-13 have distinct and important roles in the effector phase of the immune response to *N. brasiliensis*, and Th2 cells are thought to be the main source of these cytokines, it is increasingly clear that Th2 cells are not the only significant source of these cytokines. Although IL-4 and IL-13 production is required for worm expulsion, IL-4 and IL-13 from T cells is not required. When IL-4/IL-13-deficient or Stat6-deficient T cells are transferred into RAG-deficient mice, which cannot expel the worms, mice receiving deficient T cells expel the worms normally (Voehringer et al., 2004). Thus, there are other innate cell sources of IL-4 and IL-13, which play an important role in worm expulsion.

To track cells making IL-4 mRNA *in vivo,* Mohrs *et al.* designed an IL-4 reporter mouse (4get), which contains an IRES-eGFP knocked-into the IL-4 locus, allowing for normal expression of IL-4 from that locus as well as the eGFP marker, thus allowing for the detection of cells making IL-4 mRNA by flow cytometry (Mohrs et al., 2001). In addition to Th2 cells, eosinophils and basophils are the predominant IL-4 producing cells in the lung following *N. brasiliensis* infection (Voehringer et al., 2004). Eosinophils and basophils are constitutively GFP^+, even in the absence of Stat6, indicating that Stat6 does not regulate IL-4 transcription in eosinophils and basophils. Stat6 is important, though,

for eosinophil and Th2 cell recruitment to the lungs during a *N. brasiliensis* infection, because 4get/Stat6$^{-/-}$ and 4get/Rag$^{-/-}$ mice have greatly reduced numbers of eosinophils in the lung. In contrast, basophil recruitment to the lung is normal in 4get/Stat6$^{-/-}$, but impaired in 4get/Rag$^{-/-}$, suggesting that adaptive immunity is necessary for basophil recruitment to infected tissues (Voehringer et al., 2004). Importantly, using bone marrow chimeras they show that recruitment of eosinophils and Th2 cells to the lung following *N. brasiliensis* is dependent on Stat6 from hematopoietic cell(s) (Voehringer et al., 2004). Thus, IL-4/IL-13 is expressed in non-T cells, and while T cells provide important signals for effector cell recruitment, an innate cell population provides critical IL-4/IL-13 for worm expulsion. On the other hand, in the OVA-induced allergic lung disease model, which requires a sensitization phase, followed by multiple intranasal challenges of OVA, T cell-derived IL-4/IL-13 is required to reach optimal levels of eosinophil and basophil recruitment as determined by transferring knockout or wild-type T cells into Rag$^{-/-}$ mice. Consequently, when airway hyperresponsiveness is measured, Rag$^{-/-}$ mice receiving IL-4/IL-13 deficient T cells have reduced pulmonary resistance compared with the mice that receive wild-type T cells (Voehringer et al., 2006). These data imply that in a model that relies on repetitive challenge, and a recall response, T cell-derived IL-4/IL-13 plays a more significant role in amplifying cell recruitment and a type-2 immune response.

There appears to be a hierarchical arrangement for tissue recruitment of effector cells. Basophils can be recruited to tissues by activated T cells in a manner independent of Stat6, whereas eosinophil and Th2 cell recruitment requires Stat6-dependent signals from a hematopoietic cell type. Elucidation of these early steps in innate cell recruitment and activation will be important in understanding initiation of type-2 immunity.

If eosinophils and basophils are a significant source of IL-4 and possibly IL-13 early in immune response, then one or both represent innate cell types important in initiating type-2 immunity and mediating type-2 effector function. The role of eosinophils is much debated, although is has been noted that eosinophils have direct cytotoxic activity against parasitic worms (David et al., 1980; Ramalho-Pinto et al., 1978). To test whether eosinophils are required as an innate source of IL-4 for type-2 effector function, ΔdblGATA mice that contain a GATA-1 promoter mutation and have no eosinophils were infected with *N. brasiliensis.* There is no required role for eosinophils in Th2 cell or basophil recruitment into the lung, IgE production, or worm expulsion (Voehringer et al., 2006). Consequently, basophil-derived IL-4 is likely necessary for initiating effector responses. Basophils are a rare cell population, and isolating sufficient numbers of them for transfer experiments to determine if IL-4 from basophils is sufficient has not been possible. Additionally, there are no basophil deficient mice to test if they are necessary. Experiments depleting basophils using a novel antibody made by immunizing rats with a basophil-enriched population indicated that basophils are required for an IgE-mediated chronic allergic skin inflammation (Obata et al., 2007). However, conclusive experiments await the development of new genetic mouse models.

Alternatively Activated Macrophages

Alternatively activated macrophages are recognized as functional effector cells in both helminth and allergic models. Arginase-1 positive macrophages are induced in helminth infections, as well as during allergy and asthma (Gordon, 2003; Nair et al.,

2006). Significantly, inhibition of arginase-1 expression attenuated allergic lung inflammation in a mouse model (Yang et al., 2006). There is also data to suggest that memory Th2 cells could induce alternative macrophage activation, and that these macrophages mediated immunity against an intestinal helminth (Anthony et al., 2006). Additionally, researchers are exploring arginase-1 polymorphisms in human asthma (Li et al., 2006). Interestingly, alternatively activated macrophages are also associated with tumor growth and progression (Mantovani et al., 2002). In fact, SHIP (Src homology 2-containing inositol-5′-phosphatase)-deficient mice have more alternatively activated macrophages *in vivo* and corresponding enhanced tumor growth (Rauh et al., 2005). Taken together, these data implicate alternatively activated macrophages as important effector cells in allergic and tumor immunity, and previous work suggests that IL-4 and IL-13 are required to induce alternatively activated macrophages (Nair et al., 2003).

Clearly, coordination of various innate cell populations with adaptive immune cells is essential for development of a type-2 immune response. Understanding the signals required for organizing these different cell types will be instrumental in understanding how both immunity to pathogens as well as pathologies such as asthma and allergies arise. The following chapters will delineate a novel mechanism for initiating eosinophil and basophil recruitment, as well as alternative macrophage activation that relies on recognition of a highly conserved and abundant molecule chitin.

Chapter II:

AMCase and Ym2 are non-inflammatory Stat6-dependent genes

Abstract

Chitin is a major component of fungal cell walls and the exoskeleton of crustaceans and insects. Vertebrates cannot synthesize chitin, however, they express two chitinases and several chitinase-like proteins with possible roles in immune defense against fungal and helminth parasite infections. We found that the acidic mammalian chitinase (AMCase) and the chitinase-like proteins Ym1/2 were expressed in the lung of mice infected with the helminth parasite *Nippostrongylus brasiliensis*. The expression was dependent on the presence of the transcription factor Stat6 and the adaptive immune system. Transgenic mice overexpressing Ym2 and AMCase in the lung did not show any signs of spontaneous inflammation or tissue damage in the lung. Furthermore, administration of antibodies specific for Ym2 and AMCase did not prevent lung inflammation in a murine asthma model or during *N. brasiliensis* infection. Taken together, these experiments suggest that AMCase and Ym2 are not proinflammatory mediators of type-2 immunity.

Introduction

The initiation and coordination of type-2 immune responses is critically dependent on the transcription factor Stat6, which regulates the expression of several genes downstream of the IL-4/IL-13 receptor and thereby mediates effector cell recruitment and immunoglobulin isotype switching (Finkelman et al., 2004). When the type-2 immune response and inflammatory mediators are not regulated properly allergy and asthma can develop. Consequently, the tight control of inflammatory cell recruitment and activation is essential for appropriate immune regulation. Eosinophil recruitment to the lung tissue during helminth infection is dependent on Stat6 expression from a bone marrow derived cell type (Voehringer et al., 2004). Stat6-dependent genes that regulate eosinophil recruitment into tissues include the chemokines CCL11 and CCL24 (eotaxin-1 and –2) that act on CCR3, a chemokine receptor preferentially expressed on eosinophils in the mouse (Grimaldi et al., 1999).

Chitin, the second most abundant polysaccharide on earth, is a component of fungal cell walls and parasites, including the eggshells and sheathes of microfilarial worms. The enzymes that degrade chitin in these parasites are often expressed during different phases of the parasitic life cycle to degrade the chitin containing structures during development or to break down chitinous structures of insect hosts and pathogens. Mammals cannot synthesize chitin, however all the mammals studied express the enzymes that degrade chitin as well as closely related proteins that have lost enzymatic activity. These chitinases and chitinase-like proteins belong to the glycoside hydrolase family 18 chitinases (GH18) and are highly conserved in mammals, but their functions are largely unknown (Bussink et al., 2007; Funkhouser and Aronson, 2007).

Interestingly, it has been shown that alternatively activated macrophages express high levels of the chitinase-like proteins Ym1 and Ym2 in a Stat6- and CD4 T cell-dependent manner (Nair et al., 2003; Raes et al., 2002; Ward et al., 2001; Webb et al., 2001; Welch et al., 2002). Ym1/2 proteins are associated with hyalinosis, an eosinophilic cytoplasmic degenerative change in epithelial cells. They are expressed at high levels and lead to crystal formation in the lungs of aging 129/B6, $p47^{phox}$ -, SHIP-deficient and CD40L-deficient mice, as well as soluble TNFR-II transgenic and motheaten viable (me^{V}/me^{V}) mice (Guo et al., 2000; Harbord et al., 2002; Rauh et al., 2005; Ward et al., 2001).

In contrast to Ym1/2, chitotriosidase and acidic mammalian chitinase (AMCase) are two mammalian chitinases that retain enzymatic activity. Researchers speculate that they arose from a gene duplication event, and the other chitinase-like proteins such as Ym1/2 were subsequent gene duplications followed by loss of enzymatic function mutations (Bussink et al., 2007). Chitotriosidase and AMCase are expressed in the blood or gastrointestinal tract and lung, respectively (Boot et al., 2001; Suzuki et al., 2002; Zhu et al., 2004). Allelic variants of chitotriosidase are linked to genetic resistance against filiariasis in southern India, suggesting a potential protective role for this enzyme against helminth infections (Choi et al., 2001). Moreover, genetic studies found that the GH18 family of chitinases in human are closely associated with the cluster of MHC paralogs on chromosome 1, and the authors speculated that expansion of the chitinase family may have coincided with the evolution of adaptive immunity (Funkhouser and Aronson, 2007). AMCase is implicated in the inflammatory response of the lung during experimentally induced allergic asthma in mice, and intraperitoneal administration of a

steric inhibitor or polyclonal antibodies against AMCase blocks eosinophil recruitment to the lung and reduces airway hyperreactivity (Zhu et al., 2004). Additionally, AMCase and Ym1 appear to be a generalized feature of nematode infections, with induction observed at sites of infection and in the lung following infection with *Litomosoides sigmodontis* and *Nippostrongylus brasiliensis* (Nair et al., 2005).

Consequently, when we identified AMCase and Ym1/2 using global gene expression profiling as being Stat6- and Rag-dependent genes expressed in the lung after infection with the helminth parasite *Nippostrongylus brasiliensis,* we sought to uncover their potential functions in a type-2 immune response. We were able to manipulate AMCase and Ym2 levels using specific antibodies to AMCase and Ym1/2, as well as transgenic mice over-expressing AMCase or Ym2.

Results

Identification of chitinase expression in the lungs of Nippostrongylus brasiliensis *infected mice.*

Stat6-deficient mice are resistant to lung eosinophilia induced by helminth parasite infections or various murine asthma models (Mathew et al., 2001; Voehringer et al., 2004; Yang et al., 2003). To identify new Stat6-dependent genes that regulate lung eosinophilia, we compared the gene expression profile in the lungs of wild-type and Stat6-deficient mice infected with the helminth parasite *N. brasiliensis.* About 30 genes were expressed at significantly higher levels in wild-type compared to Stat6-deficient mice (Fig. 1). Among these genes were AMCase and Ym1/2, both belonging to a small family of mammalian chitinase-like proteins. Literature search revealed the existence of

Figure 1

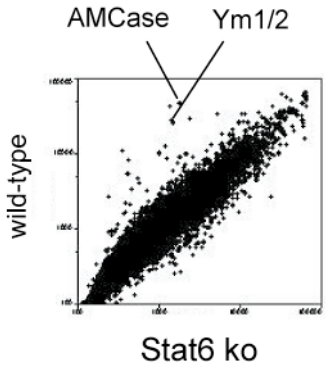


Figure 1 | Microarray analysis of total lung tissue from wild-type and Stat6-deficient mice 9 days after *N. brasiliensis* infection. cDNA samples were labeled with Cy3 and Cy5 reactive dyes and competitively hybridized to a spotted oligonucleotide array as described in Materials and Methods. The scatter plot shows intensity of hybridization signals on a log scale. Each dot represents one gene on the array.

9 chitinase-like proteins in the mouse genome, clustered on chromosomes 1 and 3. Alignment of the protein sequence indicates high homology among all family members (Bussink et al., 2007; Funkhouser and Aronson, 2007). However, only AMCase and chitotriosidase exhibit enzymatic activity due to the presence of two critical amino acid residues (Asp and Glu) in the active center of these enzymes (Boot et al., 2001; Boot et al., 1995), which are mutated in other family members. Furthermore, AMCase and chitotriosidase are the only members of this family that contain a C-terminal chitin-binding domain.

To further analyze the function of these chitinase-like proteins, we generated recombinant proteins in insect cells. As shown in Fig. 2A, AMCase, but not Ym2 recombinant protein was immunoprecipitated using chitin beads, indicating that the C-terminal chitin-binding domain of AMCase that is missing in Ym2 is required for high affinity binding of chitin. As expected, only recombinant AMCase but not Ym2 contained enzymatic activity (Fig. 2B). Our preparation of AMCase showed maximal enzymatic activity at neutral pH and 37°C. Preincubation of AMCase with rabbit antiserum to AMCase inhibited enzymatic activity of AMCase (Fig. 2C).

