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Mechanisms regulating airway
responses to the fungal allergen *Alternaria alternata*

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Abstract

Mechanisms regulating airway responses to the fungal allergen *Alternaria alternata*

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Human asthma is a heterogeneous disease characterized by the expression of both Th2 and Th17 cytokines. *In vitro* and *in vivo* studies have shown a reciprocal regulation between Th2 and Th17 pathways, suggesting a potential induction of neutrophil-promoting Th17 inflammation in the absence of a Th2 response. *Alternaria alternata* is a clinically relevant allergen that is associated with severe and fatal asthma exacerbations. Exposure to *A. alternata* is characterized by a predominant Th2 response, but can also induce the production of factors associated with Th17 responses (e.g., CXCL8) from epithelial cells. Using a mouse model, we found that wild-type mice develop an eosinophilic Th2 airway disease in response to *A. alternata* exposure, while IL-4-, IL-13-, and STAT6-deficient mice exhibit a primarily neutrophilic response. Neutrophilic asthma in STAT6^{-/-} mice was accompanied by elevated lung levels of TNF- α , CXCL1, CXCL2, and CXCL5, and was steroid-resistant. Neutralization of

Th17 signaling only partially reduced neutrophil numbers and total airway inflammation. Airway neutrophilia developed in RAG-deficient and CD4-depleted Balb/c mice, suggesting that the suppression of neutrophil responses is dependent on Th2 cytokine production by T cells and that airway neutrophilia is primarily an innate response to allergen. These results highlight the importance of combination therapies for treatment of asthma and establish a role for factors other than IL-17 as targets for neutrophilic asthma.

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Dedication

To my husband Daniel and my sons, Derek and Balian,
for the unwavering love and happiness they bring to my life.

Chapter 1

Introduction

Asthma and the Immune System

Asthma is a chronic inflammatory airway disease that affects an estimated 300 million people worldwide and is responsible for nearly 250,000 deaths every year¹. In 2009, approximately 8% of the US population suffered from asthma, accounting for nearly \$56 billion in annual costs related to medical expenses, loss of productivity, and premature deaths¹. Dysregulated immune responses to allergens result in changes to airway structure and function including thickening of the airway epithelium, mucus hypersecretion, reversible airway narrowing, and bronchial hyperresponsiveness. Commonly known as airway remodeling, these changes present clinically as wheezing, dyspnea, sputum production, and airflow obstruction². Severe asthma is characterized by exacerbations in response to allergens that lead to smooth muscle contraction, airway edema, mucus plugging, and, if left untreated, death. Asthma is a complex heterogeneous disease driven by cells of the innate and adaptive immune systems. The contribution of specific immune cells to the pathogenesis of asthma will be further discussed below.

Innate Immune Regulation in the Airways

Airway Epithelial Cells

Airway epithelial cells (AEC) are the first line of defense against inhaled environmental pathogens and particulates. Initially thought to act solely as a physical mucosal barrier combined with mucociliary clearance capacity³, epithelial cells have the ability to detect and respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns

(DAMPs) in the lung and orchestrate innate immune responses⁴. However, the ability to initiate immune responses is also the reason that AECs are critical components of respiratory diseases such as chronic obstructive pulmonary disorder and asthma. Holgate *et al.*⁵ first implicated AECs as major players in asthma fifteen years ago. Since then, research studies have demonstrated that a variety of factors including genetic polymorphisms, common environmental risk factors, barrier function, and aberrant immune responses all contribute to the sensitization of AECs to allergen and initiation of acute innate responses.

Airway epithelial cells can recognize allergens via pattern recognition receptors on their cell membrane including Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectins, and Protease-activated receptors (PAR)^{6, 7}. Signaling through these receptors induces the production of various chemokines and cytokines that recruit immune cells to the airways. In response to house dust mite (HDM), epithelial cells produce CC chemokine ligands such as CCL2 and CCL20 that induce monocyte and immature dendritic cell (DC) migration to the lungs^{8, 9}. AECs can also recruit CCR4⁺ Th2 cells, eosinophils, and neutrophils via expression of CCL17 and CCL22¹⁰, eotaxin¹¹, and CXCL1 and IL-8^{12, 13}, respectively. Following recruitment to the airways, immune cells are further activated by the epithelial-derived cytokines thymic stromal lymphopoietin (TSLP), IL-25, and IL-33, as well as additional cytokines and factors including granulocyte-macrophage colony-stimulating factor (GM-CSF) and reactive oxygen species (ROS)^{14, 15, 16}.

TSLP, IL-25, and IL-33 are important modulators of mucosal immune responses that further highlight the key role of airway epithelial cells in asthma. Although these cytokines share almost no homology to one another or to their respective family members, they all share the ability to promote Th2 immune responses. TSLP induces maturation of DCs increasing their co-

stimulatory molecules and ability to promote CD4⁺ T cell proliferation and skewing towards a Th2 phenotype¹⁷. TSLP can also directly induce production of IL-4 by CD4⁺ T cells, and IL-5 and IL-13 by mast cells^{16, 18}. IL-25 can act directly on T cells to promote differentiation and maintenance of Th2 cells, and increase the survival and cytokine production of eosinophils^{19, 20, 21}. Both IL-25 and IL-33 can induce the production of IL-5 and IL-13 by group 2 innate lymphoid cells (ILC2)^{16, 22}. Additionally, IL-33 directly acts on Th2 polarized T cells to increase production of IL-5 and IL-13, increase the survival and maturation of mast cells, increase IL-4, IL-6, and IL-13 production by basophils, and activate eosinophils^{23, 24, 25, 26}. The effects of these epithelial-derived cytokines are further highlighted by the fact that genetic polymorphisms in the *TSLP*, *il25*, and *il33* genes are risk factors for asthma²⁷.

Apart from pro-inflammatory cytokines and chemokines, AECs produce the cytokine epidermal growth factor (EGF), which in conjunction with transforming growth factor beta 1 (TGF- β) produced by eosinophils and myofibroblasts, promotes the synthesis of extracellular matrix components (ECM) and collagen^{28, 29}. ECM and collagen deposition leads to thickening of the epithelial basement membrane that is a common feature of airway remodeling. Furthermore, exposure of AECs to epithelial-derived EGF and IL-13 produced by ILC2 and Th2 cells can promote goblet cell metaplasia resulting in mucus production and airway obstruction^{30, 31, 32}.

Interestingly, airway epithelial cell sensitization to allergens can be also be influenced by environmental risk factors such as viral infections and exposure to air pollutants. For example, respiratory syncytial virus infection or exposure to cigarette smoke increases AEC expression of TLR4^{33, 34} which reduces the threshold of allergen recognition. Exposure to air pollution with

NO₂ and cigarette smoke also induces increased ROS and pro-inflammatory cytokine production by epithelial cells^{4, 35}.

Dendritic Cells

Dendritic cells are professional antigen presenting cells (APC) that have the ability to sample, phagocytize, process, and present antigens on the major histocompatibility complex II (MHC II) to naïve CD4⁺ T cells. Presentation of antigens through MHC II, along with upregulation of costimulatory molecules such as CD80, CD86, and CD40, and pro-inflammatory cytokine production are necessary in driving T cell differentiation towards the various effector helper subsets. In the context of allergic asthma, DC migration in response to allergens is mediated by epithelial cell production of the chemokines CCL2 and CCL20, followed by maturation of DCs by various factors including GM-CSF, TSLP, and IL-33³⁶. After maturation, CD11c⁺ DCs migrate to the lung draining lymph nodes where TSLP-induced expression of OX40L drives Th2 cell differentiation¹⁷. DCs can also produce a variety of factors including CCL17 and CCL22, IL-8, and eotaxin 2 that recruit T cells, granulocytes, and eosinophils into the airways³⁷.

Multiple studies have provided convincing evidence that DCs play a key role in the development of asthma³⁸. Allergen exposure results in significant increases in the number DCs in the lungs^{8, 39}. Hammad *et al.* showed that depletion of CD11c^{hi} DCs prior to intranasal sensitization with HDM protected mice from development of airway inflammation⁴⁰. van Rijt *et al.* found that acute depletion of CD11c⁺ DCs during OVA challenge, abolished CD4⁺ Th2 cell production of IL-4 and IL-13, eosinophilic inflammation, goblet cell hyperplasia, and AHR⁴¹. Th2 responses and asthma development was restored by adoptive transfer of CD11c⁺ DCs, a

finding further supported by Lambrecht *et al.* showing that intratracheal delivery of antigen-pulsed bone marrow-derived DCs was sufficient to induce allergic Th2 responses in response to allergen challenge⁴².

