Immunoregulation and the Role of IL-17 During Chronic Pulmonary Exposure to *Aspergillus fumigatus*

by

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To Penney and Owen

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AAMΦ	alternatively-activated macrophage
Af	*Aspergillus fumigatus*
APC	antigen presenting cell
BM	bone marrow
CCR	C-C motif receptor
CXCR	CXC chemokine receptor
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescent-activated cell sorting
FcR	Fragment, crystalizable (region) receptor
FSC	forward-scatter
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IL	interleukin
IL-17$^{-/-}$	IL-17 knockout
LN	lymph node
MΦ	macrophage
MACS	magnetic-activated cell sorting
MBP	major basic protein
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PAS	periodic acid-Schiff
PBS	phosphate buffer saline
PRR	pattern-recognition receptor
sigF	siglec F
SSC	side-scatter
TGF	transforming growth factor
T_H	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
T_{reg}	regulatory T cell
WT	wild-type

Chapter 1
Background

Allergic airway disease is a chronic inflammatory condition characterized by airway hyperresponsiveness and reversible airway obstruction in response to repeated antigenic stimulation (1). The literature cites numerous sources and possible sources of disease: viruses (2, 3), allergens (4), and occupational compounds (5) have been implicated as potential sources, while a wide variety of factors from viruses, to common bacteria (6), to sex hormones (7) have been shown to help drive the initial allergic response. Of particular interest is hypersensitivity to fungi (8), as 20-25% of asthmatics are skin-test positive towards fungal allergens (9, 10), and there is a strong correlation between sensitivity to fungal allergens and disease severity (11).
Askdhaksdh

Fungi and Disease

Aspergillus fumigatus, an airborne fungus, is the focus of extensive research due to its ubiquitous nature. *A. fumigatus* disseminates itself via conidia – hydrophobic (12, 13) spores which are readily and frequently inhaled into airways (14) – and large doses are not uncommon (15). Once the conidia reach a warm, moist environment such as the lungs, they lose their hydrophobic properties (16) and begin to germinate, express genes, and generate structural components (17). The conidia first swell, then extend germ tubes, and finally develop invasive hyphae (18). Even relatively large doses of conidia pose

little threat to immunocompetent individuals, however, as macrophages and neutrophils efficiently neutralize germinating conidia in the lung (19, 20), and resting conidia are cleared from the lung by macrophages without the induction of an inflammatory response (21, 22).

Therefore *A. fumigatus* typically causes disease in one of two ways: by infecting immunocompromised individuals or by inducing a hypersensitivity reaction in healthy individuals through repeated exposures or colonization. In individuals with compromised immune systems – from organ transplantation (23, 24), HIV infection (25), or steroid therapy (19), for example – a lack of immune response leads to incomplete *A. fumigatus* killing (26) and can facilitate fungal invasion of the tissue (27). On the other hand, hypersensitivity can occur when the immune system is primed towards *A. fumigatus* antigens and mounts an inappropriate response. Hypersensitivity pneumonitis (28, 29) and allergic asthma (9, 10, 30) resulting from repeated *A. fumigatus* exposure are both examples of this phenomenon.

There are a number of anti-fungal treatments used to counter *A. fumigatus* and other invasive fungi by enhancing or augmenting the host immune system or by killing the fungus outright. Examples include Voriconazole (31-33), Amphotericin B (33, 34), Pentraxin 3 (35), and new lipopeptides (36). However, such therapies often have unwanted side-effects and are geared primarily at countering invasive fungal disease rather than hypersensitivity. In the end, pulmonary hypersensitivity is not a fungal disease but an *immune* disease, and adequate clinical treatment will require a more thorough understanding of the immune response to *A. fumigatus.*

Model of Hypersensitivity

Our laboratory has designed a model of *Aspergillus fumigatus*-induced pulmonary hypersensitivity based on previous observations by Mairi Noverr, Tobias Rodriguez, and Andrew Shreiner. Repeated exposure to *A. fumigatus* conidia in wild-type C57BL/6 mice generates a hypersensitivity response that is CD4 T cell-dependent (37). This response is characterized by the production of T_H2-type cytokines, eosinophilia, mucus hyper-secretion, and tissue remodeling.

The standard model of T_H2-type pulmonary hypersensitivity involves sensitization and challenge of mice with **ovalbumin (OVA)**. Variations of this model involve sensitization with adjuvants (38, 39), immunization routes (39-41), and genetic background (42-47) which result in a wide and inconsistent spectrum of immune responses (48). In addition, the OVA model, while informative, is not necessarily physiologically relevant as it does not mimic environmental conditions that result in pulmonary hypersensitivity. Our *A. fumigatus* model, on the other hand, is both physiologically relevant and provides a consistent response among genetic backgrounds.

Innate Immune Response to *Aspergillus Fumigatus*

The immune response to *A. fumigatus* is characterized by complex interactions between the innate immune response and the adaptive immune response, both of which are quickly activated by the presence of fungi (24, 27). The reaction must be complex, because *Af* is not an inert particle but a dynamic organism capable of reacting to its environment and even the host immune system (49). The **innate immune system** acts as the first line of defense against pathogens and encounters an exceptionally wide array of microbes and particles (50-52). In the case of *Aspergillus fumigatus*, a complex collection of cells types, pathways, and cytokines compromise the initial reaction to germinating

conidia and developing hyphae. Macrophages (53), neutrophils (54), dendritic cells (55), monocytes (56), eosinophils (57), natural killer cells (58) all play a role in host defense, as do aspects of the complement system (59) such as mannan-binding lectin (60) and Pentraxin 3 (35).

Macrophages are the primary cells responsible for clearing *A. fumigatus* conidia from the lung. Macrophages bind the conidia with DC-SIGN, a lectin-like attachment site (53, 61) and engulf *A. fumigatus* in an oxidase-dependant manner (14). When low numbers of conidia enter the lungs, alveolar macrophages quickly and efficiently phagocytize and kill conidia before the spores can germinate, neutralizing any potential threat without triggering an inflammatory response (21, 22). Greater numbers of conidia may necessitate an aggressive inflammatory response, but macrophages are still an essential factor in host defense. Both macrophages and dendritic cells bind *A. fumigatus* in the early stages of infection (53); inhibition of macrophage function allows conidia to germinate and hyphae to form (19, 62). Moreover, alveolar macrophages can activate and produce cytokines independently of toll-like receptor (TLR) signaling (63, 64), perhaps in response to the ability of *A. fumigatus* to dampen TLR2 and TLR4 signaling (65). This independence is restricted to alveolar macrophages, however, and macrophages from other sites are typically TLR- and MyD88-dependent (66, 67).

Similar to macrophages, **dendritic cells** bind *A. fumigatus* in the early stages of infection but can also initiate an adaptive immune response by trafficking to the mediastinal lymph node to induce T cell activation (55). Dendritic cells can consume fungi through several mechanisms and even discriminate between fungal stages, with distinct subpopulations showing different roles in fungal defense (68). Dendritic cells seem to act as a central fulcrum for the adaptive immune response, dictating which T

helper (T_H) responses will be used. Dendritic cells can balance T_H1 and T_H2 via IL-12 and IL-13 (69), and dendritic cells activated through dectin-1 – a **pathogen-associated molecular pattern (PAMP)** receptor – can induce a T_H17 response (70) and even convert regulatory T cells into T_H17 effector cells (71). In addition, **monocytes**, often overlooked in inflammatory responses, have a significant role in the clearance of *Aspergillus* from the lung by facilitating a T cell response (56). They respond to a number a different pathogens (72), and like dendritic cells they are not homogenous (73, 74), with different subgroups producing TNF or inhibiting germination of *A. fumigatus* (75).

Once an inflammatory response has been initiated, **neutrophils** become the primary defense against invasive aspergillosis. Neutrophils are essential during an acute response to *A. fumigatus* (54) and are even more essential than macrophages during early infection (76). Neutrophils restrict hyphal invasion of local tissue, and poor activation or influx of neutrophils is associated with invasive aspergillosis (62, 77). Neutrophils help control *Aspergillus* infection via traditional phagocytosis and cytokine release, but they are also capable of releasing Pentraxin 3 lectin (78). In addition, they can form neutrophil extracellular traps in response to both conidia and hyphae (79), though the signaling mechanism for this process is not well understood. Both the activity and total numbers of neutrophils are tightly regulated by multiple mechanisms, as neutrophils have the capacity to cause significant amounts of damage to inflamed tissue (80, 81).

Eosinophils also respond to *Aspergillus fumigatus*-induced inflammation, and like neutrophils they are tightly regulated due to their potential for tissue damage. They have been implicated in a number of inflammatory defense processes towards helminths, bacteria, viruses, fungi, injured tissue, and tumors, but have also been implicated in several forms of immune dysregulation such as allergies and pulmonary remodeling (82).

Eosinophils are multi-functional; they express TLRs (83), act as antigen presenting cells (84), and secrete over thirty different inflammatory and regulatory cytokines (85, 86), including T_H1-associated cytokines (87-89). Unlike T cells, eosinophils store cytokines in granules and release them at inflammatory sites rather than generating them de novo (90). This allows a rapid response against potential pathogens, though the release of cytokines stored in granulocytes is regulated by the local milieu. Against *A. fumigatus*, eosinophils react to the β-glucan subunits expressed in germinating conidia (57), but their primary role seems to be combating invasive fungal hyphae that have already established a foothold within the host tissue.

A key component of the innate response is the expression of **indoleamine-2,3-dioxygenase (IDO)** (91). IDO is a metabolic enzyme that catabolizes L-tryptophan to N-formylkynurenine, the process of which can suppress the proliferation and activity of immune cells as well as microbes. Its expression is highly responsive to the immune system (92), and it exerts a significant, corresponding effect on the immune system, particularly neutrophils (93). IDO plays a complex role in the control of both the innate and adaptive immune responses; IDO is upregulated by proinflammatory cytokines and IFN-γ (94), but it simultaneously drives the generation of regulatory T cells (T_{reg}) (95). Moreover, lack of IDO abrogates T_H2 hypersensitivity in the lung (96), and allergy – via IDO production – can be controlled through glucocorticoid-induced tumor necrosis factor receptor (GITR) (97), a surface receptor frequently associated with regulatory T cell function. Eosinophils constitutively express IDO, and it is possible that they use IDO-mediated suppression of the T_H1 to maintain a T_H2-type immune response (98). To further complicate matters, T_H17 cells also inhibit IDO expression through IL-17 and IL-

23 (94) and can negatively affect clearance os *A. fumigatus* from the lung by altering the inflammatory program of neutrophils (99).

The major method of by which the immune system is activated is the use of PAMP receptors such as **toll-like receptors (TLRs)** and **dectin-1** (100). *Aspergillus fumigatus* expresses a number of ligands for PAMP receptors including chitin, β-glucans, and galactomannan (101). Moreover, TLR polymorphisms affect susceptibility to invasive fungal disease (102), highlighting the central role of TLRs in fungal defense. Mice deficient in TLR and MyD88 – the primary adapter protein for TLR signaling – can survive inhaled *A. fumigatus* conidia but at a lower rate than wild-type mice (103), and MyD88 signaling is particularly important in the early pulmonary response to *Aspergillus* (104) due to its control of neutrophil activity (54). PAMP receptors can have a pathogenic role in disease, however, as TLR signaling has been implicated in chronic inflammation (105), and dectin-1 can drive a potentially autoimmune-inducing T_H17 response (106). On the other hand, repeated TLR signaling can lead to hyporesponsiveness as has been found with TLR2 (107), TLR5 (108), TLR7, TLR8 (109), and TLR9 (110).

In response to *Aspergillus fumigatus*, **TLR2** and **TLR4** play a central role (111, 112). There is a synergistic effect between both receptors (113), and a blockade of the TLRs reduces TNF-α levels during *A. fumigatus* infection (114). Both are capable of activating macrophages during infection (115), though macrophages can also be activated in a TLR-independent manner (64). TLR2 can stimulate both T_H2 responses (116, 117) as well as the induction and expansion of regulatory T cell populations (118, 119). TLR2 is involved in the immune response to both conidia and hyphae (115) while TLR4 induces the response to conidia only (120). It's therefore likely that TLR4 plays a more prominent

role in early *A. fumigatus* infection while TLR2 acts during late fungal invasion. TLR9 may also be activated by Pentraxin 3 (35), and various references have shown that *A. fumigatus* can with TLRs 1, 3, 6, and 10 on neutrophils as well (54).

Also playing a key role in innate and adaptive immunity to *A. fumigatus* is **dectin-1**, a PAMP receptor which has been shown to play an essential role in the control of fungal infections (121). Originally dectin-1 was thought to be expressed primarily by monocytes, macrophages, and neutrophils (122), but one recent study has shown a significant role for dectin-1 signaling in dendritic cells in response to *A. fumigatus* (123).

The primary role of dectin-1 is the binding of β-glucan, an essential component of germinating conidia (124, 125), and like other PAMP receptors it is capable of triggering phagocytosis, inflammation, and cytokine release (126-128). One notable characteristic of dectin-1 is its ability to enhance TLR signaling (129). In fact, dectin-1 is *dependent* on TLR signaling, as dectin-1/TLR collaboration via the Syk kinase is required for proper signaling (130), and in monocytes and macrophages dectin-1 works with TLR2 and TLR4 to induce cytokine production (131). Dectin-1 signaling results in a myriad of different cytokines and adaptive immune pathways: dectin-1 can activate the cytotoxic T cell response (132), can regulate downregulate IL-12 and upregulate IL-23 (133), can generate IL-2 and IL-10 production (106), can induce regulatory T cell differentiation and tolerance (118), can promote a T_H17-type response via IL-6 and IL-23 production (106, 134), and can induce dendritic cells to convert T_{reg} to T_H17 cells (71).

The Adaptive Immune Response

Though the innate immune response plays a central role in immunity to *Aspergillus fumigatus*, equally important is the **adaptive immune response**. Similar to

the innate immune response, the adaptive immune response is activated quickly by *A. fumigatus* (24, 27). Traditionally, the adaptive immune response was viewed as a balance between the response to intracellular pathogens (T_H1) (135) and the response to parasites (T_H2) (136). In the last few years the regulatory and T_H17 responses have added an additional layer of complexity to our understanding of the adaptive immune response (Fig. 1-1). During the response to *A. fumigatus*, all four of the major arms of the adaptive response can be engaged depending on the viability of the conidia (137), which fungal components are present (138), or, as we will see shortly, how long the host has been exposed to the organism.

The **T_H1** adaptive immune response has been repeatedly shown to be of great importance in defense against invasive *A. fumigatus*. Progressive invasion of infected tissue has been associated with decreased IFN-γ production and poor T cell proliferation (139), and inhibition of IFN-γ and TNF-α enhance fungal invasion (140, 141). In reaction to inhalation, the T_H1 response peaks one week after initial infection (137) and IFN-γ production is capable of inhibiting early T_H2 hypersensitivity (142). Dendritic cells in particular appear to play a major role in driving the T_H1 defense against *A. fumigatus* (143). Conidia inhalation results in the expression of TNF-α, IFN-γ, IL-12, and IL-18 (144). These cytokines play a variety of roles in this anti-fungal response: TNF-α drives neutrophil recruitment (145), IFN-γ and GM-CSF enhances the fungal killing by neutrophils (146, 147), IL-12 helps fight fungal invasion (148, 149), and IL-18 sustains the T_H1 anti-fungal response (150). In addition, IL-6 and IL-15 have been shown to play roles in the T_H1 defense against *A. fumigatus*, both by enhancing the neutrophil response to the infection (151-153).

Antagonistic to the T_H1 pathway (154) is the **T_H2** pathway, which provides host defense against parasites and extracellular pathogens (155). *A. fumigatus* antigens are capable of inducing a T_H2 type response, though it is hyphal extracts and not conidia that do so (156). This supports the observation that the T_H2 response is dependent on high levels of fungal growth (157) though, oddly, killed fungal spores result in more IL-4 and IL-13 production than live conidia (137). The presence of a T_H2 response is most likely useful in combating late-stage fungal invasion, but its presence can have a number of unwanted effects. IL-4, IL-5, and IL-13 made in response to fungi can result in asthma (158, 159), and eosinophils, like neutrophils, can cause damage to tissue at the site of inflammation (80, 81). Moreover, early T_H2 cytokines such as IL-4 can dampen the T_H1 response and increase susceptibility to *Aspergillus* (160). Aside from the classic T_H2 cytokines, IL-25 is also emerging as a major player in the T_H2 pathway: IL-25 can control T_H2 memory cells (161), as it can drive (162), regulate (163), and amplify the T_H2 response (164, 165).

A third factor in the adaptive immune response to *Aspergillus fumigatus* is the recently discovered **T_H17** response. Though the signature cytokine of T_H17, IL-17A (also known simply as IL-17), was first discovered in 1995 (166), it wasn't until 2005 that researchers recognized that T_H17 was a wholly separate branch of adaptive immunity (167, 168). Since then T_H17 has become the focus of intense study, with many speculating that it serves as a bridge between the innate and adaptive immune responses (169). A wide array of microbes induces T_H17, and in response T_H17 T cells can produce and induce a wide array of cytokines (170). Similar to T_H2, T_H17 and T_H1 are reciprocally regulated (167, 171); also similar to T_H2, T_H17 has been linked to a number of immune diseases. In particular, T_H17 has been implicated in forms of autoimmunity

that were once considered T_H1 disorders (172). Likewise, many disorders that were once considered to be T_H2 mediated are now being reassessed. It has been found that IL-17 levels correlate with allergy severity (173) and are increased in human asthma (174, 175). In particular, IL-17 affects neutrophil recruitment during asthma and other inflammatory diseases (176), though it has also been shown that IL-17 can be a negative regulator of asthma in mice (39). In general, T_H17 may worsen inflammation by downregulating IDO and the T_{reg} response (177). That being said, the T_H17 response has demonstrated a number of protective effects (178) including defense against systemic candidiasis (179), and in the absence of a T_H1 response the T_H17 is often the most viable alternative (99). In particular, IL-17 can upregulate the expression of TLRs (180), and IL-22 – another T_H17 cytokine – is capable of upregulating the innate immune response (181-183).

Against *A. fumigatus* T_H17 plays a major role in deciding the composition of the immune response. Though it play a role in driving some anti-fungal components of the immune system (184-187), the T_H17 response's major role seems to be inhibiting T_H1 and T_{reg} function and preventing proper clearance of germinating conidia. IL-23 and IL-17 have both been shown to promote inflammation while simultaneously inhibiting fungal resistance (99, 188). Structural components of the *A. fumigatus* cell are largely responsible for this process: dectin-1 binding of these ligands induces a T_H17 – but not a T_H1 – response in dendritic cells (70, 134).

Finally, **regulatory T cells** (**T_{reg}**) play a central role in controlling the immune response to foreign microbes such *Af*. T_{reg} – which are maintained by the Foxp3 transcription factor (189, 190) – are capable of dampening inflammation caused by T_H1 (191, 192), T_H2 (193, 194), and T_H17 (190, 195, 196) CD4 T cells. Interestingly, T_{reg} have been shown to play a role in limiting inflammation during the immune response to

Aspergillus fumigatus. The regulatory response towards *Af* occurs early in the infection (94) and is most likely induced by components of the fungal cell wall binding TLR2 and dectin-1 (118). The reason for the induction of the regulatory response is not entirely clear, though at least one report suggests that limiting inflammation may aid in fungal clearance (197).

Recent reports have added extra depth to our understanding of T_{reg}, particularly the similar manner in which T_{reg} and T_H17 CD4 T cells arise and are regulated. The two cell types reciprocally develop (198) and Foxp3 and ROR-γτ – the primary transcription factor for T_H17 CD4 T cells – are mutually antagonistic (199, 200). In mice both cell types arise from naïve CD4 T cells exposed to TGF-β, though the presence of IL-10 skews differentiation towards T_{reg} while IL-6 drives the generation of T_H17 (201). Surprisingly, it has been recently demonstrated that regulatory T cells are capable of converting to T_H17 T cells under certain conditions (202, 203). The T_{reg}/T_H17 balance appears to be controlled by retinoic acid (204) which induces Treg formation (205) as well as IDO which blocks the conversion of T_{reg} to T_H17 (206, 207).

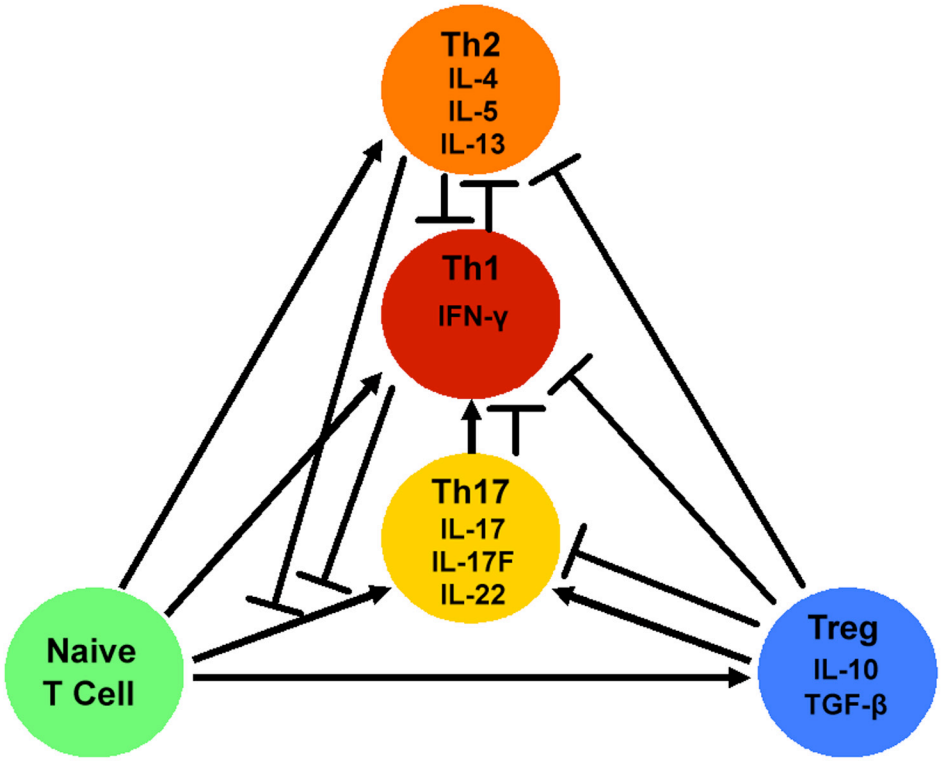

Figure 1-1. Development and regulation of the CD4 T cells. T_H1, T_H2, T_H17, and T_{reg} develop from naïve T cells (black arrows). Treg can further develop into T_H17 which can develop into T_H1. T_{reg} can inhibit the activity of all three effector cells types, and T_H1 and T_H2 are reciprocally regulated. T_H17 has been shown to inhibit T_H1 activity, and T_H1 and T_H2 CD4 T cells are capable of hindering T_H17 development but not activity. Also listed are major cytokines of each T_H type.

Hypothesis

We hypothesize that IL-17 plays a role in driving chronic allergic inflammation in the lung during repeated exposure to *Aspergillus fumigatus*. Thus, we believe that removal of IL-17 will abrogate inflammation after the initial T_H2-driven hypersensitivity response.

Study Objectives

1. To characterize the immune response to *Aspergillus fumigatus* conidia during the acute and chronic phases of inflammation
2. To determine the role of IL-17 during the hypersensitivity response to *Af* conidia.
3. To determine the role of IL-17 in pulmonary eosinophilia during the hypersensitivity response.

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Chapter 2
Methods

Mice. Wild-type (C57BL/6J) mice were obtained from the Jackson Laboratories (Bar Harbor, ME). IL-17$^{-/-}$ mice were a generous gift from Kate Eaton at the University of Michigan and have been described previously (1). Mice were housed under pathogen-free conditions in enclosed filter-topped cages. Clean food and water were given *ad libitum*. The mice were handled and maintained using microisolator techniques, with daily veterinarian monitoring. All studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

Aspergillus fumigatus. Strain ATCC 13073 was grown on Sabouraud dextrose agar (SDA) (Difco) for 14 days. Conidia were harvested by washing plates with sterile phosphate-buffered saline (pH 7.4) with 0.1% Tween 80 (PBS-Tween), followed by filtration of the suspension through two layers of sterile gauze to remove hyphae. Conidia were washed in PBS-Tween, counted with a hemacytometer, diluted to 10^8 spores/ml in sterile PBS-Tween, and stored at 4°C for up to 4 months.

Intranasal Challenge. Mice were injected intraperitoneally with anesthetic (.4 mg/ml Xylazine (Lloyd Laboratories, Shenandoah, IA) + 10 mg/ml ketamine (Fort Dodge, Fort Dodge, IA) in sterile saline (Hospira Inc., Lake Forest, IL)) based on weight. Following

sedation, 20 µl of *Aspergillus fumigatus* suspension was applied intranasally for a total of 2×10^6 conidia per mouse per challenge.

Lung Histology. Lungs were fixed by inflation with 10% neutral buffered formalin (Sigma). After paraffin embedding, 5-µm sections were cut and stained with hematoxylin and eosin or periodic acid-Schiff (PAS) to detect mucus (McClinchey Histology Lab, Stockbridge, MI).

Lung Digest for Whole Lung Leukocyte Enrichment. Lungs from each mouse were excised, washed in PBS, minced, and digested enzymatically for 30 minutes in 15 ml/lung of digestion buffer (RPMI, 5% fetal calf serum, 1 mg/ml collagenase (Boehringer Mannheim Biochemical, Chicago, IL), and 30 µg/ml DNase (Sigma Chemical Co., St. Louis, MO)) as previously described (2). After erythrocyte lysis using NH_4Cl buffer, cells were washed, resuspended in complete media, and centrifuged for 30 minutes at 2000 x *g* in presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Total lung leukocyte numbers were assessed in the presence of trypan blue using a hemocytometer.

Lymph Nodes. The draining mediastinal lymph node was excised from the thoracic cavity, placed in 1ml of RPMI (5% fetal calf serum) in a six-well plate (Corning Incorporated, Corning, NY), and ground with the flat edge of a 1ml syringe. The cell suspension was then transferred through a 100 micron screen and washed with 2 ml of RPMI. After erythrocyte lysis using NH_4Cl buffer, cells were washed, resuspended in complete media, and counted with a hemocytometer prior to analysis.