To confirm the results from the microarray experiment we performed semiquantitative RT-PCR analysis on total lung samples from wild-type, Stat6-deficient or Rag-deficient mice on day 9 after *N. brasiliensis* infection. Ym2 expression was strongly induced after infection of wild-type mice. In contrast, Stat6- or Rag-deficient mice had drastically reduced expression of Ym2 compared to wild-type mice following infection with *N. brasiliensis*. AMCase and Ym1 were expressed at low levels in non-infected lungs but were upregulated after infection in a partially Stat6- and Rag-

Figure 2

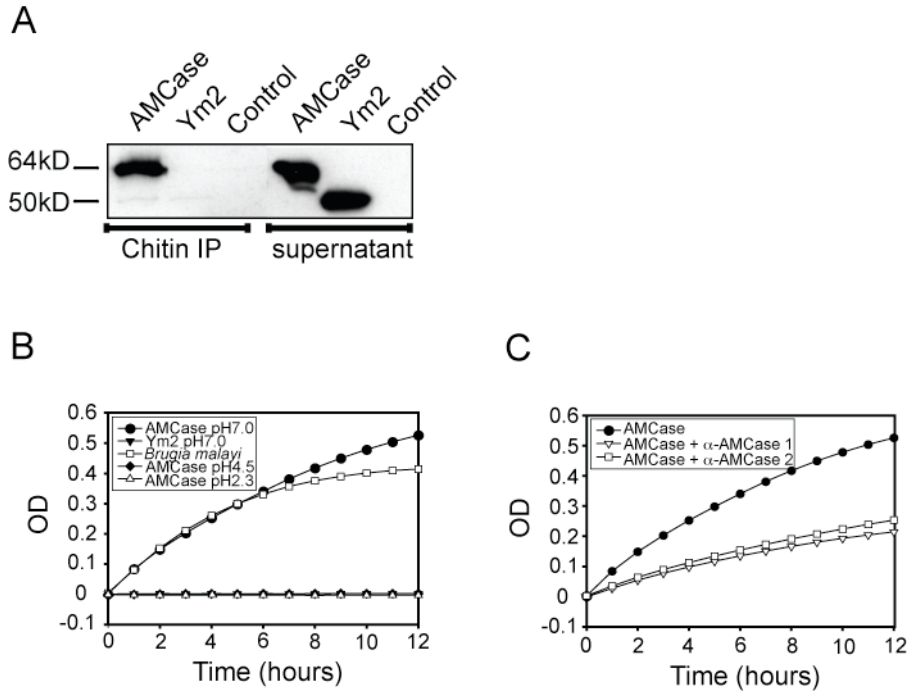


Figure 2 | Activity of recombinant AMCase and Ym2. (A) Both AMCase and Ym2 were expressed as V5-His-tagged recombinant proteins in S2 insect cells. The proteins were precipitated with chitin or agarose beads and detected by Western-Blot with anti-V5 antibody. (B) Chitinase activity at 37°C of recombinant AMCase and Ym2 was assessed using a chromogenic chitin substrate over the course of 12 hours at varying pH. A chitinase from *Brugia malayi* was used as a positive control. Ym2, AMCase at pH 4.5 and pH 2.3 do not exhibit enzymatic activity. (C) Polyclonal antibodies were generated for AMCase and Ym2. 1µg of recombinant AMCase was pre-incubated with the 3µg of one of the two different AMCase antibodies. The AMCase plus antibody was then subject to the same chitinase assay as in (B).

dependent manner (Fig. 3A). Expression levels of Brp39, another chitinase-like protein, remained unchanged after *N. brasiliensis* infection, and were not regulated by Stat6 (Fig. 3A). Chitotriosidase was undetectable in the lung (data not shown). Kinetic analysis revealed transiently increased expression levels for both AMCase and Ym2 after *N. brasiliensis* infection, which returned to baseline levels by 6 weeks after infection (Fig. 3B). Western blot analysis confirmed the RT-PCR data, showing that protein levels of Ym1/2 and AMCase increased with *N. brasiliensis* infection in the lung and that their expression was Stat6- and Rag-dependent (Fig. 3C).

Functional Assessment of AMCase and Ym2 in type-2 inflammation

To study whether AMCase or Ym2 played a pro-inflammatory role during a type-2 immune response *in vivo*, we took two approaches: (1) administration of anti-AMCase and anti-Ym2 antibodies during the OVA-induced asthma model and during the course of an infection with *N. brasiliensis* and (2) generation of transgenic mice, which over-express these proteins in the lung under control of the surfactant protein C promoter.

Antibody administration of a combination of anti-AMCase and anti-Ym2 during the challenge phase of OVA-induced asthma did not lead to significant differences in effector cell recruitment to the lung (Fig. 4A, B), airway hyperreactivity (Fig. 4C), or serum IgE levels (Fig. 4D). Similarly, antibody administration during *N. brasiliensis* infection had no effect on lung or bronchoalveolar lavage (BAL) eosinophilia (Fig. 5).

The transgenic lines SPY (over-expressing Ym2) and SPAM (over-expressing AMCase) were generated to test whether either chitinase-like protein could induce inflammation in the lung. Both transgenics overexpressed their respective protein under

Figure 3

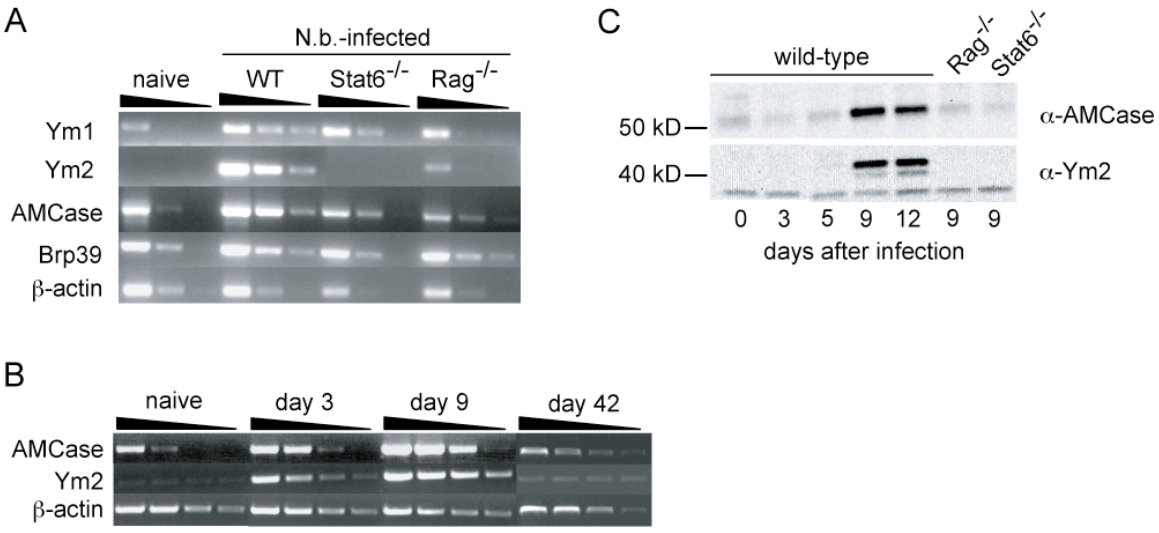


Figure 3 | Analysis of chitinase expression in the lung. (A) Semiquantitative RT-PCR analysis of Ym1, Ym2, AMCase and Brp39 expression in the lung of naïve and *N. brasiliensis* infected wild-type, Stat6-deficient and Rag-deficient mice. Represents serial 2-fold dilutions for each sample. (B) Kinetic RT-PCR analysis of AMCase and Ym2 expression in the lung. Represents serial 2-fold dilutions for each sample. (C) Western blot of total lung tissue samples at various days after *N. brasiliensis* infection of wild-type, Stat6- and Rag-deficient mice.

Figure 4

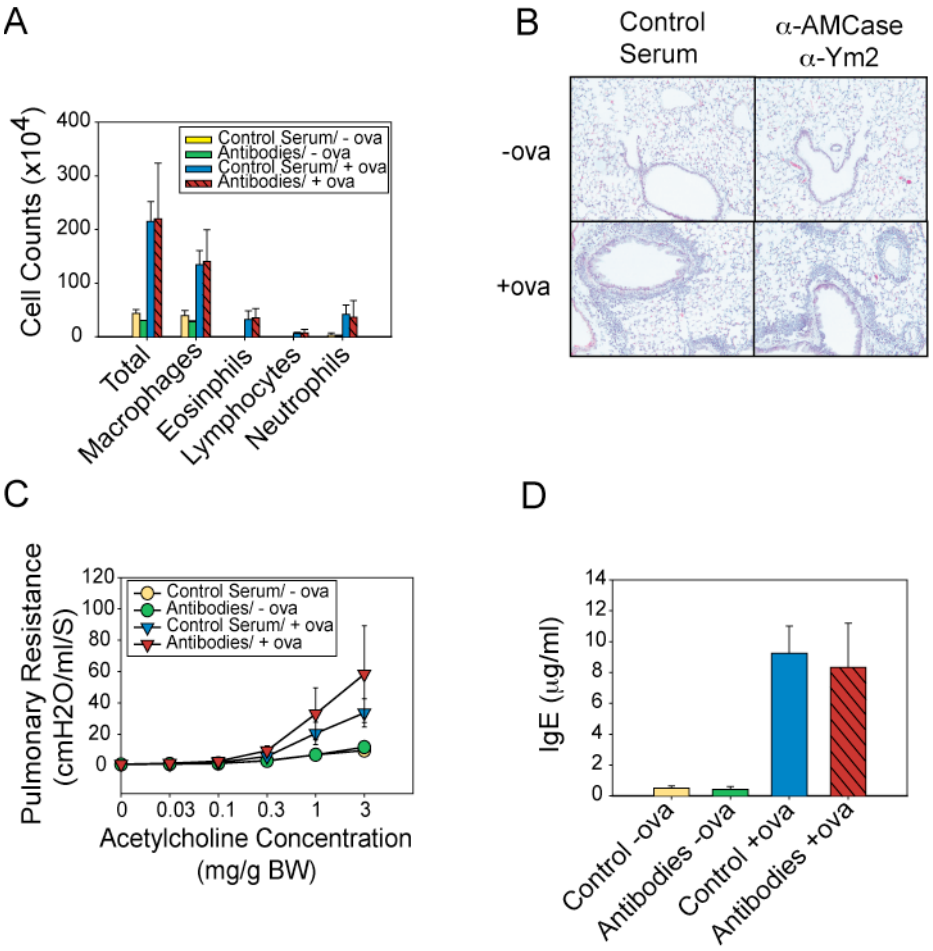


Figure 4 | Anti-AMCase and anti-Ym2 treatment does not ameliorate the asthmatic response in mice. Balb/c mice were primed on days 0, 7, and 14 with OVA/alum intraperitoneally. On days 21, 22, and 23 the mice were challenged intranasally with OVA and treated intraperitoneally with either control serum or AMCase + Ym2 antisera. On day 24 (A) cells were enumerated in bronchoalveolar lavage, (B) lung sections were stained with H&E, (C) airway hyperreactivity was measured following acetylcholine administration, and (D) serum IgE was determined by ELISA. Error bars are standard deviation.

Figure 5

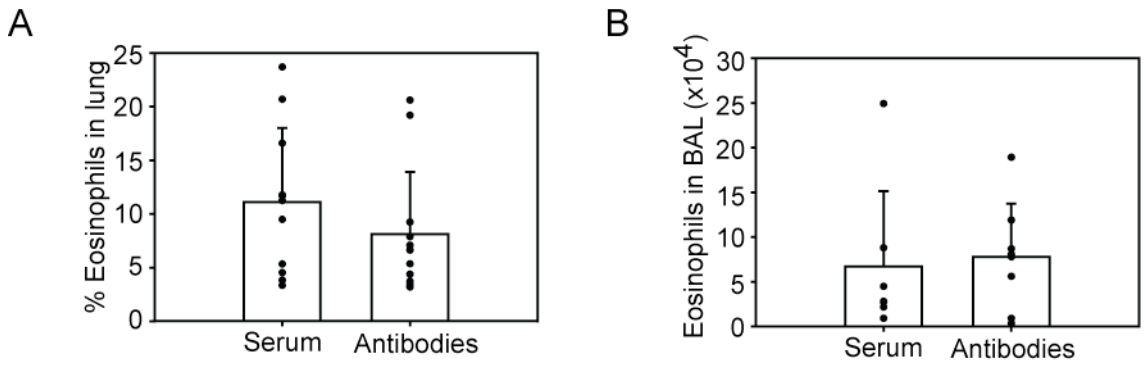
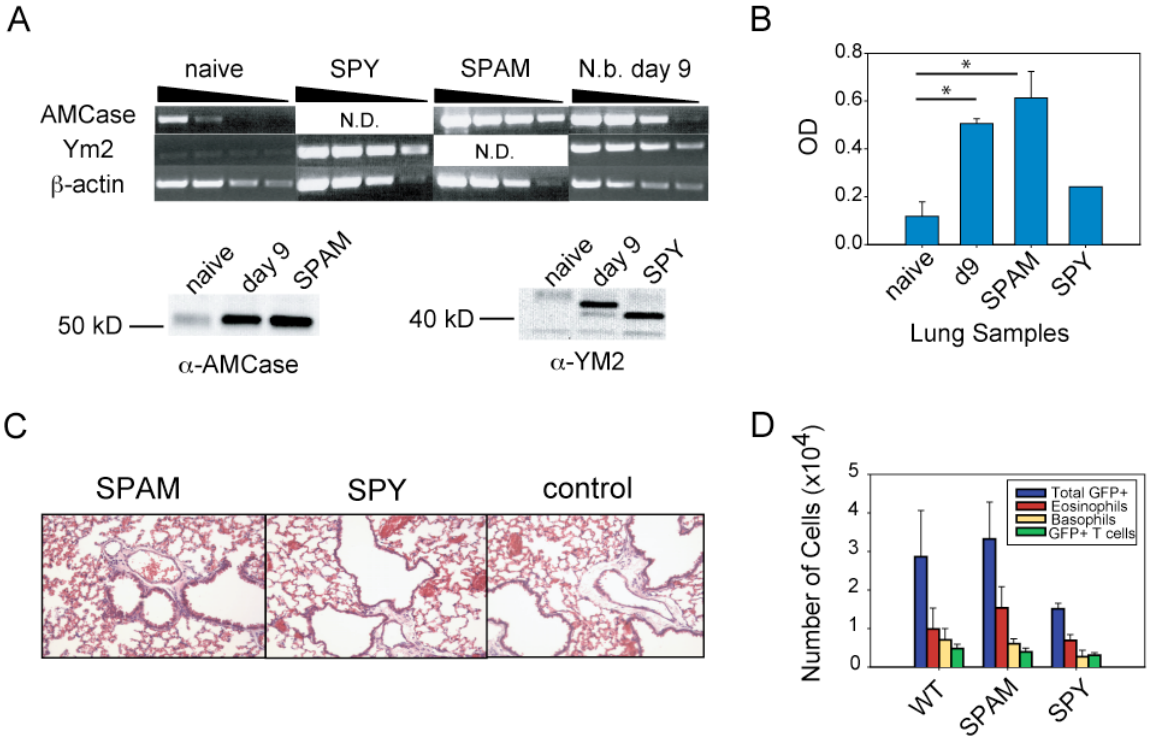


Figure 5 | Anti-AMCase and anti-Ym2 treatment does not decrease lung or bronchoalveolar lavage eosinophilia during *N. brasiliensis* infection. Mice were infected with *N. brasiliensis* and given 250 μL each of anti-Ym2 and anti-AMCase antibody or 500 μL control rabbit serum intraperitoneally on days 2, 4, 6, and 8. Mice were analyzed on day 9 after infection by flow-cytometry to determine the frequency of eosinophils in the (A) lung and (B) bronchoalveolar space (BAL). 3 pooled experiments. Dots represent individual mice, bars represent average, and error bars are standard deviation.

control of the surfactant protein C promoter. Two transgenic lines were generated for each protein, and one was selected from each based on high level of overexpression in the lung and minimal aberrant transgene expression in other tissues (data not shown). Ym2 and AMCase were highly overexpressed on both the RNA and protein level comparable to acutely *N. brasiliensis* infected mice (Fig. 6A). Lung tissue from SPAM but not SPY mice showed elevated chitinase activity similar to *N. brasiliensis* infected lung, indicating that AMCase expressed as a transgene in SPAM mice acts as a functional chitinase (Fig. 6B). Despite high overexpression, both transgenic mice showed no signs of spontaneous inflammation or increased cellular infiltrate in the lung (Fig. 6C, D). Furthermore, microarray analysis comparing whole lungs of transgenic and negative littermates indicated no significant changes in the gene expression profile in either SPY or SPAM, except high expression of Ym2 or AMCase transgenes in transgenic littermates (data not shown). Infection of SPY, SPAM or wild-type littermates with *N. brasiliensis* induced comparable effector cell recruitment to the lung and no difference in serum IgE levels (Fig. 7A). All mice expelled *N. brasiliensis* from the intestines with similar kinetics (data not shown). To analyze whether constitutive expression of Ym2 or AMCase in the lung was sufficient to induce eosinophil recruitment *in vivo*, we transferred eosinophils isolated from IL-5 transgenic mice into SPY, SPAM or negative littermates and analyzed the frequency of eosinophils in the lung 16 hours later. Using this approach, we could not detect significant differences in eosinophil recruitment (Fig. 7B). Taken together, these experiments suggest that neither Ym2 nor AMCase promote inflammation or act as chemoattractants *in vivo*.