Eosinophils

Considered a prototypical hallmark of chronic airway disease and a central effector cell, eosinophils are present in the sputum of up to 80% and 50% of corticosteroid naïve and treated patients, respectively^{43, 44}. Originating in the bone marrow, eosinophil infiltration and maintenance in the airways is driven by IL-5 and chemokines including the eotaxins CCL11, CCL24, and CCL26^{36, 45}. Activation of eosinophils leads to their degranulation and release of basic granular proteins such as eosinophil cationic protein, eosinophil peroxidase, and major basic protein resulting in airway epithelial cell and tissue damage⁴⁶. Eosinophils can also release a plethora of mediators including IL-13, TGF- β , matrix metalloproteinases, and cysteinyl leukotrienes that increase vascular permeability, smooth-muscle thickening, epithelial mucus secretion, and peribronchial fibrosis all resulting in airway remodeling and airway hyperresponsiveness⁴⁵. Moreover, eosinophils can promote Th2 responses through direct effects on DCs. In a model of peanut food allergy, activation and CCR7-dependent migration to mediastinal lymph nodes of CD103⁺ DCs was controlled by eosinophil peroxidase⁴⁷. Additionally, Mattes *et al.* showed that eosinophils could modulate IL-13 production by CD4⁺ T cells to induce AHR⁴⁸.

The importance of eosinophils in the pathogenesis of asthma has been further supported by the development of transgenic mice that specifically lack eosinophils. Lee *et al.* created a transgenic mouse with expression of the diphtheria-toxin A chain under the control of the

eosinophil peroxidase promoter (*PHIL*) and showed that eosinophils were required for mucus accumulation and AHR development⁴⁹. Humbles *et al.* deleted the GATA-1 binding site on the GATA-1 promoter (Δ bdl), which resulted in ablation of lung eosinophils⁵⁰. Although the initial study with Δ bdl GATA mice showed no significant differences in AHR and mucus production between transgenic and wildtype mice, Walsh *et al.* later determined that this effect was strain dependent. Compared to Balb/c Δ bdl GATA mice, eosinophil-deficient C57BL/6 Δ bdl GATA mice had decreased AHR and Th2 cytokine production⁵¹. Furthermore, acute transfer studies using *PHIL* mice demonstrated that adoptive transfer of both OVA-specific Th2 effector cells and eosinophils was required to restore Th2 airway inflammation and AHR⁵². Taken together, these studies support the importance of eosinophils as central mediators of chronic airway disorders.

Neutrophils

Neutrophils, a type of polymorphonuclear granulocyte, are the most abundant type of white blood cell in mammals. Neutrophils are the first line of defense against pathogens, rapidly mobilizing to tissues after activation of PRRs, particularly TLRs⁵³. Chronic neutrophilic inflammation is strongly associated with a range of lung diseases including COPD, cystic fibrosis, and severe asthma⁵⁴. Neutrophil recruitment into the airways is mediated by various factors including *CXC* chemokines signaling through the CXCR1 and CXCR2 receptors such as CXCL1, CXCL2, and CXCL5, IL-8, IL-1 β , TNF- α , and leukotriene B₄. Once in the airways, neutrophils are activated by the combined recognition of pathogens through their PRRs and lymphoid cell-derived pro-inflammatory cytokines such as IFN- γ , GM-CSF, and TNF- α ⁵⁵. Upon activation, neutrophils release granule proteins including neutrophil elastase, myeloperoxidase

(MPO), matrix metalloproteinases (MMPs), and ROS. Both neutrophil elastase and MPO are increased in human asthma and contribute to peribronchial fibrosis and AHR via induction of goblet cell hyperplasia and smooth muscle cell proliferation^{56, 57, 58}. MMPs, especially MMP-8 and MMP-9, are significantly upregulated in the BAL fluid of patients with severe asthma. Additionally, MMP-9 has been shown to contribute to allergen-induced nonspecific AHR in airways of mice after allergen challenge^{59,60}. Neutrophils can also produce a variety of pro-inflammatory cytokines including TGF- β and oncostatin M both of which stimulate proliferation of fibroblasts and smooth muscle cells resulting in airway remodeling^{61, 62}. Neutrophils have the ability to themselves recruit additional neutrophils to sites of inflammation via the production of several neutrophil attracting factors including IL-8 and TNF- α ⁵³. This is relevant given that the concentration of IL-8 in the bronchoalveolar lavage fluid of asthmatic patients correlates to the degree of bronchial hyperreactivity⁶³.

The mechanisms controlling the persistence of neutrophils during chronic airway diseases are still being explored. However, studies have shown that IL-17A is an important factor mediating chronic neutrophilia in non-allergic asthma⁶⁴. Additionally, several studies have suggested that chronic neutrophilia in the airways is a result of a decreased susceptibility to apoptosis mediated by increased airway expression of GM-CSF, IFN- γ , LTB₄, and LPS⁶⁵. Interestingly, in a model of systemic sepsis, IL-33 contributed to neutrophil infiltration via inhibition of the G protein-coupled receptor kinase 2, which prevents the downregulation of CXCR2 receptor on circulating neutrophils⁶⁶. It is possible that this also happens in asthma where allergens induce potent upregulation of IL-33 in the airways. Although the exact mechanisms driving chronic airway neutrophilia in lung disorders is still not completely

understood, there is enough evidence showing that neutrophils play an important role in these disorders and should be further explored as therapeutic targets.

Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are a family of non-T, non-B lymphocytes present in mucosal and lymphoid. ILCs are morphologically similar to lymphocytes, but lack rearranged antigen-specific receptors and lack any cell-surface markers associated with other immune cells^{67, 68}. The ability of ILCs to rapidly respond to environmental and pathogenic factors has made them critical components of tissue homeostasis, repair, and innate immunity. ILCs are categorized into three major groups based on their cytokine production capacities: 1) ILC1 produce IFN- γ , 2) ILC2 produce IL-5, IL-9 and IL-13, and 3) ILC3 produce IL-17A and IL-22⁶⁷.

ILC2s are present in the lungs of mice and humans and are important in the pathophysiology of allergic airway inflammation. Defined as lineage negative and c-Kit, Sca-1, IL-7R and IL-33R positive cells, ILC2s in the lungs, bronchoalveolar lavage (BAL) fluid, and mediastinal lymph nodes rapidly produce IL-5 and IL-33 in response to the epithelial-derived cytokines IL-25 and IL-33^{69, 70}. Intranasal administration of papain or HDM, protease-containing allergens, to Rag1^{-/-} mice induces IL-5 and IL-13 production by ILC2s resulting in airway eosinophilia, mucus production, and AHR⁷¹. These Th2 responses are significantly reduced in Rag2^{-/-}IL2rg^{-/-} mice, which lack ILC2s, and can be rescued by adoptive transfer of ILC2. ILC2 expression of IL-13 serves both as a mediator of AHR and early source of cytokines for polarization of Th2 cells. Additionally, ILC2s can produce amphiregulin, a TGF- β -like molecule that acts on fibroblasts and epithelial cells to induce airway remodeling^{72, 73}. Aside from epithelial cells, other immune cells including mast cells, eosinophils, and macrophages can

modulate ILC2s activation through the production of mediators such as leukotriene D4 (LTD4) and prostaglandin D₂ (PGD₂) following allergen challenge^{74,75}.

Recently, ILC3s were implicated in the development of non-allergic, obesity-associated asthma. Kim *et al.* found increased numbers of ROR γ ⁺CD44⁺CCR6⁺ ILC3s in mice with obesity-induced airway disease and AHR⁷⁶. These ILC3s responded to IL-1 β expressed in response to high-fat diet and produced IL-17. The increased IL-17 lung expression resulted in AHR, a feature that was independent of adaptive immune cells. Kim *et al.* also showed increased numbers of ILC3s in the BAL fluid of obese individuals suffering from severe asthma.

T Cell Responses in Asthma

Th2 Cells

Early studies in patients found increased numbers of IL-4-producing CD4⁺ T cells in the BAL fluid of mild to moderate asthmatics compared to healthy controls⁷⁷. The number of Th2 cells present in airways of asthmatics has also been shown to positively correlate to disease severity⁷⁸. Since then, the role of Th2 cells as central mediators of allergic asthma was further confirmed by mouse models of asthma in response to the antigen ovalbumin (OVA). In these studies, acute or genetic depletion of CD4⁺ T cells protected mice from asthma development, while adoptive transfer of skewed Th2 cells was sufficient to restore eosinophilic infiltration and mucus production^{79, 80}. Given that Th2 cells mediate airway inflammation mainly through the secretion of the prototypical cytokines IL-4, IL-5, and IL-13, the function of these cytokines will be discussed below.

IL-4 & IL-13

IL-4 and IL-13 are considered the central type 2 cytokines responsible for the induction and maintenance of allergic asthma. IL-4 binds to both the type I and type II receptors, which are composed of the common subunit IL-4R α plus the common gamma chain (γ C) and the IL-13R α 1 subunits, respectively. IL-13 binds only to the type II receptor. Binding of these receptors results in phosphorylation of the JAK tyrosine kinases and the signal of transducer and activator of transcription 6 (STAT6) factor, nuclear translocation, and gene transcription⁸¹.