Blood Collection and Serum Separation. Blood was collected by retro-orbital vein bleed at the time of harvest. Serum was collected after centrifugation for 2 min at 6000 rpm in Microtainer tubes (BD Pharmingen). Remaining blood was collected in K2E tubes (BD PharMingen), measured for volume and centrifuged for 10 min at 10,000 RPM. After erythrocyte lysis using NH_4Cl buffer, cells were washed, resuspended in complete media, and counted with a hemocytometer prior to analysis.

Bone Marrow Collection. During harvest, leg shanks were removed from mice and stripped of tissue using a standard razor. Marrow was flushed from femurs and tibias using complete media in a 10cc syringe (BD Pharmingen) tipped with a $25^{5/8}$g needle (BD Pharmingen). Cells were drawn into a 10cc syringe and expressed through a 21g needle to disperse the cells. After erythrocyte lysis using NH_4Cl buffer, cells were washed and resuspended in complete media prior to analysis.

Viable Conidia. Following digestion of the lung, a 100 µl aliquot was taken for analysis. The sample was serially diluted and 10 µl of undiluted, 1:10, and 1:100 diluted digest was plated on SDA media in duplicate. Hyphal foci were counted to determine the number of colony forming units, and the number from each duplicate was averaged to yield a total number of viable conidia per 10 µl of digest per mouse.

Flow Cytometry for Cell Surface Molecules. Cells were washed and resuspended at a concentration of 10^6 cells/25 µl FA buffer (Difco) + 0.1% NaN_3, Fc receptors were blocked by the addition of unlabeled anti-CD16/32 (Fc block; BD Pharmingen, San

Diego, CA). After Fc receptor blocking, $0.5\text{-}1\times10^6$ cells were stained in a final volume of 50 µl in 96-well round-bottom plates (Corning Incorporated, Corning, NY) for 30 minutes at 4°C. Cells were washed twice with FA buffer, resuspended in 120 µl of 4% formalin (Sigma), and transferred to 12 x 75-mm^2 polystyrene tubes (Becton Dickinson, Franklin Lakes, NJ). A minimum of 100,000 events were acquired on a FACSCanto flow cytometer (BD PharMingen) using Cell-Quest software (BD Pharmingen). Acquired data was analyzed with FlowJo software (Tree Star, Stanford, CA).

Flow Cytometric Leukocyte Differential Analysis. Lymphocytes, neutrophils, and eosinophils were distinguished by virtue of unique combinations of FSC and SSC profiles and CD11c and Gr1 surface expression, as previously described (3). Mature eosinophils were separated from background cells by gating for siglec F^+ forward-scatter^{low} populations. Non-granulocytic myeloid cells were separated into distinct groups by first gating out eosinophils and leukocytes using forward and side scatter, then removing neutrophils and immature eosinophils by gating out GR1^{high} CD11c^{low} cells. The remaining cell populations were then divided into four subgroups for further analysis. Alternatively activated macrophages (AAMΦ) staining consisted of IL-5R and CD206 cross staining (4). Basophils were identified as FSC^{low} SSC^{low} Gr1– CD11c– CD3– CD19– CD49b+ FcεRI+ cells as described previously (5). For eosinophil maturation, lung leukocytes were stained and FSC^{low} SSC$^{mid\text{-}high}$ cells were examined for IL-5R and CCR3 expression. IL-5R^{high} eosinophils were considered naïve while IL-5R^{low} eosinophils were considered mature (6). Graphs were made from multiple pooled experiments where $n \geq 6$.

Intracellular Flow Cytometry. Cells were first stimulated for six hours with PMA (50ng/ml) and Ionomycin (1μg/ml) then stained for CD4 and CD45 (cytokines) or were stained for CD4, CD45, and CD25 (for T_{reg}) without prior stimulation. Cells were first stained for surface markers and washed twice with FA buffer as described above. Cells were then resuspended for 30 minutes in 50 μl of Cytofix/Cytoperm^{TM} (BD PharMingen) for cytokine staining or Fixation/Permeablization solution (eBioscience) for Foxp3 staining. Cells were then washed twice with permeablization solution and stained with fluorescently labeled antibodies for IFN-γ, IL-4, IL-10, IL-17 (BD PharMingen) or Foxp3 (eBioscience). Cells were washed twice more with permeablization solution, resuspended in 125 μl of FA buffer, and transferred to 12 x 75-mm^2 polystyrene tubes for analysis.

Morphological Leukocyte Differential Analysis. Macrophages, neutrophils, lymphocytes, and eosinophils were visualized by standard morphological criteria in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides (Shandon Cytospin, Pittsburgh, PA). For Wright-Giemsa staining, the slides were fixed for 2 min with a one-step, methanol-based Wright-Giemsa stain (Harleco; EM Diagnostics, Gibbstown, NJ) followed by steps 2 and 3 of the Diff-Quik whole-blood stain kit (Diff-Quik, Baxter Scientific, Miami, FL). A total of 200 to 300 cells were counted from randomly chosen high-power microscope fields for each sample.

RNA Isolation and cDNA Generation. Following lung leukocyte enrichment or bone marrow isolation, cells were resuspended in 2 ml of Trizol (Invitrogen). Cells were incubated for 5 min at room temperature then combined with 600μl of chloroform and

shaken for one minute. Following a 3 min incubation at room temperature, samples were spun for 15 min at 13,000 RPM at 4^{o} C and the resultant supernatant transferred to DNAse/RNase free tubes (Corning). Equal volumes of isopropanol were added, and the samples mixed gently prior to a 20 min incubation at RT. Samples were spun for 15 min at 13,000 RPM at 4^{o} C, the supernatant decanted, 1 ml of 80% ethanol added, and spun again for 10 minutes at 13,000 RPM at 4^{o} C. The supernatant was again decanted and the pellet allowed to air dry. 100-600ul DEPC water was added to each pellet depending on the size of the pellet and contains 1ul/ml RNAasin. A primer cocktail (Promega AccessRT kit) was then added to10ul of RNA samples at 0.1ug/ul (total RNA 1ug/reaction) and run on a thermocycler (Applied Biosystems) as per the manufacturers instructions.

qPCR. cDNA was mixed with SYBR (Applied Biosystems) and GATA-1 primers (Integrated DNA Technologies) as per the manufacturers instructions. GATA-1 primers: FWD 5'-GCCTGCCATTGGCCCCTTGT-3' and REV 5'-CCTGTCCTGTCCCTCCGCCA-3'. Samples were run on a 7300 Real Time System (Applied Biosystems) and analyzed using 7300 system SDS Software (Applied Biosystems). Quantitation was via $2^{-\Delta\Delta CT}$.

$CD4^{+}$ T Cell Enrichment. $CD4^{+}$ T cells were enriched from splenocyte populations and from combined lung and LDLN populations via magnetic-activated cell sorting (MACS). For MACS, cell suspensions were stained with CD4 (L3T4) Microbeads (Miltenyi Biotec, Auburn, CA) and enriched via positive selection on a MidiMACS separator (Miltenyi Biotec). Enriched populations were >85% pure as assessed by flow cytometry.

Adoptive Transfer. Enriched $CD4^+$ T cells were suspended in PBS at $0.8\text{-}1\times10^7$/ml and 1ml of cell suspension was adoptively transferred to naïve recipient mice via lateral tail vein injection.

Statistical Analyses. All values are reported as mean ± standard error of the mean unless otherwise noted. 3-6 independent experiments were pooled to generate the mean unless otherwise noted. Differences between two groups were evaluated with a two-tailed Student's *t* test; $p<0.05$ were considered statistically significant. Correlation between cell types and conidia clearance was determined using Ordinary Least Squares Regression analysis. Slope, y-intercept, R^2 value, and p-value were determined for each data set. $p<0.05$ were considered statistically significant.

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Chapter 3
Characterizing the Hypersensitivity Response

Introduction

Aspergillus fumigatus is a common mold and the focus of extensive research due to its ubiquitous nature. *A. fumigatus* disseminates via conidia – hydrophobic spores – which bud off fungal hyphae and are distribute through the air. Once the conidia reach a warm, moist environment such as the lungs, they lose their hydrophobic properties and begin to germinate (1). Following conidial swelling and germ tube extension, the fungus develops invasive hyphae (2) and the cycle repeats itself.

Conidia are readily and frequently inhaled into airways at a rate of several thousand a day (3), and pulmonary exposure to large doses is not uncommon (4). Though *A. fumigatus* can pose a serious threat to immunocompromised individuals, even relatively large doses of conidia pose little danger to immunocompetent hosts as the immune system is capable of clearing conidia from the lungs before the fungus can take hold. The immune response to inhaled *Aspergillus fumigatus* is characterized by a complex interaction between the innate and adaptive immune response, both of which are quickly activated by the presence of fungi (5, 6). Macrophages and neutrophils efficiently neutralize low doses of germinating conidia in the lung (7, 8), and resting conidia are cleared from the lung by macrophages without the induction of an inflammatory response (9, 10). However, exposure to large numbers of viable conidia results in a complex collection of cell types, pathways, and cytokines in response to the fungus. Macrophages

(11), neutrophils (12), dendritic cells (13), monocytes (14), eosinophils (15), and natural killer cells (16) all play a role in host defense, as do aspects of the complement system (17).

No less important is the adaptive immune response. The Th1 adaptive immune response is important in defense against invasive *A. fumigatus*, with progressive invasion of infected tissue associated with decreased IFN-γ production and poor T cell proliferation in individuals with compromised immune systems (18). In addition, inhibition of IFN-γ or TNF-α enhances fungal invasion (19, 20). The T_H1 response is the primary reaction to large doses of inhaled conidia: following a thirty second inhalation of aerosolized *Af* spores, the Th1 response in mice peaks one week after initial infection resulting in the expression of TNF-α, IFN-γ, IL-12, and IL-18 (21).

Chronic exposure to *A. fumigatus* conidia is common but little is known about the host response under such conditions. Much of the host response literature for *A. fumigatus* has focused on the response to acute conidia exposure, usually in immunocompromised animals, due to the clinical impact of invasive aspergillosis. Other studies have examined the hypersensitivity response to conidia or *Aspergillus* antigen extracts in mice previously sensitized systemically to *Aspergillus* (22-24). Our laboratory has previously reported that two intranasal exposures to *A. fumigatus* conidia without a sensitizing event do not result in an adaptive immune response (25, 26). However, repeated pulmonary exposure to *A. fumigatus* conidia results in a strong T_H2-mediated hypersensitivity response consisting of eosinophilia, mucus hyper-secretion, IgE secretion, and cytokine production (27).

Our initial objective was to determine if additional challenges – four challenges and eight challenges – would stimulate an adaptive response and, if so, analyze the

development of the response in the lungs, including pulmonary inflammation, CD4 T cell polarization and conidial clearance. Here we show that the reaction to repeated pulmonary exposure is a dynamic adaptive immune response characterized not only by a T_H2 hypersensitivity response, but early regulatory T cell activity and a mounting T_H17 reaction. Moreover, repeated exposure does not result in increased mortality, and hypersensitivity does not significantly enhance clearance of *A. fumigatus* from the lung.

Aims

1. Determine the cellular composition during acute and chronic *Af* conidia-driven inflammation
2. Determine the adaptive response to chronic pulmonary exposure to *Af* conidia
3. Determine whether hypersensitivity hinders the immune system's capacity to clear conidia from the lungs

Results

Chronic pulmonary exposure to *Aspergillus fumigatus* conidia results in pulmonary inflammation.

To determine the host response to chronic *Af* conidia challenge, mice were challenged with zero, two, four, or eight weekly inoculums of 2×10^6 live *Aspergillus fumigatus* conidia and harvested 24 hours after final challenge. The survival rate over the course of 8 weeks was 100% (Fig. 3-1A). Lungs of challenged mice were excised, minced, and digested to quantify both the pulmonary inflammatory response and levels of viable conidia. Small aliquots were plated on SDA in a ten fold dilution series and mold colonies counted. We found that 24 hours after intranasal challenge low numbers of

viable conidia could still be detected in the lung (approximately 1%, Fig. 1B) and potentially serve as a source of continued antigen or inflammatory stimulus. Two challenges stimulated the influx of a small number of leukocytes into the lungs, the most numerous of which were neutrophils (Fig. 3-3B). Repeated challenges gradually augmented pulmonary inflammation but did not significantly alter the efficacy of clearance, as mice exposed eight times to conidia still had levels in the lungs similar to those that had been challenged two or four times with conidia (Figs. 3-3A & 3-1, respectively). Repeated challenge also caused an expansion of the lymphocyte populations in the draining lymph node (mediastinal lymph node), most notably between the second and fourth challenge (Fig. 3-3A). Thus, chronic exposure promoted development of an inflammatory response but did not enhance or impede conidial clearance.

We next examined the composition of the inflammatory response during chronic *Af* exposure. Whole lungs from mice challenged zero, two, four, and eight times were examined histologically in H&E and PAS stained sections. Following two challenges, granulocytic infiltrates were evident around the airways, with minimal changes in goblet cells (PAS+, Fig. 3-2A). After four challenges, the inflammatory infiltrate had markedly increased, including large numbers of eosinophils, and goblet cell metaplasia was evident (as indicated by large numbers of PAS+ cells in the airway epithelium). In addition, at this time point, there were a large number of multinucleated giant cells in the leukocyte infiltrates (Fig. 3-2B). By eight challenges, the inflammatory response had decreased, including fewer multinucleated giant and metaplastic goblet cells. Thus, chronic exposure to *Af* conidia induced an increasingly stronger pulmonary inflammatory response through

four conidia challenges that leveled off or began to resolve even in the face of continued *Af* challenges.

To further analyze the kinetics of the pulmonary inflammatory response, leukocytes were isolated from enzymatically dispersed lungs of challenged mice. Consistent with our histological analysis, we observed a significant difference between the number of cells found in untreated mice and those challenged twice (Fig. 3-3A). The influx of cells in the lung was almost exclusively granulocytes (Fig. 3-3B), indicative of primarily an innate response. However, mice challenged four times with conidia had a six fold increase in the number of recruited leukocytes in the lungs compared to two challenges. The inflammatory infiltrate was comprised of various myeloid cells and lymphocytes, with eosinophils being the dominant cell type. CD4 numbers did not increase significantly following two challenges, but four challenges yielded a five-fold increase in the total number of CD4 T cells (Fig. 3-3B). There was a similar influx of B cells into the lungs; however, CD8 T cell numbers remained relatively low. After eight challenges, the total number of myeloid cells was lower while CD4 T and B cells remained elevated. In the draining lymph nodes, there was an expansion of the CD4 T and B cell populations between two and four challenges that persisted through eight challenges (Fig. 3-3B). Thus, the influx of eosinophils, together with increased numbers of CD4 T cells and B cells in the lungs and mediastinal nodes, suggested the potential development of a Th2 response to *Af* conidia challenge during repeated exposure that wanes as the number of exposures continues.

Precursor cells, macrophages, and dendritic cell populations expand in response to conidia challenge.

Our next objective was to analyze the kinetics of the non-granulocyte myeloid populations during chronic *Af* conidia exposure. This heterogeneous population includes antigen-presenting cells, effector cells and regulatory myeloid cells. $CD45^+$ cells isolated from the lung were stained with anti-Gr1 and anti-CD11c, and lymphocytes and mature eosinophils were removed from the cell pool by excluding cells with a low side-scatter profile (Fig. 3-4A). Cells expressing high levels of Gr1 but low levels of CD11c were gated out to remove neutrophils and immature eosinophils. The remaining cell population clustered into four distinct subpopulations, which we called Groups I-IV (Fig. A2-2).

Each of these subpopulations was analyzed for expression of CD16/32, Ly6C, CD80, as well as its autofluorescence and forward scatter/side scatter profile. CD16/32 (FcR) expression was found on all groups, indicating all groups are of myeloid lineage. Ly6C, a marker of late myeloid precursors, was highest in Group III. This, along with the large size-distribution of the subpopulation and lack of autofluorescence indicated differentiating precursor cells (Fig. 3-4B). Likewise, the lack of Ly6C expression, high autofluorescence, and expression of CD80 – a marker of maturity – indicated that Group II was composed of macrophages. Group I was composed of dendritic cells, as supported by the CD80 expression, lack of autofluorescence, and moderate Ly6C expression. We could not specifically define Group IV by cell sub-type, only by surface phenotype. Group IV cells expressed relatively high levels of Ly6C, expressed no CD80 and were moderately autofluorescent. These cells are most likely macrophage precursors based on their scatter profile as well as their moderate autofluorescence. None of the populations contained alternatively activated macrophages: all stained negative for IL-5Rα and CD206 (Fig. 3-5). Likewise, all of the subgroups stained negative for pan NK markers.

There was a nearly five-fold increase in dendritic cell (Group I) numbers between two challenges and four challenges, with total numbers dropping significantly by eight challenges (Fig. 3-6). Group III numbers showed a similar pattern, while macrophage (Group II) numbers doubled between two and four challenges and stayed high over the course of exposure. Levels of Group IV cells remained unchanged over time. Following exposure to conidia, the dendritic cells increased expression of CD11b, indicating that new cells were coming in to the lung (28). Similarly, the macrophage population had increased CD11b and decreased siglec F expression over time, again indicating an influx of new cells. Group IV non-granulocytic myeloid cells (NGMC), whose numbers did not change over time, increased CD11b expression concurrent with the development of peak inflammation. This reinforces the idea that Group IV is composed of precursor cells transitioning into a more mature cell type. Since the 'newness' of the cell population changes but total levels remain the same, the Group IV is likely a transitory population for monocytes on their way to becoming more mature myeloid cells.

Chronic Af challenges result in an influx of antigen presenting cells into the lungs.

The numbers of antigen presenting cells frequently increases during inflammation, so we wished to see if this was true in our model. The generation of Th2 responses has recently been linked to basophils (29), so we asked whether the number of basophils in the lungs increased concomitant with the development of the Th2 response. Basophils were identified using antibodies against CD49b and FcεRI as described previously (30) (Fig. 3-7A). Consistent with their potential role in the development of the Th2 component of the response to *Af* conidia, the numbers of basophils in the lungs increased at two challenges (prior to the appearance of IL-4+ CD4 T cells, Fig. 3-7B) and

continued to increase between two and four challenges, the period when the Th2 response fully developed.

CD4 T cells in the lung and lymph nodes show different activation and function after two, four, and eight challenges.

T cells are essential to inflammatory response resulting from four intranasal challenges; depletion of CD4 T cells completely abrogates hypersensitivity and eosinophil influx (27). We wished to determine whether repeated exposure resulted in CD4 T cell activation. CD4 T cells were identified in both the lung and lymph node by CD45 and CD4 staining. Cells were additionally stained with fluorescently labeled antibodies specific for CD44 and CD69, both of which are markers of T cell activation. CD4 T cells that were $CD44^{high}$ and $CD69^{+}$ were counted as activated. In the lung, just two exposures to conidia resulted in an increase in the percentage of CD4 T cells that were activated (Fig. 3-8A). The percentage of activated CD4 T cells did not significantly increase between two and four challenges, but the increase in total numbers of activated cells was significant (Fig. 3-8B). Between the four-challenge and eight-challenge time points the percentage and total number of activated CD4 T cells increased slightly but not significantly, supporting the idea of a sustained adaptive immune response over the course of exposure. In the lymph node, the percentage of activated T cells remains constant throughout the course of exposure. This is consistent with previous reports that have found that CD4 T cells activate in the lungs but not the lymph nodes in response to fungi (31). Therefore, pulmonary exposure to *A. fumigatus* results in T cell activation in the lungs but not the lymph node. However, it is unlikely that activation alone results in

hypersensitivity as CD4 T cell activation can be observed after two challenges when there is no hypersensitivity response.

To determine whether a lack of regulatory cells is responsible for hypersensitivity we determined the percentage of T_{REG} within the pool of CD4 T cells. Similarly to T cell activation, the percentage of CD4 T cells with a regulatory phenotype ($CD25^+$ $Foxp3^+$) was constant in the lung-associated lymph nodes but dynamic in the lungs. In the lungs, the percentage of cells expressing a T_{REG} phenotype increases significantly in mice challenged twice and then receded during hypersensitivity (Fig. 3-9). The sudden increase in regulatory T cells during the initial exposure may explain the relative lack of an inflammatory response seen after two challenges. Interestingly, the total number of CD4 T cells does not increase significantly in mice challenged twice versus untreated mice, while the percentage of T_{REG} in the lung increases nearly 50%. This indicates that the small number of CD4 T cells entering the lungs during the first two challenges (Fig. 3-9) are almost exclusively T_{REG} or that some of the endogenous T cells in the lungs are being converted to regulatory function in response to low doses of *A. fumigatus* conidia. Thus, the initial exposure to the fungus induces tolerance rather than inflammation, but this response is eventually replaced by an aggressive inflammatory reaction. Though the total number of regulatory T cells increases during hypersensitivity as has been reported previously (32), the Effector:Treg ratio begins to grow as expansion of the effector T cell pool outpaces that of regulatory T cells.

These observations logically led us to examine the cytokine production over the course of exposure. We examined CD4 T cells cytokine production by flow cytometry to determine which cytokines were being expressed during each phase of the response.

During the initial response to conidia challenge there is a burst of IL-10 production, supporting the conclusion that early inoculums of *A. fumigatus* drive tolerance (Fig. 3-10A). Predictably, production of IL-4 spikes after four challenges when there is acute hypersensitivity and a significant influx of eosinophils, and there is a corresponding drop in the percentage of IL-10-producing CD4 T cells. However, we were surprised by the relative lack of change in IFN-γ production at all time points as well as the significant increase in IL-17 production that accompanied hypersensitivity. While the total number of IFN-γ producing cells did increase, the percentage expressing the cytokine stayed almost constant through four challenges. It is not until 8 challenges that a significant increase in the percentage of CD4 T cells expressing IFN-γ is seen. In sharp contrast to this, there is constant increase in the percentage of CD4 T cells expressing IL-17 over the course of exposure, and between two challenges and four challenges there is a ten-fold jump in the number of these cells (Fig. 3-10A). Production of IL-17 is high after four challenges and remains high at the late time point suggesting that IL-17 and the T_H17 response may play a role in driving chronic inflammation. This is reinforced by the presence of IFN-γ/IL-17 double-producing CD4 T cells. While the number of IFN-γ/IL-10 and IL-10/IL-17 producers rises slightly with multiple challenges, there is a striking and significant increase in the number of IFN-γ/IL-17 producers between two and four challenges (Fig. 3-10B). IFN-γ/IL-17 double-positive CD4 T cells are associated with inflammation in a number of autoimmune models (33-35), and their presence suggests a role for IL-17 in maintaining an inflammatory response to *A. fumigatus*.

Precursor levels are associated with decreased fungal clearance during the hypersensitivity response.

When measuring the number of viable conidia in the lung, we noticed that the number measured in each lung following four challenges was highly variable. While other time points had relatively tight clustering of data points, the counts following four challenges varied from no detectable conidia to 1.5×10^5 viable spores. Given the wide distribution of still-viable conidia, we wondered if there was a positive correlation between a lack of fungal clearance and a particular cell type. We therefore generated an XY scatter plot for each cell type showing both the number of conidia detectable in each lung versus the number of that cell. Each mouse is represented as a single point within the graph. Regression analysis was then used to determine whether there was a positive or negative correlation between each cell type and the number of detectable conidia and whether that correlation was significant (Fig. 3-11).

Most cell types showed little correlation with a lack of clearance. Neutrophil, dendritic cell, macrophage, and CD4 T cell numbers had virtually no relation to the number of conidia that could be isolated from the lung, while CD8 T cells and B cells showed a negative correlation that did not quite reach statistical significance. Similarly, there was a modest correlation between conidia and eosinophils that also did not reach statistical significance ($p = .063$). However, there was a strong correlation between Group III myeloid precursors and fungal levels ($p = .036$), which is unsurprising given the central role of monocytes in the CD4 T cell response during fungal infection (14). Most surprising though was Group IV, which maintained a relatively constant number of cells over the course of exposure (Fig. 3-11) and had an extremely high correlation with the number of viable conidia ($p = .002$). Like the number of conidia, the number of Group IV cells varied dramatically at the four challenge time point. It is unclear though if the correlation is causative. It is plausible that a cell population would be increased in

response to lingering conidia rather than being the source of poor clearance. However, these data do indicate which cell types are most likely playing a central role – either positive or negative – in defense and clearance.

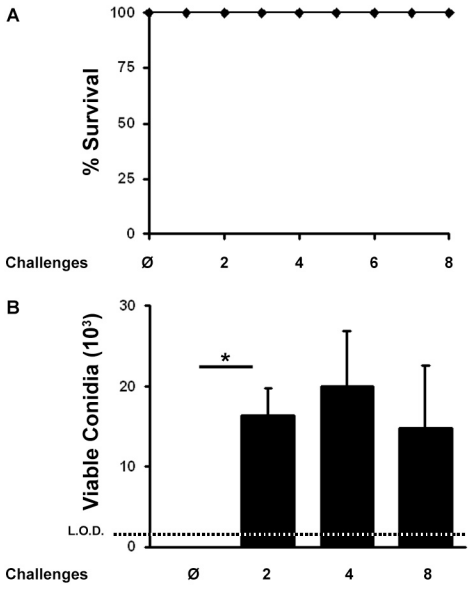

Figure 3-1. Conidia introduced via weekly intranasal challenge do not induce death and can be detected twenty-four hours after exposure. (A) Mice were challenged intranasally with 2 x10^6 live conidia once a week for eight weeks. Shown is the survival of all mice in the cohort over the course of eight weeks. **(B)** Following digestion of the lungs and aliquot of digest was serially diluted and plated on SDA media and hyphal foci were counted. The graph shows the average viable conidia per lung detected 24 hours after zero, two, four, and eight challenges. Starred bars indicate $p < .05$.