Figure 6 | Analysis of naïve Ym2- and AMCase-transgenic mice. (A) Transgenic mice were engineered to overexpress AMCase or Ym2 under control of the surfactant protein C promoter, and are referred to as SPAM and SPY, respectively. Transgene expression was assessed by semi-quantitative RT-PCR and Western blot analysis of lung tissues from naïve and *N. brasiliensis*-infected wild-type mice, and naïve SPAM and SPY mice. The expression of both transgenes was comparable to day 9 *N. brasiliensis*-infected wild-type mice at both mRNA and protein levels. (B) Total lung tissue chitinase activity of naïve SPAM and SPY transgenic mice was compared to naïve and day 9 *N. brasiliensis* infected wild-type mice. Lung tissue supernatants were incubated with chromogenic chitin substrate at 37°C. 3 mice per group were analyzed. Error bars represent standard deviation. * $p<0.005$. (C) Histological analysis of lung tissue from SPY and SPAM transgenic mice and transgene-negative littermates. Lung tissue was fixed in paraformaldehyde, embedded in paraffin, sectioned and stained with H&E. Magnification is 10X. (D) SPAM and SPY mice were crossed to the 4get mice. Numbers of IL-4-expressing cells were enumerated in the lungs of naïve SPAM/4get, SPY/4get, and WT 4get mice by flow cytometry. Eosinophils were GFP^+, $CD4^-$, Siglec F^+, SSC^{hi}. Basophils were GFP^+, $CD4^-$, IgE^+, $ckit^-$. Th2 cells were GFP^+, $CD4^+$. 3 mice per group, and the experiment was repeated 2 times with similar results. Error bars represent standard deviation.

Figure 6


A
naive
SPY
SPAM
N.b. day 9
AMCase
Ym2
β-actin
N.D.
N.D.
naive
day 9
SPAM
50 kD
α-AMCase
naive
day 9
SPY
40 kD
α-YM2
B
OD
0.8
0.6
0.4
0.2
0.0
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naive
d9
SPAM
SPY
Lung Samples
C
SPAM
SPY
control
D
Number of Cells (x10^4)
5
4
3
2
1
0
Total GFP+
Eosinophils
Basophils
GFP+ T cells
WT
SPAM
SPY

Figure 7

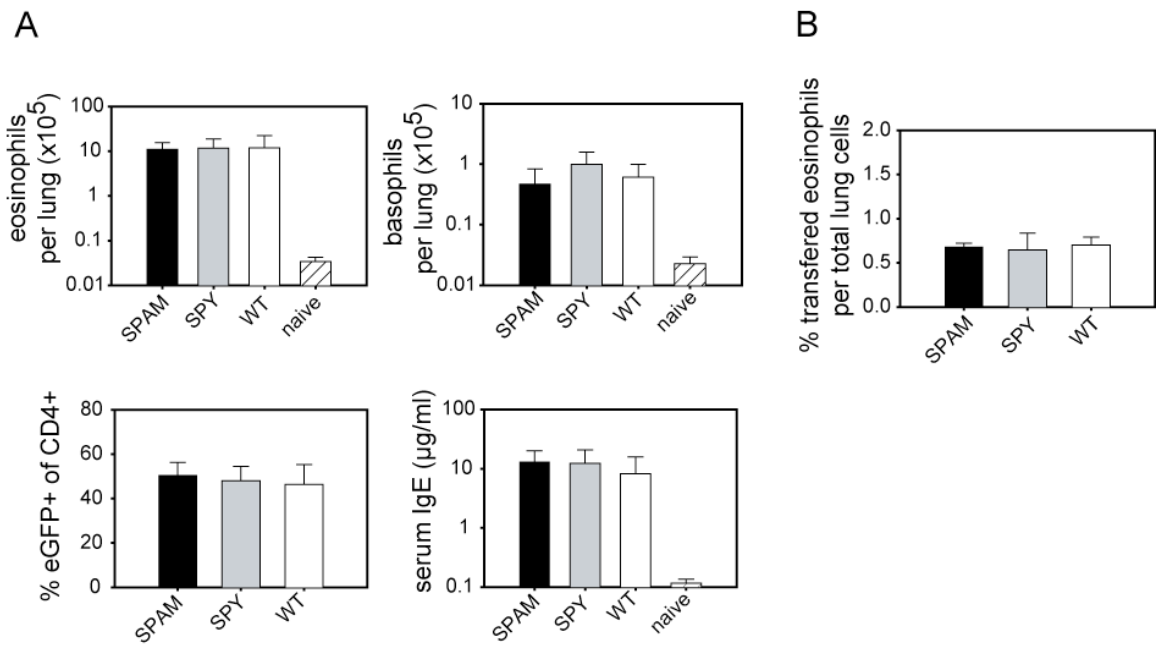


Figure 7 | Analysis of the immune response to *N. brasiliensis* in SPY and SPAM mice. (A) SPY and SPAM transgenic mice or negative littermates were infected with *N. brasiliensis* and analyzed 9 days later for effector cell recruitment to the lung tissue and total serum IgE levels. (B) To analyze potential eosinophil chemotactic activity of Ym2 and AMCase *in vivo*, $5x10^7$ eosinophils isolated from the peritoneum of IL-5 transgenic mice were labeled with CFSE and transferred intravenously into SPY and SPAM transgenic mice or negative littermates. Lungs of transferred mice were analyzed 16 h later by flow cytometry. 3 mice per group, representative of 2 independent experiments. Error bars are standard deviation.

Discussion

We identified both AMCase and Ym2 as being Stat6-dependent genes expressed in the lung after infection with *N. brasiliensis.* Using multiple different approaches, including antibody blockade during an asthma protocol or *N. brasiliensis* infection, as well as transgene overexpression we were unable to identify a role for Ym2 or AMCase in promoting inflammation or inducing effector cell recruitment.

Others have suggested that AMCase acts as a proinflammatory mediator of allergic lung inflammation. AMCase levels are increased in the lung of asthmatic mice and humans (Zhu et al., 2004) and blocking chitinase activity with anti-AMCase antibodies or allosamidin, a steric AMCase inhibitor, abrogates the asthmatic phenotype in mice (Zhu et al., 2004). This study implies that AMCase activity is involved in mediating effector cell recruitment and airway hyperreactivity. However, in addition to the lack of spontaneous inflammation observed in mice that constitutively overexpressed AMCase or Ym2 in the lung (Fig. 6), in our hands, blocking AMCase and Ym2 activity *in vivo* did not result in amelioration of the asthma response in mice (Fig. 4).

AMCase and Ym2 belong to a family of enzymes that degrade chitin, a common structural component of fungi and worms. Researchers have speculated that another enzymatically active chitinase-like protein, chitotriosidase found in mouse and human has antimicrobicidal activity. A host genetic polymorphism in chitotriosidase is associated with filarial infection (Choi et al., 2001), and alterations in circulating chitotriosidase activity may impact infection with filarial worms. However, we found that *N. brasiliensis* worm migration to the intestines and clearance in SPAM and SPY transgenic mice occurred with the same kinetics as in wild-type mice, suggesting that

high levels of AMCase and Ym2 in the lung do not have a direct toxic effect on the worms themselves. Moreover, we observed that expression of AMCase and Ym2 protein following *N. brasiliensis* infection in wild-type mice did not occur in the lung early during infection when the worms are present in the lung, but is rather expressed late in the infection (Fig. 3C). By this time the worms have migrated out of the lung, and the time point corresponds with the beginning of the contraction phase of the immune response in the lung. Normal worm clearance and effector cell recruitment in the transgenic mice will be discussed further in Chapter IV.

In addition to chitin, other carbohydrate polymers like peptidoglycan, β-glucans, and mannans constitute important structural components of microbial cell walls. Higher organisms synthesize none of these polymers including chitin. Thus, they could represent important pathogen associated molecular patterns, the recognition of which induces a rapid innate immune response. In fact, it has been shown that the fruit fly *Drosophila melanogaster* critically depends on the expression of certain peptidoglycan recognition proteins (PGRPs) to provide resistance against bacterial infections (Choe et al., 2002; Michel et al., 2001). However, these proteins seem to play a less critical role in the mammalian immune system, since mice deficient for PGRP-L or PGRP-S show only minor defects in immune responses against various bacterial and fungal pathogens (Dziarski et al., 2003; Xu et al., 2004)

β-1,3 and β-1,6 glucans, both major components of fungal cell walls are recognized in mammals by various receptors, including dectin-1, complement receptor 3 and scavenger receptors. It has recently been shown that dectin-1 in combination with

TLR2 plays an important role in β-glucan recognition of fungi by the innate immune system (Brown et al., 2003; Gantner et al., 2003; Saijo et al., 2007; Taylor et al., 2007).

Members of the chitinase-like family of proteins are lectins with affinity for *N*-acetylglucosamine and *N*-glucosamine. While most of the previous reports on AMCase and Ym1/2 have focused on the association of these proteins with inflammation, and have implicated them in the pathogenesis of allergic lung disease, we are unable to find corroborating evidence for this hypothesis. It is possible that the functions of chitinase-like proteins, such as AMCase and Ym2 lie in their ability to bind to polymers of *N*-acetylglucosamine (chitin), as well as other sugars. Investigations into their functions in the presence of chitin or even endogenous ligands will be important for elucidating their functions.

Materials and Methods

Mice. BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Interleukin 4 reporter mice (4get mice) have been described (Mohrs et al., 2001). These mice express an eGFP reporter gene under control of an IRES element that has been inserted after the stop codon of the IL-4 gene by homologous recombination. Stat6$^{-/-}$ mice (Kaplan et al., 1996), Rag$^{-/-}$ mice (Mombaerts et al., 1992) and IL-5 transgenic mice (Lee et al., 1997) were backcrossed at least 10 generations to BALB/c. Mice were maintained in the specific pathogen-free animal facility at UCSF according to institutional guidelines.

Microarray analysis. Total RNA was isolated from the lungs of wild-type and Stat6-deficient mice 9 days after *N. brasiliensis* infection using the Total RNA Isolation Kit (Fluka, Buchs, Switzerland). cDNA was generated using the Superscript™ reverse transcriptase kit (Invitrogen, Carlsbad, CA) and coupled to Cy3 and Cy5 fluorescent dyes (CyScribe^{TM} dye labeling kit, Amersham Biosciences, Peapack, NJ). Probes were hybridized to spotted glass oligonucleotide (70-mer) arrays, which cover just over 16,400 unique genes (Mouse Genome Set Version 2.0, Qiagen, Germany). The arrays were prehybridized in 1% BSA (Invitrogen, Carlsbad, CA), 5 x SSC, 0.1% SDS for 2 hr at 42°C, washed in water and hybridized with the Cy3/Cy5-labeled probes in 2x hybridization buffer (Genesystems, Rockville, MD) at 53°C for 45 hr. Slides were washed sequentially in 0.03%SDS/1 x SSC, 0.2 x SSC and 0.05 x SSC, dried and scanned on an Axon 4000B scanner. Data were normalized and analyzed using Genepix 3.0 software (Axon Instruments, Inc., Molecular Devices, Union City, CA).

RT-PCR analysis. cDNAs were prepared from total lung RNA generated using the total RNA isolation kit (Fluka, Buchs, CH) and Superscript™ reverse transcriptase kit (Invitrogen). RT-PCR was performed with the following primer pairs: Ym1: 5'-tggaattggtgcccctacaa-3' and 5'-aacttgcactgtgtatattg-3'; Ym2: 5'-aaccgtcagacattcatta-3' and 5'-tggtccttccagtaggtaata-3'; Brp39: 5'-agagctgctctgcgtacaag-3' and 5'-agtttctctctgcctggctg-3'; AMCase: 5'-tcacaggtctggctcttctg and 3'-catatgtcatgacatggatg; β-actin: 5'-atggatgacgatatcgct-3'and 5'-atgaggtagtctgtcaggt-3'. PCR conditions were 35 cycles with 30 sec 94°C, 30 sec 58°C, 60 sec 72°C followed by a final elongation for 10 min at 72°C.

Generation of recombinant proteins. Full-length cDNA for Ym2 and AMCase were cloned in pMT/V5-His expression vector (Invitrogen, Carlsbad, CA). S2 insect cells were cotransfected with the expression constructs and pCo-Blast selection plasmid conferring Blasticidin resistance. After establishing stably transfected cell lines protein production was induced for 3 days with 0.7 mM $CuSO_4$ and culture supernatants or control supernatants from untransfected S2 cells were purified over Ni^{2+} columns.

Analysis of chitinase activity. Chitinase activity was determined by incubation of recombinant proteins or supernatants from dispersed lung tissue with 270 μM p-nitrophenyl β-D-N,N',N''-triacetylchitotriose (Sigma-Aldrich) in McIlvain Buffer pH 7.0 (100 mM citric acid, 200 mM sodium phosphate) at 37°C. *Brugia malayi* chitinase was used as a positive control (New England Biolabs, Beverly, MA). Substrate turnover by 1 μg AMCase in 100 μl volume was analyzed on an ELISA reader at 405 nm.

Immunoprecipitation and Western Blot. Recombinant V5-His-tagged proteins were precipitated overnight with 50μL Chitin (New England Biolabs) or Agarose beads and detected by Western-Blot with anti-V5 antibody. Western blot analysis was also performed on whole lung supernatants from *N. brasiliensis* infected mice and transgenic mice. 5μg of total protein from lung tissue was loaded per lane of a 4-20% Tris-glycine gel and the proteins were blotted with purified anti-AMCase or anti-YM2, followed by goat-anti-rabbit-HRP (Promega).

Generation of anti-Ym1/2 and anti-AMCase antibodies. Antibodies were generated using the Invitrogen custom antibody service (Invitrogen, Carlsbad, CA). The anti-AMCase antibody was generated against the peptide DKADGLYPVADDRNAFWQC. The anti-Ym1/2 antibody was generated against KDRPTEGSFKPGNIDPC. Briefly, the peptides were conjugated to KLH and the KLH-peptide was injected subcutaneously into three dorsal sites of New Zealand white rabbits with Freund's adjuvant at weeks 0, 2, 7 and 9. Serum was collected at weeks 0, 4, 8 and 10. Terminal bleed of the anti-AMCase antibody was peptide-affinity purified, and the terminal bleed of the anti-Ym1/2 antibody was purified over a Protein G column.