The importance of these cytokines in asthma pathogenesis has been confirmed through various animal models. Acute blockade of IL-13 as well as genetic deletion of the genes encoding IL-4R α and STAT6 protect mice from allergen-induced airway inflammation in response to OVA challenge^{82, 83, 84}. Constitutive expression of IL-13 and IL-4 in the lungs is sufficient to induce an asthma-like disease characterized by eosinophilic inflammation, mucus hypersecretion, and subepithelial fibrosis⁸⁵. Additionally, single nucleotide polymorphisms (SNPs) in the *IL4* and *IL13* genes are positively correlated with pulmonary fibrosis, hyper-IgE, atopy, and asthma⁸¹.

IL-4 plays a critical role in the polarization of CD4⁺ T cells into effector Th2 cells by upregulation of the transcription factor GATA-3. GATA-3 binding promotes the expression of various Th2 genes including *IL4*, *IL5*, *IL9*, and *IL13*^{83, 86}. Importantly, IL-4 is required for allergen-specific IgE responses. IL-4 both induces class switching in B cells towards IgE synthesis and up-regulates the IgE receptors on mast cells, B cells, and basophils⁸⁷. Subsequent allergen encounter results in crosslinking of the IgE receptors leading to the release of pro-inflammatory mediators such as histamine, leukotrienes, and prostaglandins⁸⁸.

IL-4 and IL-13 have some overlapping functions. Both cytokines contribute to the development of alternatively activated macrophages through the transcription of arginase 1,

Retnla, and Chi3L3⁸⁹. Additionally, IL-4 and IL-13 regulate the migration of immune cells to tissues by inducing the expression of the vascular cell adhesion molecule-1, VCAM-1, in endothelial cells^{90, 91}.

IL-13 is the main cytokine mediating goblet cell hyperplasia, tissue fibrosis, and AHR. Blockade of IL-13 is sufficient to prevent mucus production in response to allergen challenge, while administration of recombinant IL-13 or genetic overexpression of IL-13 is sufficient to induce mucin gene expression on airway epithelial cells leading to mucus secretion⁹². Tissue fibrosis is regulated by IL-13 through a variety of mechanisms including induction of TGF- β production by epithelial cells, upregulation of arginase I by alternatively activated macrophages, and proliferation of myofibroblasts^{93, 94, 95}. The exact mechanisms by which IL-13 mediates AHR are still not fully understood. However, it is suggested that induction of mucin genes in epithelial cells, regulation of epithelial-derived contractile mediators, as well as direct effects on airway smooth muscle cells all contribute to the development of AHR⁹⁶.

IL-5

IL-5 binds to the IL-5R, consisting of the IL-5R α and the common cytokine β -chains, and signals through the STAT5 transcription factor to mediate multiple functions in various cell types. The main role of IL-5 in allergic diseases is the regulation eosinophil survival and function. IL-5 promotes the terminal differentiation of eosinophil progenitors in the bone marrow⁹⁷, mediates the survival of mature eosinophils by promoting migration to tissues⁹⁸, inhibits apoptosis by blocking the activation of the proapoptotic protein Bid⁹⁹, and promotes eosinophil activation and degranulation¹⁰⁰. The importance of IL-5 has been supported by the presence of high levels of this cytokine in the serum and BAL fluid of patients with atopic

asthma¹⁰¹. Additionally, transgenic overexpression of IL-5 in the lung epithelium of mice¹⁰², as well as the inhalation of recombinant IL-5 by asthma patients¹⁰³, results in increased eosinophil numbers, goblet cell hyperplasia, and AHR.

Th17 Cells

Recently, Th17 cells and IL-17A have been implicated as important mediators of non-allergic, neutrophilic asthma. Several groups have found increased numbers of IL-17A-producing CD4⁺ T cells in the lungs of asthmatic patients and shown a positive correlation between the levels of IL-17A and disease severity^{104, 105}. McKinley *et al.* showed that, in response to allergen challenge, adoptive transfer of polarized antigen-specific Th17 cells resulted in steroid-resistant lung neutrophilia and AHR¹⁰⁶. Additionally, IL-17-deficient mice exhibit reduced neutrophilia and airway remodeling^{107, 108}.

IL-17A directly promotes airway epithelial cell expression of IL-8, IL-6, and GM-CSF, which act on neutrophils to induce migration, elastase release, and anti-apoptotic survival, respectively^{109, 110, 111}. IL-17A can also promote the gene expression of MUC5AC and MUC5B in epithelial cells leading to mucus secretion¹¹². Additionally, IL-17A acts on airway smooth muscle cells to induce production of proinflammatory cytokines and chemokines such as IL-6, IL-1 β , CCL11, and CXCL8, inhibit cell apoptosis, and promote proliferation and migration¹¹³. Overall, these studies suggest a critical role of IL-17A in the promotion of chronic airway responses to allergens.

Asthma Endotypes

The traditional mouse model of allergic asthma consists of two phases: 1) sensitization with an intraperitoneal injection of the adjuvant aluminum hydroxide (alum) in combination with the antigen OVA, and 2) challenge with multiple intranasal doses of the aerosolized antigen OVA. This model gives rise to a predominant Th2 response characterized by eosinophil infiltration, AHR, and pulmonary pathology that is dependent on antigen-specific adaptive immune responses¹¹⁴. Although this model has been instrumental in advancing our understanding of the mechanisms driving asthma development, the method of sensitization is not physiologically relevant and the disease phenotype does not mirror the heterogeneity of that observed in patients. McGrath *et al.* found that only 36% of asthmatics have sputum eosinophilia (defined as greater than 2% eosinophils)¹¹⁵. Recent animal models have attempted to correct the deficiencies of the OVA/alum model by: 1) sensitizing and challenging mice solely through the intranasal route, and 2) studying the responses elicited by different allergens. These models have yielded asthma phenotypes that are heterogeneous and closer resemble human asthma. To better understand the clinical implications of these models, it is necessary to know the different endotypes observed in asthmatic patients.

For years, the most common way of classifying asthma was based on the inflammatory cell profile observed in induced sputum. These asthma phenotypes include: 1) eosinophilic or Th2-high, 2) neutrophilic or Th2 low, 3) mixed eosinophilic and neutrophilic, and 4) paucigranulocytic, where there are no detectable inflammatory cells¹¹⁶. In 2011, experts from the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology produced a consensus report called PRACTALL which redefines the classification of asthma based on both the inflammatory phenotype as well as the pathophysiological mechanisms driving disease¹¹⁷. The PRACTALL report suggests using the

following 7 parameters to define various asthma endotypes: clinical characteristics, biomarkers, lung physiology, genetics, histopathology, epidemiology, and treatment response. Based on these parameters several asthma endotypes were defined including aspirin-sensitive, allergic bronchopulmonary mycosis (ABPM), adult allergic asthma, preschool wheezer, severe late-onset hypereosinophilic, and asthma in cross-country skiers. These parameters will be critical in helping stratify patients based on both their inflammatory profiles and pathophysiological responses to better target asthma therapies.

Treatments for Human Asthma

The current standard therapies for asthma include a combination of inhaled corticosteroids and β_2 -agonists, which are nonspecific anti-inflammatory and bronchodilator drugs, respectively¹¹⁸. Although these therapies have been successful in managing the symptoms of asthma, they do not address the underlying cause. Additionally, long-term use of high-dose inhaled corticosteroids can cause systemic side effects including impaired growth in children, skin thinning, and cataracts¹¹⁹. Furthermore, approximately 5-10% of patients, mostly those with severe asthma, do not respond to steroid treatments. Even though it is a small percentage of the total patient population, this group accounts for about 50% of the total cost of asthma as measured by work productivity, activity impairment, and emergency hospital visits¹²⁰. Thus, there has been an increased effort to develop therapies that target the specific molecular pathways responsible for disease initiation and maintenance.

To date, only three alternative therapies have received approval by the Food and Drug Administration (FDA) for the treatment of severe asthma. Omalizumab is a humanized anti-IgE monoclonal antibody approved in 2003¹²¹. Bronchial thermoplasty, approved in 2010, is a

procedure that involves the delivery of heat energy to the bronchi with the purpose of destroying the smooth muscle lining to attenuate bronchoconstriction¹²². Finally, mepolizumab, approved in 2015, is an anti-IL-5 antibody targeting eosinophilic inflammation in severe asthma¹²³. Emerging therapies for severe asthma are mostly divided between those targeting Th2 and non-Th2 pathways and will be briefly discussed below.