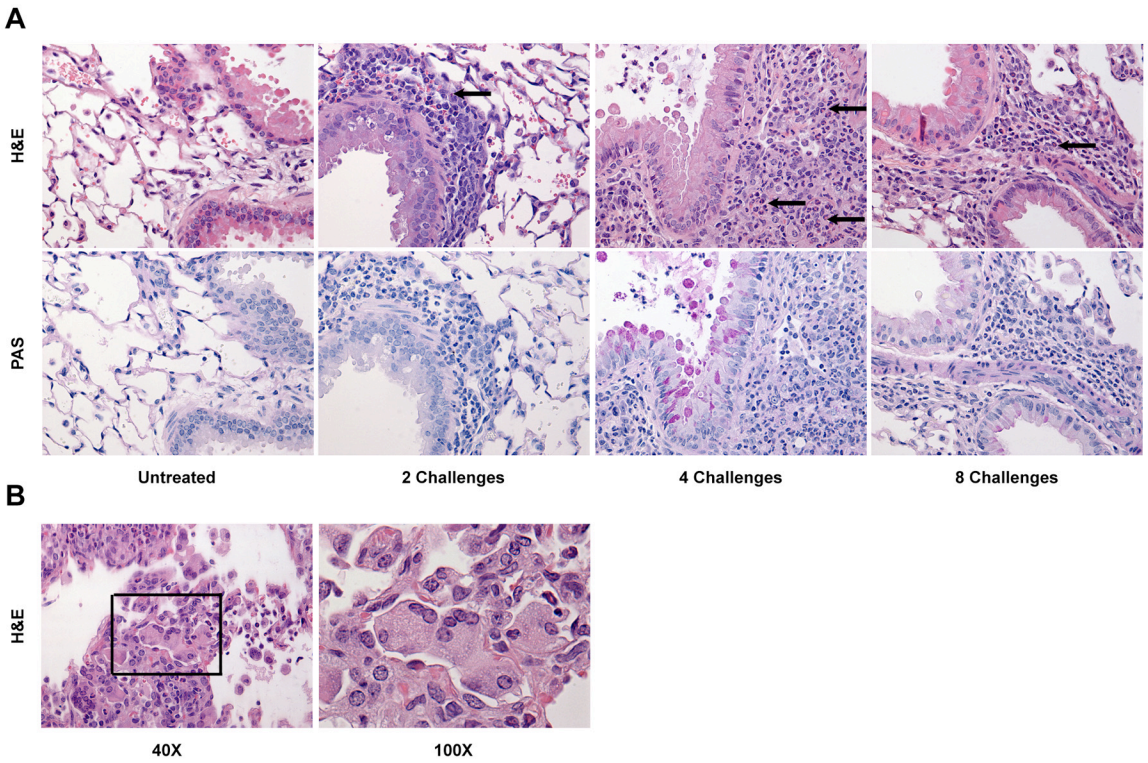

Figure 3-2. Hypersensitivity in the lung is accompanied by cellular infiltrate around the airways and mucus hyper-secretion. Lungs from mice challenged zero, two, four, or eight times were fixed in formalin and embedded in paraffin blocks. Histological slices were then stained with H&E or PAS. **(A)** Slides were examined at 40X magnification. Arrows indicate eosinophil and neutrophil infiltrate. In PAS staining, bright purple cells indicate mucus secretion. **(B)** In mice challenged four times with conidia, multinucleated macrophages were observed in the lungs.

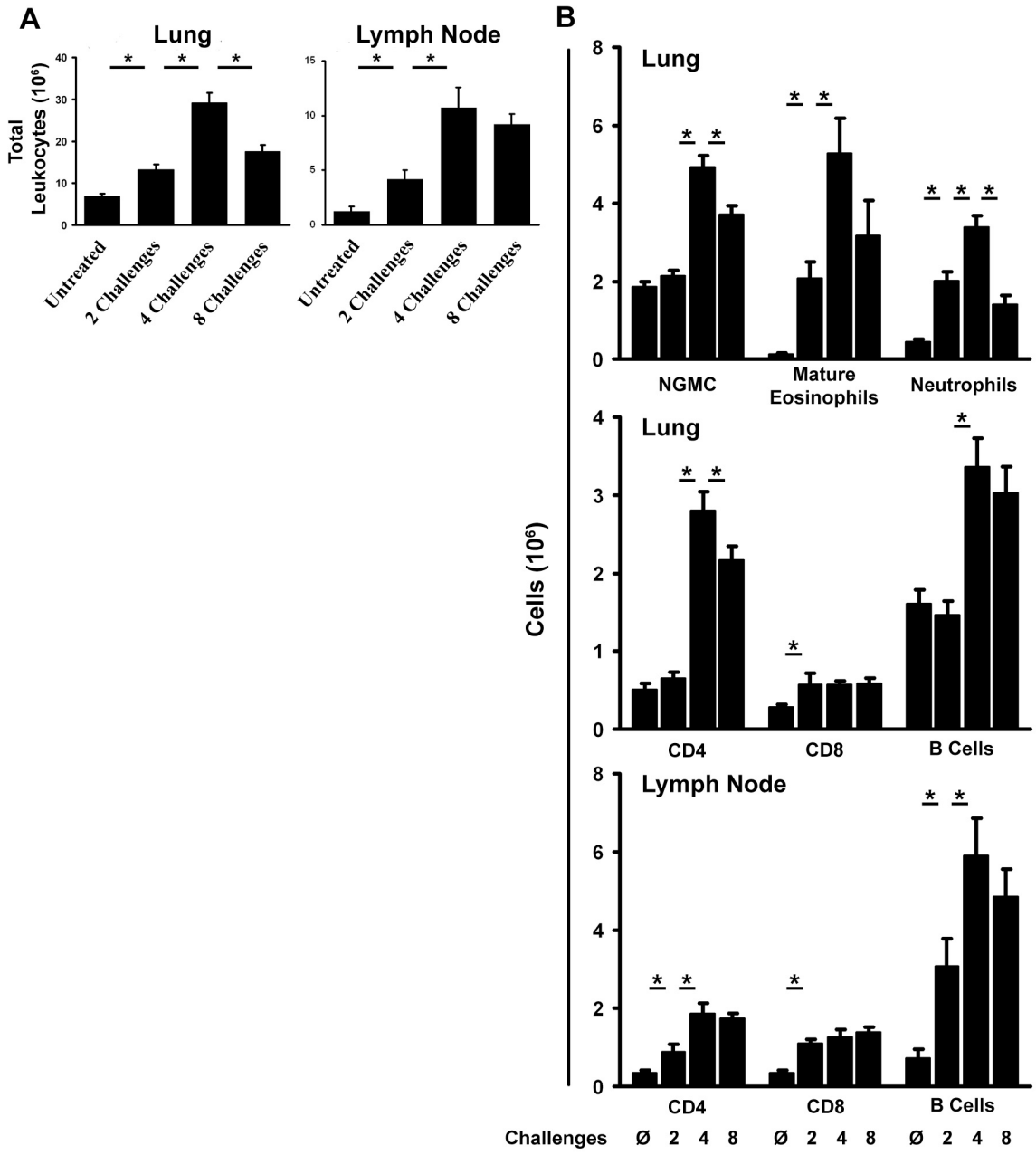

Figure 3-3. Repeated intranasal exposure to *Aspergillus fumigatus* conidia generates a hypersensitivity response in C57BL/6 mice. (A) Total cells in both lungs and lymph node was calculated by counting total live cells in both tissue types using trypan exclusion and multiplying by the percentage of cells that were $CD45^{+}$. **(B)** Cell populations were identified as described in Figure A-1. The non-granulocytic myeloid cell (NGMC) population consists of the total numbers pooled from Groups I, II, III, and IV. All bars represent the average of at least four separate experiments.

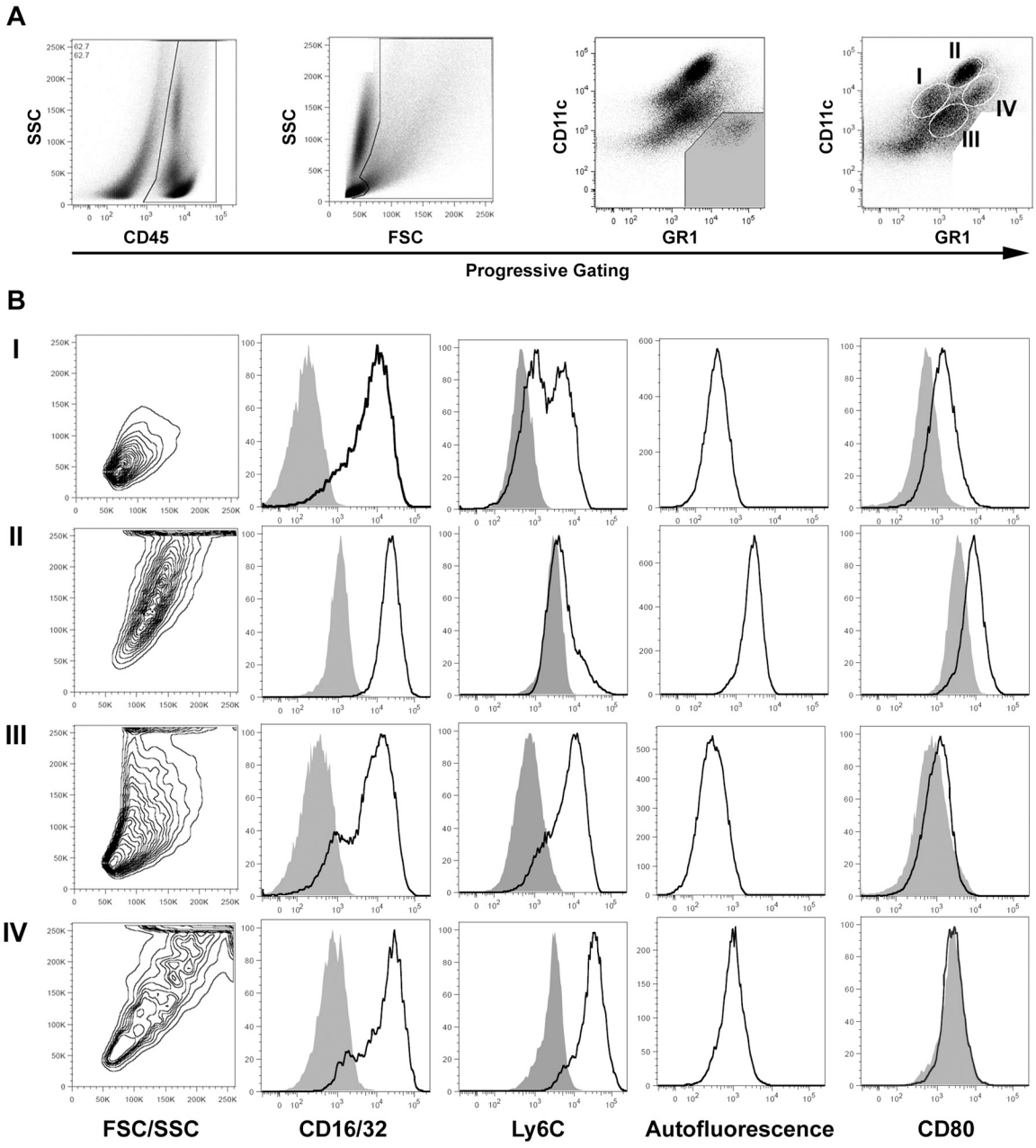

Figure 3-4. Subsets of myeloid cells within the lung have distinct expression patterns of cell surface markers. (A) Live cells from the lung were stained with a spectrum of fluorescently labeled antibodies and analyzed using FLOW cytometry. $CD45^{+}$ cells were first gated, and forward-scatter/side-scatter analysis was used to separate myeloid cells from lymphocytes (FSC^{low} SSC^{low}) and eosinophils (FSC^{low} $SSC^{mid\text{-}high}$). Neutrophils ($CD11c^{low}$ $GR1^{high}$) were then removed. The remaining cell population clusters into four distinct subpopulations labeled as Group I, II, III, and IV. **(B)** Subpopulations were identified by examining FSC/SSC profile, surface marker expression, and autofluorescence. Black lines indicate expression; gray peaks indicate IgG controls.

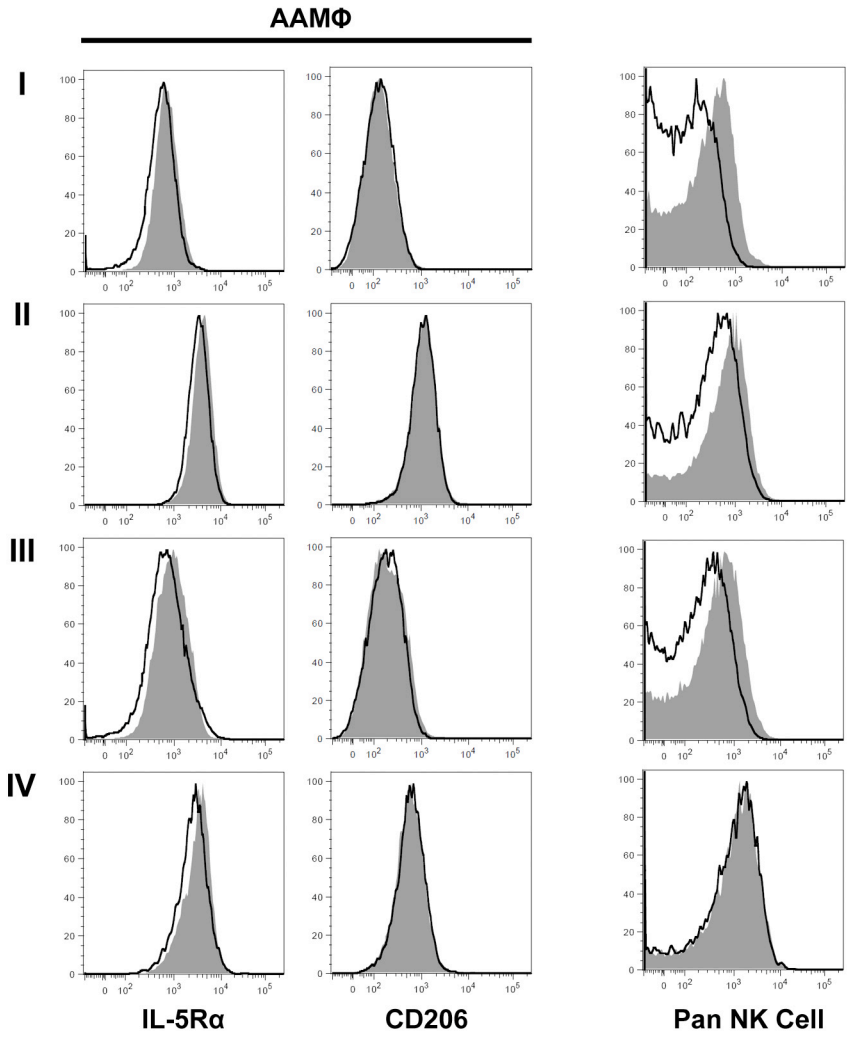

Figure 3-5. Subpopulations do not consist of alternatively activated macrophages or natural killer cells. Each subpopulation was examined for expression of IL-5Rα and CD206 or expression of pan NK surface markers. Black lines indicate expression; gray peaks indicate IgG controls.

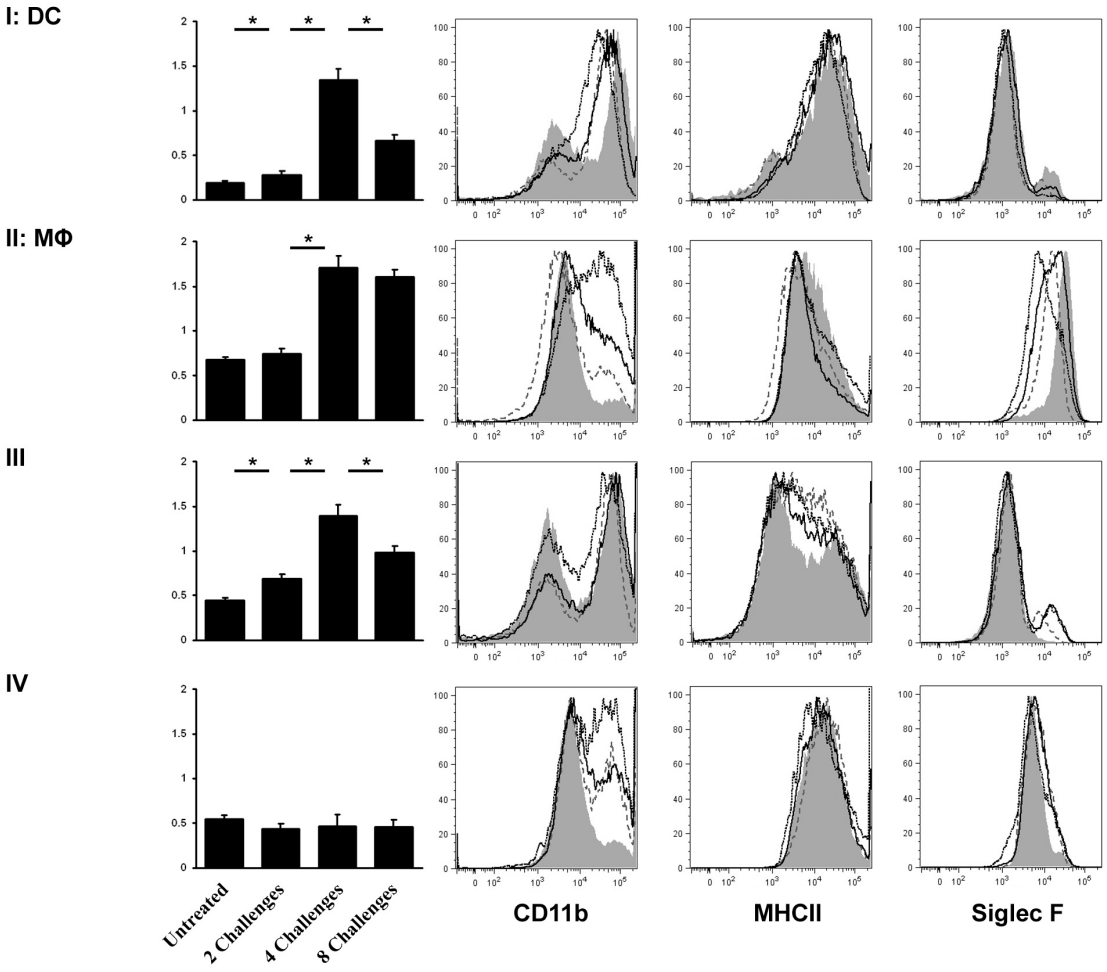

Figure 3-6. New cells are recruited to the lung during peak inflammation. Each subpopulation was analyzed for total cell number after each challenge as well as expression of CD11b, MHCII, and siglec F at each time point (Untreated = gray background, 2 Challenges = gray dashed line, 4 Challenges = black dashed line, 8 Challenges = solid black line).

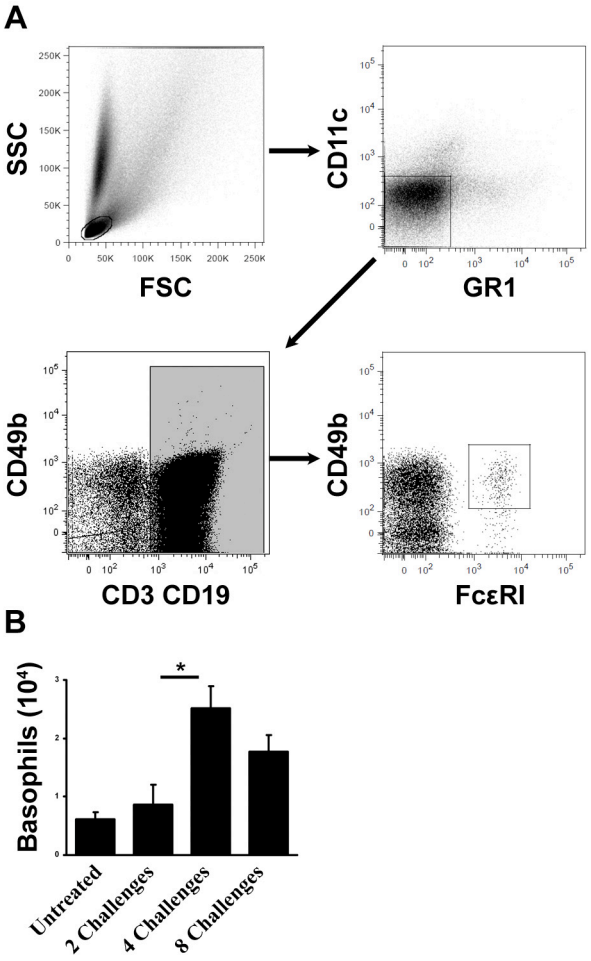

Fig. 3-7. Basophil numbers significantly increase during the hypersensitivity response. (A) Basophils were identified by gating for FSC^{low} SSC^{low} Gr1- CD11c- cells, then gating out T cells and B cells (CD3+ and CD19+ cells). The remaining cells that were CD49b+ FcεRI+ were classified as basophils. **(B)** The mean number of basophils following zero, two, four, and eight challenges is shown. Starred bars indicate $p < .05$.

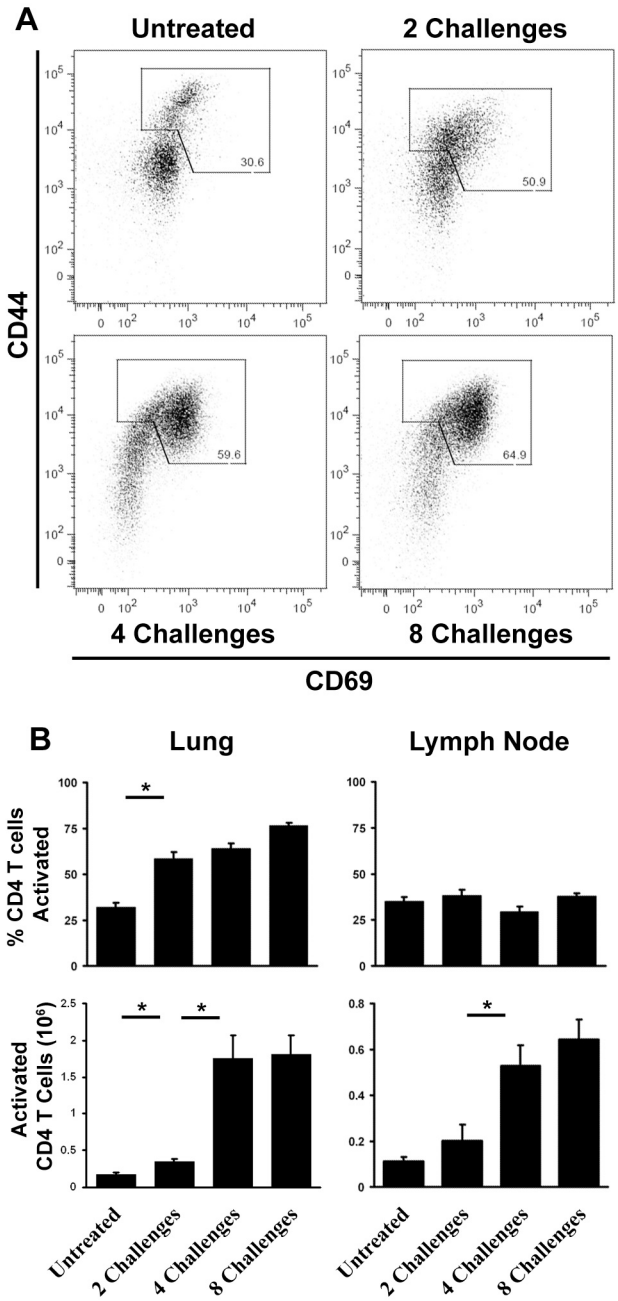

Figure 3-8. CD4 T cells are activated in response to *A. fumigatus* conidia in the lung but not the lymph node. (A) Lung cells were stained antibodies for CD45, CD4, CD44, and CD69. CD4 T cells were isolated via gating and those that were $CD44^{High}$ $CD69^{+}$ (FLOW gate) were counted as activated. Each time point is a representative concatenation of three samples. **(B)** Both the percentage and total number of activated CD4 T cells in the lung and lymph nodes was calculated for each mouse and averaged for each time point. $n \geq 6$ for each time point.

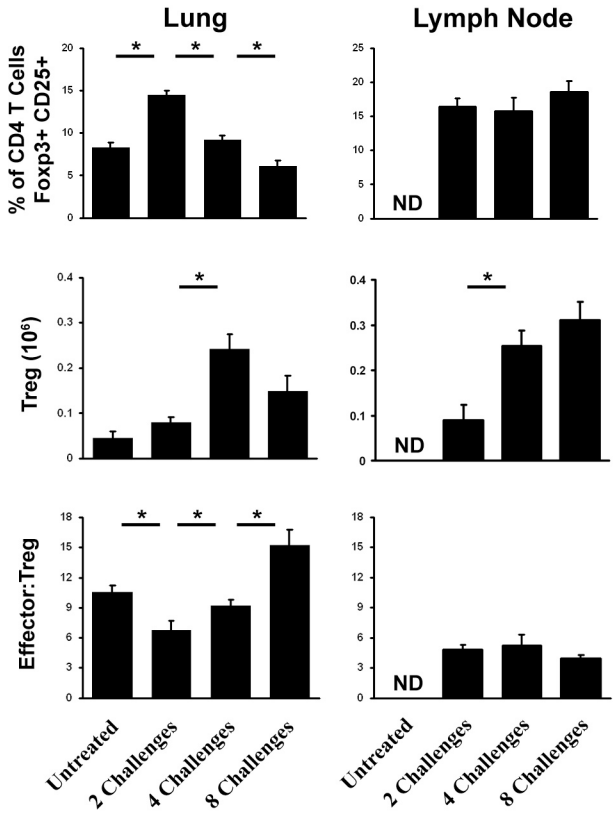

Fig. 3-9. The percentage of CD4 T cells displaying a regulatory phenotype increases during the early immune response, but total numbers increase over the course of exposure. Cells taken from both lung and mediastinal lymph node were stained for CD45, CD4, and CD25. Following permeablization cells were then stained for intracellular Foxp3 expression. Effector:Treg ratio was determined by comparing the percentage of CD4 T cells that were double-positive for CD25 and Foxp3 to those that were double-negative. Bars represent the mean of each mouse at each time point. $n \geq 6$ for each time point.