Generation of SPAM and SPY transgenic mice. The full-length cDNAs for Ym2 and AMCase were generated from total lung RNA of *N. brasiliensis*-infected BALB/c mice using the following primer pairs: 5'Ym2-SalI: 5'-gcgtcgaccatggccaagctcattc-3', 3'Ym2-BHI: 5'-cgggatccctaaagctcccctcg-3' and 5'AMCase-SalI: 5'-gcgtcgaccatggccaagctacttc-3' ,

3'AMCase-BHI: 5'-cgggatccggttcatggccagttg-3'. cDNAs were cloned into pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA), sequenced and subcloned into SalI and BglII sites of a lung-specific expression vector containing surfactant protein C promoter elements (Glasser et al., 1991). Constructs were linearized with NotI and injected in DBA/2B6 F1 oocytes. 4-5 founder mice per construct were identified and one transgenic line per construct was established based on highest mRNA expression level and specific expression of the transgene in the lung. Mice were backcrossed with 4get/BALB/c mice up to 8 generations.

***Nippostrongylus brasiliensis* infection.** Third-stage larvae (L3) of *N. brasiliensis* were recovered from the cultured feces of infected rats, washed extensively and injected (500 organisms) into mice subcutaneously at the base of the tail. Infected mice were placed on antibiotic-containing water (2 mg/l neomycin sulfate, 100 mg/l chloramphenicol) for 5 days and killed for analysis after 9 days. Some mice were treated with intraperitoneal administration of 500µl rabbit-anti-Ym2 and rabbit-anti-AMCase antibodies at day 2, 4, 6, and 8 after infection.

Asthma model. BALB/c mice were sensitized on day 0, 7 and 14 by intraperitoneal administration of 50 µg ovalbumin emulsified in 10 mg alum and challenged daily by intranasal aspiration during anesthesia of 1 mg ovalbumin from day 21 to day 23. To inhibit Ym2 and AMCase *in vivo*, mice were given daily intraperitoneal injections of 250 µl rabbit-anti-Ym1/2 and 250 µl rabbit-anti-AMCase antisera from day 20 to day 23. On day 24 mice were analyzed for airway hyperreactivity to varying concentrations of

acetylcholine, inflammatory infiltrates in the lung by hematoxylin and eosin staining of lung sections, cell content in the bronchoalveolar lavage fluid and serum IgE.

Serum IgE ELISA. Serum IgE levels were determined by ELISA using the monoclonal antibody B1E3 for coating and the biotinylated monoclonal antibody EM95 for detection.

Flow cytometry. Cell suspensions were washed with FACS buffer (PBS, 2% FCS, 1 mg/L NaN_3) and the resuspended cell pellets were incubated for 5 min with anti-CD16/CD32 mAb (2.4G2, BD Pharmingen, San Jose, CA) before staining with APC-Alexa Fluor 750-anti-CD4 (RM4-5, Caltag, Burlingame, CA), PE-anti-CCR3 (R&D Systems, Minneapolis, MN) or PE- anti-Siglec F (E50-2440, BD Pharmingen, San Jose, CA), APC-anti-ckit (2B8, BD Pharmingen, San Jose, CA), and biotinylated anti-IgE (R35-72, BD Pharmingen, San Jose, CA) followed by PerCP Cy5.5-streptavidin (BD Pharmingen San Jose, CA). Cells were suspended in 1 μg/ml DAPI in FACS buffer prior to analysis to exclude dead cells. Cell counts were determined using Counting Beads (Caltag, Burlingame, CA). Samples were acquired on a LSRII (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (Tree Star, Ashland, OR).

Eosinophil transfer. Eosinophils were isolated from the peritoneum of IL-5 transgenic mice, washed in PBS and labeled with CFSE. $5x10^7$ cells were transferred intravenously into recipient mice, which were analyzed 16 hours later for recruitment of transferred eosinophils ($CFSE^+CCR3^+SSC^{hi}$) into the lung.

Chapter III:

Chitin induces accumulation in tissue of innate immune cells associated with allergy

Summary

Allergic and parasitic helminth immunity is characterized by infiltration of tissues with IL-4- and IL-13-expressing cells, including Th2 cells, eosinophils and basophils (Ramalingam et al., 2005). Tissue macrophages assume a distinct phenotype, designated alternatively activated macrophages (Gordon, 2003). Relatively little is known regarding factors that trigger these host responses. Chitin, a widespread environmental biopolymer of N-acetyl-β-D-glucosamine, confers structural rigidity to fungi, crustaceans, helminthes and insects (Bowman and Free, 2006). Here, we show that chitin induces the tissue accumulation of IL-4-expressing innate immune cells, including eosinophils and basophils, when given to mice. Tissue infiltration was unaffected by the absence of Toll-like receptor-mediated LPS recognition and was abolished by treatment of chitin with the IL-4- and IL-13-inducible mammalian chitinase, AMCase (Zhu et al., 2004), or by injection into mice that over-expressed AMCase. Chitin mediated alternative macrophage activation *in vivo* and production of leukotriene B4, which was required for optimal immune cell recruitment. Chitin is a recognition element for tissue infiltration by innate cells implicated in allergic and helminth immunity and this process can be negatively regulated by a vertebrate chitinase.

Results and Discussion

Using infection with the migrating helminth, *Nippostrongylus brasiliensis*, to examine lung tissue responses, we confirmed prior findings that highly Stat6-dependent genes induced during infection included acidic mammalian chitinase (AMCase) and Ym1 and/or Ym2 (in human, chitinase-3-like protein 3 (CHI3L3) and CHI3L4, which could not be distinguished on the microarray) (Nair et al., 2006). These proteins belong to the nine-member family of mammalian chitinase-like proteins. As compared to other family members, which were not regulated by Stat6, we established that AMCase and Ym2 were expressed in the lung as mRNA by day 3 after infection and as protein at high levels by day 9 (Chapter 2 Fig. 1, 3). *N. brasiliensis* larvae migrate within hours from the subcutaneous inoculation site to the lung, where organisms molt and ascend the trachea by day 2 and are swallowed to reach the intestines. In mice, adult worms are expelled by day 10 by an immune response dependent upon IL-4 and IL-13 (Finkelman et al., 2004).

The finding that expression of chitinase-like proteins was Stat6-dependent in the lung raised the possibility that chitin, which constitutes a structural constituent remodeled during molting in worms (Zhang et al., 2005), might serve as a recognition element for eliciting tissue infiltration by IL-4- and IL-13-producing immune cells. To test this, we administered chitin to the lungs of 4get mice, which contain a knock-in IL-4 reporter that allows the detection of cells competent to produce IL-4 (Mohrs et al., 2001). Chitin challenge led to recruitment of eosinophils and basophils to the lungs that peaked by days 2-3 and returned to basal levels by day 9 (Fig. 1a, b). Intraperitoneal injection of chitin induced eosinophil accumulation as early as 6 hours, demonstrating that the response was not tissue-specific (Fig. 1c, d). Neutrophils were also recruited to the peritoneum early

Figure 1

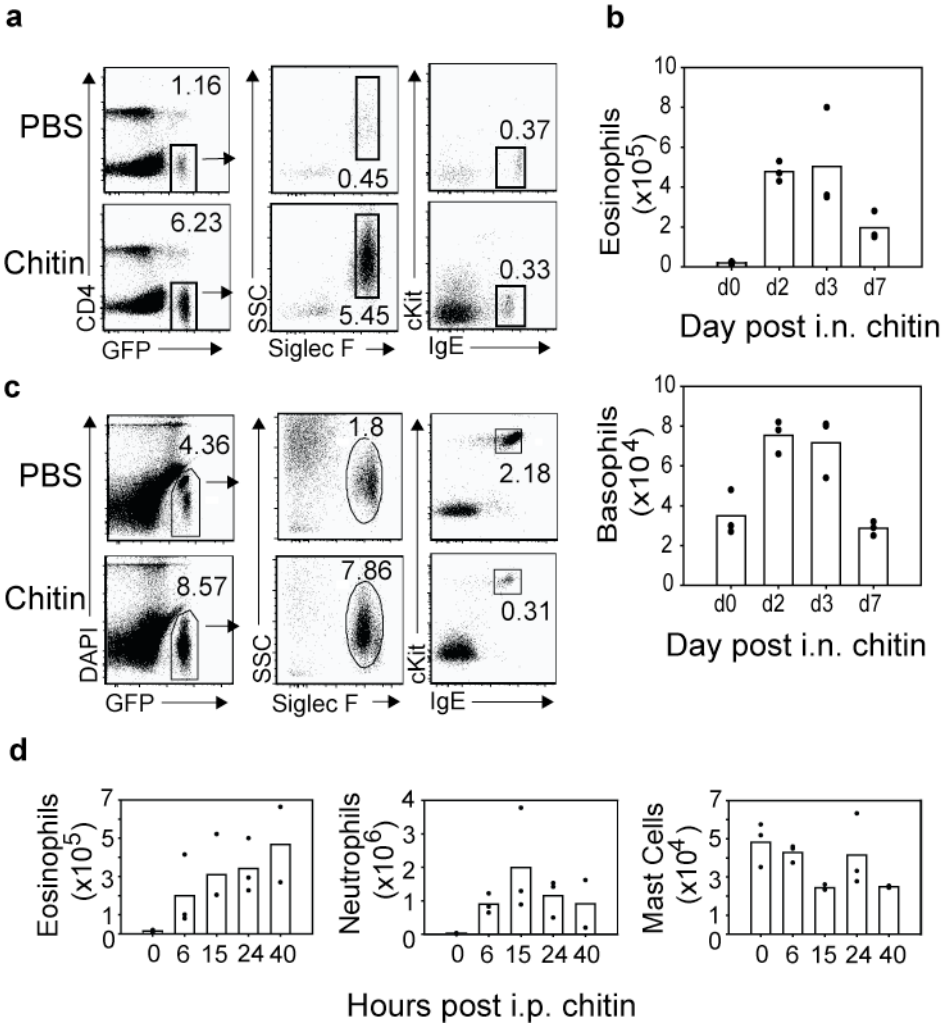


Figure 1 | Chitin induces accumulation of innate effector cells. a, FACS analysis 1 day after 2 intranasal chitin doses to 4get mice. Eosinophil (GFP^+ (Voehringer et al., 2004) Siglec F^+ (Zhang et al., 2004)) and basophil (GFP^+ (Voehringer et al., 2004), IgE^+ $cKit^-$ (Voehringer et al., 2004)) gates shown. **b,** Kinetics of chitin-induced lung eosinophils. **c,** FACS of peritoneal cells 2 days after i.p. chitin. Eosinophils and mast cells ($GFP^+IgE^+cKit^+$) gated. **d,** Kinetics of chitin-induced peritoneal cells. Data representative of 2-3 independent experiments.

in response to chitin, although there was no influx of neutrophils to the lung (Fig. 1d, 2). Unlike in the lung, mast cells ($eGFP^+$, $cKit^+$, IgE^+) represent a substantial proportion of constitutively eGFP-positive cells in the peritoneum and basophils were not recruited by chitin. Compared to LPS, which resulted in neutrophil recruitment but no eosinophil recruitment to either the lung or peritoneum, chitin induced eosinophil influx in both tissues. Neither challenge altered peritoneal mast cell numbers.

Although LPS can modulate allergic immunity in some models, chitin mediated eosinophil and basophil recruitment in TLR4-deficient (LPS unresponsive) and MyD88-deficient mice (Fig. 3, and data not shown). Next, we produced recombinant enzymatically active mouse AMCase, which binds and degrades chitin (Boot et al., 2001); mutant AMCase with alterations of the active site aspartate-154 and glutamate-158 necessary for the catalytic activity (Boot et al., 1995); and Ym2, a non-chitinolytic member of the chitinase-like family which is also Stat6-induced but lacks chitin-binding and chitinolytic functions (Jin et al., 1998). Pre-treatment of chitin with enzymatically active AMCase, but not with mutant enzymatically-inactive AMCase or with Ym2, led to loss of eosinophil and basophil recruitment to the lung, consistent with a role for intact chitin in these cellular events (Fig. 4a, b). Similar results were seen after injection of these preparations intraperitoneally to assess eosinophil recruitment (data not shown). We generated transgenic mice that over-expressed AMCase constitutively in the lung resulting in the production of enzymatically active AMCase that approximated the levels seen at day 9 after infection with *N. brasiliensis* (Fig. 4c). Analysis of 2 independent transgenic lines revealed that lung-specific expression of AMCase led to no apparent abnormalities in the mice and the lungs were histologically normal (Chapter 2 Fig. 6).

Figure 2

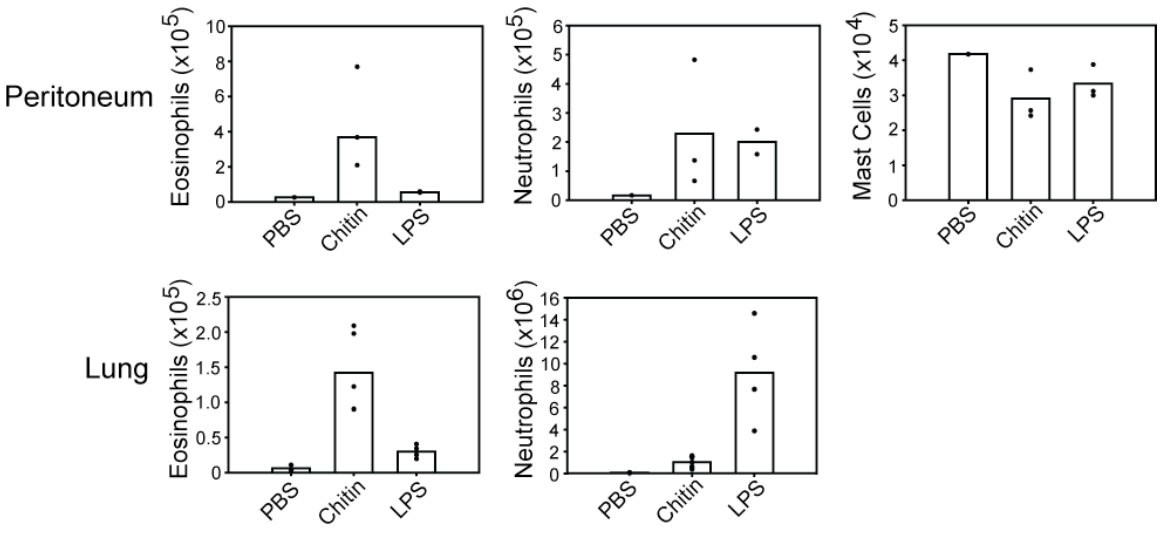


Figure 2 | Differential cell recruitment to the peritoneum and lung with chitin and LPS. Mice either received PBS, chitin or LPS (50 μg) intraperitoneally on day 0 or intranasally on day 0 and day 1. Peritoneal exudates or lungs were analyzed on day 2 for cell recruitment by flow cytometry.