Th2 Drugs

IL-4 and IL-13 are the cytokines that have received the most attention as targets for successful therapies. Multiple clinical trials have studied the efficacy of molecules and monoclonal antibodies that block the IL-4/IL-13 signaling pathway. Among these, soluble IL-4 receptors and monoclonal antibodies to IL-4 like pascolizumab and altrakincept failed to show any clinical efficacy in patients¹²⁴. Interestingly, molecules blocking IL-13 or the common IL-4R α subunit have been successful in reducing airway eosinophilia, enhancing Forced Expiratory Volume (FEV₁), and inhibiting late-phase asthmatic responses in patients with severe asthma. Examples of these drugs currently in phase I or II clinical trials include pitrakinra, tralokinumab, and lebrikizumab¹²⁵. A novel drug, SB010, is a DNA enzyme that cleaves and inactivates GATA3, the transcription factor driving Th2 polarization through expression of the *IL4*, *IL5* and *IL13* genes¹²⁶. Promising results of phase II clinical trials showed lower plasma levels of IL-5 and attenuated sputum neutrophilia in asthmatic patients receiving SB010 treatment. These studies have provided evidence that targeting only IL-4 has no clinical efficacy and future treatments should be aimed at blocking both IL-4 and IL-13 function.

A recent proof-of-concept study used a human anti-TSLP monoclonal antibody (AMG 157) to treat patients with mild asthma¹²⁷. Patients receiving the anti-TSLP antibody showed

decreases in sputum eosinophils, FeNO levels, and early- and late-phase FEV₁. These results are promising as targeting epithelial-derived cytokines might provide a way to treat a broader category of patients than those responsive to selective Th2 cytokines.

Non-Th2 Drugs

This subset of drugs is aimed at patients with a Th2-low phenotype associated with neutrophilia, smoking, obesity, and infection. Most of the drugs currently in clinical trials target neutrophilia. Infliximab and golimumab, both soluble receptors against TNF- α , moderately decreased asthma exacerbations in patients with adult-onset disease and did not significantly improve lung function in patients with severe uncontrolled asthma¹²⁸. Moreover, both drugs had unfavorable safety profiles, with golimumab being significantly associated with increases in systemic infections and cancer. It is still unclear whether blockade of TNF- α will be further explored as a therapeutic for asthma.

Small molecule antagonists against the CXCR2 and IL-1 receptors have also been explored as a method to decrease neutrophilia. Treatment of mild asthmatics with SCH527123, the CXCR2 receptor antagonist, resulted in reduced neutrophil levels in blood and sputum without any major safety concerns¹²⁹. The effects of anakinra, the IL-1 receptor antagonist, on neutrophil migration was tested in healthy volunteers challenged with one dose of inhaled lipopolysaccharide (LPS)¹³⁰. Neutrophilic inflammation in response to LPS challenge was reduced in response to anakinra and no serious side effects were observed.

Multiple mouse models and human studies have implicated IL-17A as a critical mediator of neutrophilic asthma making it an attractive therapeutic target. Secukinumab is a neutralizing human monoclonal antibody against IL-17A developed by Novartis. Secukinumab was tested in

a phase II clinical trial of ozone exposure, which induces acute neutrophilic airway inflammation, in healthy volunteers. Analysis of sputum samples revealed no significant differences in neutrophil counts between control and secukinumab-treated groups¹³¹. A different study, headed by Amgen and AstraZeneca, also reported no clinical efficacy of IL-17A blockade on moderate to severe asthmatics¹³². Interestingly, this study used the drug brodalumab, a human anti-IL-17 receptor A monoclonal antibody, which blocks binding of IL-17A, IL-17F, and IL-25 to their cognate receptor, suggesting that not even the pleiotropic effects of this antibody was enough to alleviate symptoms. The results of these studies are underwhelming given the mounting body of evidence suggesting a critical role of IL-17A in mediating lung neutrophilia.

Overall, the results of clinical trials with the various cytokine and receptor inhibitors suggest that finding a successful drug to treat asthma will require proper patient endotyping and stratification, and likely a drug cocktail that targets various pathways including components of the innate and adaptive immune systems.

Alternaria alternata

Alternaria alternata, an environmental saprophytic fungus, is considered one of the most potent human aeroallergens due to its strong clinical association to asthma, rhinosinusitis, onychomycosis, and ABPM^{133, 134}. *A. alternata* belongs to the genus *Alternaria*, which consists of more than 50 species of pathogenic and non-pathogenic fungi¹³⁵. *A. alternata* is commonly found in dead vegetation and soil samples from cultivated areas of grass and grain. Fungal spores constitute the main mode of dispersion and the primary source of fungal allergens. However, some *A. alternata* allergens have also been found along the whole hyphael tube¹³⁶. Exposure and subsequent sensitization to *A. alternata* is thought to occur predominantly outdoors where spore

counts are approximately 26 times higher than those observed indoors¹³⁷. Nevertheless, higher indoor levels of *A. alternata* allergens have been shown to significantly increase the odds of developing asthma symptoms¹³⁶. In patients sensitized to *A. alternata*, 100 spores per cubic meter is approximately the concentration at which allergy symptoms arise¹³⁸.

Several large epidemiological studies have assessed the incidence of *A. alternata* sensitization and found that, among asthmatics, the prevalence rates vary anywhere from 2% to 23.8%^{139, 140}. The incidence is even greater among asthmatics that have sensitization to one or more fungi where more than 60% have positive reactions to *A. alternata* allergens¹⁴¹. Children are particularly prone to sensitization with more than 38% of American asthmatic children having positive responses to *A. alternata*¹⁴². Sensitization to *A. alternata* is associated with increased asthma persistence and severity, emergency department visits, and asthma-related deaths^{134, 137}.

***A. alternata* Allergens**

A. alternata sensitization is thought to be primarily the result of IgE reactivity to proteins contained in the fungus. To date, 17 allergens derived from *A. alternata* have been isolated and characterized. Alt a 1 is the major allergen with an 80% sensitization prevalence among individuals with *A. alternata* reactivity¹³⁴. Alt a 1 is located in the cell wall of spores and is thought to be involved in spore germination. The other allergens include Alt a 2, Alt a 3, Alt a 4, Alt a 5, Alt a 6, Alt a 7, Alt a 8, Alt a 9, Alt a 10, Alt a 12, Alt a 13, Alt a 14, Alt a 15, Alt a 70 kDa, Alt a TCTP, and Alt a NTF2¹³⁴. These allergens, which include heat shock and ribosomal proteins, enolases, mannitol dehydrogenases, and serine proteases, serve various biochemical functions during *A. alternata*'s lifecycle. Interestingly, serine proteases, like Alt a 15, have the

ability to initiate allergic responses via the cleavage of PAR-2 receptors in airway epithelial cells¹⁴³. Thus, although Alt a 15 is considered a minor allergen based on its IgE reactivity, it could be an important player in inducing allergic responses to *A. alternata*. Future studies are needed to further determine the role and importance, if any, of all the other allergens in the development of allergic responses to *A. alternata*.

Immune Responses to *A. alternata*

Studies over the last several years have implicated airway epithelial cells as critical drivers of innate immune inflammation in response to *A. alternata*. Exposure of airway epithelial cells to *A. alternata* extract results in the rapid production of various pro-inflammatory cytokines including TSLP, IL-33, GM-CSF, IL-6, IL-8, and IL-18, as well as the eosinophil-recruiting chemokines eotaxin and RANTES^{144, 145, 146, 147, 148}. The exact mechanisms driving the activation of epithelial cells is not fully understood, however there is strong evidence suggesting a dependency on ATP release and PAR-2 activation. Exposure of airway epithelial cells to *A. alternata* results in the rapid release of ATP, which in turn increases intracellular Ca²⁺ concentrations and release of IL-33. IL-33 production is mediated by ATP-dependent activation of the P2 purinergic receptors given that epithelial cells treated with siRNAs against these receptors produce significantly lower levels of IL-33¹⁴⁸. The response to *A. alternata* has been suggested to be dependent on the activation of the PAR-2 receptors in epithelial cells by *A. alternata* serine and cysteine proteases. Blockade of PAR-2 activation via protease inhibitors or heat-inactivated fungal extracts result in significant decreases of intracellular Ca²⁺ concentrations, subsequent production of IL-33, IL-6, and IL-8, and immune cell recruitment^{144, 149, 150, 151}. However, Denis *et al.* and Doherty *et al.* showed that wildtype mice treated with heat-

inactivated *A. alternata* extract and PAR-2-deficient mice, respectively, had comparable levels of lung eosinophils and pro-inflammatory cytokine production compared to mice with intact PAR-2 signaling suggesting that the response to *A. alternata* is not entirely dependent on PAR-2 activation^{152, 153}. Aside from the production of pro-inflammatory cytokines, *A. alternata* can directly induce epithelial cell gene expression of mucin genes and the resistin-like molecule FIZZ1 that promote epithelial cell thickening and airway fibrosis¹⁵³.

IL-33 has been singled out as the cytokine predominantly responsible for driving the initiation of immune responses after *A. alternata* exposure. ST2^{-/-} mice, which lack the IL-33 receptor, have significant decreases in total BAL eosinophils^{147, 148, 154}. IL-33-mediated lung eosinophilia is independent of adaptive immunity and driven by lung resident ILC2 production of IL-5 and IL-13^{154, 155, 156}. Interestingly, ILC2 accumulation in the lungs after *A. alternata* exposure is dependent on the expression of the receptor for advanced glycation end-products (RAGE) by lung stromal cells, while ILC2 proliferation and expression of the EGF receptor ligand amphiregulin are regulated by STAT6^{156, 157}.