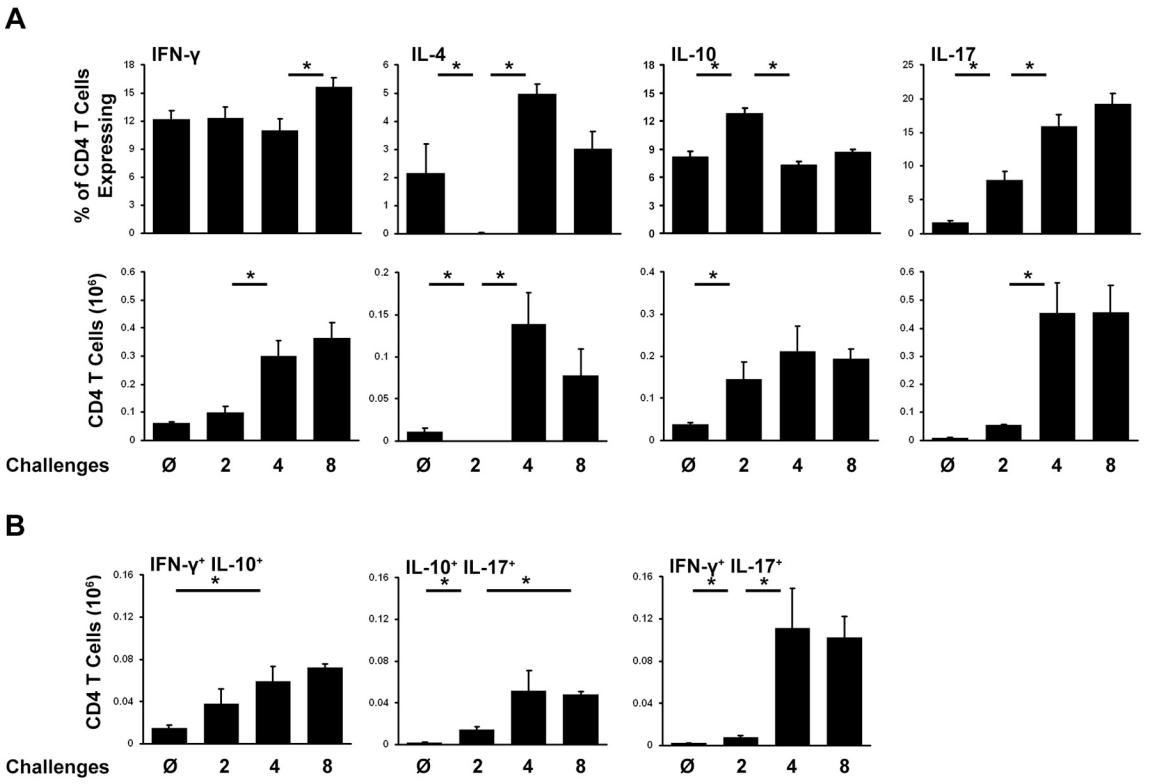

Figure 3-10. Each stage of the immune response to *A. fumigatus* displays a distinct CD4 cytokine profile in the lung. (A) Cells taken from lung were stimulated for six hours with PMA and Ionomycin and then stained with fluorescently labeled antibodies specific for CD45 and CD4. Following permeablization cells were then stained for intracellular IFN-α, IL-4, IL-10, and IL-17 expression. The mean percentage and mean total number of CD4 T cells expressing each cytokine is shown. **(B)** The mean number of CD4 T cells expressing multiple cytokines is shown. $n \geq 6$ for each time point.

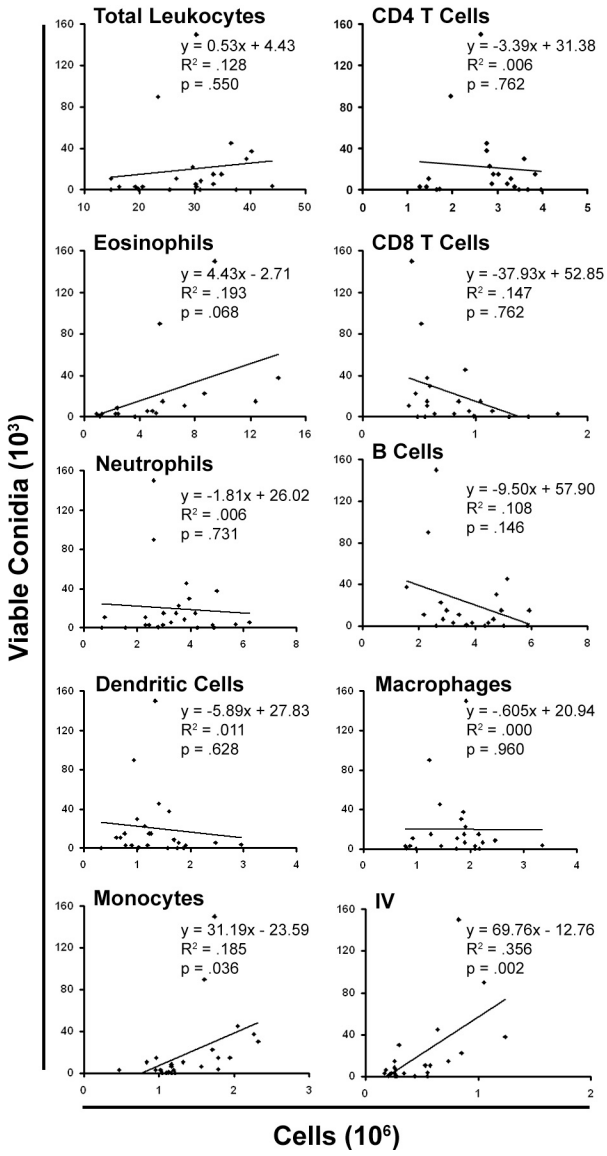

Figure 3-11. Poor clearance of conidia is positively associated with specific cell populations during peak inflammation. Following 4 conidia challenges, the number of viable conidia per mouse was compared to individual cell populations in each lung. Each point represents the number of viable conidia vs. the number of cells in an individual mouse. Slope, y-intercept, R^2 value, and p-value were determined using regression analysis.

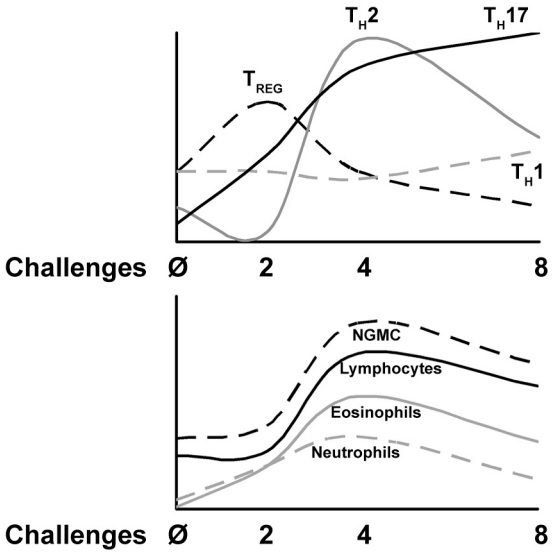

Figure 3-12. Repeated exposure to low levels of pulmonary exposure conidia results in hypersensitivity and a dynamic immune response. The rise and fall of the four main branches of the adaptive immune response as well as various cell populations is shown. Both graphs are in arbitrary units.

Discussion

We have demonstrated in this study that chronic exposure of an immunologically intact host to *Af* conidia does not result in invasive aspergillosis or fatal disease but does result in the development of chronic pulmonary inflammation and a CD4 T cell response that is dynamic in its polarization, switching from an innate response and high regulatory:effector CD4 T cell ratio to a low regulatory:effector ratio and an adaptive response that shifts in its ratio of T_H1:T_H2:$T_H17$cells as the number of conidia challenges increases (Fig. 3-12). However, it should be noted that all three types of CD4 T cell responses are present in the lungs at some level at four and eight challenges. While the number of IFN-γ/IL-10 and IL-10/IL-17 producing CD4 T cells rises slightly with multiple challenges, there is a striking increase in the number of IFN-γ/IL-17 producing CD4 T cells between two and four challenges (Fig. 3-10B). IFN-γ/IL-17 double-positive T cells are associated with inflammation in several models of autoimmunity and graft rejection (33-36) and their presence suggests a role for IL-17 in maintaining the inflammatory response to *A. fumigatus*.

Viable conidia could be detected 24 hrs after introduction following each challenge, providing a sustained and repeating source of antigen or inflammatory stimuli during the evolving CD4 T cell response. Neutrophils, a major cell type during the early phases of the response, are well-known to play a central role in host defense against *Aspergillus* (12, 37-40) and were seen at all stages of the response, particularly early stages prior to engagement of the adaptive response. Eosinophils, the dominant cell type during hypersensitivity, have been repeatedly found in T_H2 responses. The chronic inflammatory response included the recruitment of antigen-presenting cells such as

basophils, dendritic cells, macrophages, CD4 T cells, and B cells, all of which are capable of driving T_H2, T_H1, T_H17 and T_{reg} responses. In addition, exposure to *A. fumigatus* conidia or fungal glucan can induce regulatory responses via TLR2 and dectin-1 (41, 42). This then raises an interesting question: why is there a marked transition from tolerance and mild inflammation to induction of T_H2, T_H1 and T_H17 responses? Expansion of the regulatory T cell population is important for limiting disease because T_{reg} cells ultimately aid in the clearance of fungi by limiting Th1 inflammation (43) or dampening T_H2 hypersensitivity reactions (44, 45). In aspergillosis studies using high doses of conidia (10^8 spores per mouse) there is a strong T_H1 response that occurs one week after the initial exposure (46) rather than the T_H2 and T_H1/T_H17 responses that we have reported here during chronic *Af* conidia exposure.

There are several possible explanations then for the transition from tolerance to hypersensitivity that we observed during chronic *Af* conidia exposure. One possibility is that the accumulation of innate cells eventually overwhelms the regulatory response. Chitin – which is generated following conidial germination – can drive an accumulation of innate cells, stimulating inflammation and ultimately leading to an allergic response (47). We show here that viable conidia are still present twenty-four hours after challenge, and we have previously shown that germinating fungi can be detected in the lungs even when low concentrations of conidia are used. Thus, it is possible that chitin production results in an innate response that outpaces the tolerance response and eventually results in the engagement of T_H2 adaptive immunity. Another possibility is that control of the T_H1 response is what ultimately drives the development of the T_H2 response. The T_H1 response has repeatedly been shown to cause tissue damage (48), so it is reasonable to suspect that the immune system would dampen the T_H1 response to a dose of conidia that

can easily be cleared by the innate immune system. Such T_H1 suppression could in turn allow the expansion of a T_H2 response in reaction to multiple challenges, as T_H1 and T_H2 responses are often reciprocally regulated. Moreover, T_H1 and T_H17 are also reciprocally regulated (49) which could explain the increased number of IL-17 producing CD4 T cells.

Similar to the development of the T_H2 response, the T_H17 adaptive immune response during chronic *Af* conidia exposure may be a result of a combination of factors. The initial T_H17 adaptive immune response may be triggered simply by the presence of conidia. Like regulatory T cells, T_H17 is promoted by fungal cell wall components via dectin-1 (50, 51). In addition, T_{reg} can facilitate the differentiation of T_H17 cells (52, 53), and regulatory T cells themselves can be converted to T_H17 cells (54), a process that is facilitated by DC (55). Moreover, IL-10 inhibits production of IFN-γ but not IL-17 (56). Thus, the initial T_H17 response seen following two challenges may be driven by regulatory T cell conversion to T_H17. This process would be aided by the dampened T_H1 response, as the development of T_H17 cells is inhibited by T_H1 and T_H2 cytokines. On the other hand, CD4 T cells already committed to the T_H17 lineage are resistant to T_H1- or T_H2-mediated suppression (57) which could explain why we observed that IL-17 producing CD4 T cells continue to expand even during the T_H2-driven inflammatory response that follows four challenges.

The T_H17 T cells in our study are also likely playing an active role in shaping the reaction to conidia. Not only does the T_H17 arm of the adaptive response regulate T_H1 differentiation (58, 59), but it can dampen production of indoleamine-2,3-dioxygenase (IDO) which enhances fungal clearance by inhibiting inflammation. This could explain why after eight challenges the adaptive response is still not capable of efficiently clearing

conidia (60). There have been no reports to our knowledge suggesting that the T_H17 response regulates T_H2 responses, so the presence of T_H17 does not explain the dampening of the hypersensitivity response between four and eight challenges. In contrast, previous studies have indicated that T_H2 airway inflammation is enhanced by T_H17 (61). How then, does the T_H17 response ultimately replace the T_H2 reaction seen earlier during chronic *Af* conidia exposure? Rather than suppressing T_H2, it may be that T_H17 becomes the dominant adaptive response through attrition. Repeated exposure to an antigen leads to restimulation-induced cell death of CD4 T cells, but it has been reported that in autoimmune disease models T_H17 cells are resistant to this form of apoptosis (62).

Thus, the emergent T_H17 response may arise as an imperfect immune compromise when dealing with low levels of repeated conidia. While a T_H2 response does little to aid in the clearance of non-hyphal *A. fumigatus* and hinders function of the lungs, a persistent T_H1 response could result in severe damage to the tissue. T_H17 hinders T_H1-mediated clearance of fungi (60), but on its own the T_H17 response has anti-fungal properties (63-66). Therefore it is quite possible that repeated pulmonary exposure to *Af* conidia eventually leads to an immune homeostasis where the ultimate response creates the least damage while still controlling microbial load (conidia germination).

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Chapter 4
The Role of IL-17 in Hypersensitivity

Introduction

In the previous chapter we showed that repeated pulmonary exposure to *Aspergillus fumigatus* conidia led C57BL/6 mice to develop pulmonary hypersensitivity with increased production of IL-17 over the course of infection. IL-17 and the T_H17 response have an unclear relationship with allergy and hypersensitivity, and given the robust IL-17 response seen in mice challenged multiple times we wished to elucidate the relationship between IL-17 and hypersensitivity response to *Aspergillus fumigatus* conidia. To do this we used an identical challenge protocol to the one used in Chapter 3, only in addition to wild-type mice we used a previously characterized IL-17 knockout strain on a C57BL/6 background (1).

IL-17 was discovered over fifteen years ago (2), but it was not until 2005 that the scientific community realized that it is part of an adaptive response wholly separate from the classic T_H1 and T_H2 branches previously characterized (3, 4). Until that time researchers had labored under the belief that the T_H1 and T_H2 responses balanced one another and constituted the sum total of the adaptive response (5). Which of these two branches was dominant depended on the pathogen of interest: T_H1 dealt with intracellular pathogens (6), and T_H2 dealt with extracellular pathogens (7). However, with the discovery of T_H17 it became clear that old paradigm was obsolete, and new research has endeavored to determine the role of T_H17 in the newly emerging model.

The role of the T_H17 response has been viewed in a number of ways. It is sometimes seen as a bridge between innate and adaptive immunity (8, 9), as a protective mechanism for mucosal surfaces (10, 11), and as the source of many chronic inflammatory diseases once thought to be mediated by the T_H1 response (12). Of particular interest is the emerging role of T_H17 in diseases that were once thought to be mediated by the T_H2 adaptive response. T_H17 and IL-17 have been shown in numerous studies to play a significant role – both positive and negative – in both airway hypersensitivity as well as fungal disease.

In response to fungus and other pathogens, T_H17's role is highly varied, as T_H17 cytokines have been repeatedly shown to possess a wide variety of anti-microbial activity. IL-17 and IL-22 have been shown to work together to enhance the expression of anti-microbial peptides (13-15), and IL-17 and IFN-γ together can induce the production of defensins in humans (16). IL-17 is necessary for defense against systemic candiasis (17) and has been previously shown to have a variety of anti-fungal properties (18-21). Unfortunately, the T_H17 response tends to be inferior to T_H1 when it comes to fungal defense and can hamper the T_H1 response (21). It has even been speculated that microbes such as fungi – which promote T_H17 differentiation via dectin-1 signaling – may preferentially activate the T_H17 response over T_H1 as a form of immune evasion (22).

The involvement of T_H17 in hypersensitivity appears to be a double-edged sword as well. IL-17 is increased in human asthma (23), and IL-17 levels correlate with the severity of asthma as well as other allergies (24, 25). Moreover, IL-17 has been shown to directly affect neutrophil recruitment during OVA-induced asthma in mice (26). However, it has also been shown that IL-17 can be a negative regulator of asthma in mice (27), and IL-17 depletion can exacerbate eosinophilia in lungs (26). Based on these

observations, the role of IL-17 in airway hypersensitivity seems to be time-dependent. At some stages of inflammation T_H17 is a detriment and at others it is a benefit. This concept meshes nicely with the dynamic cytokine response we demonstrated in the last chapter, and as such, we wished to see what effect a lack of IL-17 production would have on the hypersensitivity response.

Aims

1. Determine if IL-17 drives accumulation of leukocytes in the lung during hypersensitivity
2. Determine if IL-17 alters the adaptive immune response to *Af*
3. Determine if IL-17 inhibits clearance of conidia from the lung

Results

IL-17 exacerbates hypersensitivity but is not responsible for mucus hyper-secretion.

IL-17 has shown to be a hindrance to fungal clearance, but it has also been shown to be essential to defense against several pathogens. More importantly, the T_H17 response has been shown to play a minor role in fungal defense under some circumstances. To see whether a lack of IL-17 increased the mortality rate of C57BL/6 mice, mice that were to be challenged eight times were checked daily for fatalities. Over the course of five separate runs of at least three mice, not a single death in either the wild-type or IL-17 knockout mice was observed (Fig. 4-1). Moreover, there were no signs of mouse morbidity (data not shown).

Using flow cytometry, cell populations were identified using a battery of fluorescently labeled antibodies specific for cell surface markers as previously described

in Chapter 3. In the lung, there was no significant difference in the total leukocyte number following zero, two, and eight challenges in wild-type and IL-17$^{-/-}$ mice (Figure 4-2A). However, there was a thirty-three percent reduction in the total leukocyte number in IL-17$^{-/-}$ mice following four challenges. The extra cells present at this time point consisted of neutrophils, eosinophils, macrophages, dendritic cells, CD4 T cells, and B cells (Fig. 4-2B). There was no difference between CD8 T cells nor, surprisingly, myeloid precursors at this time point (Group III). Interestingly, the only population that showed a significant difference after eight challenges was the macrophage populations, which remains high over the course of exposure in wild-type mice. Conversely, more differences were observed in the lymph node following eight challenges as opposed to four. Though there was a significant difference in total lymphocyte number between wild-type and IL-17$^{-/-}$ mice following four challenges, the difference in cell number was due entirely to an expansion of the B cell population (Figure 4-2B). In contrast, there are significantly fewer CD4 T cells present in the mediastinal lymph node of IL-17$^{-/-}$ mice following eight challenges along with fewer B cells and CD8 T cells.

Basophils have been recently shown to play a central role in driving the T_H2 response (28). We wished to compare the levels in WT and IL-17$^{-/-}$ mice to determine if altered basophil levels accounted for the disparity in cell numbers seen during peak inflammation. To our surprise, basophil levels were nearly identical in the WT and IL-17$^{-/-}$ strains at every time point (Fig. 4-3).

H & E staining confirmed what flow cytometry had already told us, namely that lack of IL-17 attenuates cellular infiltration into the lungs. Cellular infiltrate could still be observed around the airways, particularly after four challenges, but the intensity of cellular infiltrate was greatly reduced in mice lacking IL-17 (Fig. 4-4A). Interestingly,

PAS staining revealed that IL-17$^{-/-}$ mice still showed mucus hyper-secretion in the airways, though its prevalence and intensity were slightly reduced (data not shown). Likewise, the lungs of IL-17$^{-/-}$ mice also contained multi-nucleated macrophages, though again, they were less frequent than in their wild-type counterparts (Fig. 4-4B). Finally, as a simple visual confirmation of our observations, we stained leukocytes from digested lung with a Wright-Giemsa stain and compared cellular composition. As seen with FLOW cytometry, both mouse strains contained similar ratios of cell types, though WT mice had a higher percentage of eosinophils during the hypersensitivity response following four challenges (Fig. 4-5).

IL-17 does not drive T cell activation or production of IL-4, but may play a role in the regulatory response.

Given the central role of CD4 T cells in hypersensitivity, we wished to see whether a lack of IL-17 production affected CD4 T cell activation or the presence of regulatory T cells in either lung or mediastinal lymph node. We found that there was no significant difference in percentage of CD4 T cell activation following four challenges in either the lung or lymph node (Figure 4-6A). There was a small but significant difference in the lungs following eight challenges, but at no time point in the lymph node was the difference significant. In contrast, there was a significant difference in the percentage of CD4 T cells expressing the CD25+ Foxp3+ regulatory phenotype in the lungs during the initial immune response. Unlike wild-type mice where a greater percentage of CD4 T cells expressed CD25 and Foxp3 in response to conidia, IL-17$^{-/-}$ mice did not see an increase in the percentage of T_{reg} within the pool of CD4 T cells (Figure 4-6B). However,

within the lymph node there were no statistically significant differences between wild-type and IL-17$^{-/-}$ mice at any time point.

Similarly, there was very little difference in CD4 T cell cytokine expression between wild-type and IL-17$^{-/-}$ mice. The percentage of CD4 T cells expressing IFN-γ or IL-4 showed no significant differences at any time point (Figure 4-7). Predictably, there were no detectable IL-17-producing CD4 T cells in the IL-17$^{-/-}$ mice. Given the disparity in T_{reg} percentage, it is perhaps unsurprising that there was a difference in IL-10 production following two challenges. The discrepancy between the two strains may help explain the presence of hypersensitivity and eosinophilia in WT and not IL-17$^{-/-}$, as we have previously shown that IL-10$^{-/-}$ mice have a dampened inflammatory response to repeated *Af* conidia challenge (29).

IL-17 hinders clearance of viable conidia from the lung, but clearance is not associated with a particular cell type.

One of the biggest differences seen between wild-type and IL-17$^{-/-}$ mice is the ability to clear fungi (21). Therefore we wished to determine if IL-17 was a hindrance or benefit to clearance of conidia in the lung. Whereas wild-type mice were unable to efficiently clear conidia after even eight challenges, we found that after four challenges IL-17$^{-/-}$ mice were significantly better at clearing conidia than their wild-type counterparts (Fig. 4-8). After two challenges – and before the adaptive immune system has been activated – both mouse strains had identical clearance of *A. fumigatus* from the lung. However, by four challenges the IL-17$^{-/-}$ mice had significantly lower numbers of viable conidia in the lung. Moreover, while there is a significant difference between the two mouse strains during hypersensitivity, the IL-17$^{-/-}$ mice are significantly better at

clearing conidia after four challenges than they are following two challenges. This enhanced clearance is maintained throughout the course of the exposure.

Given the enhanced clearance in IL-17$^{-/-}$ mice, we wanted to see if there was the same correlation between viable conidia and cell types as in wild-type mice. As before, XY scatter plots were generated from mice that had been challenged four times and regression analysis was used to determine if there is any relationship between cellular levels and viable conidia. Unlike wild-type mice, there is no significant correlation between any cell type and the number of viable conidia in the lung (Fig. 4-9). As with wild-type mice there is a strong but not statistically significant negative correlation between the number of B cells and viable conidia. Similarly, there was a positive correlation between the number of viable conidia and the number of Group III and Group IV cells. These relationships did not reach significance, however, though the trends were similar to the relationship seen in WT mice following four challenges.

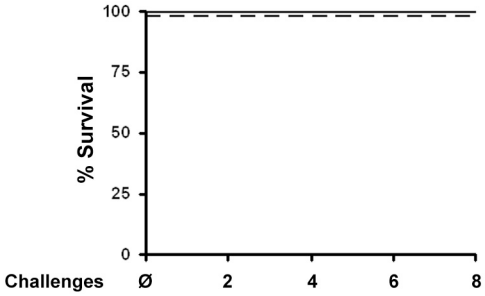

Figure 4-1. IL-17$^{-/-}$ do not have increased susceptibility to lethal fungal infection. Mice were challenged intranasally with 2 x10^{6} live conidia once a week for eight weeks. Shown is the survival of all mice in the cohort over the course of eight weeks. Solid line indicates WT; dashed line indicates IL17$^{-/-}$.

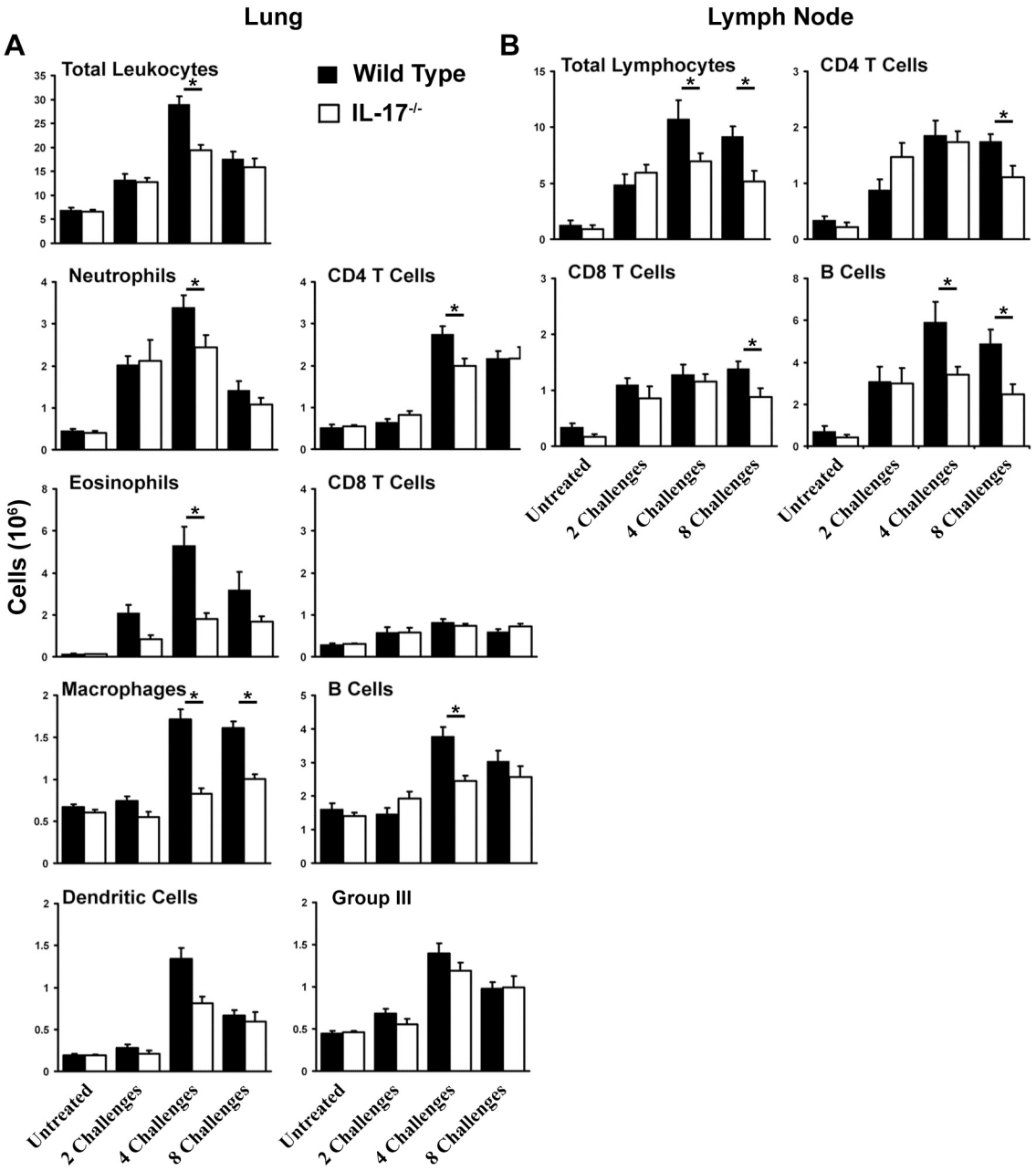

Figure 4-2. Lack of IL-17 attenuates hypersensitivity response but does not dampen the chronic inflammatory response. (A) Total cells in the lungs node were calculated by counting total live cells in both tissue types and multiplying by the percentage of cells that were $CD45^{+}$. Cell populations were identified as described in Figure A-1. All columns represent the average of at least four separate experiments. Black columns = WT mice; white columns = $IL\text{-}17^{-/-}$ mice. Starred bars indicate $p < .05$.
(B) Lymphocyte numbers in the mediastinal lymph node.