Figure 3

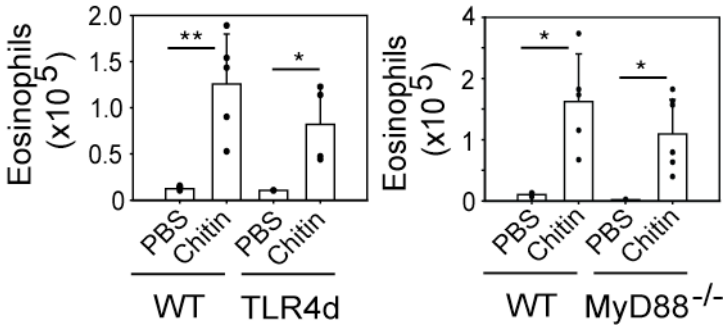
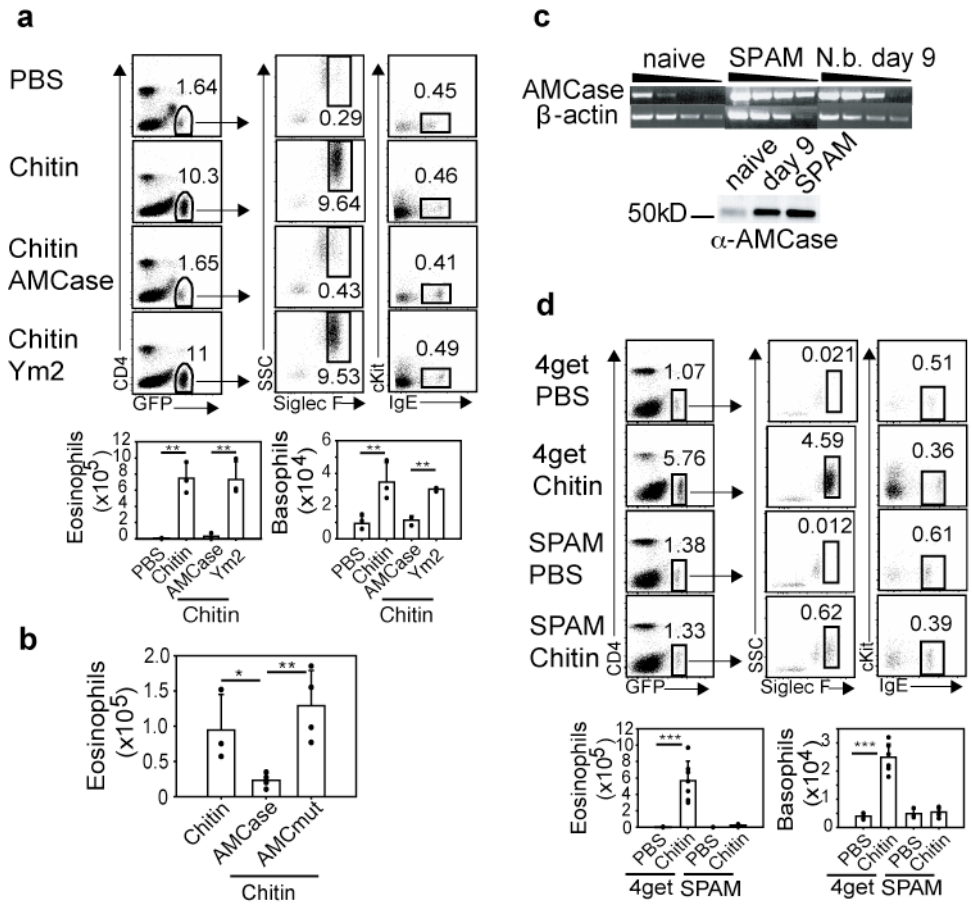


Figure 3 | Chitin induces eosinophil recruitment independent of TLR recognition.

Wild-type, TLR4d mice or MyD88$^{-/-}$ mice received chitin intranasally on day 0 and day 1. Lung eosinophils were quantitated by flow cytometry on day 2. * $p<0.05$, ** $p<0.01$. P-values were determined using Student's t-test. Error bars represent standard deviation. Shown is representative of 2 independent experiments, with an n=3-4 per group.

Figure 4 | AMCase prevents chitin-induced eosinophil and basophil recruitment. a, Chitin or chitin preincubated with recombinant AMCase or Ym2 was administered to 4get mice on day 0 and day 1 and recruitment of lung eosinophils and basophils was determined on day 2. **b,** Chitin was preincubated with PBS, recombinant AMCase, or recombinant mutant enzymatically-inactive AMCase (AMCmut) and given to 4get mice. **c,** AMCase expression in SPAM transgenic mice assessed by RT-PCR and Western blot analysis of lung tissues from naïve, day 9 *N. brasiliensis*-infected wild-type and naïve SPAM mice. **d,** SPAMx4get transgenic mice (SPAM) or wild-type 4get mice were given intranasal chitin and lung eosinophils were quantitated on day 2 by flow cytometry. * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ P-values were determined using Student's t-test. Error bars represent standard deviations. In FACS plots, numbers represent percentage of gated cells from total live cells. Shown is representative of three independent experiments, with n=3-4 per group.

Figure 4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Chitin
Chitin AMCase
Chitin Ym2
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0.29
0.45
10.3
9.64
0.46
1.65
0.43
0.41
11
9.53
0.49
CD4
GFP
SSC
Siglec F
cKit
IgE
Eosinophils (x10^5)
Basophils (x10^4)
PBS
Chitin
AMCase
Ym2
Chitin
b
Eosinophils (x10^5)
Chitin
AMCase
AMCmut
Chitin
c
naive
SPAM
N.b. day 9
AMCase
β-actin
naive
day 9
SPAM
50kD
α-AMCase
d
4get PBS
4get Chitin
SPAM PBS
SPAM Chitin
1.07
0.021
0.51
5.76
4.59
0.36
1.38
0.012
0.61
1.33
0.62
0.39
CD4
GFP
SSC
Siglec F
cKit
IgE
Eosinophils (x10^5)
Basophils (x10^4)
PBS
Chitin
4get
SPAM

These mice were crossed to 4get mice, thus allowing analysis of the recruitment of IL-4-competent cells to the lung. When challenged with chitin, mice over-expressing AMCase (SPAMx4get) had attenuated inflammatory responses (Fig. 4d). These experiments suggest that chitin itself robustly elicits infiltration by IL-4-competent innate cells following introduction into tissues and that the Stat6-inducible chitinase, AMCase, abrogates this activity.

Although AMCase expression was Stat6- and Rag-dependent, eosinophil recruitment mediated by chitin *in vivo* was independent of Stat6 and Rag (Fig. 5a, b), suggesting an early innate response. Basophil recruitment to the lung was also independent of Stat6 and Rag (data not shown). By RT-PCR analysis of whole lung 2 days after intranasal chitin, we found no evidence for upregulation of eotaxin-1 or eotaxin-2 (data not shown). Additionally, we could consistently document eosinophil chemoattractant activity in supernatants of bone marrow-derived macrophages and the RAW267.4 macrophage cell line after incubation with chitin that was unaffected by the addition of neutralizing antibody against eotaxin (data not shown). However, eosinophil chemoattraction by macrophages was inhibited if the chitin was pre-treated with enzymatically active AMCase, consistent with our *in vivo* results, and was also inhibited when the macrophages were pre-treated with an inhibitor of leukotriene production, MK886 (Fig. 6a). Leukotriene B4 (LTB_4) is a potent chemoattractant for eosinophils (Huang et al., 1998) and is induced from canine phagocytes by chitin (Usami et al., 1998). Additionally, the high-affinity receptor for LTB_4, BLT1, is an important mediator of early effector T cell recruitment to the lung in an allergic asthma model (Tager et al., 2003). To establish a role for LTB_4 in the chitin-mediated effects we observed, we

Figure 5

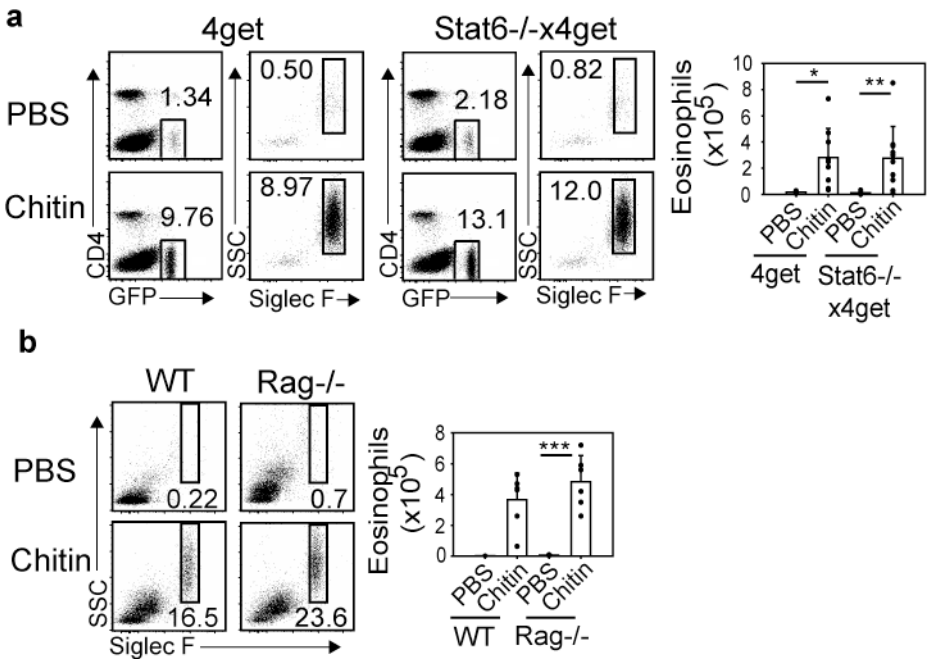


Figure 5 | Chitin-induced eosinophil recruitment is independent of Stat6 and Rag. a, Stat6$^{-/-}$x4get and 4get mice received chitin or PBS, and the lungs were analyzed for eosinophils. **b,** Rag$^{-/-}$ and wild-type mice received chitin or PBS, and lungs were analyzed for eosinophils. Eosinophils were identified as DAPI^{-}, FSC^{lo}, CD4^{-}, Siglec F^{+}, SSC^{hi}. P-values were determined using Student's t-test. Error bars represent standard deviations. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ Numbers in FACS plots are percentages of gated cells from total live cells. Experiments represent 2-3 independent experiments with n=3-4 per group.

Figure 6

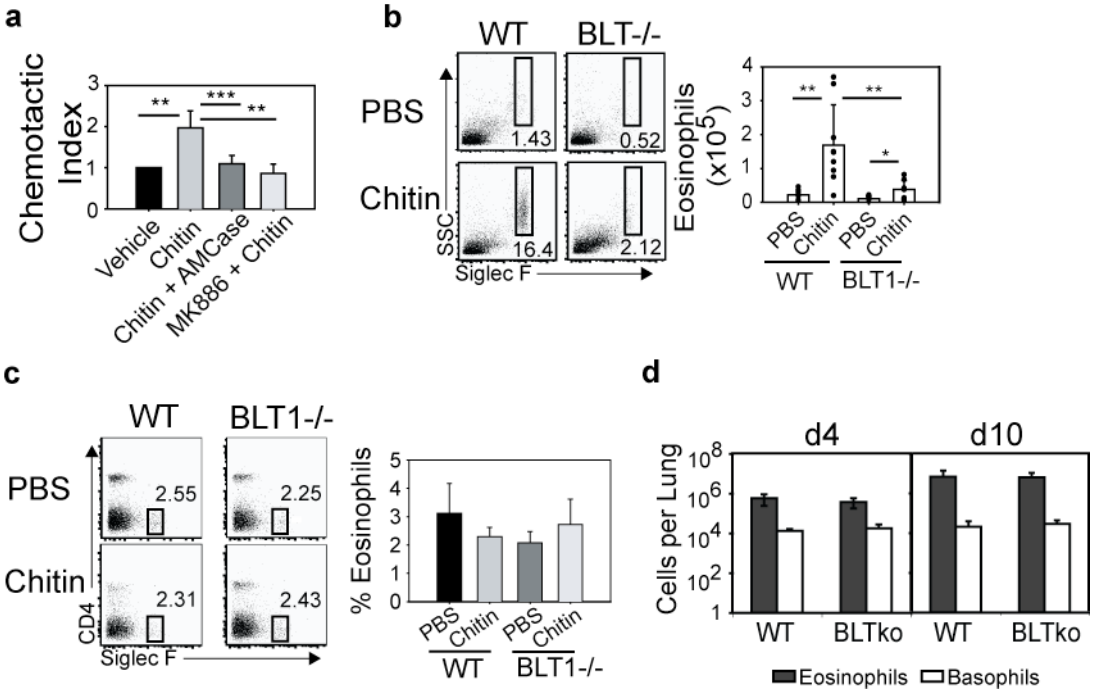


Figure 6 | Chitin-induced eosinophil recruitment is dependent on BLT1 expression on eosinophils. **a,** Transwell analysis of eosinophil recruitment by RAW267.4 macrophages incubated with vehicle, chitin, or AMCase-treated chitin. Where indicated, macrophages were pretreated with vehicle or MK886 (10 μM) for 10 min at 37°C prior to addition of chitin. **b,** BLT1$^{-/-}$ mice and WT C57BL/6 mice received intranasal chitin on day 0 and day 1. Lung eosinophils were quantitated on day 2. **c,** Blood eosinophils were analyzed from PBS and chitin-treated mice from part **b** by flow cytometry. Graph represents average of percent eosinophils of 5 mice per group. **d,** BLT1$^{-/-}$ and WT C57BL/6 mice were infected with *N. brasiliensis*. Lung eosinophils and basophils were quantitated on day 4 and day 10. Numbers in FACS plots represent percentages of eosinophils from live cells. P-values were determined using Student's t-test. Error bars represent standard deviations. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ Results representative of 2 independent experiments, with an n=5 per group.

challenged mice deficient in BLT1 with chitin. As compared to controls, chitin-induced eosinophil and basophil recruitment to the lung was significantly attenuated in the BLT1 knockout mice (Fig. 6b, and data not shown), despite the equivalent percentages of eosinophils present in the blood of the knockout mice as compared to wild-type mice (Fig. 6c). Contrary to the significant defect in eosinophil recruitment following intranasal chitin, we did not observe impaired recruitment in BLT1 knockout mice after *N. brasiliensis* infection (Fig. 6d). This potentially reflects the complexity of inflammatory signals generated by a whole worm that are not present in chitin alone.

To determine whether macrophages play a role *in vivo*, we used clodronate liposomes to deplete peritoneal macrophages prior to the administration of chitin. Treatment with clodronate markedly curtailed eosinophil recruitment to the peritoneum by chitin (Fig. 7a). Mast cells are also potent producers of leukotrienes. Following administration of chitin to mast cell-deficient Kit W-sash mice (Wolters et al., 2005), eosinophil recruitment to the lungs was unaffected, suggesting that mast cells are not required for a chitin-mediated response (Fig. 7b).