Aside from its effects on epithelial cells and ILC2s, *A. alternata* can directly activate eosinophils and dendritic cells. Eosinophils can adhere to *A. alternata* via binding of CD11b to the β -glucan present in the fungi's cell wall. Direct contact to *A. alternata* induces the degranulation of eosinophils including the release of eosinophil-derived neurotoxin and major basic protein¹⁵⁸. Aspartate proteases found in *A. alternata* can cleave PAR-2 receptors in eosinophils to induce cell surface expression of CD63 and CD11b, as well as production of IL-8^{159, 160}. Moreover, direct stimulation of bone-marrow derived DCs with *A. alternata* results in upregulation of MHC II and the costimulatory molecule OX40 ligand. *A. alternata*-activated DCs promote CD4⁺ T cell polarization into Th2 effector cells¹⁶¹. Taken together, these studies

implicate IL-33 and innate immune cells as critical regulators of the allergic airway responses observed after *A. alternata* exposure.

Questions to Address

Asthma is a complex disease that can be triggered by a variety of allergens and environmental factors. Initially thought to be a predominant Th2 response, studies over the last several years have shown that asthma is a heterogeneous disease mediated by both Th2 and non-Th2 pathways and characterized by the presence of a variety of inflammatory cell types including eosinophils, neutrophils, ILCs, and T cells. The complicated nature of this disorder has resulted in very few successful new therapies. Thus, there is a need to further understand the mechanisms driving innate and adaptive immune responses to specific allergens and, based on this data, develop novel drugs that will target the needs of individual patients.

Alternaria alternata is a clinically relevant allergen associated with severe and fatal asthma. Multiple studies have shown the molecular pathways driving the initial recognition and subsequent activation of innate immune cells in response to *A. alternata*. However, little is known about the role of the adaptive immune system and the pathways mediating chronic airway responses to *A. alternata*. It is important, therefore, to understand exactly what cell types and cytokine pathways regulate the persistence of chronic inflammation. The answers to these questions will be critical in determining the best therapeutic strategies for patients with sensitization to *A. alternata*. In Chapter 2, we investigate the role of the STAT6 signaling pathway in a chronic asthma model in response to *A. alternata*. We show that STAT6 signaling and CD4⁺ T cells are required for persistent lung eosinophilia. Additionally, in the absence of

the primary Th2 response, *A. alternata* induces a neutrophilic innate immune response that is steroid-resistant.

Chapter 2

STAT6 regulates the development of eosinophilic versus neutrophilic asthma in response to *Alternaria alternata*

Introduction

Asthma is a chronic inflammatory disease of the airways that can develop in response to a multitude of allergens. Fungi constitute one of the most important immunogens, with over 80% of asthmatics in the United States having sensitization to one or more fungi; of these, 75% have reactivity to *Alternaria alternata*¹³⁶. Large epidemiological studies have shown a strong correlation between *A. alternata* sensitization and an increased risk of severe and fatal asthma^{137, 162}. Mice exposed to *A. alternata* develop an allergic airway response characterized by increases in lung expression of the type 2 cytokines IL-4 and IL-13, eosinophil infiltration, and high levels of serum IgE^{153, 154, 161}.

IL-4 and IL-13 are critical to the development of allergic airway responses and are highly elevated in the bronchoalveolar lavage (BAL) fluid and sputum of asthmatic patients^{163, 164}. Recognition of these cytokines by a shared receptor unit, IL-4R α , results in the phosphorylation of the signal transducer and activator of transcription factor 6 (STAT6)^{165, 166}. STAT6 activation leads to cytokine specific responses with IL-4 driving the differentiation of Th2 cells and recruitment of eosinophils to the airways⁸³, and IL-13 inducing goblet cell hyperplasia and airway hyperresponsiveness (AHR)¹⁶⁷. Given their importance in the pathogenesis of asthma, IL-4 and IL-13 have become targets for the development of monoclonal antibodies to modulate asthma. Clinical trials targeting IL-4 and IL-13 have had mixed results and overall only a minimal effect in disease burden¹⁶⁸.

In addition to the Th2 pathway, IL-17 and the Th17 pathway have been shown to play a key role in severe asthma pathology^{169, 170}. IL-17A mediates the development of neutrophilic airway inflammation via upregulation of CXCL chemokines^{171, 172}, AHR^{106, 173}, and corticosteroid resistance^{106, 174}. Moreover, Th17 cytokines can contribute to airway inflammation by collaborating with other cytokines, such as tumor necrosis factor- α (TNF- α), to up-regulate the expression of neutrophil-attracting factors^{175, 176}.

Human asthma presents as a heterogeneous disease with various distinct phenotypes, including differential expressions of Th2 and Th17 signatures^{177, 178, 179}. IL-17A expression and neutrophils are present at high levels in patients with severe, persistent asthma^{180, 181}. Interestingly, IL-4 negatively regulates the differentiation of Th17 cells both *in vitro*^{182, 183} and *in vivo*^{184, 185}, suggesting a possible correlation between blockade of the Th2 pathway and a potential increase in Th17-dependent neutrophilic airway inflammation^{177, 185, 186}. The crosstalk between Th2 and Th17 pathways and the development of different asthma phenotypes is still not completely understood. In this study, we used STAT6-deficient mice to show that: 1) IL-4 and IL-13 are necessary for the development of airway eosinophilia in response to *A. alternata*, and 2) blockade of STAT6 signaling promotes the development of non-atopic, neutrophilic asthma. Our findings suggest that patients with sensitization to *A. alternata* will benefit from therapies that target both eosinophilic and neutrophilic inflammatory responses.

Materials and Methods

Mice

Six- to 8-week-old male and female Balb/c, Rag1^{-/-}, IL-4^{-/-}, and STAT6^{-/-} mice were obtained from Jackson Laboratories. IL-13^{-/-} mice were a gift from Andrew McKenzie (MRC

Laboratory of Molecular Biology, Cambridge, United Kingdom). All animals were bred and housed in specific pathogen-free conditions at the Benaroya Research Institute animal facility and all experiments were performed as approved by the Benaroya Research Institute Institutional Animal Care Committee.

Animal Model

Mice received intranasal (i.n.) administration of 50 µg *Alternaria alternata* extract (lots no. 188817 and 252218, Greer, Lenoir, NC) in a total volume of 50 µl PBS on days 0 and 1, rested, and challenged with 25 µg *A. alternata* extract on days 17, 18, and 19. Control mice were given PBS only. Mice were sacrificed 24 hours after the final challenge and inflammation was assessed as described below. For *in vivo* depletion of CD4⁺ cells, 200 µg of anti-CD4 antibody (clone: GK1.5, UCSF Monoclonal Antibody Core) in a total volume of 200 µl PBS was injected intraperitoneally (i.p.) on days -7, 0, 7, 14. For IL-17RA blockade, STAT6^{-/-} mice were injected i.n. on days 0, 1, 17, 18, 19 with 200 µg of M751 (anti-IL-17RA antibody, Amgen) or 200 µg rIgG in a total volume of 200 µl PBS; followed 2 hours later by i.n. administration of *A. alternata* extract as described above. For steroid sensitivity experiments, on days 17, 18, and 19, 2 hours prior to each *A. alternata* challenge dose, mice were given i.n. doses of 40 µg of Dexamethasone (Bimeda-MTC Animal Health, Inc.) in a total volume of 50 µl PBS or PBS only for controls.

Assessment of Airway Inflammation

At 24 hours after the final challenge, mice were euthanized by i.p. lethal injection of 1 ml of 2.5% Avertin in PBS. Bronchoalveolar lavage (BAL) fluid was collected by intratracheal insertion of a catheter and four lavages of 1 ml of PBS. Lungs were perfused with 5 ml PBS; the upper right lobe was removed and frozen in RNAlater (Thermo Fisher Scientific) for RNA

isolation. The left lung was excised, digested with Liberase TM (Roche), and single-cell suspensions obtained. The remaining lung tissue was removed and placed in 10% neutral buffered formalin (Leica Biosystems), embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H&E) and Periodic Acid Schiff (PAS) stains. BAL TNF- α was detected by ELISA (R&D Systems). Lung tissue and BAL cellular differential counts were determined by flow cytometry analysis. For quantitative RT-PCR, RNA was isolated with the NucleoSpin RNA kit (Clontech). cDNA was synthesized using PrimeScript Reverse Transcriptase (Takara) and mRNA expression levels assessed using the SYBR Premix Ex Taq II (Takara) according to the manufacturer's instructions. The level of mRNA was normalized to Gapdh expression, and the results were analyzed by the $2^{-\Delta\Delta C_t}$ method.