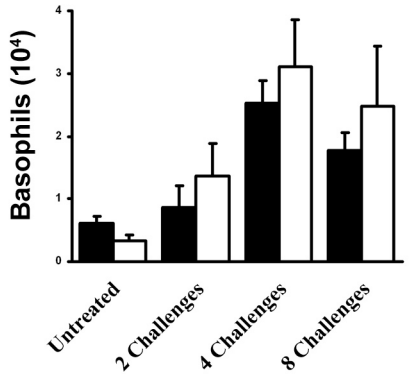

Figure 4-3. Basophil levels are unaffected by the lack of IL-17 during acute and chronic inflammation. Basophils were identified by gating for FSC^{low} SSC^{low} Gr1- CD11c- cells, then gating out T cells and B cells (CD3+ and CD19+ cells). The remaining cells that were CD49b+ FcεRI+ were classified as basophils. The mean number of basophils following zero, two, four, and eight challenges is shown. Black columns = WT mice; white columns = IL-$17^{-/-}$ mice. Starred bars indicate $p < .05$.

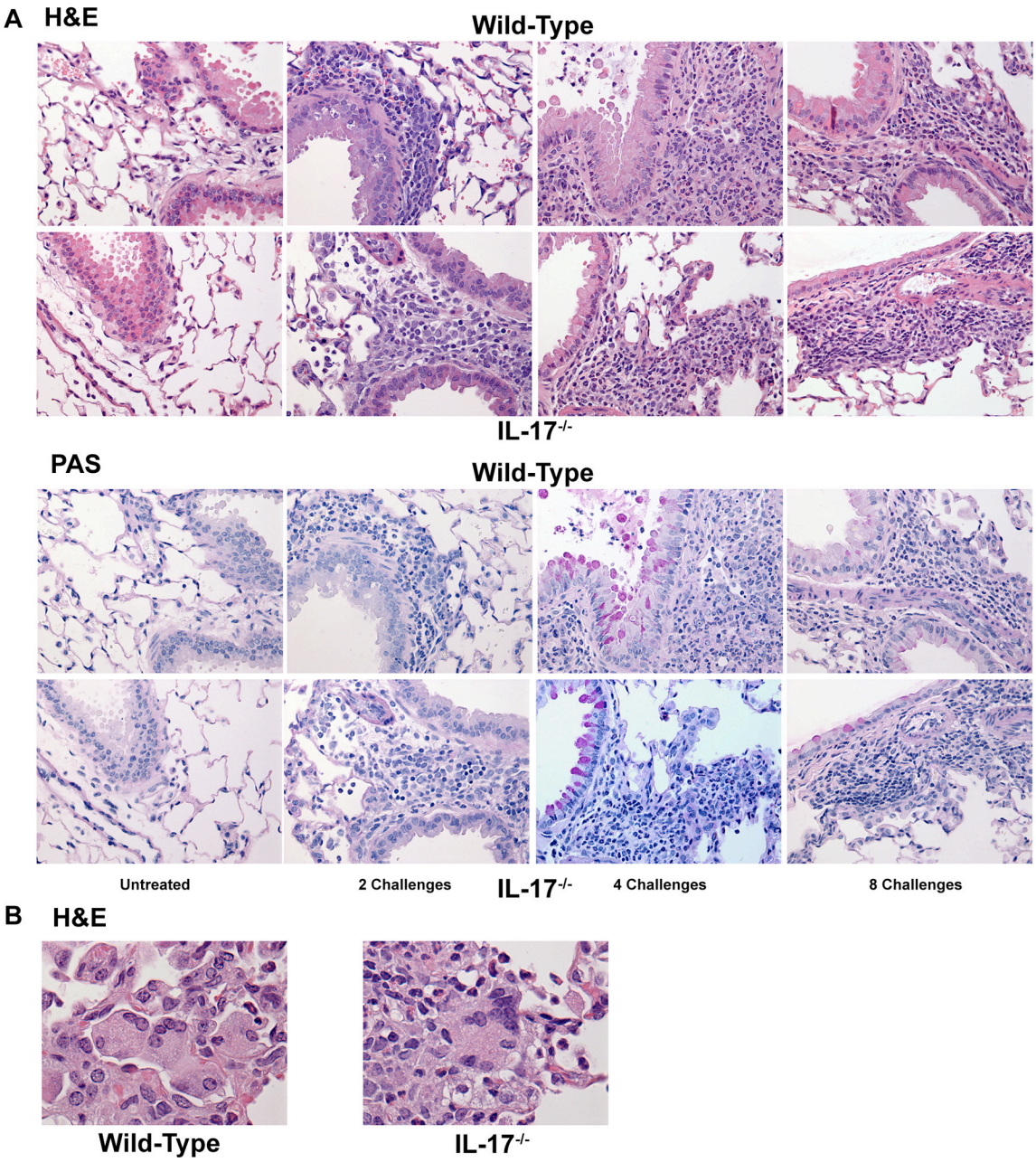

Figure 4-4. IL-17$^{-/-}$ mice show reduced cellular infiltrate but are still capable of mucus-hyper-secretion and multi-nucleated macrophage formation. Lungs from mice challenged zero, two, four, or eight times were fixed in formalin and embedded in paraffin blocks. Histological slices were then stained with H&E or PAS. **(A)** Slides were examined at 40X magnification. Arrows indicate eosinophil and neutrophil infiltrate. In PAS staining, bright purple cells indicate mucus secretion. **(B)** In mice challenged four times with conidia, multinucleated macrophages were observed in the lungs. Magnification is 100X.

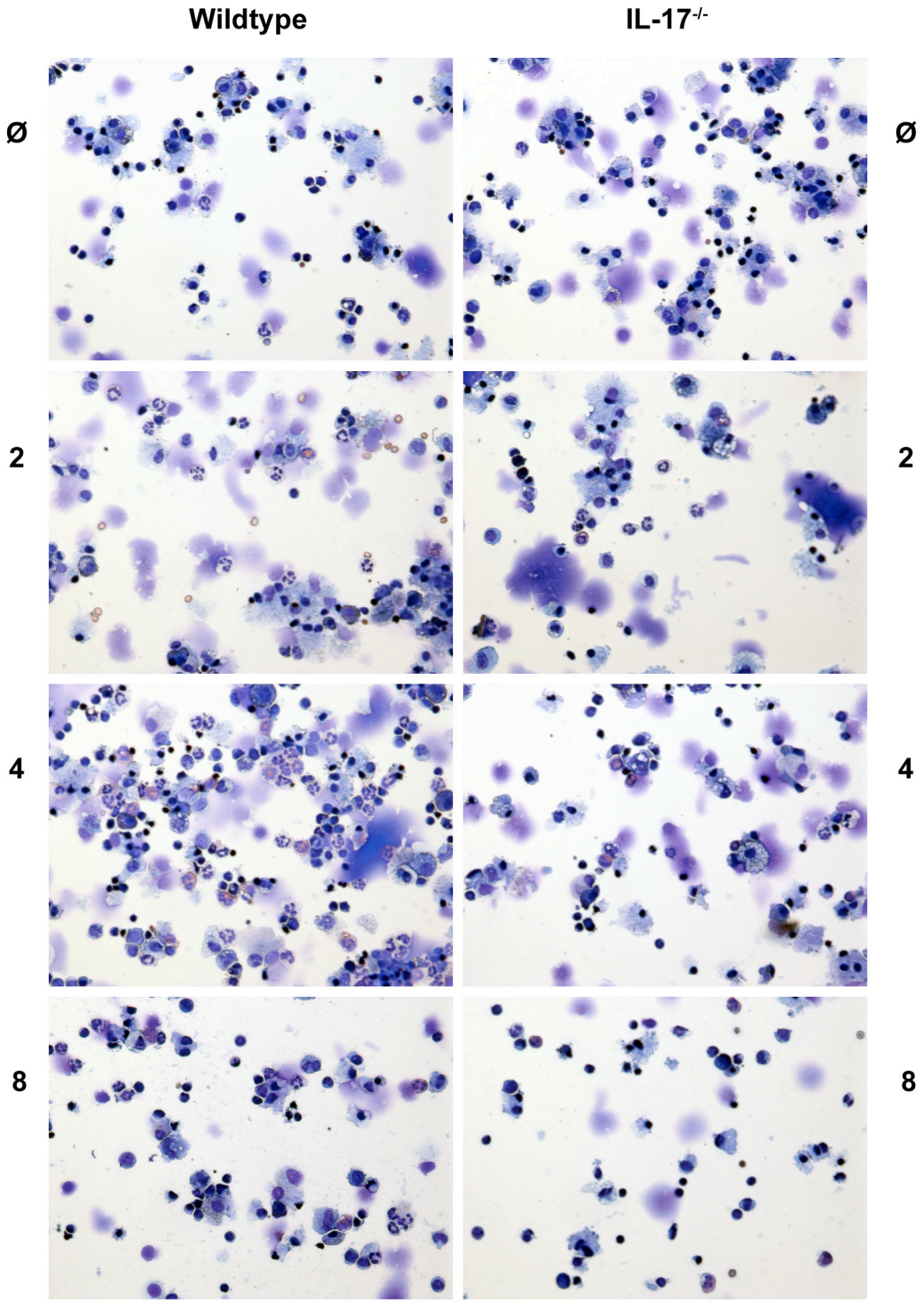

Figure 4-5. Reduced eosinophil infiltrate can be observed in IL-17$^{-/-}$ mice following four challenges with *Af* conidia. Aliquots of leukocytes from WT and IL-17$^{-/-}$ mice challenged zero, two, four, and eight challenges were stained with a Wright-Giemsa stain set and observed at 40X magnification.

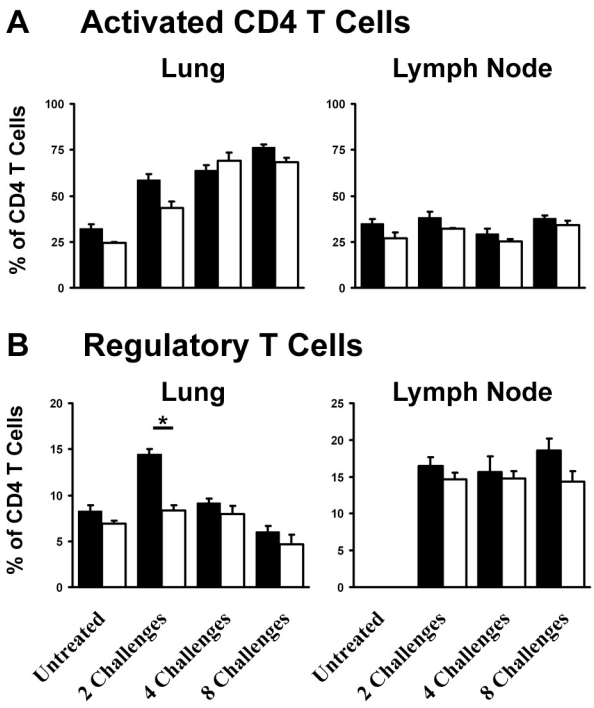

Figure 4-6. T cell activation is unaffected in IL-17$^{-/-}$ mice, but there is a decrease in the CD4 regulatory phenotype following two challenges. (A) Lung cells were stained antibodies for CD45, CD4, CD44, and CD69. CD4 T cells were isolated via gating and those that were CD44^{High} CD69^{+} (FLOW gate) were counted as activated. Each time point is a representative concatenation of three samples. Both the percentage and total number of activated CD4 T cells in the lung and lymph nodes was calculated for each mouse and averaged for each time point. $n \geq 6$ for each time point. **(B)** Cells taken from both lung and mediastinal lymph node were stained for CD45, CD4, and CD25. Following permeablization cells were then stained for intracellular Foxp3 expression. Effector:Treg ratio was determined by comparing the percentage of CD4 T cells that were double-positive for CD25 and Foxp3 to those that were double-negative. Bars represent the mean of each mouse at each time point. $n \geq 6$ for each time point. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

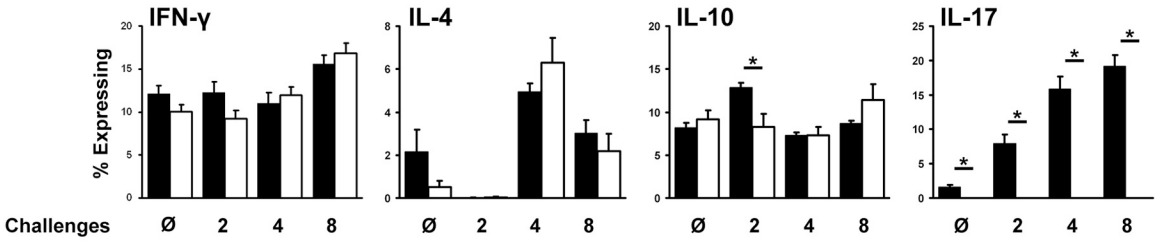

Figure 4-7. IL-17$^{-/-}$ mice do not produce IL-10 in response to initial conidia challenges. Cells taken from lung were stimulated for six hours with PMA and Ionomycin and then stained with fluorescently labeled antibodies specific for CD45 and CD4. Following permeablization cells were then stained for intracellular IFN-α, IL-4, IL-10, and IL-17 expression. The mean percentage and mean total number of CD4 T cells expressing each cytokine is shown. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

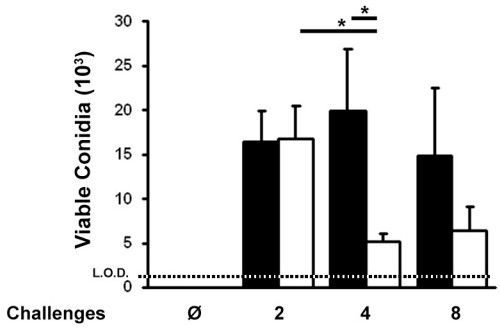

Figure 4-8. IL-17$^{-/-}$ mice have enhanced clearance of conidia following four and eight challenges. Following digestion of the lungs an aliquot of digest was serially diluted and plated on SDA media and hyphal foci were counted. The graph shows the average viable conidia per lung detected 24 hours after zero, two, four, and eight challenges. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

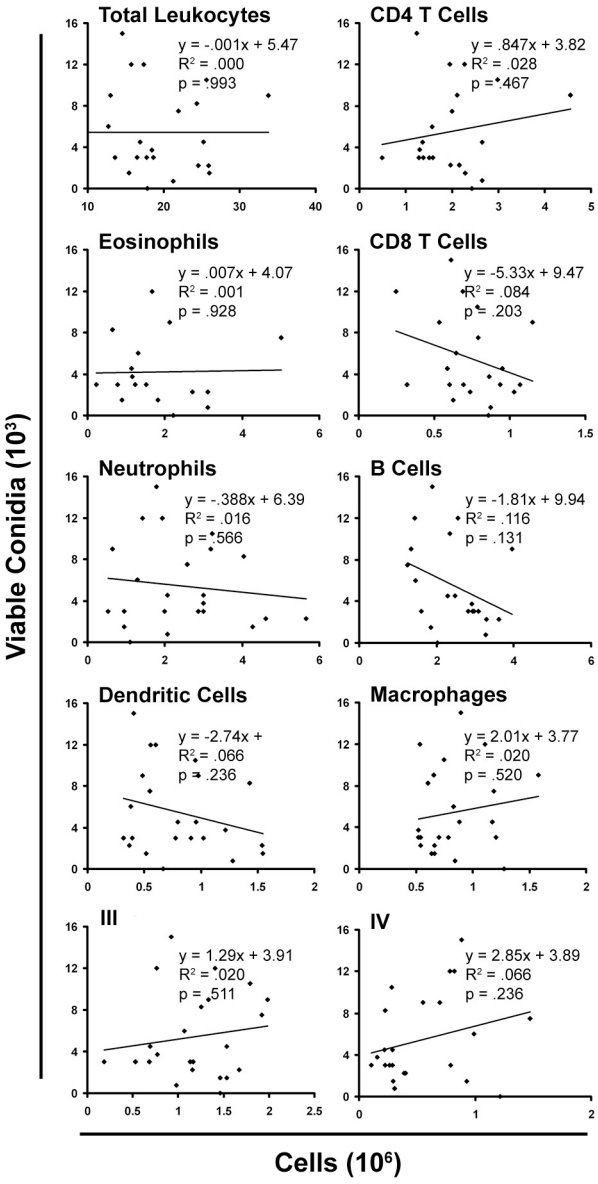

Figure 4-9. Cell numbers do not correlate with conidia clearance in IL-17$^{-/-}$ mice during peak inflammation. Following 4 conidia challenges, the number of viable conidia per IL-17$^{-/-}$ mouse was compared to individual cell populations in each lung. Each point represents the number of viable conidia vs. the number of cells in an individual mouse. Slope, y-intercept, R^2 value, and p-value were determines using regression analysis.

Discussion

We show here that IL-17 plays a significant role in exacerbating hypersensitivity to *Aspergillus fumigatus* conidia. Whereas wild-type mice have a burst of cellular infiltration after four challenges that recedes over the next several weeks, cellular levels in IL-17$^{-/-}$ mice plateau after four challenges and stay relatively constant with the exception of the neutrophil population. In the lymph node, CD4 and CD8 numbers are almost identical. Differences are due almost entirely to B cell levels which is not unexpected given the role of IL-17 in driving germinal center B cell differentiation (30).

What *is* surprising is the abrogation of eosinophilia in the lung following four challenges. Several previous studies have examined the role of IL-17 in pulmonary hypersensitivity models using modified protocols of the traditional OVA-asthma model. In almost every case it was found that IL-17 knockout mice had similar (1, 26, 31) or increased (27) levels of eosinophils in the bronchoalveolar lavage fluid. The only time eosinophil abrogation has been observed is in mice unable to produce the IL-17 receptor (IL-17R). However, IL-17 and IL-25 share the IL-17RA (32), and the knockout strain used was IL-17RA$^{-/-}$. IL-25 plays a central role in T_H2 allergic inflammation (33-36), so it is unsurprising that knocking out the IL-25 receptor would reduce eosinophilia in the lung. Thus, the attenuation of eosinophilia in response to *A. fumigatus* conidia represents a novel observation within the scientific field.

Despite the importance of an IL-17 role in eosinophilia, there are other differences between the wild-type and IL-17$^{-/-}$ mice that provide tantalizing clues as to the role of T_H17 in the pulmonary hypersensitivity response. First, and least surprising, lack of IL-17 results in a significantly reduced number of 000.neutrophils. This is to be expected as several previous studies of IL-17 and asthma have found that IL-17 knockout

or depletion abrogates neutrophilia (26). Puzzlingly though, there was no difference in precursor levels at any time point despite a proven role for IL-17 as a monocyte chemotactic agent (37), yet there were significant differences in numbers of dendritic cells after four challenges and macrophages after four and eight challenges. Since precursors are required for both dendritic cells and macrophages, why then are the numbers between the two mouse strains virtually identical? These data suggest the intriguing possibility that IL-17 may play a role in cellular differentiation.

Of course, it is also possible that differences in total cell numbers and cellular composition directly affect differentiation and that IL-17 is merely an upstream mediator not a direct effector of the process. Certainly, the role of IL-17 in cellular trafficking and signal induction is not disputed. IL-17 is capable of upregulating production of a number of CXCR3 agonists including CXCL9, CXCL10, and CXCL11 (8). It can enhance epithelial release of β-defensins, I-CAM-1, IL6, IL-8, CXCL1, CCL20, G-CSF, MUC5B, and MUC5AC (38), induce GM-CSF production (39) (which in turn enhances T_H17 development (40)), and can even enhance chemokine gene expression by stabilizing mRNA transcripts (41). It is therefore possible that IL-17 is affecting differentiation and cytokine production by altering the quantity of cells as well as their function. Indeed, it has been previously shown that IL-17, IL-23, and IL-25 can all alter eosinophil cytokine production and release (42), and GM-CSF can alter the function and survival of both eosinophils and neutrophils (43).

The same logic can be applied towards the clearance of conidia. We show that after repeated exposure IL-$17^{-/-}$ mice are significantly more efficient at clearing conidia from the lungs. Again, this is most likely due to a downstream consequence of IL-17 production rather than a direct effect on local cells. Previous reports have shown that IL-

17 can dampen the killing efficacy of neutrophils by altering IDO production (21). Likewise, IL-17 can worsen inflammation and downregulate regulatory T cells (44), T_{REG} in particular having been shown to play a role in fungal clearance (45). However, differences in T_{REG} levels are only seen after two challenges when there is no difference in fungal clearance but are identical after four and eight challenges when fewer viable conidia can be detected in IL-$17^{-/-}$ mice. Thus, differences in clearance are most likely due to IDO levels or some other mechanism that is controlling the efficacy of fungal clearance.

Additionally, there is the role of IL-17 and the T_H17 response in cytokine production and the transition from a tolerant response to a hypersensitivity reaction. While the hypersensitivity response seen in wild-type mice was absent following four challenges, mice showed an inflammatory response and greater cellular infiltrate than was found following two challenges. In IL-$17^{-/-}$ mice the response following four challenges was remarkably similar to the eight-challenge response, suggesting that while IL-17 drives the hypersensitivity response it is not responsible for the induction of the adaptive immune response. It is also possible that it plays no role in the resolution of the T_H2 response, though without being able to generate hypersensitivity it is impossible to know for sure.

This isn't to say that the T_H17 response may not be playing a role in driving and/or resolving the hypersensitivity response to *Af* conidia. IL-23, which is responsible for the maintenance of T_H17 T cells (46) – and whose receptor is expressed on a number of different cell types – is perfectly viable in IL-$17^{-/-}$ mice and acts upstream of IL-17 (47, 48). Moreover, IL-23 has been repeatedly linked to autoimmune diseases and injurious inflammation: brain inflammation (49), autoimmunity (50), and inflammatory bowel

disease (51) have all been linked to IL-23. IL-23 levels also correlate with severity in several diseases (10, 18, 52) and the T_H17 response may worsen inflammation by regulating IDO, kynurenines, and T_{regs} levels (44). Thus it is possible that IL-23 may be responsible for initiating or resolving hypersensitivity and IL-17 serves to exacerbate the response.

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Chapter 5
The Role of IL-17 in Eosinophil Development, Maturation, and Trafficking

Introduction

Eosinophils have been associated for years with defense against parasitic infections (1). More recently, details of their role in combating various pathogens has become more apparent. Not only can they combat vegetative fungi (2), but eosinophils express TLRs (3) and can act as APCs (4). However, it has also long been known that eosinophils play a central role in several pulmonary hypersensitivity responses (5-7) as well as tissue remodeling (8). Moreover, the cellular components that make eosinophils so effective against parasites can also result in damage to host tissue and impair function. Major Basic Protein (MBP) is a prime example. MBP is crystallized in eosinophil granules (9) and is toxic to parasites (10) and bacteria (11). Unfortunately, it is also cytotoxic to airway epithelium (12, 13) and activates remodeling factors (14). Other granulocyte factors such as eosinophil peroxidase, eosinophil cationic protein, and eosinophil-derived neurotoxin (15, 16) have anti-pathogenic effects (17) at the expense of tissue damage and mutagenesis (18-20). Thus, the presence of eosinophils in the lungs is necessary during parasitic or hyphal infection, but the hypersensitivity response provides little tangible benefits to individuals experiencing chronic exposure to conidia. The lack of eosinophilia in IL-17$^{-/-}$ mice in response to chronic *Af* exposure therefore makes IL-17 a tantalizing target for clinical therapy.

However, in order to provide a more tangible benefit, the underlying mechanism must be elucidated. Three primary possibilities exist for IL-17's role in driving eosinophilia: IL-17 enhances bone marrow development of eosinophils, IL-17 directly or indirectly alters eosinophil maturation and activation at the inflammatory site, or IL-17 plays a central role in eosinophil trafficking to the lungs. Another possibility not examined here is that IL-17 prolongs the eosinophil lifespan in the lung.

Several previous studies have delved into the development of eosinophils from bone marrow precursors. A 1997 study by Murali et al. showed that bone marrow eosinophilia could be detected in mice with allergic bronchopulmonary aspergillosis (21), and since then the mechanism of development has been elucidated. Eosinophils arise from eosinophil-basophil precursors (22) with transcription factor composition and order affecting development (23). Actual development is controlled by the GATA-1 (24), PU.1 (25), and c/EBP (26) transcription factors, with GATA-1 being the most important (27). Surface marker expression is also distinct from other progenitor groups allowing for identification via flow cytometry (28). IL-17 has been shown to induce production of GM-CSF – a potent stimulator of granulocyte differentiation – in a dose-dependent manner (29), though conversely it has been found that IL-17 may combat GM-CSF signaling (30). Along similar lines, it has been previously shown that IL-17 depletion can enhance eosinophil production in the bone marrow during OVA-induced asthma (31). Either way, a substantial link has already been established between IL-17 and eosinophil production in the bone marrow.

Though there is no direct evidence that IL-17 affects eosinophil maturation or activation to our knowledge, several reports show a role for IL-17 in controlling the eosinophil response. IL-17, along with IL-23 and IL-25, can affect eosinophil cytokine

production and release (32). In addition, chemokines can promote the activation and function of eosinophils (33), and IL-17 has been found to enhance chemokine expression by stabilizing cellular mRNA (34). Thus it is possible that IL-17 may affect eosinophils indirectly through the release of chemokines, or we may observe a direct IL-17 role in eosinophil activation.