The capacity of chitin to mediate effects on macrophages suggested that these cells might represent sensors for chitin in tissues. A signature gene induced in alternatively activated macrophages is arginase I (Gordon, 2003; Nair et al., 2006). Arginase I enzymatically cleaves arginine to generate L-ornithine, a precursor for polyamines and proline, which have been implicated in cell proliferation and collagen production, respectively. To assess whether chitin induces alternative macrophage activation *in vivo*, we generated mice containing an IRES-driven fluorescent eYFP reporter introduced at the 3'-end of the *arginase I* gene (H.-E. Liang and R.M. Locksley,

Figure 7

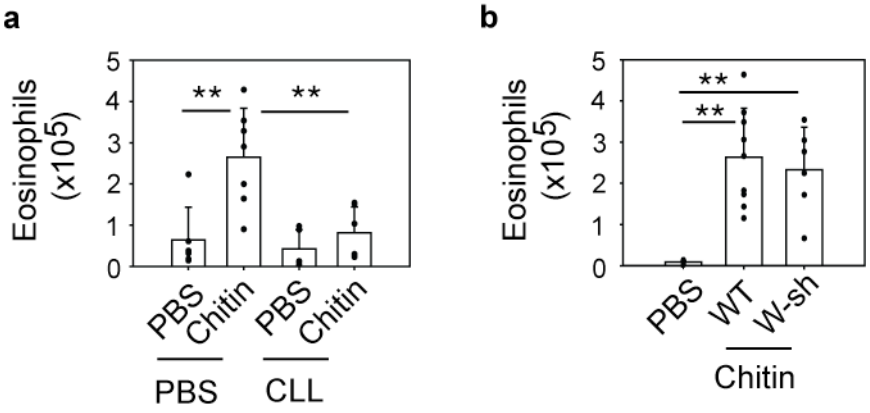


Figure 7 | Macrophages are required for chitin-induced eosinophil recruitment. a, 4get mice were treated intraperitoneally with clodronate liposomes (CLL) or PBS on days 0 and 3. On day 4 mice received chitin i.p. and were analyzed 6 hrs later for eosinophil recruitment to the peritoneum. **b,** Kit W-sh or wild-type controls received chitin intranasally on day 0 and day 1. Lung eosinophil recruitment was analyzed on day 2. P-values were determined using Student's t-test. Error bars represent standard deviations. * $p<0.05$, ** $p<0.01$. Experiments represent 2-3 independent experiments with n=3-4 per group.

manuscript in progress). Under resting conditions, no eYFP-positive macrophages were identified in the lungs or peritoneum of arginase I reporter mice, designated YARG (Fig. 8a, data not shown). On day 9 after *N. brasiliensis* infection, however, large numbers of eYFP-positive macrophages (CD11b+, CD11c-, Gr1-) were present in both tissues (Fig. 8a, b, data not shown). Thus, parasitic infection elicits accumulation of arginase I-positive macrophages, in agreement with prior studies (Gordon, 2003; Nair et al., 2006). Next, we injected chitin into YARG mice. As early as 6 hours, and sustained up to 7 days, arginase I-positive cells with surface markers consistent with macrophages appeared in the lungs surrounding a chitin particle and in the peritoneum (Fig. 9a, 10a, b, data not shown). Further, when sorted into transwell dishes, arginase I-positive macrophages induced by chitin attracted eosinophils in a manner inhibited by MK886, consistent with a role for these cells in mediating cell recruitment by LTB_4 *in vivo* (Fig. 9b). Additionally, we crossed YARG and SPAM mice, and challenged the lungs of these mice with chitin. In contrast to YARG mice, which developed alternatively activated macrophages by day 2, YARG x SPAM mice developed greatly attenuated numbers of these cells (Fig. 9c). Similar results occurred if chitin was digested with AMCase before challenging animals (Fig. 9d). Arginase I was induced initially in the peritoneum in macrophages with the characteristic phenotype of resident cells ($CD11b^{hi}$, $F4/80^{hi}$, $Ly6C^{-}$, $CD62L^{-}$). Macrophages with the surface phenotype of inflammatory macrophages ($CD11b^{+}$, $F4/80^{+}$, $Ly6C^{+}$, $CD62L^{+}$) did not acquire arginase I expression until later in the response, indicating that resident macrophages respond early to chitin (Fig. 10).

Whereas previous reports implicated AMCase as a proinflammatory mediator of allergic inflammation (Zhu et al., 2004), transgenic mice over-expressing AMCase

Figure 8

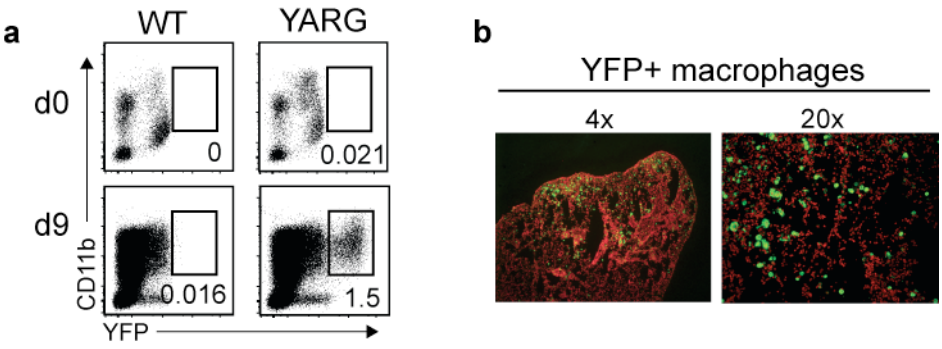
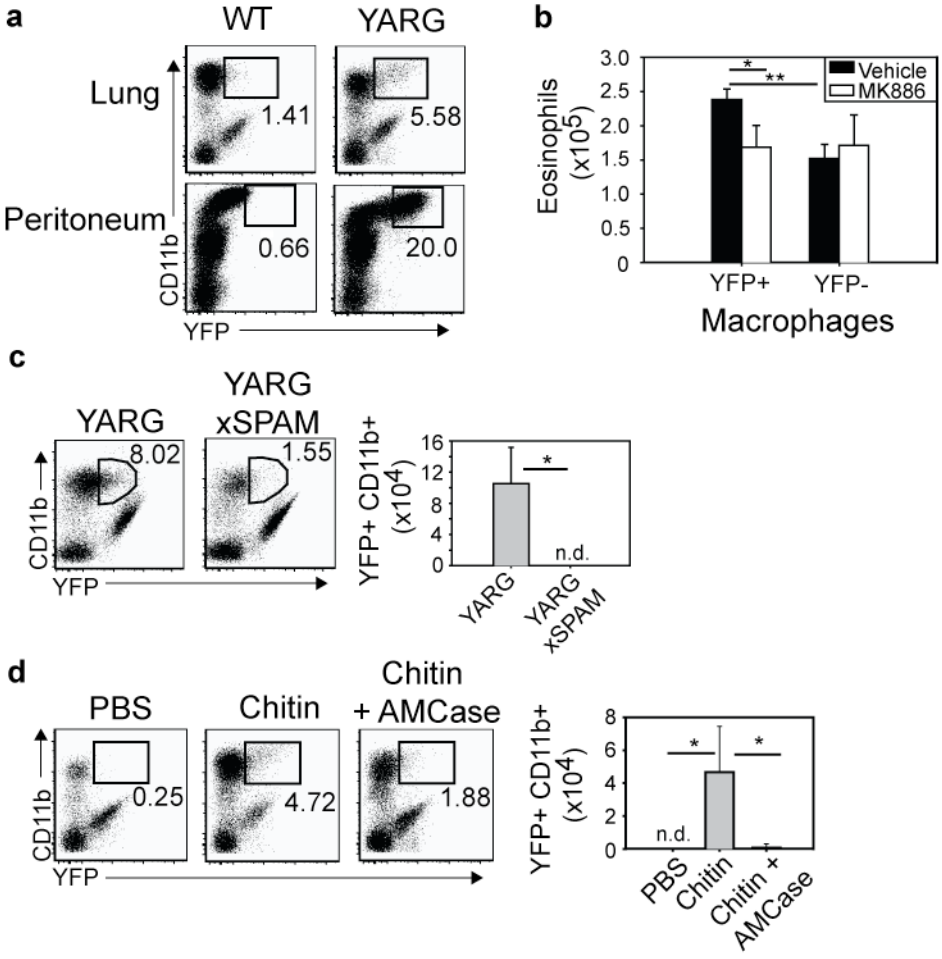


Figure 8 | YARG mice report alternative macrophage activation. a, YARG or wild-type littermates were infected with *N. brasiliensis*. CD11b^{+} lung macrophages were analyzed for YFP expression on days 0 and 9. **b,** Day 9 *N. brasiliensis*-infected lung from YARG mice was stained with anti-GFP (green) and nuclei were counterstained with DAPI (red). Lens magnification indicated. In FACS plots, numbers represent percentage of gated cell from total live cells.

Figure 9 | Chitin induces alternatively activated macrophages. a, Chitin administered to lung or peritoneum of YARG or wild-type mice. $CD11b^+$ macrophages were analyzed for YFP expression on day 2. **b,** Sorted YFP^+ and YFP^- macrophages were treated with vehicle or 10 μm MK886 for 10 min/37°C before analysis for eosinophil chemotaxis using a transwell assay. Results average of three independent experiments. **c,** YARGxSPAM transgenic mice were compared with YARG littermates following intranasal chitin. **d,** YARG mice received PBS, chitin, or chitin pretreated with AMCase on day 0 and day 1. On day 2 lung macrophages were analyzed for YFP induction by flow cytometry. * $p<0.05$, ** $p<0.01$ P-values determined using Student's t-test. Error bars represent standard deviations. In FACS plots, numbers represent percentage of gated cell from total live cells. For quantitation of total YFP^+ cells the number of background YFP^+ events detected in wild-type littermates was subtracted from the number of YFP^+ cells detected in YARG mice. n.d. = none detected. Each experiment represents two independent experiments with n=3 per group.

Figure 9


a
WT
YARG
Lung
Peritoneum
1.41
5.58
0.66
20.0
CD11b
YFP
b
Eosinophils (x10^5)
Vehicle
MK886
YFP+
YFP-
Macrophages
c
YARG
YARG xSPAM
8.02
1.55
CD11b
YFP
YFP+ CD11b+ (x10^4)
n.d.
YARG
YARG xSPAM
d
PBS
Chitin
Chitin + AMCase
0.25
4.72
1.88
CD11b
YFP
YFP+ CD11b+ (x10^4)
n.d.
PBS
Chitin
Chitin + AMCase

showed no signs of spontaneous inflammation. Rather, our data support a role for AMCase in the feedback attenuation of chitin-induced allergic innate immune responses by enzymatically degrading chitin, thus removing the stimulus for further eosinophil and basophil recruitment. We tried to test whether AMCase was important during the contraction phase of the immune response to *N. brasiliensis* by administering neutralizing antibodies to AMCase on days 7, 9, 11, 13, and 15 after infection. This treatment was associated with statistically increased numbers of basophils, and a trend towards increased eosinophils and Th2 cells in lung tissue (Fig. 11). These data further support our hypothesis that AMCase is part of a negative feedback loop that digests chitin, thus relieving the stimulus for persistent inflammatory recruitment.

Although inhibition of LTB_4 rendered mice unable to control the helminth *Strongyloides venezuelensis* (Machado et al., 2005), *Nippostrongylus* was expelled from BLT1-deficient mice (Fig. 6d), suggesting that additional pathways, such as activation of cytokine secretion by helminth proteases (Phillips et al., 2003), may contribute to the immune response against these complex pathogens. Prior studies have suggested that chitin may skew immunity away from Th2-mediated allergic responses, although differences in animal models, chitin preparations and delivery, and the potential for contamination by LPS and other microbial activating ligands makes direct comparisons with our studies difficult (Shibata et al., 2000).

Chitin is the second most abundant biopolymer in nature, with estimates of billions of metric tons produced annually in oceans alone. Despite the prodigious production by phytoplankton and crustaceans, marine sediments contain only trace

Figure 10

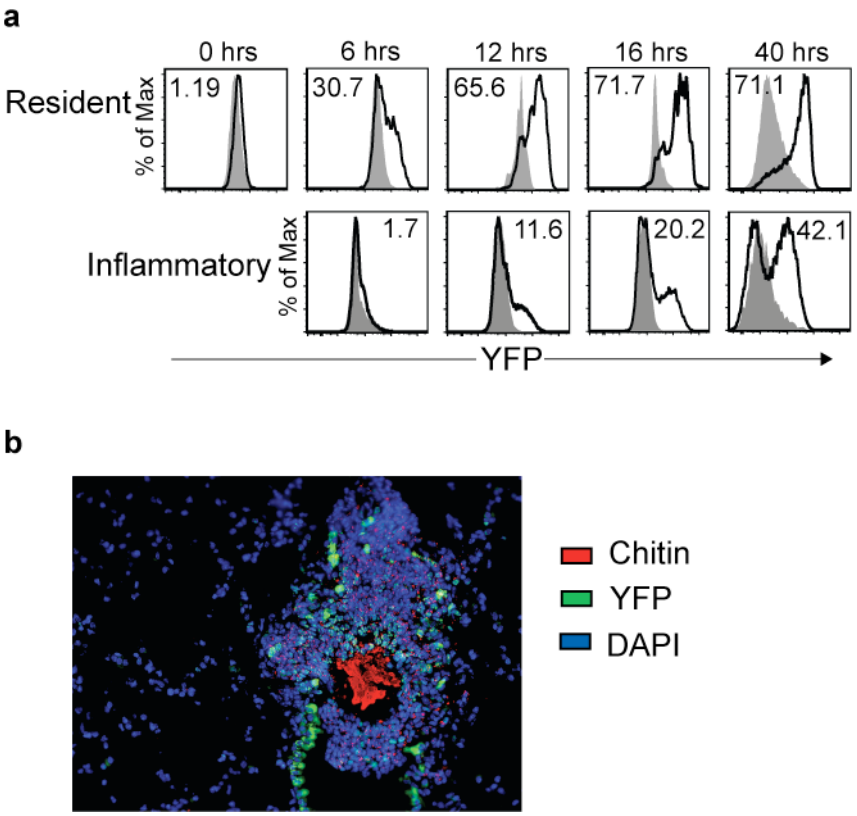


Figure 10 | Characterization of resident and inflammatory alternative macrophage activation. **a,** YARG mice or negative littermates received chitin and were analyzed for YFP expression. Filled histograms represent negative littermates. Solid line represents YFP in the YARG littermate. Numbers in the histogram are the percentage YFP+. Resident and inflammatory macrophages were subset using surface markers described in the text. Each experiment represents two independent experiments with n=3 per group. **b,** 13 hours after intranasal chitin lungs from YARG mice were stained with chitin-binding domain labeled with rhodamine to stain chitin (red), anti-GFP to stain YARG^{+} cells (green), and nuclei were counterstained with DAPI (blue). 20x lens magnification shown.