Antibodies and Flow Cytometry

The following antibodies were purchased from BD Biosciences: purified CD16/CD32 (Fc block), anti-SiglecF PE (E50-2440), and anti-IFN γ FITC (XMG1.2). The following antibodies were purchased from BioLegend: anti-Ly6G FITC (1A8), anti-CD19 APC (6D5), anti-CD45.2 PB (104), and anti-CD4 BV650 (RM4-5). The following antibodies were purchased from eBioscience: anti-CD8 PE-Cy5 (53-6.7), anti-CD11c PE-Cy7 (N418), anti-CD45.2 PerCP-Cy5.5 (104), anti-CD3 eFluor 450 (17A2), anti-MHC II Alexa Fluor 700 (M5/114.15.2), anti-CD11b APC-eFluor 780 (M1/70), anti-IL-17A PerCP-Cy5.5 (17B7), anti-TCR β Alexa Fluor 700 (IM7), anti-IL-13 PE (eBio13A), and anti-IL-4 PE-Cy7 (BVD6-24G2). Gating: Eosinophils: CD45+CD11c+SiglecF+; Neutrophils: CD45+CD11c-CD11b+Ly6G+, T cells: CD45+CD4+TCR β +

Single-cell suspensions were stained with antibodies and analyzed on a BD LSR II flow cytometer (BD Biosciences), and data was analyzed with FlowJo (Tree Star). For T cell

intracellular staining, lung cells were cultured with phorbol 12-myristate 13-acetate (1:2000, Sigma) and ionomycin (1:500, Sigma) in the presence of GolgiStop (BD Biosciences) for 3 hours. Cells were then stained for surface markers and intracellular cytokines (Cytofix/Cytoperm; BD Pharmigen).

Measurement of airway hyperresponsiveness

Airway responsiveness was measured 24 hours after the last challenge dose by invasive pulmonary function testing using the flexiVent system (Scireq, Montreal, Quebec, Canada). Briefly, mice were anesthetized and paralyzed with i.p. injections of 50 mg/kg pentobarbital (Sigma-Aldrich) and 0.6 mg/kg vecuronium bromide, followed by tracheostomy and intubation to the flexiVent ventilator. After baseline measurement, mice were challenged for 10s with saline aerosol, and at 4.5 minute intervals, with methacholine (MCh) at increasing concentrations (3.125, 12.5, and 50 mg/ml). For each MCh dose, the peak response was calculated as the mean of the six maximal values and used for calculation of airway resistance ($\text{cmH}_2\text{O}\cdot\text{s}\cdot\text{mL}^{-1}$) and airway elastance ($\text{cmH}_2\text{O}\cdot\text{ml}^{-1}$).

Bone marrow chimeras

Balb/c CD45.1 and STAT6^{-/-} CD45.2 mice were used for reciprocal chimeras. Donor bone marrow was isolated from tibias and fibulas. Recipient mice were irradiated twice (4 hours apart) with 450R, followed by tail vein injection of 1×10^6 donor cells. Mice were allowed to reconstitute for eight weeks before the initial *A. alternata* dose.

Statistical Analyses

A Student's unpaired *t* test was used to determine statistical significance between two groups. Multivariable analysis was determined using one or two-way ANOVA with Bonferroni's posttest. Data are presented as means \pm SEM. Differences between groups were considered

significant at the following values: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. All statistical analyses were performed using GraphPad Prism 6 software.

Results

Deficiency in Th2 cytokine signaling during A. alternata experimental asthma results in airway neutrophilia

Exposure to *A. alternata* induces airway inflammation characterized by increases in Th2 cytokines and infiltration of eosinophils^{153, 154, 161}. Recent reports of a house dust mite (HDM) murine model of asthma and an OVA-atopic march model have shown the development of a Th17 allergic phenotype after blockade of Th2 cytokines^{177, 185}. Considering the heterogeneity of Th2 and Th17 signatures in human asthma^{177, 178, 179} and the *in vitro* regulation of IL-4 and IL-13 in IL-17 production^{182, 183}, we sought to determine the effects of IL-4 and IL-13 blockade in *A. alternata*-induced asthma. WT, IL-4^{-/-}, IL-13^{-/-}, and STAT6^{-/-} mice were intranasally sensitized with two consecutive doses of *A. alternata* extract (ALT), rested for two weeks, and challenged three times. Disease development was assessed 24 hours after the last challenge dose (Fig. 2-1A). Total cell infiltration in the bronchoalveolar lavage (BAL) and lungs of all three KO strains was comparable to WT mice (Fig. 2-1B). However, while WT mice developed a predominant Th2 inflammation characterized by greater than 50% of cells in the BAL consisting of eosinophils, IL-4^{-/-} and IL-13^{-/-} mice developed a mostly neutrophilic phenotype with eosinophils consisting of only a small percentage of total cell infiltrate. STAT6^{-/-} mice developed a primarily neutrophil (~40% of BAL cells) response with no detectable eosinophils (Figs. 2-1C and 2-1D). The change in lung inflammation between WT and KO strains was accompanied by decreases in lung expression of IL-4, IL-5, and IL-13 in IL-4^{-/-} and STAT6^{-/-} mice, as well as increases in IL-

17A and IFN- γ in IL-4^{-/-} and STAT6^{-/-} mice, respectively (Fig. 2-1E). IL-13^{-/-} mice had comparable levels of IL-4 and IL-5 as that observed in WT mice, suggesting that IL-4 and IL-5 alone are not sufficient to drive airway eosinophilia.

Histologically, all mice displayed severe peribronchial and perivascular inflammatory infiltrates, as seen by H&E staining (Fig. 2-1F). IL-13- and STAT6-deficiency completely abrogated mucus production, as assessed by PAS staining and Gob5 expression (Figs. 2-1E & F). IL-4^{-/-} mice had diminished epithelial thickening and decreased mucus production, which correlated with a decrease in lung Gob5 expression (Figs. 2-1E & F).

Excessive constriction of the airways following challenge with an allergen is a critical feature of asthma. Given that airway neutrophilia is associated with severe bronchoconstriction and persistent asthma¹⁸⁷, WT and STAT6^{-/-} mice were challenged with methacholine to assess whether STAT6^{-/-} mice develop increased AHR. Airway reactivity was determined as a measure of lung resistance and elastance (Fig. 2-1G). ALT induced significant AHR in WT mice both at medium and high doses of methacholine (12.5 mg/ml and 50 mg/ml). In contrast, STAT6^{-/-} mice did not display reactivity to methacholine at any of the doses tested (Fig. 2-1G), showing that neutrophilia in response to ALT is not accompanied by airway hyperreactivity. Taken together, these results suggest that STAT6 signaling is necessary for airway eosinophilia; and in the absence of this signaling pathway, *A. alternata* induces a neutrophilic airway response.

T cells are required for persistent airway eosinophilia but not neutrophilia in response to A. alternata exposure

To identify the cell types involved in the response to *A. alternata*, bone marrow chimera studies were performed. Host mice were irradiated and reconstituted with donor cells to create

the following chimeric mice: WT \rightarrow WT, WT \rightarrow STAT6^{-/-}, and STAT6^{-/-} \rightarrow WT. Eight weeks after irradiation, mice were challenged with ALT as previously described. In agreement with prior findings¹⁵³, STAT6 signaling deficiency in hematopoietic cells resulted in an inhibition of eosinophil infiltration into the BAL. Interestingly, these mice exhibited a neutrophil phenotype similar to that observed in full STAT6^{-/-} mice (Fig. 2-2A), suggesting that the regulation of airway eosinophilia vs neutrophilia by STAT6 occurs in hematopoietic cells.

Examination of various BAL cell types revealed no major differences in percent and total cell numbers between WT and KO mice (Fig. 2-6A). However, given that Th2 cells have long been recognized as critical mediators of allergic asthma¹⁰⁵, we sought to determine the role of T cells in *A. alternata* airway responses. ALT exposure induced significant infiltration of CD4⁺ T cells in the BAL and lungs, with comparable numbers observed in WT and STAT6^{-/-} mice (Fig. 2-2B). Although both strains had similar amounts of activated T cells, as seen by CD44⁺ staining, STAT6^{-/-} mice had reduced numbers of proliferating CD4⁺Ki67⁺ T cells (Fig. 2-2C). Additionally, STAT6^{-/-} mice had a higher percentage of IFN- γ ⁺ and IL-17A⁺ lung CD4⁺ T cells and a decrease in IL-4⁺IL-13⁺ double producers (Fig. 2-2D). To further determine the contribution of the adaptive immune system, disease development in response to ALT was assessed in RAG^{-/-} mice. Although not statistically significant, there was a trend towards reduced cell infiltrates in the BAL of RAG^{-/-} mice compared to WT mice (Fig. 2-2E). Interestingly, RAG^{-/-} mice developed a primarily neutrophilic response similar to that observed in STAT6-deficient mice (Fig. 2-2F). Lung mRNA expression showed decreases in IL-4 and IL-13, and no changes in IL-5 or IL-17A (Fig. 2-2G). Similar results were obtained in WT mice depleted of CD4⁺ T cells (data not shown), suggesting that CD4⁺ T cells are necessary for the maintenance of airway eosinophilia in response to *A. alternata*. On the other hand, CD4⁺ T cell depletion in STAT6^{-/-}

mice only marginally affected total BAL cellularity and neutrophil numbers (Figs. 2-6B & C), suggesting that the neutrophil phenotype is primarily an innate immune response.