Probably the most logical possibility, however, is that IL-17 affects eosinophil trafficking to the lungs. Leukocyte trafficking to specific tissue is controlled by chemokines and integrins (35), and eosinophils are no exception. Eosinophils constitutively express CCR1 and CCR3 (36, 37), and knockout of CCR3 or eotaxin-1 or -2 dampens pulmonary eosinophilia (38). At least one group has concluded that the three necessary factors for eosinophilia are primed CD4 T cells, IL-5, and eotaxin-1 (39), though there is still some debate as to the role and importance of eotaxin-1 versus eotaxin-2 (40). This reliance on chemotactic agents suggests a possible role for IL-17 in eosinophil chemotaxis, as the cytokine has previously been shown to induce epithelial release of a number of molecular signals including I-CAM-1, IL-8, IL-6, CXCL-1, CCL20, and G-CSF (41). IL-17 is a direct chemotactic agent for monocytes (42) and upregulates production of CXCL 9, 10, and 11 (43). IL-17 has also been shown to play a role driving granulocytic influx during allergic responses (31), though to date only neutrophil levels have been altered by IL-17 knockout or depletion (41). It is therefore reasonable to think that IL-17 may be playing a role in controlling eosinophil trafficking to the lung.

Aims

1. Determine the effect, if any, of IL-17 on eosinophil development from bone marrow cells during the pulmonary hypersensitivity response.
2. Determine the effect, if any, of IL-17 on eosinophil maturation and activation during the pulmonary hypersensitivity response.
3. Determine the effect, if any, of IL-17 on eosinophil trafficking during the pulmonary hypersensitivity response.

Results

IL-17$^{-/-}$ mice are capable of generating eosinophils in the bone marrow in response to *Aspergillus fumigatus* conidia.

There are several possible explanations for the differences seen between wild-type and IL-17$^{-/-}$ mice during the hypersensitivity response; our final goal was to determine the source of these differences. In particular, we wished to explore the novel observation that IL-17$^{-/-}$ mice lacked the eosinophilia found in their wild-type counterparts. To do this, we first examined bone marrow development in both mouse strains during the hypersensitivity response and prior to conidia challenge. Previous reports have shown that the composition of bone marrow can be analyzed by examining surface expression of CD31 and Ly6C (44, 45). Cells in the resulting flow plot are dispersed into six distinct gates (Fig. 5-1). Cellular distribution is slightly different in wild-type and IL-17$^{-/-}$ mice that are untreated; however during peak hypersensitivity the cellular populations converge and are nearly indistinguishable. By pooling several experiments we were able to statistically compare the composition of each group following four conidia challenges. We found that there was no significant difference in the bone marrow between WT and knockout mice during the hypersensitivity response (Fig. 5-2). To further verify similar

production of eosinophils in both mouse strains we generated cytospin stains from bone marrow cells with a Wright-Giemsa stain. Eosinophils were observable in low quantities in both mouse strains regardless of *Af* exposure, and there was no obvious increase in the percentage of eosinophils in the bone marrow cells (Figure 5-3).

Interestingly, we saw something different when looking at GATA-1 mRNA production in the bone marrow. mRNA expression of GATA-1 – the primary transcription factor for eosinophil development – was unchanged in WT mice during the hypersensitivity response, indicating that the hypersensitivity response is not driving excess production of eosinophils. However, IL-17$^{-/-}$ mice showed a significant drop in GATA-1 mRNA production relative to wild-type mice during the same time period (Fig. 5-4). It is therefore possible that IL-17 is playing a role in driving, or at least maintaining, eosinophil differentiation during the hypersensitivity response. In either case, the reduction of GATA-1 expression in IL-17$^{-/-}$ mice has a rather mild effect on eosinophil production, as eosinophils are still observable in IL-17$^{-/-}$ in the bone marrow and blood (Figs. 5-3 & 5-9).

Lack of IL-17 does not alter the maturation or the activation of eosinophils in the lung during hypersensitivity.

A second possibility is that the lack of IL-17 is somehow affecting the potential in the lung for immature eosinophils to fully develop or for mature eosinophils to become activated. To test this, we stained cells from digested lungs with a specific set of antibodies to examine changes in eosinophil development. Of particular interest were CCR3 – a trafficking receptor for a number of chemokines including eotaxin – and the IL-5 receptor (IL-5R). CD45+ cells were first separated from the pool of total lung cells,

and then eosinophils were further isolated from the leukocyte population by gating for FSC^{low} cells with a $SSC^{mid\text{-}high}$ profile (Fig. 5-5A). The eosinophil population was then analyzed by looking at expression of IL-5R and CCR3. We found that eosinophils in the lung could be subdivided into three distinct subgroups based on their IL-5R expression. These groups were labeled as IL-$5R^{high}$, IL-$5R^{mid}$, and IL-$5R^{low}$. Expression of siglec F (sigF) in each group showed that cells with high IL-5R expression had almost no sigF expression, while those that were IL-$5R^{low}$ almost universally expressed sigF (Fig. 5-7).

We then compared the cellular distribution of cells within these three gates in wild-type and IL-$17^{-/-}$ mice during the hypersensitivity response. As a positive control we performed an identical stain set on wild-type C57BL/6 mice that had previously been infected with *Cryptococcus neoformans*, which results in a strong T_H2 response and extreme eosinophilia. There were no obvious differences between WT and IL-$17^{-/-}$ mice that had been untreated, and there were no obvious differences between strains following four challenges (Fig. 5-5 B). However, differences could be seen between mice that were untreated, had been challenged four times, or had been infected with *C. neoformans*. In agreement with previous data suggesting that *low* IL-5R expression is associated with activation, mice that were untreated had the highest percentage of IL-$5R^{high}$ cells, while those infected with *C. neoformans* had nearly ninety percent of their eosinophils in the IL-$5R^{low}$ gate (Fig. 5-6 B). Using exclusively forward- and side-scatter analysis, there was a significant increase in the percentage of leukocytes that were eosinophils between untreated mice and those challenged four times (Fig. 5-6 A). Additionally, there was a significant decrease in percentage in IL-$17^{-/-}$ mice following four challenges compared to WT mice, an observation consistent with other data. The distribution of eosinophil IL-5R expression between time points was significantly different for both strains of mice, but

there was no difference between the strains (Fig. 5-6 B). Moreover, within each of the three gates there was no difference in surface expression of siglec F between wild-type and IL-17$^{-/-}$ mice following four challenges (Fig. 5-7). Thus, IL-17 does not play a direct role in driving eosinophil activation or maturation.

IL-17 drives cellular recruitment to the lung during acute hypersensitivity to Aspergillus fumigatus conidia.

The most logical remaining explanation for the reduced numbers of eosinophils in the lungs is trafficking. Previous reports have shown that IL-17 is responsible for inducing the release of a number of chemokines from epithelial cells, and it possible that a lack of IL-17 prevents cells from reaching the lung during infection (42, 43). Cell surface expression of trafficking markers on non-granulocytic myeloid cells in the lung supports this. In the lungs, CD11b expression shows that the percentage of new cells is much greater in WT mice than in IL-17$^{-/-}$ mice (Fig. 5-8). This is particularly true in macrophages and Group IV cells. Moreover, siglec F – which is expressed on resident macrophages – is reduced in wild-type mice, further supporting this observation. There are also small differences in dendritic cell expression, but these are not nearly as pronounced. Similarly to eosinophils, differences in NGMC between the two mouse strains seems to be limited to cellular levels and not activation. CD80 and CD86 expression is identical to that seen in untreated mice and is the same in both WT and IL-17$^{-/-}$ mice following four challenges (Fig. 5-8).

To see if inhibited trafficking to the lung was responsible for the dearth of eosinophils observed during the inflammatory response in IL-17$^{-/-}$, we took whole blood from mice challenged four times and analyzed the number of leukocytes and the cellular

composition. Following isolation from the blood, cells were gated for CD45 expression and then isolated via CD11b surface expression. $CD11b^{+}$ cells were then broken down into two groups: eosinophils – SSC^{high} – and small myeloid cells – FSC^{low} SSC^{low} – consisting of neutrophils and monocytes (Fig. 5-9 A). In uninfected mice, there was no significant difference seen between WT and IL-$17^{-/-}$ with regards to total leukocytes, eosinophils, or small monocytes. However, there was a striking and significant difference between cell levels in WT and IL-$17^{-/-}$ mice that had been challenged four times (Fig. 5-9 B). Moreover, while there was a significant difference between untreated and four-challenge IL-$17^{-/-}$ cell levels for all cell types, there was a much more modest – and not statistically significant – difference between untreated and four-challenge wild-type mice. The data therefore show a buildup of leukocytes in the blood of IL-$17^{-/-}$ mice that is not present in WT mice during hypersensitivity. This suggests then that the lack of IL-17 is altering pulmonary levels of leukocytes by preventing extravasation from the blood to the tissue at the site of infection.

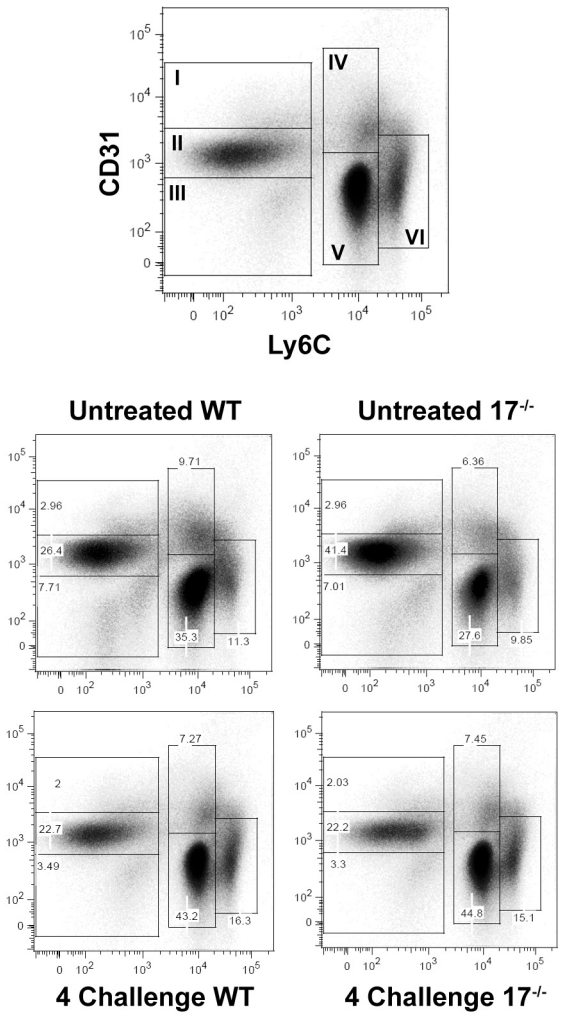

Figure 5-1. WT and IL-17$^{-/-}$ mice show identical bone marrow differentiation in response to repeated pulmonary conidia challenge. Bone marrow isolated from WT and IL-17$^{-/-}$ mice challenged zero and four was stained with Ly6C and CD31. Cells segregated into six distinct populations designated I-VI.

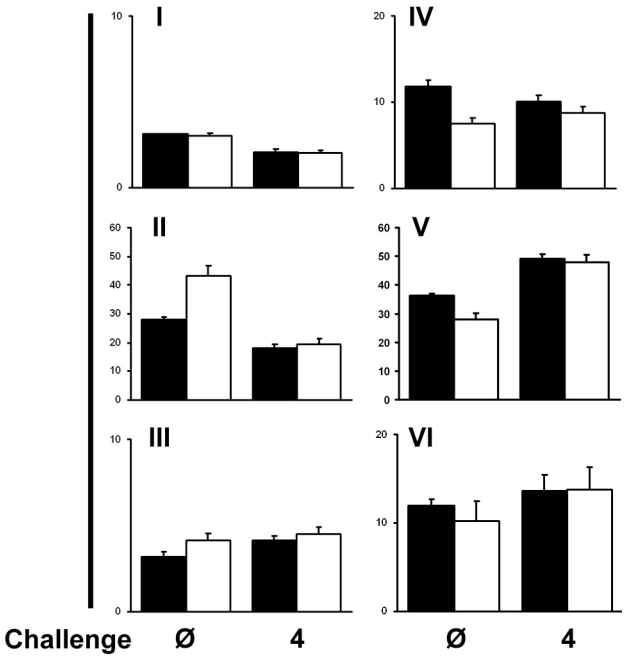

Figure 5-2. Bone marrow differentiation is unchanged in IL-17$^{-/-}$ mice during peak inflammation. The total percentage of cells within each gate was averaged for each mouse strain following zero or four challenges. For untreated mice, n = 3; for mice challenged four times n ≥ 11.

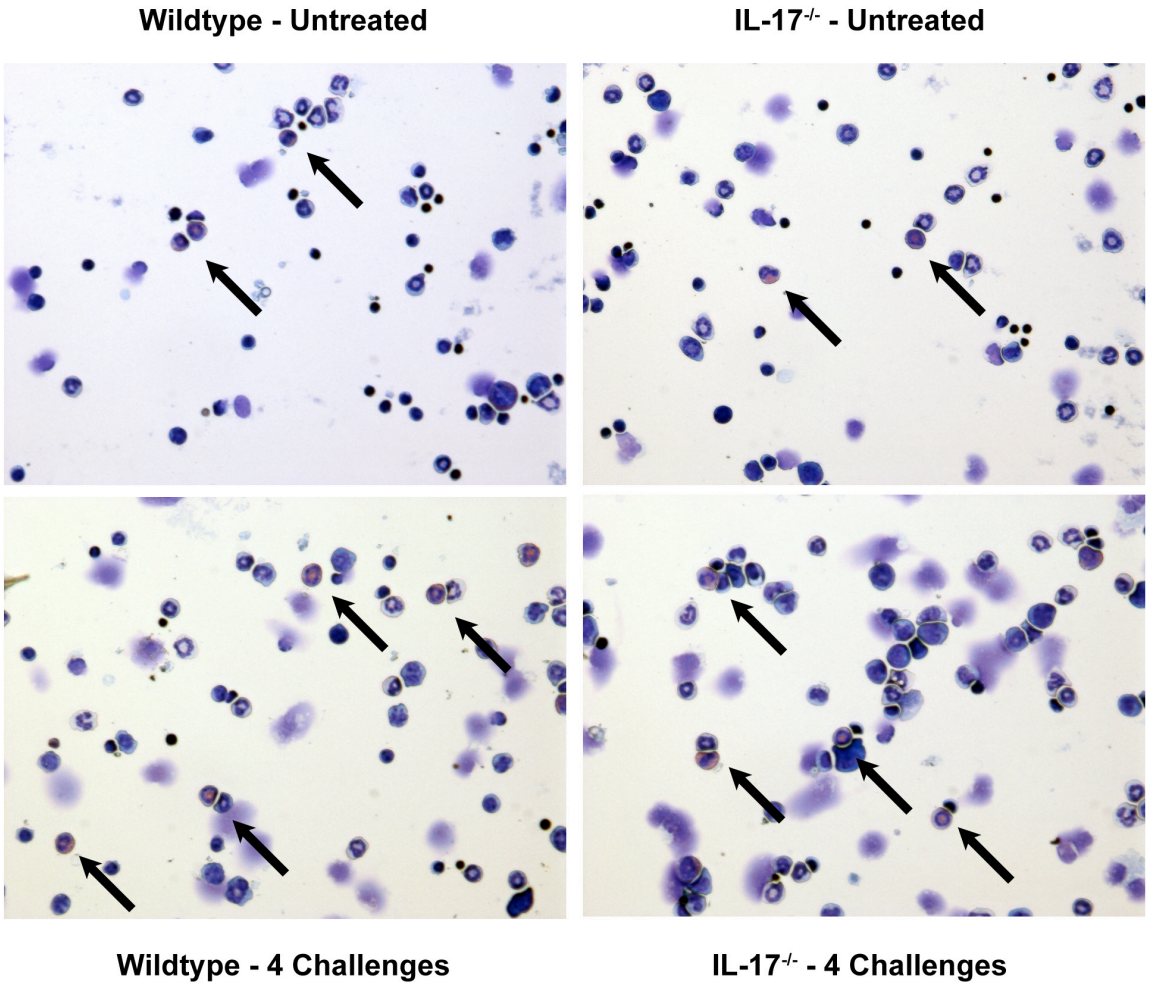

Figure 5-3. WT and IL-17$^{-/-}$ mice bone marrow shows no obvious phenotypic differences following chronic exposure to conidia. Aliquots of leukocytes from WT and IL-17$^{-/-}$ mice challenged zero, two, four, and eight challenges were stained with a Wright-Giemsa stain set and observed at 40X magnification.

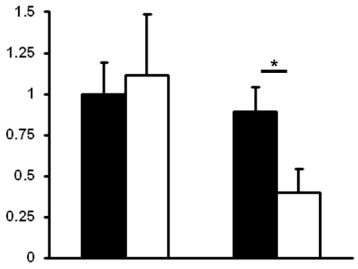

Figure 5-4. Bone marrow from IL-17$^{-/-}$ mice shows a significant reduction in production of GATA-1 mRNA. RNA from WT and IL-17$^{-/-}$ bone marrow was isolated after zero and four challenges. Following conversion to cDNA, GATA-1 levels were measured for each individual mouse. For untreated mice, n = 3; for mice challenged four times $n \geq 6$. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

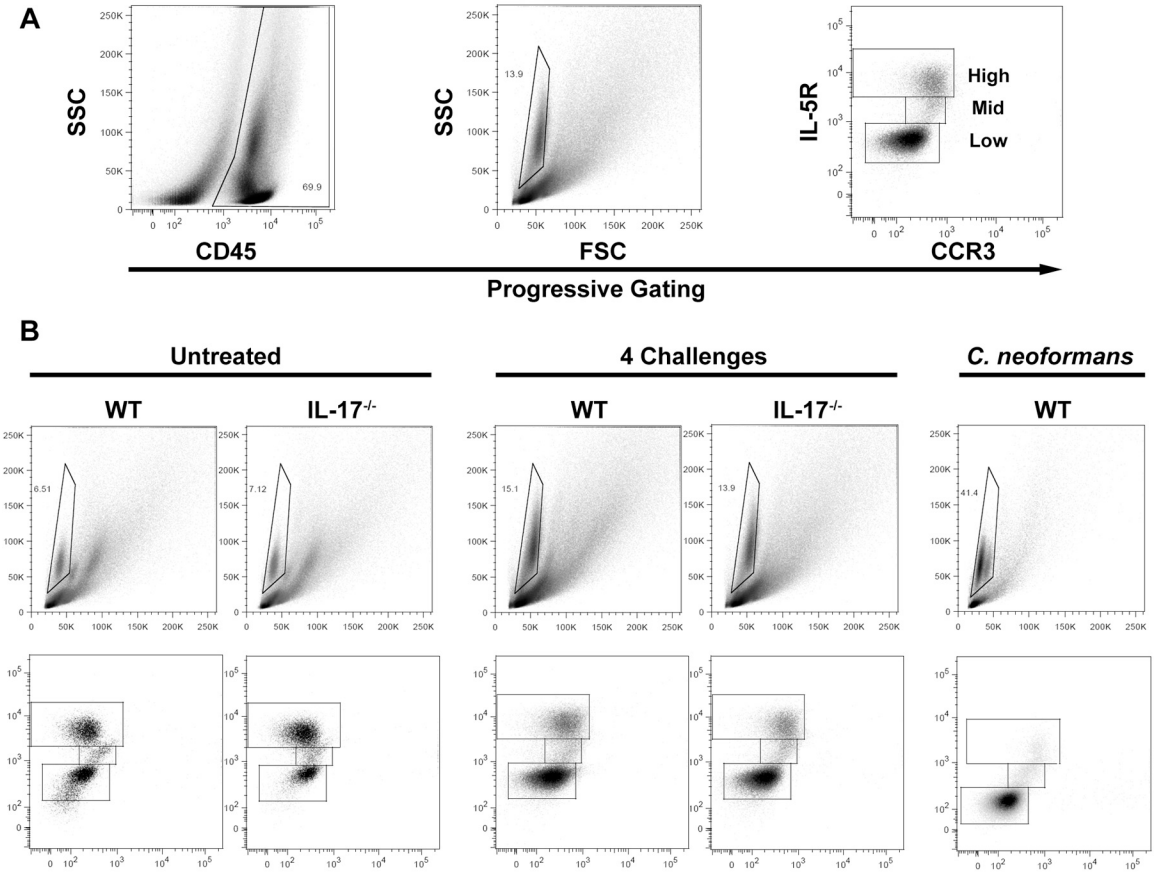

Figure 5-5. Lung eosinophils show increased maturity in response to antigen challenge. (A) Eosinophils were separated from the cellular background by selecting CD45+ cells that were FSC^{low} $SSC^{mid\text{-}high}$. The enriched population was then analyzed for IL-5R and CCR3 surface expression. The eosinophil population was then divided into three distinct gates: High, Mid, and Low. (B) The FSC/SSC profile and IL-5R/CCR3 expression of lung eosinophils in WT and IL-$17^{-/-}$ mice was compared following zero and four challenges. The expression profile of lung eosinophils from C57BL/6 mice infected with *C. neoformans* is also shown and serves as a positive control.

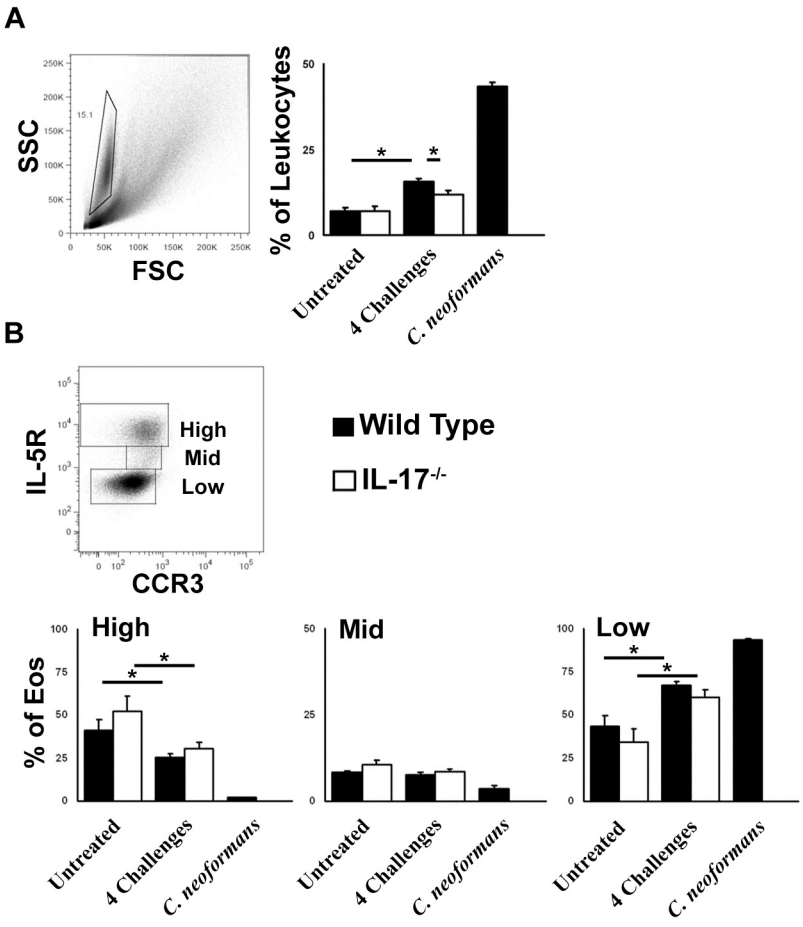

Figure 5-6. Lung eosinophils maturity is unaffected by a lack of IL-17. (A) Using FSC/SSC profiles, the percentage of lung CD45+ cells that were eosinophils was plotted. (B) Within the eosinophil population, the percentage of cells that fell within the High, Mid, and Low gates was plotted for WT and IL-17$^{-/-}$ mice following zero and four challenges, as were eosinophils from *C. neoformans*-infected mice. For untreated mice, n = 3; for mice challenged four times n ≥ 11. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

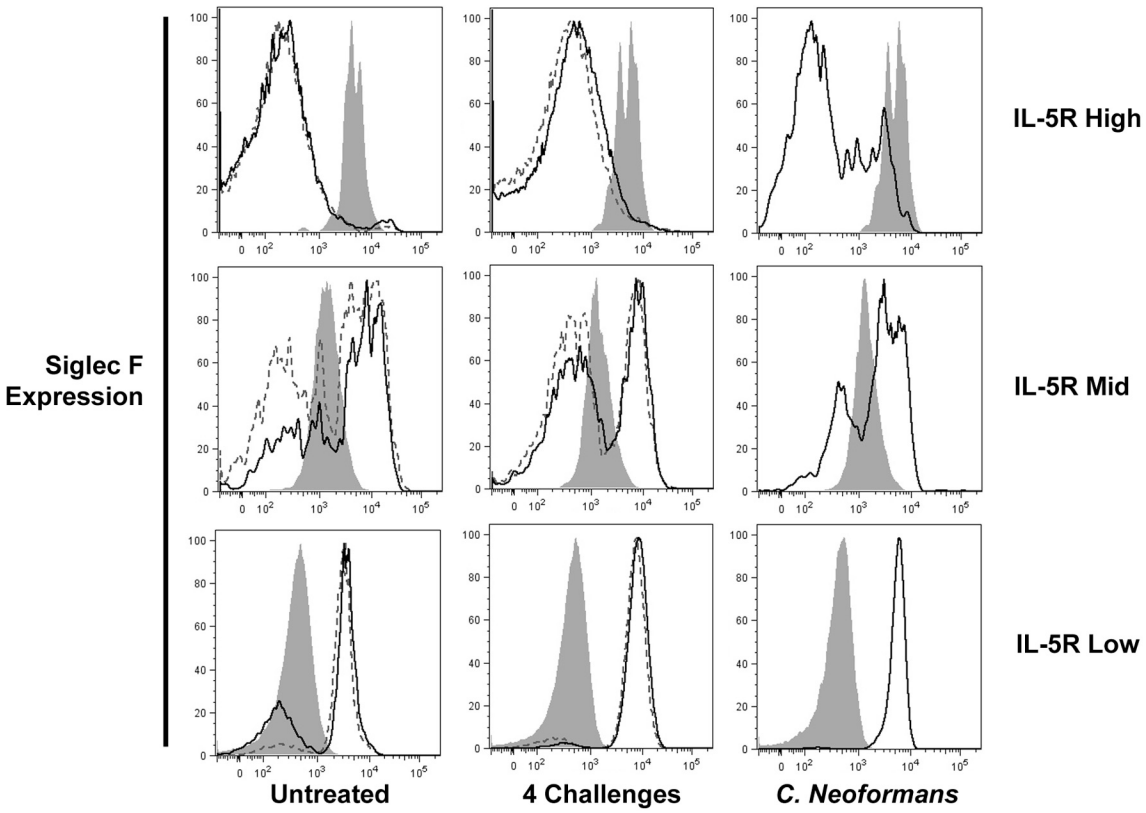

Figure 5-7. Expression of siglec F on eosinophils is governed by cell maturity and is unaffected by fungal exposure or the presence of IL-17. Cells within the High, Mid, and Low gates were analyzed using histograms for their expression of siglec F. Solid black lines = WT mice; dashed gray lines = IL-17$^{-/-}$ mice, gray peaks = IgG controls.