Figure 11

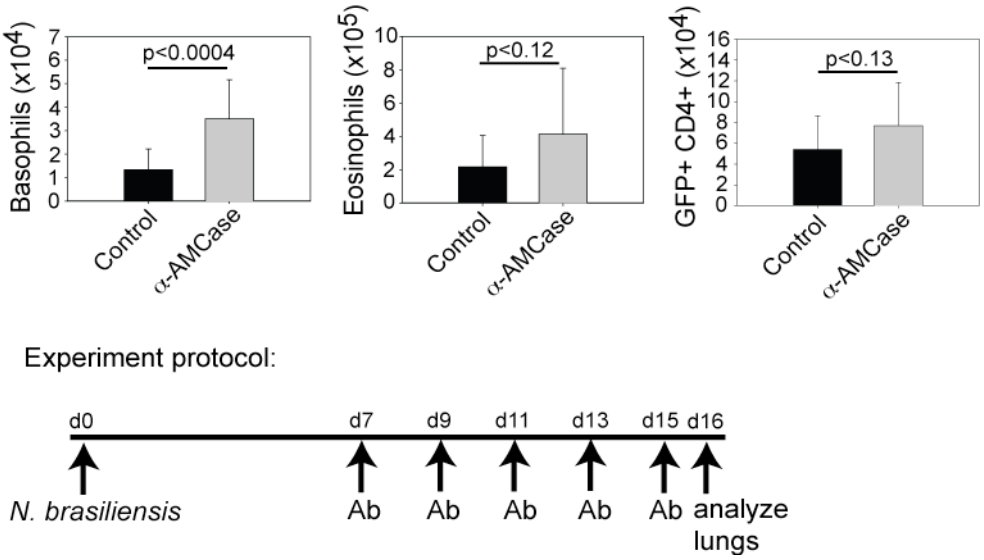


Figure 11 | Inhibition of AMCase after *N. brasiliensis* leads to prolonged inflammation. 4get mice were infected with *N. brasiliensis* and on days 7, 9, 11, 13, 15 either control rabbit serum or anti-AMCase serum was administered intraperitoneally. Lung basophils, eosinophils, and Th2 cells were enumerated on day 16 by flow cytometry. P-values determined using Student's t-test. Error bars represent standard deviations. Graphs indicate summary of 3 independent experiments with n=3-4 per group.

amounts due to degradation by chitinolytic marine bacteria (Keyhani and Roseman, 1999). Chitin provides osmotic stability and tensile strength to fungal cell walls (Bowman and Free, 2006) and scaffolds the rigid exoskeleton in insects (Merzendorfer and Zimoch, 2003). Nematode chitins are important for eggshell integrity and for structure of the rigid pharynx, including the buccal cavity and grinder, a specialized cuticle that is shed and resynthesized during molting (Zhang et al., 2005). The use of chitin by fungi, worms and insects may have driven evolutionary pressure to maintain chitin-recognition molecules in vertebrates akin to those in plants and protochordates (Hernandez Prada et al., 2006; Kaku et al., 2006). Intriguingly, these data support a role for chitin as a molecular pattern recognized in tissues and linked with the accumulation of innate cells implicated in helminth and allergic immunity, including alternatively activated macrophages, eosinophils and basophils (Gordon, 2003; Voehringer et al., 2006). Occupations predicted to have high environmental chitin levels, such as shellfish processors, are marked by high incidence of asthma, suggesting that this pathway may play a role in human allergic disease (Cartier et al., 2004).

Materials and Methods

Mice. BALB/c mice, C57BL/6 mice, TLR4-deficient mice (C.C3-Tlr4^{Lps-d}/J) were purchased from Jackson Laboratories (Bar Harbor, ME). MyD88-deficient mice (Adachi et al., 1998), mast cell-deficient Kit (W-sh) mice (Wolters et al., 2005), IL-4 reporter mice (4get mice) (Mohrs et al., 2001), and BLT1$^{-/-}$ mice have been described (Tager et al., 2000). Stat6$^{-/-}$ mice, Rag$^{-/-}$ mice and IL-5 transgenic mice were backcrossed 10 generations to 4get/BALB/c (Voehringer et al., 2006).

For creating SPAM transgenic mice the full-length cDNA for AMCase was isolated from total lung RNA of *N. brasiliensis*-infected BALB/c mice using the following primer pair: 5'AMCase-SalI: 5'-gcgtcgaccatggccaagctacttc-3', 3'AMCase-BHI: 5'-cgggatccggttcatggccagttg-3'. cDNAs were cloned into pCR2.1 TOPO vector (Invitrogen), sequenced and subcloned into SalI and BglII sites of a lung-specific expression vector containing surfactant protein C promoter elements (Glasser et al., 1991). The construct was linearized with NotI and injected in DBA/2B6 F1 oocytes. Founder lines with tissue-specific AMCase expression were selected for further study. One line with high lung-specific expression was selected to backcross 8 generations onto the BALB/c 4get background.

For generation of YFP-Arginase-1 Reporter Mice (YARG) a 6 kb BamHI-HindIII fragment was obtained by PCR amplification from 129/SvJ genomic DNA containing exons 4-8 and 3 kb of 3' untranslated sequence of the *arg1* gene. BamHI and BglII sites were introduced downstream of the endogenous stop codon and upstream of the 3' untranslated region within exon 8 using PCR-mediated mutagenesis, and the mutated fragment was inserted into pKO-Select-DT (Lexicon) containing diphtheria toxin (DT) α

chain for negative selection. A bicistronic reporter cassette (Mohrs et al., 2001) containing IRES linked to eYFP (Clontech) was placed 3' of a floxed-neomycin-resistant cassette derived from pL2neo2. The positive selection/reporter cassette was cloned into the BamHI and BglII sites in the mutated *arg1* gene to generate the final targeting construct. PrmCre ES cells, which express the Cre recombinase under control of the protamine promoter, were electroporated with the NotI-linearized targeting construct and selected in 300 μg/ml G418. Resistant ES clones were screened for homologous recombination by Southern blot. Two independent clones were injected into C57BL/6 blastocysts to generate chimeras. The floxed neomycin-resistant cassette was deleted in the male germline after breeding male chimeras to wild-type B6 females. Heterozygous F1 animals were bred to B6 mice and F2 offspring were screened for the presence of the reporter and the absence of the PrmCre transgene. YARG mice were backcrossed onto C57BL/6 background for at least 6 generations. Mice were maintained in the specific pathogen-free animal facility at UCSF according to institutional guidelines.

Chitin administration. Chitin (New England Biolabs) was washed three times in PBS and large aggregates settled for 2 min. Suspended chitin was collected and diluted 1:4 in PBS. For intranasal administration anesthetized animals aspirated 50 μl of chitin on consecutive days, and were analyzed on day 2. When 50 μl was dried down and weighed we confirmed that mice were receiving 50 – 100 μg of dry chitin per dose. Where designated, 200 μl of chitin was injected once i.p. In designated experiments, chitin was preincubated with 100 μg recombinant AMCase, Ym2, or mutant AMCase for 1 hr/37°C.

Flow cytometry and cell purification. Cell suspensions were washed with FACS buffer (PBS, 2% FCS, 1 mg/L NaN3) and the resuspended cell pellets were incubated for 5 min with anti-CD16/CD32 mAb (2.4G2, BD Pharmingen) before staining with APC-Alexa Fluor 750-anti-CD4 (RM4-5, Caltag), PE-anti-Siglec F (E50-2440, BD Pharmingen), APC-anti-ckit (2B8, BD Pharmingen), PerCP cy5.5-anti-CD11b (M1/70, BD Pharmingen), APC-anti-F4/80 (BM8, eBiosciences), and biotinylated anti-IgE (R35-72, BD Pharmingen) followed by PerCP Cy5.5-streptavidin (BD Pharmingen). For some experiments cell were stained with APC-anti-CD62L (BD Pharmingen) or biotinylated anti-Ly6C (BD Pharmingen) followed by APC-streptavidin. Cells were suspended in 1 μg/ml DAPI in FACS buffer to exclude dead cells. Cell counts were determined using Counting Beads (Caltag). For quantitation of total YFP+ cells in YARG mice the number of background YFP+ events detected in wild-type littermates was subtracted from the number of YFP+ cells detected in YARG mice. Samples were acquired on a LSRII (Becton Dickinson) and analyzed with FlowJo software (Tree Star).

For purification of YFP+ and YFP- macrophages, cells were washed from the peritoneum of YARG mice with 10 ml PBS/10% FCS and cell suspensions were stained with PE-anti-CD11b (M1/70, Caltag) and APC-anti-F4/80 (BM8, eBiosciences) in PBS/1% FCS. Cells were suspended in 1 μg/ml DAPI in PBS/1%FCS prior to sorting (MoFlo; DakoCytomation).

Transwell Assay. RAW264.7 macrophage cell line or bone marrow-derived macrophages were distributed at $1x10^6$ cells/well in 24-well plates in serum-free DMEM media. The following morning adherent cells were stimulated with chitin (0.8 μg)

(Sigma) or PBS (vehicle control). Optimal chitin concentration was determined separately in a dose response curve. For macrophages sorted from YARG mice, $5x10^5$ cells/well were distributed to 24-well plates in RPMI/10%FCS and used immediately in transwell assays. $1x10^6$ splenocytes from IL5 transgenic x 4get mice were placed in the top well of the transwell chamber (Costar, 5.0 μm pore size, Corning Inc.) at 37°C/5% CO_2 for 3 hrs. Cells that migrated to the bottom well of the transwell chamber were enumerated using counting beads (Caltag) and stained for Siglec-F-PE. MK886 was purchased from Cayman Chemical. Chemotactic index is the number of cells that migrate to the stimulus divided by the number of cells that migrate to vehicle. Experiments with less than 120,000 migrated eosinophils were excluded due to viability concerns.

Immunohistochemistry. Lungs were isolated from YARG mice and processed as described (Reinhardt et al., 2006) using rabbit anti-GFP polyclonal Ab (ab 6556; Novus Biologicals) and chitin-binding domain probe labelled with rhodamine (New England Biolabs).

***In vivo* depletion of macrophages.** Clodronate was a gift from Roche Diagnostics GmbH, Mannheim, Germany. Clodronate liposomes were prepared as described (Van Rooijen and Sanders, 1994) and 200 μl of liposomes or PBS was injected i.p. on days 0 and 3. Chitin was administered on the next day and peritoneal cell recruitment was analyzed 6 hrs later.

***Nippostrongylus brasiliensis* infection**. Third-stage larvae (L3) of N. brasiliensis were recovered from the cultured feces of infected rats, washed extensively and injected (500

organisms) into mice subcutaneously at the base of the tail. Infected mice were placed on antibiotic-containing water (2 g/l neomycin sulfate, 100 mg/l chloramphenicol) for 5 days and killed for analysis after 9 days.

RT-PCR analysis. cDNAs were prepared from total lung RNA generated using the total RNA isolation kit (Fluka, Buchs, Switzerland) and Superscript™ reverse transcriptase kit (Invitrogen, Carlsbad, CA). RT-PCR was performed with the following primer pairs: Ym1: 5'-tggaattggtgcccctacaa-3' and 5'-aacttgcactgtgtatattg-3'; Ym2: 5'-aaccgtcagacattcatta-3' and 5'-tggtccttccagtaggtaata-3'; Brp39: 5'-agagctgctctgcgtacaag-3' and 5'-agtttctctctgcctggctg-3'; AMCase: 5'-tcacaggtctggctcttctg and 3'-catatgtcatgacatggatg; β-actin: 5'-atggatgacgatatcgct-3'and 5'-atgaggtagtctgtcaggt-3'. PCR conditions were 35 cycles with 30 sec 94°C, 30 sec 58°C, 60 sec 72°C followed by a final elongation for 10 min at 72°C.

Generation of recombinant proteins. Full-length cDNA for Ym2 and AMCase were cloned in pMT/V5-His expression vector (Invitrogen, Carlsbad, CA). S2 insect cells were cotransfected with the expression constructs and pCo-Blast selection plasmid conferring Blasticidin resistance. After establishing stably transfected cell lines protein production was induced for 3 days with 0.7 mM $CuSO_4$ and culture supernatants or control supernatants from untransfected S2 cells were purified over Ni^{2+} columns.

Site-directed mutagenesis of AMCase. Two critical amino acids of the catalytic center of AMCase, aspartate and glutamate at position 154 and 158, were mutated in AMCase to asparagine and glutamine using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenesis was performed in two steps using the following

primers: aspartate to asparagine 5'-ctggactggcagtacccaggctcacgtgggagc-3', 5'-gctcccacgtgagcctgggtactgccagtccag-3', and glutamate to glutamine 5'-gatggactgaacctggactggcagtacccaggc-3', 5'-gcctgggtactgccagtccaggttcagtccatc-3'.

Generation of anti-AMCase antibodies. Antibody was generated using the Invitrogen custom antibody service (Invitrogen, Carlsbad, CA). The anti-AMCase antibody was generated against the peptide DKADGLYPVADDRNAFWQC. Briefly, the peptide was conjugated to KLH and the KLH-peptide was injected subcutaneously into three dorsal sites of New Zealand white rabbits with Freund's adjuvant at weeks 0, 2, 7 and 9. Serum was collected at weeks 0, 4, 8 and 10. Terminal bleed of the anti-AMCase antibody was peptide-affinity purified.

Analysis of chitinase activity. Chitinase activity was determined by incubation of recombinant proteins or supernatants from dispersed lung tissue from control or SPAM mice with 270 μM p-nitrophenyl β-D-N,N',N''-triacetylchitotriose (Sigma-Aldrich) in McIlvain Buffer pH 7.0 (100 mM citric acid, 200 mM sodium phosphate) at 37°C. *Brugia malayi* chitinase was used as a positive control (New England Biolabs, Beverly, MA). Substrate turnover by 1 μg AMCase in 100 μl volume was analyzed on an ELISA reader at 405 nm.

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Chapter IV:

Chitin and chitinases in allergic inflammation

Summary

Our studies suggest a pathway by which inhaled chitin, a common environmental biopolymer and potentially a component of many aerosolized antigens, induces alternative macrophage activation and LTB_4 from resident macrophages, thereby provoking the infiltration of eosinophils and basophils, both of which are innate IL-4-expressing cells that are highly associated with allergic immune responses (Voehringer et al., 2006). In turn, through expression of IL-4/IL-13 and activation of Stat6-dependent genes, infiltrating cells can induce AMCase expression from lung cells, including macrophages and epithelial cells (Homer et al., 2006). AMCase enzymatically attacks chitin, thus relieving the stimulus for further eosinophil and basophil recruitment (Figure 1). The function of Ym2 remains unclear at this time and further studies will be needed to determine if Ym2 has a role in carbohydrate recognition or tissue remodeling.