Differential gene expression profile in lungs of WT and STAT6^{-/-} mice after A. alternata challenge

To further determine the factors contributing to the airway responses to *A. alternata*, the gene expression of various critical lung cytokines and chemokines were examined in WT and STAT6^{-/-} mice. Preliminary kinetic studies showed no differences in gene expression between WT and STAT6^{-/-} mice during the sensitization phase, but significant differences were observed 24 hours after the first challenge dose (Fig. 2-7A & B). Thus, an extended gene expression profile was further assessed at this time point. Compared to WT mice, STAT6^{-/-} mice had increased lung expression of the neutrophil-attracting chemokines CXCL1, CXCL2, CXCL5, as well as increased expression of the IFN- γ -driven chemokines CXCL9, and CXCL10 (Fig. 2-3A). Furthermore, expression of the Th2-driven genes CCL11, and the alternative activating macrophage factors Arg1, Chi3l3 (Ym1), and Fizz1 were significantly downregulated in STAT6^{-/-} mice (Fig. 2-3B). No significant differences were observed in GM-CSF, IL-1 α , and IL-18 expression at this time point (Fig. 2-7C). Interestingly, STAT6-deficient mice had increased lung expression of the Macrophage-inducible C-type lectin (Mincle) and higher levels of TNF- α in BAL fluid (Figs. 2-3C & D), possibly implicating other innate cells, such as macrophages and DCs as drivers of the neutrophil infiltration observed in the absence of STAT6 signaling.

Neutrophilic asthma in STAT6-deficient mice is partially dependent on IL-17A

IL-17A has been implicated as a key driver of neutrophilic asthma in both mice and humans^{171, 175, 188}. Additionally, *A. alternata* exposure induces significant increases in lung tissue expression of IL-17A in both WT and STAT6^{-/-} mice and an increase in the number of CD4+IL-17A+ cells in the lungs of STAT6^{-/-} mice. Thus, to assess the role of IL-17A in the neutrophilic response, STAT6^{-/-} mice were treated with anti-IL-17RA antibody one hour prior to each challenge dose. Compared to control animals receiving anti-IgG antibody, treatment with the anti-IL-17RA antibody achieved a 50% reduction in total cell numbers in the BAL (Fig. 2-4A) and a decrease in the percent and total cell number of neutrophils and lymphocytes (Figs. 2-4B & C). The number of CD4+ IFN- γ + and CD4+IL-17A+ lung cells was slightly increased after anti-IL-17RA treatment (Fig. 2-4D). Histological analysis revealed diminished peribronchial cellular infiltrates and decreased epithelial thickening after blockade of the IL-17RA receptor (Fig. 2-4E). These results suggest that neutrophil inflammation in response to *A. alternata* is partially dependent on IL-17A.

Airway neutrophilia in response to A. alternata is steroid-resistant

To date, corticosteroids are the mainstream treatment for asthma due to their potent anti-inflammatory activity¹⁸⁹. However, severe, persistent asthma with neutrophil infiltrates is commonly steroid-resistant¹⁹⁰. To assess the effect of steroids on *A. alternata*-induced asthma, WT and STAT6^{-/-} mice were treated with dexamethasone one hour prior to each challenge dose. Dexamethasone significantly reduced the total number of cell infiltrates, specifically eosinophils and CD4+ T cells, in the BAL of WT mice compared to controls (Fig. 2-5A, B & D). In contrast, STAT6^{-/-} mice treated with dexamethasone had comparable levels of total BAL cellularity,

percent, and number of neutrophils as that observed in control mice (Figs. 2-5A & C). Although not statistically significant, there was a trend towards decreased IL-4, CCL11, and Gob5 expression in lungs of WT mice treated with dexamethasone (Fig. 2-5E). No differences were observed in expression levels of IL-5 and IL-13. In STAT6^{-/-} mice, dexamethasone treatment resulted in decreased lung expression of IFN- γ , but no changes in IL-17A, CXCL1 or CXCL5 (Fig. 2-5F). H&E staining in WT mice revealed diminished epithelial thickening and decreased cellular infiltrates after dexamethasone treatment compared to control (Fig. 2-5G). No changes were observed in PAS staining (Fig. 2-5G). STAT6^{-/-} mice did not display any changes in epithelial thickening or cell infiltrate as seen by H&E stain (Fig. 2-5H). These results demonstrate that eosinophil inflammation in response to *A. alternata* is steroid sensitive, whereas the neutrophilic response is steroid-resistant.

Discussion

Alternaria alternata is a clinically relevant allergen that has been associated with severe and fatal cases of asthma^{136, 137, 162, 191}. Studies into the pathogenesis of *A. alternata* have largely focused on the innate immune response during sensitization and the role of the epithelial derived cytokines IL-33 and TSLP^{70, 153, 161, 192}. Herein we focus on the role of the adaptive immune system and the STAT6 signaling pathway in driving the persistence of pulmonary inflammation after *A. alternata* challenge, including the development of a previously unidentified airway neutrophilic response.

The transcription factor STAT6 is required for IL-4 and IL-13 signaling, and is essential for the induction of Th2-dependent airway responses^{82, 193}, as well as the late effector phases of asthma^{166, 194}. We and others^{70, 153, 161} have shown that upon *A. alternata* exposure, mice exhibit

increased lung expression of IL-4, IL-13, and IL-5 leading to the expansion of Th2 cells, airway eosinophilia, mucus production, and airway hyperreactivity. As expected, deficiency in the STAT6 signaling pathway resulted in a marked reduction in lung Th2 cytokine expression, eosinophilia and mucus production. Airway eosinophilia was dependent on T cells, as WT mice depleted of CD4⁺ T cells failed to develop lung eosinophilia and Th2 airway inflammation. Our data demonstrates that *A. alternata*-induced production of IL-4 and IL-13 in the airways and activation of Th2 effector cells drives persistent airway eosinophilia and lung allergic inflammation.

Interestingly, even in the absence of a Th2-like eosinophilic response, STAT6-deficient mice challenged with *A. alternata* developed substantial airway disease. This response was characterized by significant neutrophil infiltration in BAL and lungs with a higher percentage of IFN- γ and IL-17A-producing CD4⁺ T cells. This response was not mediated by IL-33, as IL-33R KO mice exposed to ALT did not develop airway neutrophilia to the extent observed in STAT6 signaling-deficient mice (Fig. 2-6D-E). Our results are similar to models of HDM-induced asthma and OVA epicutaneous sensitization and challenge where Choy *et al.* and He *et al.*^{177, 185}, respectively, showed that blockade of IL-4 and/or IL-13 results in a switch from eosinophilic to neutrophilic inflammation in the airways. Taken together, these observations suggest a critical interplay between the STAT6 signaling pathway and the development of either eosinophilic or neutrophilic asthma in response to various allergens.

Along with previous studies^{64, 171, 172}, these reports have highlighted the role of the Th17 pathway as a key driver of neutrophilic asthma pathophysiology, further supporting reports implicating IL-4 and IL-13 in the inhibition of IL-17A protein expression both *in vitro* and *in vivo*^{182, 183, 184, 185}. Although Choy *et al.* demonstrated that reciprocal regulation of Th2 and Th17

responses in the airways leads to mutually exclusive phenotypes in asthmatic patients, they found no relationship between total counts of neutrophils in blood, sputum, or lamina propria and a Th17 molecular phenotype. Here, we show that although there was a higher percentage of lung IL-17A-producing CD4⁺ T cells in STAT6^{-/-} mice compared to WT, there were no significant differences in the total lung IL-17A expression between both strains, suggesting that exposure to *A. alternata* induces IL-17A expression independent of the resulting inflammatory phenotype. Additionally, blockade of the IL-17RA receptor in STAT6^{-/-} mice failed to completely protect against neutrophil infiltration in the airways in response to *A. alternata* challenge. Moreover, neutrophil airway inflammation was observed in both RAG-deficient mice and WT mice depleted of CD4⁺ T cells, suggesting that Th17 cells are not required for the development of airway neutrophilia. It is possible that blockade of the Th2 pathway creates an environment where Th17 inflammation can exacerbate the persistence of neutrophil-promoting innate immune responses. The source of IL-17, other than CD4⁺ T cells, could be $\gamma\delta$ T cells¹⁹⁵, ILC3^{76, 196} and/or alveolar macrophages¹⁹⁷, all of which have been implicated as sources of IL-17A and neutrophil-attracting factors. Interestingly, in response to *A. alternata*, STAT6^{-/-} mice, compared to WT mice, have a lower percentage of lung IL-17A⁺ ILC3 and an increase in the total number and percentage of IFN- γ ⁺ ILC1 (Figs. 2-8A & B). ILC1 are widely found in mucosal surfaces including gut and tonsils where they respond to IL-12 by producing IFN- γ ¹⁹⁸. Additionally, higher expression of IFN- γ has been observed in patients with severe asthma and increased numbers of sputum neutrophils^{189, 199, 200}. Consistent with this, we found higher lung levels of IFN- γ expression and the IFN- γ -driven chemokines CXCL9 and CXCL10 in IL-4 and STAT6-deficient mice. Going forward, it will be crucial to determine the role, if any, of this Th1 signature in the development of airway neutrophilia, the interplay between lung Th1, Th2, and

Th17 pathways, and the potential of Th1 pathways as therapeutic targets for patients with both eosinophilic and neutrophilic asthma.