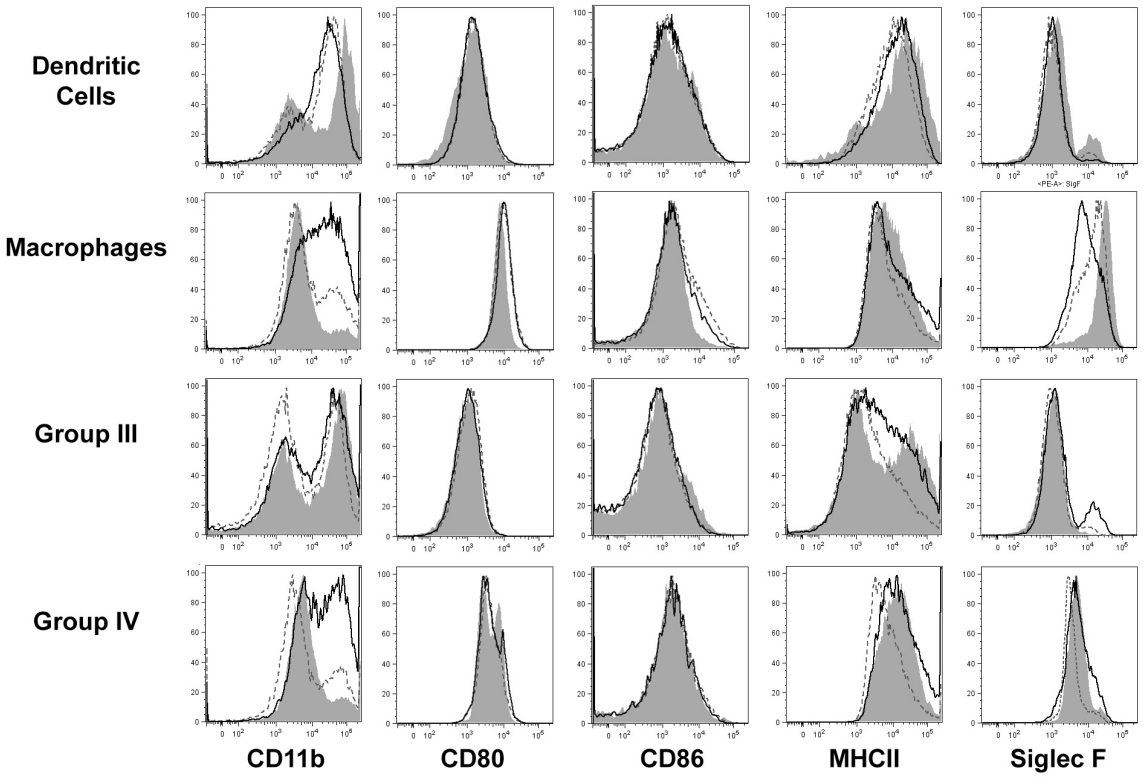

Figure 5-8. Lack of IL-17 does not alter myeloid activation but reduces the influx of new cells into the lung. Non-granulocytic myeloid cells were examined for their expression of cell surface markers. Solid black lines = WT mice 4x challenge; dashed gray lines = IL-17$^{-/-}$ mice 4X challenge, gray peaks = WT untreated mice.

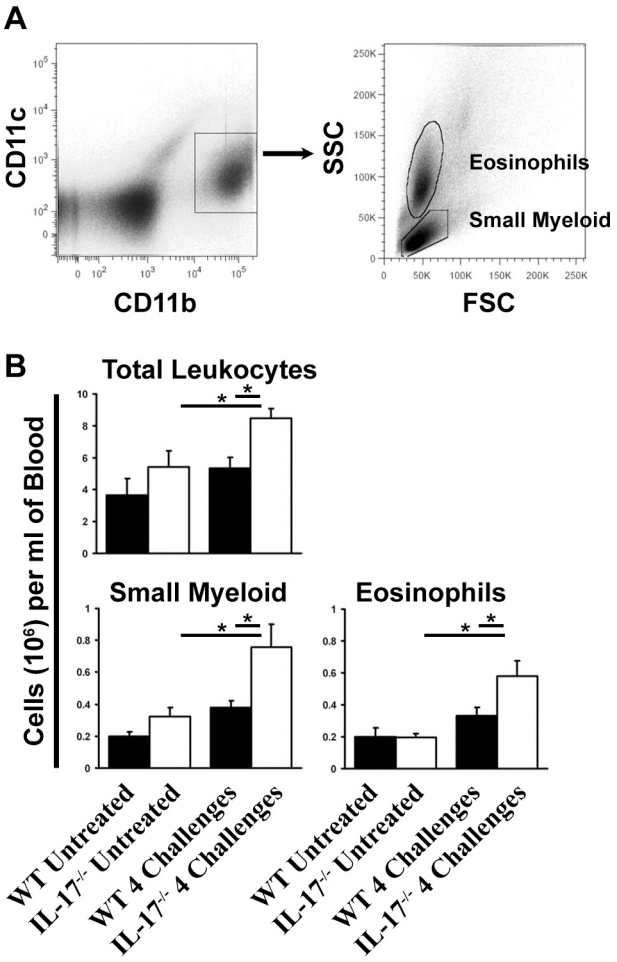

Figure 5-9. IL-17$^{-/-}$ mice show increased numbers of eosinophils and other myeloid cells in the blood during peak inflammation. Blood was collected from WT and IL-17$^{-/-}$ mice following zero and four conidia challenges. **(A)** Following cell counting, cells were stained for flow cytometry. CD45+ CD11b+ CD11c^{low} cells were gated and their FFSC/SSC profile analyzed. (B) The total number of CD45+ cells was measured in the blood of WT and IL-17$^{-/-}$ mice. Additionally, the number of eosinophils and small myeloid cells was plotted. For untreated mice, n = 3; for mice challenged four times n ≥ 11. Black columns = WT mice; white columns = IL-17$^{-/-}$ mice. Starred bars indicate $p < .05$.

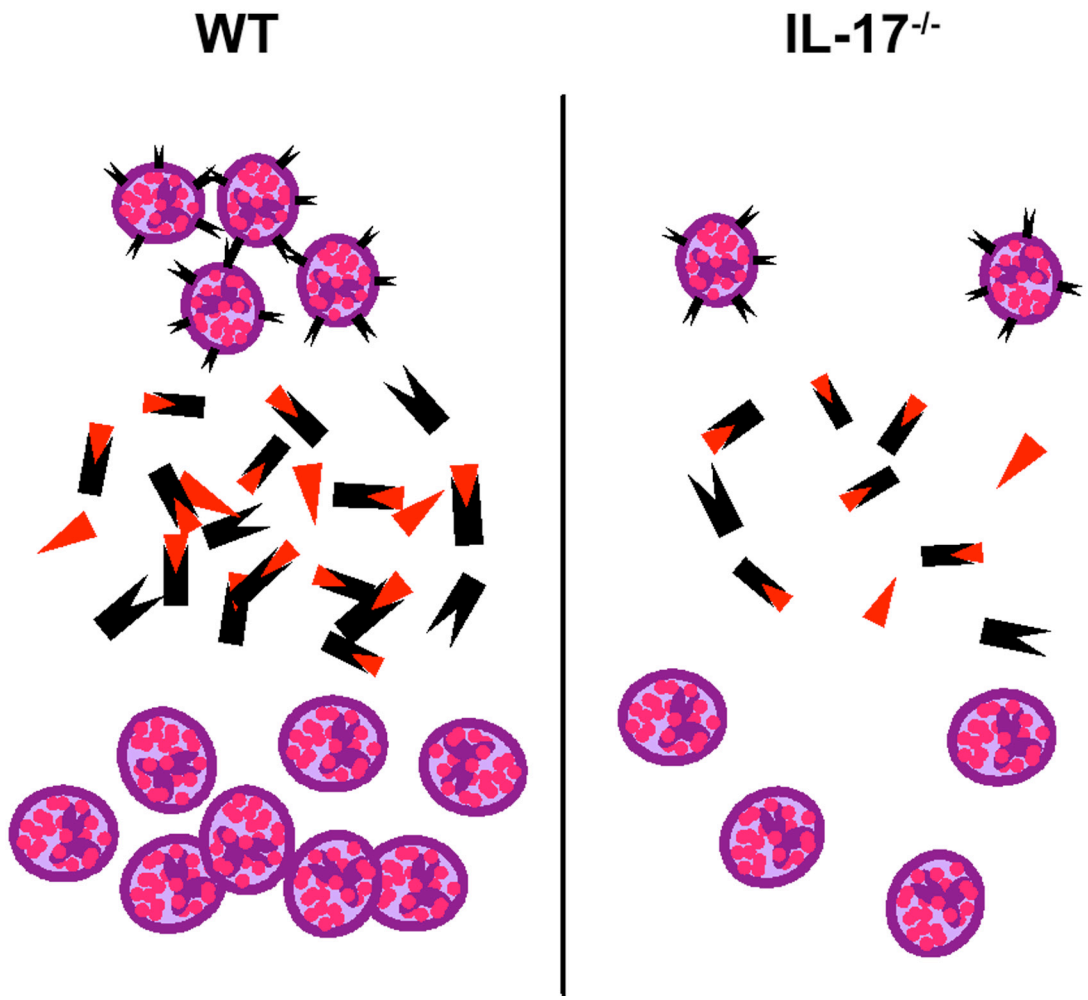

Figure 5-10. IL-17$^{-/-}$ mice are predicted to have less IL-5 in the lungs during peak inflammation. Red triangles indicate IL-5, black forks indicate IL-5R. Immature eosinophils are represented expressing IL-5R, while mature eosinophils have shed the receptor into a soluble form.

Discussion

We show here that IL-17 plays several roles in regulating eosinophil levels in the lung during the hypersensitivity response. Firstly, IL-17 has a modest role in driving, or at least maintaining, eosinophil differentiation in the bone marrow. Secondly, IL-17 has a direct role in cellular recruitment, and during conidia-induced pulmonary hypersensitivity it plays a role in eosinophil trafficking from the blood to the lungs. Thirdly, IL-17 appears to not play a role in the maturation of eosinophils, though there is a distinct possibility that it may indirectly play a role in shaping the eosinophil population in the lungs.

The data regarding bone marrow differentiation are somewhat conflicting. Based on the flow and cytospin data, one would conclude that eosinophil production from the bone marrow is unchanged in IL-17$^{-/-}$ mice. However, GATA-1 mRNA is reduced in the bone marrow. A reduction in eosinophil production makes sense of course, as eosinophils express the GM-CSF receptor (46) and GM-CSF plays a big role in eosinophil development along with IL-3 and IL-5 (46-49). How does one then reconcile the data? The most likely answer is that IL-17 *does* play a small role in BM eosinophil development, but the effects are not obvious due to the relatively modest eosinophil response. First, IL-17$^{-/-}$ mice are still plainly capable of generating eosinophils as evidenced by the cytospin data, the low levels in the lung during hypersensitivity, and the buildup of CD11b+ cells in the blood. Moreover, the change in GATA-1 levels seen in wild-type mice is negligible during hypersensitivity, and visual inspection does not show differences between either mouse strains at either time point. Thus, the hypersensitivity response does not result in the generation of many new eosinophils even in wild-type

mice, and it is likely that even reduced eosinophil production in IL-17$^{-/-}$ mice is enough to maintain levels in the blood.

This concept is bolstered by the method in which eosinophils are kept in reserve in mice. Unlike other myeloid cell types, eosinophils first traffic to the peritoneum where they pool to form a cellular reservoir (50). Even during parasitic infection it was found that the majority of eosinophils at the site of infection had come not from the bone marrow, but from the peritoneum. This then could explain why there were still eosinophils in the blood, even if production was reduced in the bone marrow: the eosinophils had been generated prior to infection and had come from the cellular reservoir. This might help explain why previous IL-17 depletion studies using OVA-sensitization have not noticed differences in eosinophil levels (41). These studies focus almost entirely on acute eosinophilia shortly after the allergic response is triggered. Since the eosinophils are coming from the peritoneum rather than the bone marrow, it's likely that cellular levels are being measured before the cellular reserve is depleted.

This of course does not entirely explain the unchanged eosinophil levels seen in IL-17$^{-/-}$ mice using OVA protocols, as we also show that IL-17 plays a role in cellular trafficking to the lung. There are several ways in which IL-17 might be affecting eosinophil trafficking. Aside from chemokine production, IL-17 might conceivably be altering expression of integrins that are used for rolling, tethering, adhesion, and transendothelial migration (51). However, given the similar integrin use of eosinophils and basophils, it is more likely that eosinophil-specific chemokines are altering recruitment as basophil levels are unchanged in IL-17$^{-/-}$ mice.

It is likely then, that IL-17 affects eosinophil trafficking by altering chemokine expression, probably eotaxin. Whereas dendritic cells have been shown to be responsible

for driving T_H1 chemokine production in response to *Aspergillus fumigatus* (52), the main recruiter of airway eosinophils is suspected to be eotaxin-2 produced by macrophages (53). Since we have shown that a lack of IL-17 results in diminished macrophage recruitment, it is possible that IL-17 drives the accumulation of macrophages which in turn drives the accumulation of eosinophils. Certainly it has been shown that monocytes – the precursors to macrophages – express the IL-17 receptor and use IL-17 as a chemotactic agent (42). However, it is clear, not only from our observations but from the literature, that some mechanistic redundancy must exist or eosinophils would not be able to reach the lungs at all. Eosinophils have been shown to upregulate expression of other chemokine receptors in response to IL-5, and IL-5 itself can as a chemoattractant, though it is less effective than the eotaxins (54). Thus, even if IL-17 does drive one aspect of eosinophil recruitment, there are likely other paths that allow some trafficking of eosinophils to the lungs. This idea is supported by the previous allergic studies that found no drop in eosinophil levels in response to OVA hypersensitivity in IL-$17^{-/-}$ and IL-17 depleted mice. It is likely that these models induced eosinophil migration through a number of different pathways so that IL-17 knockout or depletion had little effect. In response to low doses of conidia, however, IL-17 probably plays a much more central role in recruitment.

A final, more remote possibility relates to the methods used by previous researchers. Previous studies have measured eosinophils levels by visualization, relying on cytospin counts to determine eosinophil composition. Upon activation, eosinophils degranulate, releasing stored anti-microbial compounds and cytokines at the site of inflammation. It is possible that previous studies have simply been unable to differentiate

between eosinophils and neutrophils using a Wright-Giemsa stain because, without granules, the two cell types look nearly identical.

It is also possible, though, that IL-17 is affecting IL-5 levels in the lung, and this is affecting not only trafficking, but development. IL-5 plays a significant role in eosinophil development (47-49), and it is essential for expansion of the population in the bone marrow (55). One might ask then, if there is altered IL-5 production, why is the maturation of eosinophils during hypersensitivity the same in both wild-type and IL-17$^{-/-}$ mice? In order to answer that question, one must remember that the eosinophil population in IL-17$^{-/-}$ mice is one third that of WT during peak inflammation. Therefore there are three times as many eosinophils binding IL-5 in WT mice, and there are three times as many mature eosinophils releasing secreted IL-5 receptor into the milieu (56). This implies that more IL-5 is present to maintain the larger population of eosinophils in WT mice (Fig. 05-10). When we measured total cytokine production in the lung using micro array analysis, we indeed found higher levels of IL-5 in WT mice than IL-17$^{-/-}$ mice during the hypersensitivity response, though the difference did not reach statistical significance ($p > .05$) (Fig. A2-5). Taken together, it is highly possible that IL-17 affects IL-5 expression, either directly or through an intermediary. This would explain not only the difference in trafficking, but also developmental differences and differences in maturation. Given the difference in numbers for all cell types, it is likely that IL-17 acts as a global regulator of inflammation during the hypersensitivity response and downstream components of the immune system ultimately govern eosinophil recruitment and development.

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Chapter 6
Summary, Interpretation, Critical Review, and Future Directions

Summary and Interpretation

The studies in this dissertation can be broken down into three broad, yet distinct, categories: the characterization of the immune response to repeated pulmonary exposure to *Aspergillus fumigatus* conidia, the role of IL-17 in the hypersensitivity response to chronic conidia challenge, and the role of IL-17 in driving eosinophilia during hypersensitivity. In all three cases, the data presented here provides both novel insights into the mechanisms of the hypersensitivity response towards *Af*.

First and foremost, our research has elucidated a number of possible adaptive mechanisms during the immune response to *Aspergillus fumigatus*. Unlike previous studies which have shown the immune response to be purely adaptive (1-4) or T_H1 in nature (5-7), we find that chronic exposure to conidia results in a dynamic response characterized by early tolerance, subsequent T_H2 hypersensitivity, and, ultimately, moderate T_H17 inflammation. The hypersensitivity response is characterized by an influx of nearly all cells types; macrophages, dendritic cells, neutrophils, basophils, CD4 T cells, B cells, and the most dominant new population, eosinophils, all are significantly increased in the lung between two and four challenges. This hypersensitivity is accompanied by mucus hyper-secretion in the airways as well as formation of multinucleated macrophages. Moreover, repeated challenges did not improve the ability of the immune system to clear viable conidia from the lung, and the levels of several cells

types – myeloid precursors in particular – were significantly correlated to fungal levels. Perhaps most importantly, we observed IL-17 production that was increased following two challenges and continued to increase over the course of exposure. In particular, the expression of IL-17 and the poor clearance of conidia represent novel observations that lead to new lines of inquiry.

This led to our next series of experiments where we showed that IL-17 played a role in driving the hypersensitivity response. To our surprise, cellular levels were almost identical at all other time points in both mouse strains, but following four challenges IL-$17^{-/-}$ mice were unable to generate the same level of inflammation as wild-type mice. In particular, IL-$17^{-/-}$ mice showed a significant reduction in the number of eosinophils in the lungs following four challenges. To our knowledge such an observation has never been reported, as previous studies using OVA models of pulmonary inflammation similar eosinophil levels in the lungs in IL-$17^{-/-}$ and IL-17-depleted mice.

However, aside from a reduction in IL-10 and Foxp3 production following two challenges, IL-$17^{-/-}$ mice mounted a nearly identical, albeit dampened, inflammatory response to repeated conidia challenge. IL-4 was still produced following four challenges, epithelial cells still secreted mucus, and even basophil levels were unaffected. The only other difference we observed was a more effective clearance of conidia from the lung in the IL-$17^{-/-}$ mice.

The observation that eosinophilia was dampened in IL-$17^{-/-}$ mice led us to examine the mechanism behind the eosinophil accumulation in the lungs of WT mice during hypersensitivity. To do so we examined bone marrow differentiation, eosinophil maturation in the lungs, and cellular trafficking. The data regarding bone marrow differentiation was conflicting, as flow cytometric analysis and cell staining showed no

obvious differences in eosinophil development. However, expression of GATA-1 mRNA – the primary transcription factor for the eosinophil lineage (8, 9) – was reduced in IL-17$^{-/-}$ mice during peak inflammation. This is in opposition to our initial data, as well as a previous report showing that IL-17 depletion enhances GATA-1 expression (10). The most likely explanation is that IL-17 does play a role in bone marrow differentiation of eosinophils but one that is relatively minor. Though Hellings et al. found an increase in BM eosinophil differentiation when IL-17 depleted, the difference – while statistically significant – was relatively minor. In our model of inflammation the eosinophil infiltrate is relatively mild compared to other models that exhibit an intense T_H2 response. Thus, it is likely that differences in GATA-1 expression are not due to lower total transcript expression but increased expression of other transcripts that dampen the GATA-1 signal. Analysis of eosinophil maturation indicated that IL-17 played no role in eosinophil maturation, but our results predicted a decrease in IL-5 production. This hypothesis was supported, though not proven, by preliminary data from cytokine arrays. Analysis using ELISA or western blot will be needed to confirm these initial findings.

Finally, we showed that IL-17 drove the accumulation of new myeloid cells in the lung, and IL-17$^{-/-}$ mice had a buildup of eosinophils and other small myeloid cells in the blood during the inflammatory response. This indicated that the dearth of eosinophils in the lungs in IL-17$^{-/-}$ mice during the hypersensitivity response was due mainly to poor trafficking, though the data suggests IL-17 may play a smaller role in bone marrow differentiation as well as IL-5 production. It is currently known that IL-17 can affect the production of a number of chemotactic signals; IL-17 can drive IL-5 and GM-CSF production, and IL-17 itself is used as trafficking signal by monocytes. To our knowledge, however, IL-17 has not been shown to play a role in driving eotaxin

production or integrin expression. Therefore the data presented here generate new avenues of research with regards to IL-17 and cellular trafficking.

The study provided in these pages introduces several novel observations and adds to our understanding of mechanisms that have been previously shown by other researchers. Using the hypersensitivity mechanism initially developed by Mairi Noverr and Andrew Shreiner, we expand upon their initial findings about the adaptive response during hypersensitivity, particularly the role of IL-17 in driving inflammation and eosinophilia. What's more, our data are consistent with the findings of Zelante et al., namely that IL-17 negatively affects the immune system's ability to clear conidia from the lung (11). These studies also add several novel scientific methods to the field of immunology, particularly to the role of flow cytometry and cellular identification in the lungs. Not only did we identify several myeloid precursors, but we were able to independently confirm IL-5R shedding as previously reported by Liu et al. (12). Unlike previous in vitro methods, our use of flow cytometry allows in vivo tracking of cellular movement as well as characterization of eosinophil maturity in the bone marrow, blood, and peripheral tissue. Together, with CD11b and siglec F expression, this approach provides an exciting new method of examining the maturity of eosinophils in response to stimuli.

Critical Review

The data presented here provides insights into the immune system during an allergic reaction in the lungs in response to chronic allergen exposure. There are three primary human pulmonary diseases associated with *Aspergillus fumigatus* exposure in immunocompetent individuals: hypersensitivity pneumonitis (HP), allergic

bronchopulmonary aspergillosis (ABPA), and allergic asthma. The model used in these studies shares certain features with these diseases, particularly asthma, though with any mouse model there will be limitatations due to differences between mice and humans in terms of genetics, immune reactions, and basic physiology.

The mouse model of disease here has very little in common with hypersensitivty pneumonitis (also refered to as 'extrinsic allergic alveolitis'). HP is an inappropriate inflammatory response against chronic exposure to small particles, particularly organic dust. However, microorganisms such as *Af* have also been shown to be a source of disease (13-15). In terms of both physiological reaction as well as immune reaction, HP bears little resemblance to our model of disease. Inflammation with HP results in a thickening of the alveolar wall and ultimately interstitial fibrosis, neither of which are observed in our model of allergy. Moreover, other features typically associated with HP are absent as well. While CD4 T cells play a role in our model as well as HP, in HP the CD4 response is primarily T_H1 and there is a low CD4/CD8 ratio (16), whereas our model shows negligible CD8 T cell involvement.

Allergic bronchopulmonary aspergillosis is a hypersensitivity disease resulting from colonization of the airway with *Aspergillus fumigatus* (17). This occurs primarily in individuals with asthma or cytic fibrosis, most likely due to the elevated levels of mucus secretion in the lung. Our model shares some traits with ABPA, particularly an influx of innate cells into the airway as well as increased serum IgE levels. However, there are some key differences. First, we fail to see fibrosis of the lung, though we have previously shown arterial remodeling, a characteristic of ABPA (18). Second, repeated conidia challenge does not result in a colonization of the lung in our mouse model. Silver staining lung sections twenty-four hours after challenge shows that the few conidia left in the lung

are beginning to germinate but have not colonize the lung. However, it is possible that repeated exposure to germinating conidia could mimic the allergens produced by colonizing fungi. Unfotunately, ABPA is a condition which requires a previous disease – in this case asthma – in order to manifest itself. Thus the lack of a hypersensitivity reponse prior to four challenges suggests the allergic response in C57BL/6 mice is not ABPA.

Thus, allergic asthma bears the strongest relationship with our mouse model. Though Af has not been shown to be a cause of asthma, *per se*, 20-25% of individuals with asthma are skin-test positive against fungal allergens (19, 20), and previous reports have linked asthma severity to sensitivity to fungal allergens (21, 22). Asthma itself, a reversible narrowing of the airways which obstructs airflow, has a number of immunologic features consistent with what we have shown. There is peribronchial and peribronchiolar cellular infiltrate, airway eosinophilia, metaplasia and mucus hypersecretion, and an increase in T_H2 cells and cytokines in both the disease and the mouse model (23). There are some key differences, however, between our mouse model and asthma. Most importantly, mice challenged repeatedly with *Af* do not exhibit many of the physiologic changes that characterize the asthmatic response. Histological examination does not reveal a narrowing of the airways nor any remodeling. Moreover, previous studies in the laboratory using this model have shown that mice challenged multiple times with *Af* conidia do not exhibit airway resistance using methacoline challenge, though C57BL/6 mice have been shown to be poor responders during this assay (24-26). As such, we cannot say that we are modeling asthma, however mice challenged repeated with conidia develop an allergic airway disease with most of the immunologic characeristics of asthma. This is important because while asthma is defined

by physiologic responses, it is ultimately driven by by the host immune system (not always – sometimes asthma can be triggered by physical stimuli) and an understanding of the host immune response is central to any treatment. Both asthma and HP can arise from nearly identical environmental factors (27, 28), highlighting the importance of host immunity in driving disease as well as skewing the response towards a T_H1 or T_H2 reaction.

Several previous models of murine allergic airway disease have been generated by different groups in an attempt to elucidate immune pathways during pulmonary allergic reactions. The three primary methods of generating a response are repeated conidia exposure (29, 30), IP OVA sensitization and subsequent challenge (31, 32), and *Af* antigen bound to inert particles (33). The main benefit of our model is that it simulates the method by which an individual would be chronically exposed to *Af*, namely though repeated inhalation of fungal spores (34), albeit with a more strictly regimented timeline. In addition, the dose used is physiologically relevant, consistent with one found in a damp, musty environment or even a construction site (35). Thus, as compared to previously used models of pulmonary sensitization, our model more closely resembles environmental conditions encountered outside of a laboratory environment.