Prior studies identify AMCase and Ym1 induction after nematode infections and during allergic immunity (Nair et al., 2005; Sandler et al., 2003; Welch et al., 2002) and previous reports implicate AMCase as a proinflammatory mediator of allergic inflammation (Zhu et al., 2004). Transgenic mice over-expressing AMCase and Ym2 had no signs of spontaneous inflammation, however, and we could not ameliorate the course of allergic inflammation with neutralizing antibodies to AMCase and Ym2 using the OVA-induced lung inflammatory model. Rather, our data support a role for AMCase in the feedback attenuation of chitin-induced allergic innate immune responses in the lung. Evidence in support of this hypothesis is that transgenic mice that overexpress AMCase have significantly attenuated eosinophil and basophil recruitment in response to chitin. Experiments comparing AMCase transgenic mice with Ym2 transgenic mice, as

Figure 1

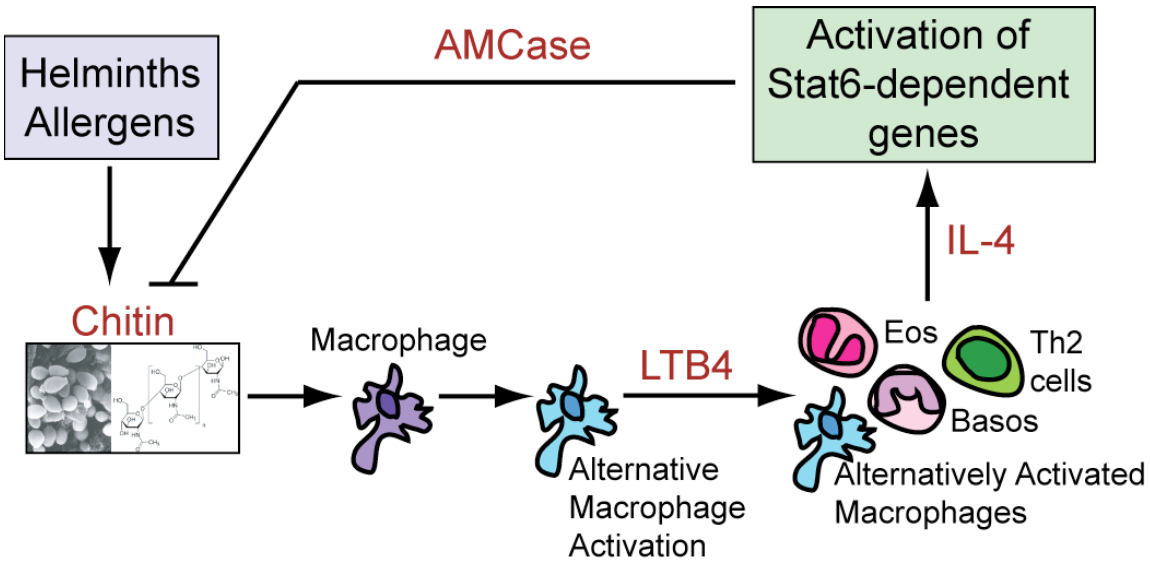

Figure 1 | Model of chitin-induced inflammation and AMCase-mediated attenuation of inflammation. Chitin, a common component of inhaled allergens and helminths, activates macrophages. Macrophages rapidly become alternatively activated and secrete LTB_4, a lipid mediator of inflammation. LTB_4, in turn, recruits innate effector cells, including eosinophils, basophils, and Th2 cells and leads to more alternative macrophage activation. Eosinophils, basophils and Th2 cells can make IL-4, which activates Stat6-dependent genes. One Stat6-dependent gene is AMCase, an enzymatically active chitinase. AMCase acts as part of a negative feedback loop to decrease inflammation by degrading chitin.

well as experiments using enzymatically inactive AMCase suggest that AMCase dampens eosinophilia by degrading chitin and making it less immunogenic.

Our hypothesis would be further supported by additional experiments that address a few key issues. First, identifying a receptor for chitin present of macrophages will be critical for further studies. Second, addressing the ability of chitin to act as an adjuvant will be essential for tying together the innate cell recruitment in response to chitin with the induction of the Stat6-dependent gene, AMCase. Third, showing that the chitin component of a helminth or allergen is an important component for the immune response to that pathogen will provide further evidence that chitin is a critical pathogen associated molecular pattern.

Receptors for chitin

Importantly, if chitin serves as a pathogen associated molecular pattern that is specifically recognized by macrophages and other cell types, then we should be able to identify a receptor specific for chitin. Macrophages express a variety of C-type lectin receptors, including the mannose receptor (MR), which is capable of mediating recognition of chitin particles by macrophages (Shibata et al., 1997). The mannose receptor recognizes mannose, fucose, and *N*-acetylglucosamine residues in a Ca^{2+} dependent manner (Lennartz et al., 1987; Wileman et al., 1986). Its role as a pattern recognition receptor is speculated due to binding of MR to a wide variety of pathogens, including *Candida albicans* (Ezekowitz et al., 1990), *Pneumocystis carinii* (Ezekowitz et al., 1991), *Mycobacterium tuberculosis* (Schlesinger, 1993), and *Trypanosoma cruzi* (Kahn et al., 1995). However, MR-deficient mice display no difference in susceptibility

to *C. albicans* or *P. carinii* infection (Lee et al., 2003; Swain et al., 2003). The important role of dectin-1 for β-glucan recognition of fungi may account for these observations (Saijo et al., 2007; Taylor et al., 2007). MR-deficient mice also cleared *N. brasiliensis* with standard kinetics and displayed normal effector cell recruitment to the lungs (T. Reese, unpublished data). Despite an as yet unidentified requirement for MR in pathogen recognition, MR does appear to be important for clearance of endogenous serum glycoproteins (Lee et al., 2002). Additionally, MR mediates delivery of mannosylated antigens to the MHC class II processing pathway and enhances presentation of antigen to T cells (Engering et al., 1997; Tan et al., 1997).

Search of the Consortium for Functional Glycomics (CFG) databases reveals a handful of C-type lectin receptors with affinity for *N*-acetylglucosamine (www.functionalglycomics.org). In addition to the mannose receptor, other members of the mannose receptor family, including DEC205 and Endo180 have affinity for *N*-acetylglucosamine. DEC205 is found primarily on DCs but also on pulmonary epithelial cells and is thought to mediate antigen uptake (Jiang et al., 1995; Witmer-Pack et al., 1995). Moreover, it binds β1-4GlcNAc. Endo180 exhibits Ca^{2+}-dependent binding to mannose, fucose, and *N*-acetylglucosamine (East et al., 2002). Within the group of Type 2 Receptors that contain C-type lectin domains some of the SIGNR proteins in mouse have affinity for *N*-acetylglucosamine. In mouse there are 5 SIGNR receptors that are homologous to human DC-SIGN (Park et al., 2001). Both SIGNR1 and SIGNR3 bind terminal mannose, fucose, and *N*-acetylglucosamine residues. SIGNR2 specifically binds terminal *N*-acetylglucosamine (Powlesland et al., 2006), however sequence alignment of

this gene suggests it might be a pseudogene, because it lacks a transmembrane domain and a cytoplasmic tail.

As mentioned in the introduction LysM domain-containing proteins may represent a conserved mechanism for chitin recognition. LysM domains are often found with amidase, chitinase, or protease domains, and were originally identified in bacteria. They bind *N*-acetyl-glucosamine-*N*-acetyl-mureine. Multiple chitin-binding proteins identified in plants contain LysM domains. Although none of the LysM domain-containing proteins from mouse or human have been described in the literature, there are sequences deposited in the GenBank database describing putative proteins with LysM domains. Given the promiscuity of many C-type lectin receptors as well as the lack of known signaling motifs in receptors like the mannose receptor, it may be difficult to identify only one receptor required to recognize chitin. Thus, exploration of novel proteins, both intracellular and membrane-bound, will likely be necessary to delineate the chitin recognition pathway.

Chitin as an adjuvant

In our model, chitin is recognized by macrophages and through the production of LTB_4 drives effector cell recruitment associated with type-2 inflammation. We also hypothesize that these effector cell types produce IL-4 and initiate Stat6-dependent gene expression, leading to AMCase upregulation, and subsequent degradation of chitin. For chitin to enhance Stat6-dependent gene expression, chitin needs adjuvant activity that promotes Th2 cell differentiation in the presence of protein antigen. Our experiments

thus far do not test this part of the hypothesis, because we used chitin alone and only focused on the earliest innate events in response to chitin.

In preliminary experiments where we tried mixing OVA protein with chitin and looking either at T cell polarization by intracellular cytokine stain or at antibody isotype switching we observed highly variable results (data not shown). We speculate that this could be because the OVA and the chitin were not conjugated together, and not all DCs that ingested OVA also took-up chitin, or vice versa. There are reports examining adjuvant activity of particulates, and it is noted that conjugation of the protein to the particulate is necessary to achieve optimal T cell and antibody responses (Fifis et al., 2004). We are currently conjugating OVA to chitin in order to overcome any potential problems with targeting the protein and the adjuvant to the same cell.

These observations with particulate adjuvants raise the question of whether the recognition of chitin by the immune system is really due to a specific receptor mediated interaction, or whether it is simply due to the particulate nature of chitin. Alum is an insoluble compound that readily forms aggregates at the injection site (T. Reese, personal observations). It is also capable of inducing inflammatory recruitment (Jordan et al., 2004). Some of these supposedly inert particulate adjuvants and alum may exert some of their inflammatory effects through inducing tissue damage, retaining antigen for longer periods of time at the injection site, or by shuttling the antigen to the appropriate endocytic pathway for enhanced antigen presentation and DC activation.

Discerning the difference between the particulate nature of chitin and the actual specific recognition of *N*-acetylglucosamine by an immune cell is an essential part of the adjuvant question regarding chitin. This question would be best answered by isolating

polymers of *N*-acetylglucosamine of defined lengths that are soluble to test whether they are still proinflammatory. These experiments are also intimately tied with the experiments that address the question of the receptor. If a receptor can be found that recognizes chitin then we can begin to delineate some of the signaling events that occur downstream of chitin recognition. Whether chitin is proinflammatory because it specifically triggers a PRR or because of its particulate nature, specifying the adjuvant activity of chitin and understanding the mechanism of action will be important for any vaccine design that uses chitin.

Importance of chitin component for fungal and helminth recognition

We demonstrate that chitin alone promoted accumulation in tissues of eosinophils, basophils, and alternatively activated macrophages. While this is an important observation, we have not shown whether the chitin component of a helminth or fungus is necessary for an immune response to that pathogen or allergen. We observed normal worm clearance kinetics and effector cell recruitment to the lungs of SPAM mice after *N. brasiliensis* infection, either at day 9 (Chapter 2 Fig. 7) or at earlier time points (data not shown). In this case, degradation of the chitin component of the worms did not affect the immune response to the worms. However, *N. brasiliensis* is a large helminth that is likely composed of many immunogenic molecules, and it may also secrete proteases to facilitate migration in tissues. As described in the introduction, proteases are suspected to play a role in initiating type-2 immunity. Additionally, we have no evidence to say whether the AMCase expressed in the SPAM mice was able to access the chitin and degrade it in the worms. We have preliminary immunohistochemistry of *N.*

brasiliensis stained with a chitin-binding domain probe labeled with a fluorophore to indicate that chitin is mostly present in the pharynx of different larval stages and in the eggs found in adult worms (data not shown). This agrees with previous reports of chitin staining in *C. elegans* (Zhang et al., 2005).

The complexity of a helminth parasite, as well as the probable requirement of chitin for worm viability suggests that *N. brasiliensis* may not be the ideal model to test the importance of the chitin component for immune recognition. There are asthma models that utilize *Aspergillus fumigatus,* a common environmental fungus that contains chitin, to induce an asthma response in mice. Upon further characterization, it became apparent to us that the *A. fumigatus* extracts used in most asthma challenges were sterile filtered and did not contain insoluble cell wall components, including chitin. Consequently, we are pursuing a newer model of allergic lung disease that uses whole *Aspergillus niger* hyphae (David Corry, Baylor College of Medicine, personal communication). The hyphae were freeze-dried using a lyophilizer, ground into a powder, and sterilized by irradiation. We have suspended this powder in PBS, and found that by staining using a chitin-binding domain probe that there is chitin in the preparation. Significantly, after as little as one to two doses of this fungal preparation we can induce lung inflammation (T. Reese, Steve Van Dyken, unpublished observations). Most other published asthma protocols that use sterile filtered *A. fumigatus* extract require multiple intranasal doses over the course of weeks or priming with the extract in alum to induce asthma. We will now pursue testing the importance of the chitin component of our *A. niger* hyphae for the induction of inflammation by depleting the preparations of chitin

using a chitinase. This will be an important step in understanding the induction of type-2 inflammation and specifically asthma.

Role of alternatively activated macrophages in type-2 immune responses

Alternatively activated macrophages are recognized as functional effector cells in both helminth and allergic models. Arginase-1 positive macrophages are induced in helminth infections, as well as during allergy and asthma (Gordon, 2003; Nair et al., 2006). Significantly, inhibition of arginase-1 expression attenuates allergic lung inflammation in a mouse model (Yang et al., 2006). There is also data to suggest that memory Th2 cells induce alternative macrophage activation, and that these macrophages mediate immunity against an intestinal helminth (Anthony et al., 2006). Additionally, researchers are exploring arginase-1 polymorphisms in human asthma (Li et al., 2006). Taken together, these data implicate alternatively activated macrophages as important effector cells in allergic immunity, and previous work suggests that IL-4 and IL-13 are required to induce alternatively activated macrophages (Nair et al., 2003).

Importantly, our data establish a possible novel role for alternatively activated macrophages. We showed that arginase-1 positive macrophages were induced as early as 6 hours after chitin administration *in vivo*. Additionally, we demonstrated by sorting out YFP^+ and YFP^- macrophages that the YFP^+ (arginase-1^+) macrophages were more significant producers of leukotriene B4 and that they recruited more eosinophils in a transwell assay (Chapter 3 Fig. 9b). There are at least two important implications from these experiments. First, chitin was able to induce alternative macrophage activation within hours, prior to induction of IL-4/IL-13, suggesting that alternative macrophage

activation can occur from the earliest innate recognition of chitin. Second, the transwell experiments place alternatively activated macrophages as not only effector cells, but also implicate them as proximal sensors of chitin that are responsible for coordinating early effector cell recruitment. Further studies will be needed to establish the signals required for alternative macrophage activation. In addition, more work will be required to elucidate their roles in coordinating innate cell recruitment and initiating effector activities.

It is important to note that we observed that LTB_4 production by macrophages was important for recruiting specifically eosinophils to the lung. LTB_4 is also a potent chemoattractant for neutrophils (Ford-Hutchinson et al., 1980), however we did not observe neutrophil recruitment to the lung at day 2 in response to chitin. Possibly, neutrophils were recruited to the lung within hours after chitin administration, but a survival factor for them was missing. The earliest we examined the lung by FACS was at day 2, so we may have missed the window of time during which neutrophils were recruited. The early alternative macrophage activation we observed may reflect a very early bias for type-2 inflammation, and the macrophages either promoted the survival of eosinophils in the lung, or produced other factors that preferentially recruit eosinophils but not neutrophils. Indeed, there is evidence to suggest that alternatively activated macrophages preferentially recruit eosinophils, and exhibit enhanced phagocytosis of neutrophils, thus clearing them from inflammatory sites (Loke et al., 2007). Undoubtedly, there are more layers of complexity involved in the preferential recruitment of eosinophils that will require examining.

Chitinases in human asthma

Following from our hypothesis that AMCase attenuates chitin-induced inflammation, is the idea that alterations in AMCase levels or enzymatic activity could have significant implications for human asthma. In fact, AMCase is elevated in bronchoalveolar lavage fluid of asthmatics (Zhu et al., 2004), and one study of AMCase polymorphisms found an association of a genetic variant with asthma (Bierbaum et al., 2005). However, this study did not tie the polymorphism in AMCase with any alterations in AMCase levels or enzymatic activity.

Lending more support to the idea that AMCase could be involved in asthma is a more recent genetic association study that also found association between a particular polymorphism and asthma. Importantly, this group expressed recombinant human AMCase proteins with the different polymorphisms, and tested the enzymatic activity of the different variants. They found that the asthma-protective variant of AMCase has significantly elevated enzymatic activity when compared with the variant of AMCase that is more prevalent in people with asthma (Max A. Seibold and Esteban Buchard, manuscript in progress). This study supports our hypothesis that AMCase functions to degrade chitin, leading to subsequent attenuation of inflammation. We speculate that one attributing factor in the development of asthma is expression in patients of a variant of AMCase that has decreased enzymatic activity. This could lead to an impaired ability in those people to degrade inhaled chitin in allergens, thus leading to protracted lung inflammation.

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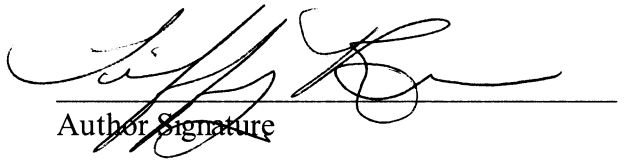

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