Our results provide several novel observations that may further aid in the study of allergic airway disease. First and foremost, we show that IL-$17^{-/-}$ mice have decreased eosinophilia during a T_H2-type response, something not seen before with knockout mice or IL-17-depleting antibodies (36) This new observation is most likely due to the method of sensitization: unlike previous studies viable conididia was used rather than fungal antigen, thereby creating a more complex and nuanced immune response. It is also possible, however, that the use of flow cytometry rather than simple cytospin allowed a

more nuanced analysis of the cellular population during inflammation. This lack of eosinophilia was accompanied by a buildup of eosinophils and total leukocytes in the blood, implying a possible role for IL-17 in extravasation. Moreover, we observed an initial regulatory response to low levels of inhaled conidia that is consistent with previous reports (37, 38) as well as observations previously made with our mouse model (39), suggesting that the regulatory response somehow plays a role in initiating or even driving the inflammatory response. Similarly, IL-17 plays a role in the hypersensitivity response by exacerbating the cellular infiltrate following four challenges, conistent with previous reports (10, 40-42). Finally, we observed a decrease in the T_H2 response following chronic exposure to conidia that correlates with an increase in the T_H17 response. This suggests a possible role for T_H17 in regulation of T_H2, something that has not been previously demonstrated in the literature. Taken together, our results show or suggest new roles for several cytokines including IL-17 and IL-10, as well as role for T_H17 and T_{reg} in initiating – and in the case of T_H17 resolving – the hyeprsensitivity response during allergic airway disease.

Future Directions

The data presented here opens up a wide range of possible future directions. Along with a continued examination of trafficking signals during inflammation, many intriguing lines of inquiry arise from observations made during the hypersensitivity response to *Aspergillus fumigatus*. Possible future directions range from hypersensitivity induction to eosinophil maturation.

The first and most obvious project involves elucidating the mechanism by which hypersensitivity is induced in the first place. Andrew Shreiner previously demonstrated

that a lack of IL-10 abrogated eosinophilia (39), and in Chapter 3 we demonstrated that the early response to conidia involved increased IL-10 production as well as an increase in the percentage and number of regulatory T cells. This suggests that the regulatory response has an early role in activating the adaptive immune response somehow. This is counterintuitive, however, as the hypersensitivity response is characterized by T_H2 cytokines and regulatory T cells have been shown to attenuate T_H2 allergic hypersensitivity (43). In fact, T_{reg} and the T_H2 responses are mutually antagonistic, as IL-4 inhibits induced-T_{reg} formation (44, 45). How then are IL-10 and regulatory T cells responsible for driving a T_H2 hypersensitivity response? Given the suppression of IFN-γ seen over the course of exposure, one possible explanation is that suppression of T_H1 creates a sort of vacuum that the T_H2 response moves in to fill. Many of these ideas could be tested using antibody depletion models or by the addition of specific cytokines during inflammation. The addition of IL-17 during the early response might accelerate or exacerbate the inflammation following four challenges. Likewise, addition of IL-17 during the hypersensitivity response may accelerate the rate at which the inflammatory response is resolved. Similarly, T_{reg} depletion prior to sensitization may attenuate inflammation given the early regulatory response in reaction to a low number of conidia challenges.

What's more, we know that IL-4 is produced during the hypersensitivity response, but we don't know how strong that expression is relative to other models such as *C. neoformans* infection. It has been previously shown that T_{reg} dampen the T_H2 inflammatory response but do so by controlling IL-4 and not IL-5, which is controlled by IFN-γ (46). This data concept is supported in our model by microarray data (Fig. A2-5) where IL-5 is shown to be upregulated in WT mice during hypersensitivity. Thus, there

are several experiments that could be introduced to try to alter the hypersensitivity response. The first, and simplest, is to use IL-5$^{-/-}$ mice and determine whether a lack of IL-5 significantly affects inflammation and subsequently use IL-5 depletion. Certainly it will reduce or abrogate eosinophilia (47, 48), but it is also possible that IL-5 plays a role in hypersensitivity given its role in chemotaxis (49, 50) and IL-13 production (51).

A similar possibility mentioned in Chapter 4 is that IL-23 plays a role in driving hypersensitivity since it functions upstream of IL-17 (52, 53). Given its links to other forms of inflammation, it would be interesting to see if it also played a role in allergy. Once again, IL-23 depletion or IL-23$^{-/-}$ mice would be useful, the latter already available as p19$^{-/-}$ mice (11). Also intriguing is the resolution of the hypersensitivity response. Between four and eight challenges the hypersensitivity response is dampened and eosinophilia is reduced. While several reports have indicated that IL-17 exacerbates T_H2 inflammation (40, 41), the addition of IL-17 at specific times can attenuate a T_H2 allergic response (10, 54). Moreover, our studies show that during chronic exposure to conidia the number of IL-17-producing CD4 T cells continues to climb, even as the hypersensitivity response is resolved. Could then IL-17 play a role in regulating T_H2? If so, this could explain why asthma is frequently 'outgrown': the T_H17 response eventually suppresses and replaces T_H2. This could be tested by depleting IL-17 once the hypersensitivity response has been induced, or by adding IL-17 prior to inflammation to try to abrogate the response.

Interestingly, we found that viable conidia seemed to be more important in priming of the hypersensitivity response than in actually triggering it. Unintentionally, we administered conidia with lower viability to two mice to examine four-challenge hypersensitivity but did this after using normal, fully viable conidia for the first two

challenges. Under these circumstances mice were still able to generate a robust hypersensitivity response. However, when one viable challenge was followed by three semi-viable challenges the response was significantly dampened. This suggests that multiple challenges of viable conidia are needed to prime the immune system for a hypersensitivity response, while a small dose is capable of initiating it. Possible future studies could examine the role of viable versus semi-viable conidia in the allergic response by alternating exposures and timing.

Also interesting is the timing of hypersensitivity. We have found that cohorts of mice display variable hypersensitivity over the course of conidia exposure which is most likely due to the viability of the conidia (55). In a recent exposure regimen we observed that mice challenged eight times had a similar pattern of expression as those exposed four times in previous assays, something we had never seen before. This anomaly occurred when we used a conidia stock that were subsequently shown to have lower viability than primary stock. Therefore it is entirely possible that the viability of the conidia determines not only the strength but also the kinetics of the hypersensitivity response. If this were the case then repeated low doses of an allergen would ultimately result in an allergic response similar to higher doses, but over a prolonged period of time and with a longer buildup. The kinetics of this process would be an interesting focus of research, especially as it pertains to asthma in the general populace. Since non-viable – or simply old – conidia are presumably responsible for the delayed reaction, one could measure kinetics of the response by mixing fresh and aged/non-viable conidia together in distinct ratios and observing the rate at which hypersensitivity is generated.

Looking at eosinophils, there are several signaling pathways to examine in regards to trafficking. Though our data suggests a decrease in IL-5 due to lack of IL-17

production, it is possible that other signals are affected. Eosinophil trafficking involves IL-4, IL-5, and IL-13 (56), and both Eotaxin-1 and -2 play central roles in eosinophil recruitment (57, 58). Moreover, now that microarrays have been used to survey of the molecular signal landscape – including leukotriene expression – (Fig. A2-5), a more thorough analysis using ELISA or western blotting might shed light onto the role of IL-17 in allergic hypersensitivity. Finally, an examination of eosinophil survival could add a final piece to the puzzle in elucidating the role of IL-17 in this model.

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Appendix 1
CD4 T Cells Are Not the Sole Contributors of IL-17

Introduction

Based on the central role of CD4 T cells in generating the hypersensitivity response, we treated – to some extent – CD4 T cells as the primary producers of IL-17. However, there are several other sources of IL-17. Lymphoid tissue inducer-like cells have been shown to be innate sources of IL-17 (1) as have γδ T cells (2). Moreover, several myeloid cell lines express IL-23R and are capable of generating IL-17 (3), and IL-17-producing macrophages have been shown to mediate allergic lung disease (4).

We therefore wished to examine whether CD4 T cells were truly the primary source of IL-17 or whether other cell types may be playing a role in production. To do this, we isolated CD4 T cells from the spleen of WT and IL-17$^{-/-}$ mice challenged four times with *Af* conidia. Cells were enriched using MACS and adoptively transferred into naïve wild-type mice. Twenty-four hours after injection mice containing transferred T cells were challenged with conidia following the standard protocol, then challenged seven days later and harvested. Cell numbers were compared between mice receiving WT 4X CD4 T cells and IL-17$^{-/-}$ 4X CD4 T cells.

Aims

1. To determine the source of IL-17 during pulmonary hypersensitivity to *Aspergillus fumigatus* conidia.

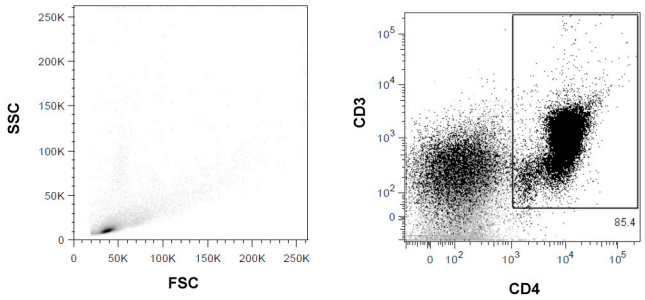

Figure A1-1. CD4 T cells for adoptive transfer are > 80% pure. Leukocytes were isolated from the spleens of WT and IL-17$^{-/-}$ mice challenged four times with Af. CD4 T cells were enriched using the MACS system and then stained for CD3 and CD4 to verify purity.

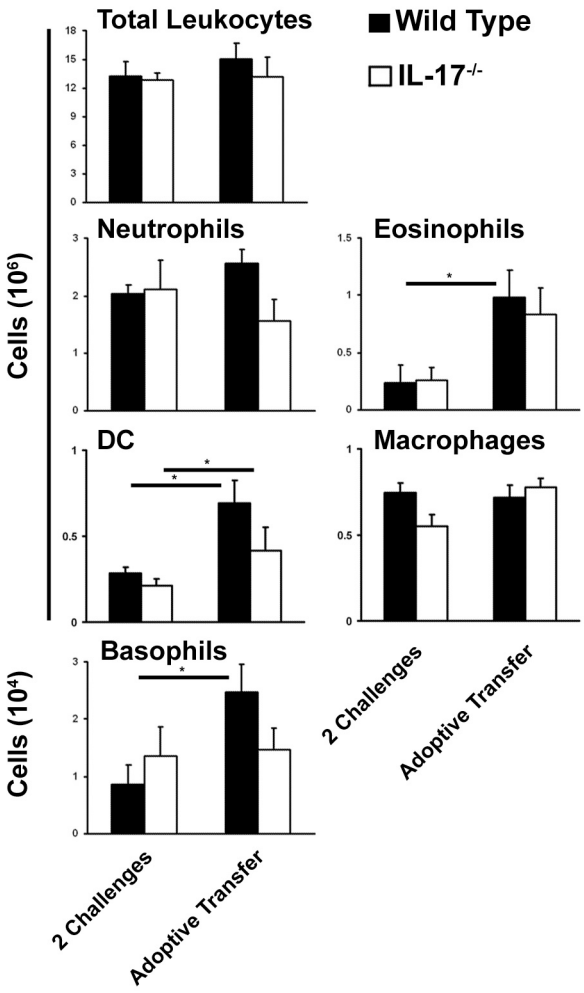

Figure A1-2. CD4 T cells from both WT and IL-17$^{-/-}$ elicit a similar early hypersensitivity response to conidia challenge. Following adoptive transfer of CD4 cells harvested from WT and IL-17$^{-/-}$ mice challenged four times with conidia, naïve WT C57BL/6 mice were challenged twice with *Af* conidia. Twenty-four hours after final challenge lungs were enzymatically dispersed, and cells were counted and analyzed by FLOW cytometry. Mice challenged twice with no adoptive transfer were used as controls. For two-challenge mice n = 3, for adoptively transferred mice n = 8. Black columns = WT mice; white columns = IL-17$^{-/-}$ 2-challenge mice and WT mice receiving IL-17$^{-/-}$ CD4 T cells and challenged twice.

Discussion

We wished to see if CD4 T cell production of IL-17 was solely responsible for the hypersensitivity response seen following four challenges. The data provided here suggest that while CD4 T cells play a role in producing IL-17, other sources may be involved as well.

Adoptively transferring CD4 T cells to naïve mice accelerated the rate at which certain cell types began to appear in the lung. In particular, dendritic cells, basophils, and eosinophils all showed a significant increase when mice were primed with transferred CD4 T cells. However, there was no significant different seen between mice that had received T cells from WT mice or IL-17$^{-/-}$ mice. This suggests that other IL-17 producing cells are responsible for the additional cells levels, while the CD4 T cells are merely accelerating the process.

One potential problem with this assay was the lag time between mouse harvest and subsequent adoptive transfer. Spleens were collected during the flow of a mouse harvest, and CD4 T cells were not purified until several hours after that. Following purification, there was another wait before the cells could be adoptively transferred. Given the long lag time and relatively short life of a CD4 T cell away from a source of stimulation, it is possible that greater differences would be seen if the cells had been enriched and transferred in a more hurried fashion.

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Appendix 2
Protocols and Odds and Ends

Introduction

The following figures contain exposure timelines, gating strategies, and other random data that did not fit directly into the narrative of this dissertation.

In the case of the microarray data, n values were too low and error bars too high for me to place the data alongside observations that were much more concrete. Nonetheless, I find the data supports assertations made in Chapters 3, 4, and 5 and could serve as a valuable jumping-off point for future studies.

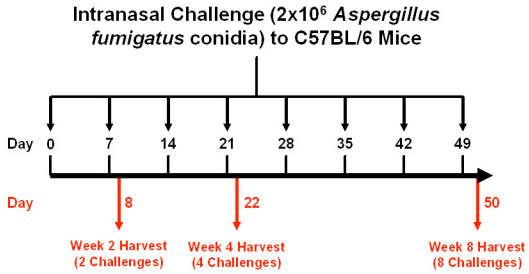

Figure A2-1. Timeline for pulmonary *Af* conidia challenge. Naïve WT and IL-17$^{-/-}$ mice were challenged weekly with 2 x 10^6 live *Af* conidia. Mice were harvest twenty-four hours after two, four, and eight challenges.

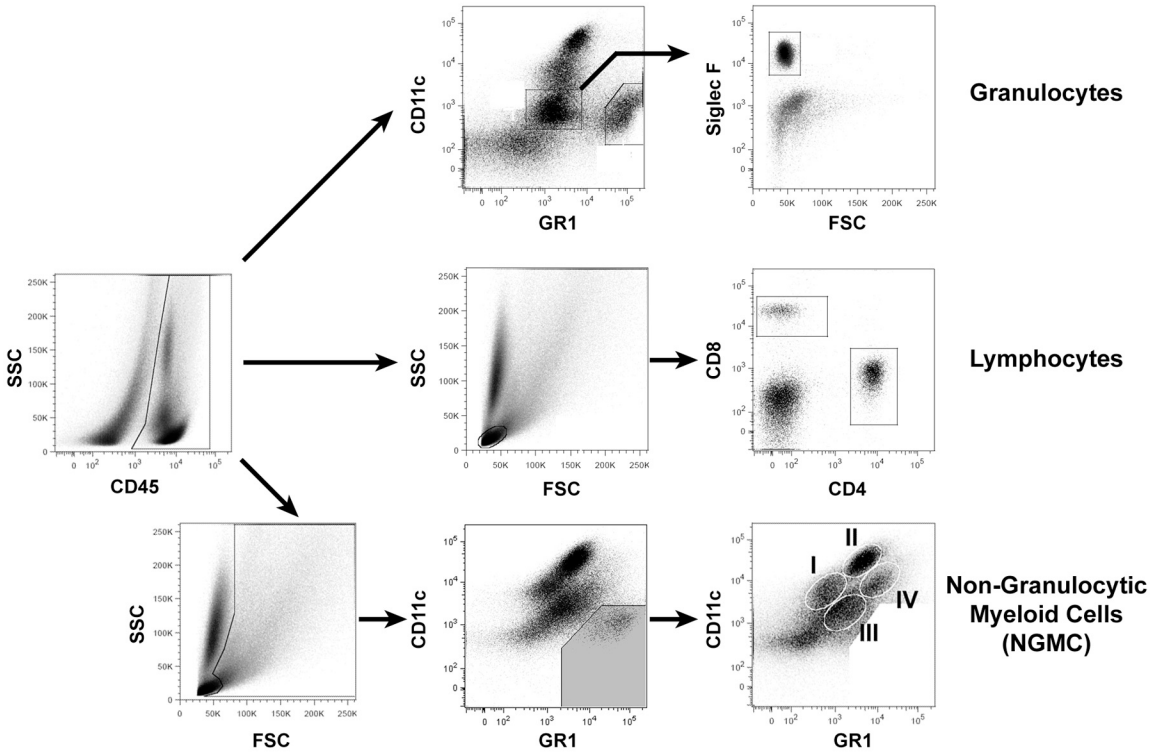

Figure A2-2. Gating strategy for enzymatically dispersed lung leukocytes. Following enzymatic digestion, purification, and counting, cells were stained with fluorescently labeled antibodies and analyzed using FLOW cytometry. All leukocyte populations were first separated from epithelial cells by CD45 gating. Eosinophils and neutrophils were identified by CD11c Gr1 gating: eosinophils are $CD11c^{mid}$ $Gr1^{mid}$ siglec F+ and neutrophils are $CD11c^{low}$ $Gr1^{high}$. Lymphocytes were identified by gating on cells with a low forward- and side-scatter profile then observing CD4, CD8, or CD19 expression. B cells (CD19) is not pictured. Non-granulocytic myeloid cells were examined by removing low forward-scatter cells and neutrophils. The remaining cells fell into four distinct groups labeled I-IV.

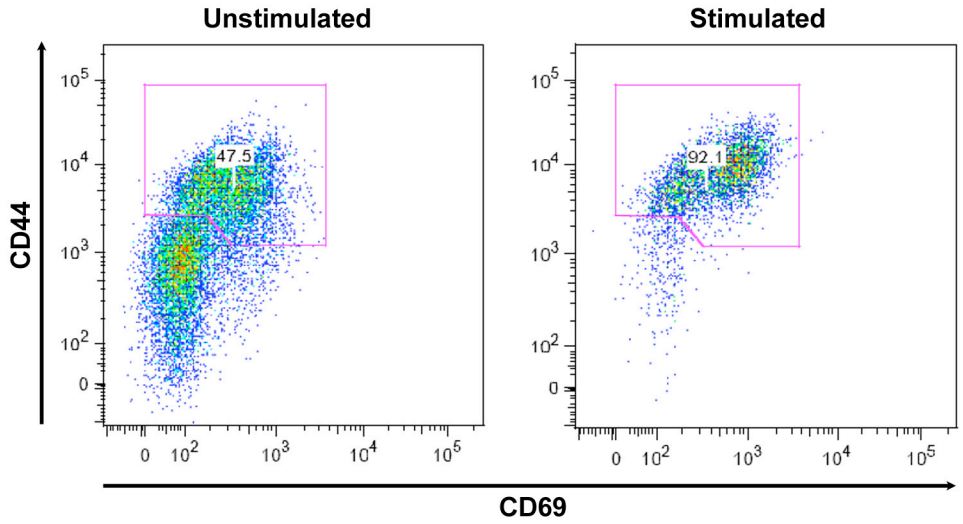

Figure A2-3. CD4 T cells stimulated with PMA and Ionomycin are activated. Cells were isolated from a naïve, unchallenged mouse. Following stimulation, cells were stained for CD4, CD44, and CD69. The change in expression indicates that (a) stimulation was successful and (b) that the activation gating strategy is viable.

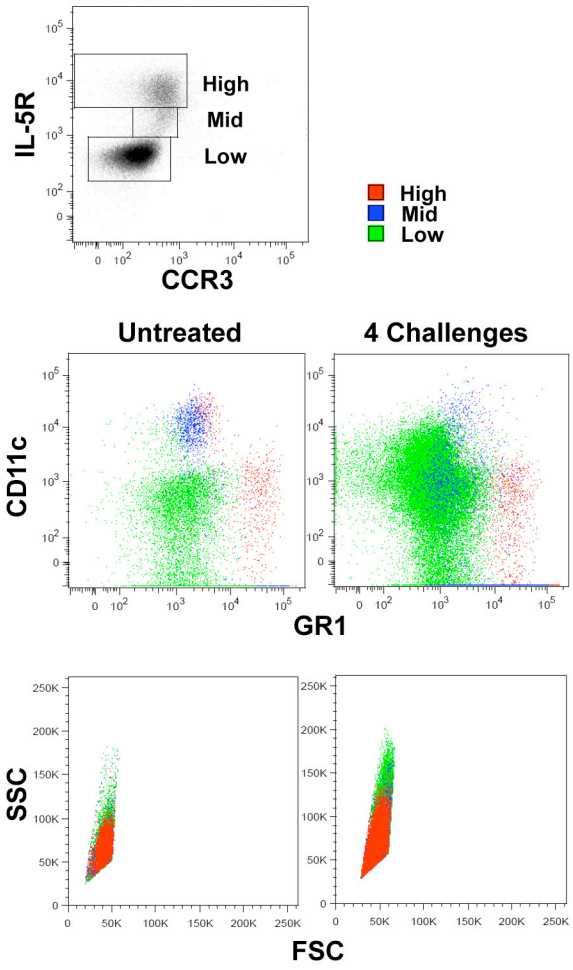

Figure A2-4. Eosinophils start Gr1+ CD11c^{low} but change their surface expression pattern as they mature. Lung eosinophils were stained with IL-5R, CCR3, CD11c, and GR1. As cells matured from IL-5R^{high} expression cells to IL-5R^{low} they migrated within the CD11c Gr1 plot. There was, however, no particular correlation between size and activation.

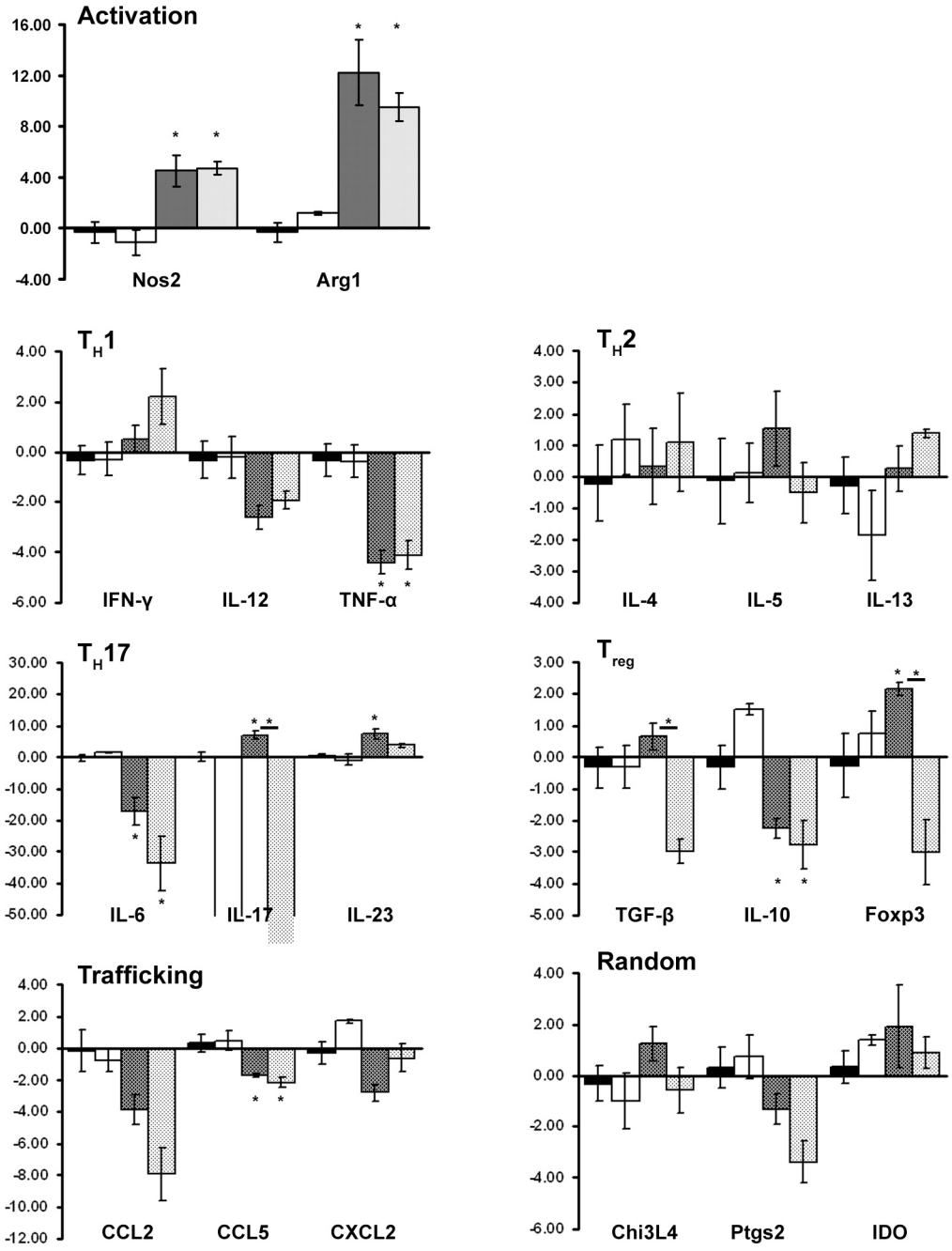

Figure A2-5. Hypersensitivity differentially alters the cytokine make-up of WT and IL-17$^{-/-}$ mice. WT and IL-17$^{-/-}$ mice challenged zero or four times were harvested and lung leukocytes isolated. Following RNA isolation and cDNA conversion, samples were run on a microarray plate containing primers for a battery of transcripts. Black columns = WT untreated, white columns = IL-17$^{-/-}$ untreated, dark cross-hatched columns = WT 4x challenges, light dotted columns = IL-17$^{-/-}$ 4x challenge. Stars indicate significant differences between untreated and 4X challenges; starred bars indicate significant differences between mouse strains ($p < .05$). n = 3 for untreated mice, = 5 for 4X challenge WT mice, and = 4 for IL-17$^{-/-}$ 4 X challenge mice.