To better understand what other factors may be mediating neutrophil inflammation in response to *A. alternata*, we measured cytokine and chemokine expression in the lungs and BAL of STAT6^{-/-} mice after challenge with *A. alternata*. As expected, we found significantly increased expression of the neutrophil-attracting chemokines CXCL1, CXCL2, and CXCL5, consistent with previous findings highlighting the role of these chemokines in neutrophilic asthma^{175, 201, 202}. This was accompanied by a complete decrease in Arg1, Fizz1, and Chi3l3, markers of alternatively activated macrophages (M2) and Th2 lung inflammation. Reduction of these markers might indicate the presence of classically activated macrophages (M1) in the lungs. Exposure to IFN- γ activates M1 macrophages to produce various Th1 cytokines including IL-1 β and TNF- α ^{200, 203}. We saw increased levels of both IFN- γ and TNF- α in STAT6-deficient mice. Additionally, various reports have demonstrated a key role for TNF- α in neutrophil-mediated inflammation. Fei *et al.*¹⁷⁵ showed that TNF- α production by lung inflammatory DCs inhibited lung IL-5 expression, induced expression of CXCL1 and macrophage inflammatory protein 2 (MIP-2), and promoted the development of airway neutrophilia at the expense of a Th2 eosinophilic response. IL-4 can also induce TNF- α mRNA destabilization leading to a reduction in neutrophil chemotaxis²⁰⁴, providing further support to the role of STAT6 signaling in the inhibition of airway neutrophilia. We also observed, in lungs of STAT6^{-/-} mice, higher expression of Mincle, a macrophage-associated receptor that senses cell death and induces infiltration of neutrophils into damaged tissue²⁰⁵. This data suggests a key role for lung macrophages as drivers of neutrophil infiltration in response to *A. alternata*. Thus, our model supports the importance of IL-17A in airway neutrophilia, but adds to previous studies by

showing that neutrophil inflammation in the lungs is a predominant innate response driven by a variety of factors that are differentially regulated in the presence or absence of the STAT6 signaling pathway.

Our findings are clinically relevant as we describe the pathology of *A. alternata*-induced asthma and the effects that different treatment options would have on disease persistence. We show that while WT mice develop significant airway hyperresponsiveness, STAT6^{-/-} mice are protected from AHR and mucus production. Even though STAT6^{-/-} mice are protected from reactivity to methacholine, *A. alternata* induces a severe lung disease with significant inflammatory cell infiltration that shares similar traits to neutrophilic asthma in humans¹⁸⁷. Additionally, consistent with other asthma models^{106, 206}, we find that lung eosinophilia in WT mice is reduced upon dexamethasone treatment, whereas lung neutrophilia in STAT6^{-/-} mice is steroid-resistant. This finding is important given that over the last decade, some clinical trials have explored the use of antibodies against IL-4, IL-13, and/or IL-5^{168, 207, 208} as alternative treatments for both steroid-sensitive and steroid-resistant asthma. In light of recent studies, including our own, it's not surprising that a majority of these clinical trials have shown minimal or no efficacy, as it is likely that some of the patients treated with antibodies targeting Th2 cytokines might be suffering from a change in asthma phenotype towards a neutrophilic airway inflammation. Thus, treatment with corticosteroids would be insufficient while alternative treatments such as IL-4 and IL-13 blockade would also be ineffective. Patients with sensitization to *A. alternata*, as well as other allergens, would benefit from therapies that address both the Th2 eosinophilic response and the innate neutrophilic inflammation. Future studies will be aimed at determining the efficacy of various combination therapies including TNF- α and CXCR2 antagonists, in controlling airway eosinophilic and neutrophilic responses.

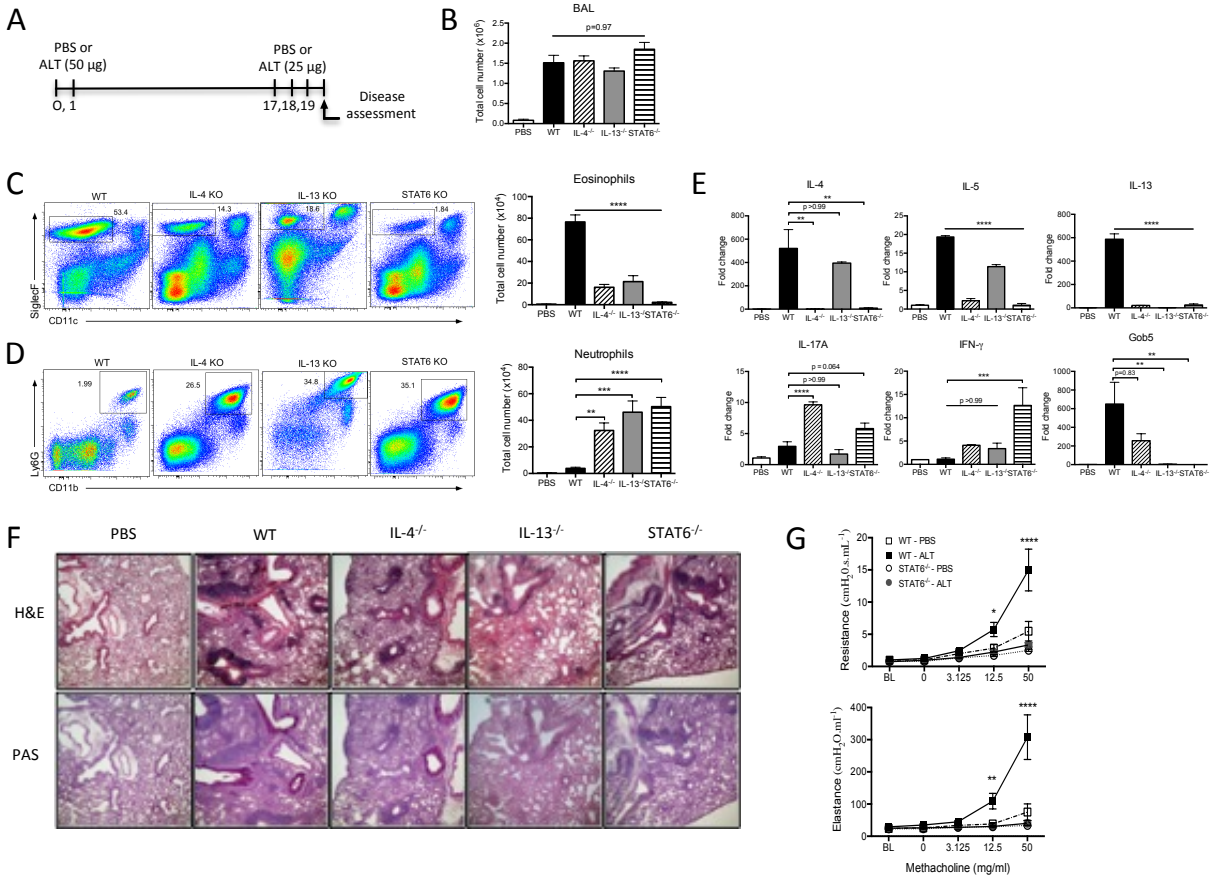


Figure 2-1. Deficiency in Th2 cytokine signaling during *A. alternata* experimental asthma results in airway neutrophilia. Model of *A. alternata* airway sensitization and challenge (A). Total cell numbers in BAL of WT, IL-4^{-/-}, IL-13^{-/-}, and STAT6^{-/-} mice (B). Percent BAL eosinophils (C) and neutrophils (D) by FACS (left) and total numbers (right). Lung mRNA expressed as fold increase over gapdh (E). Representative histology images of H&E and PAS stained lung sections (F). Lung resistance and elastance measured at baseline (BL) and increasing concentrations of methacholine (G). All values are expressed as the means ± SEM (* p<0.05, ** p<0.01, ***p<0.001). Graphs show representative data of three independent experiments (n=3-5 mice/group) (A-F) and combined data from two independent experiments (n=4 mice/group) (F).

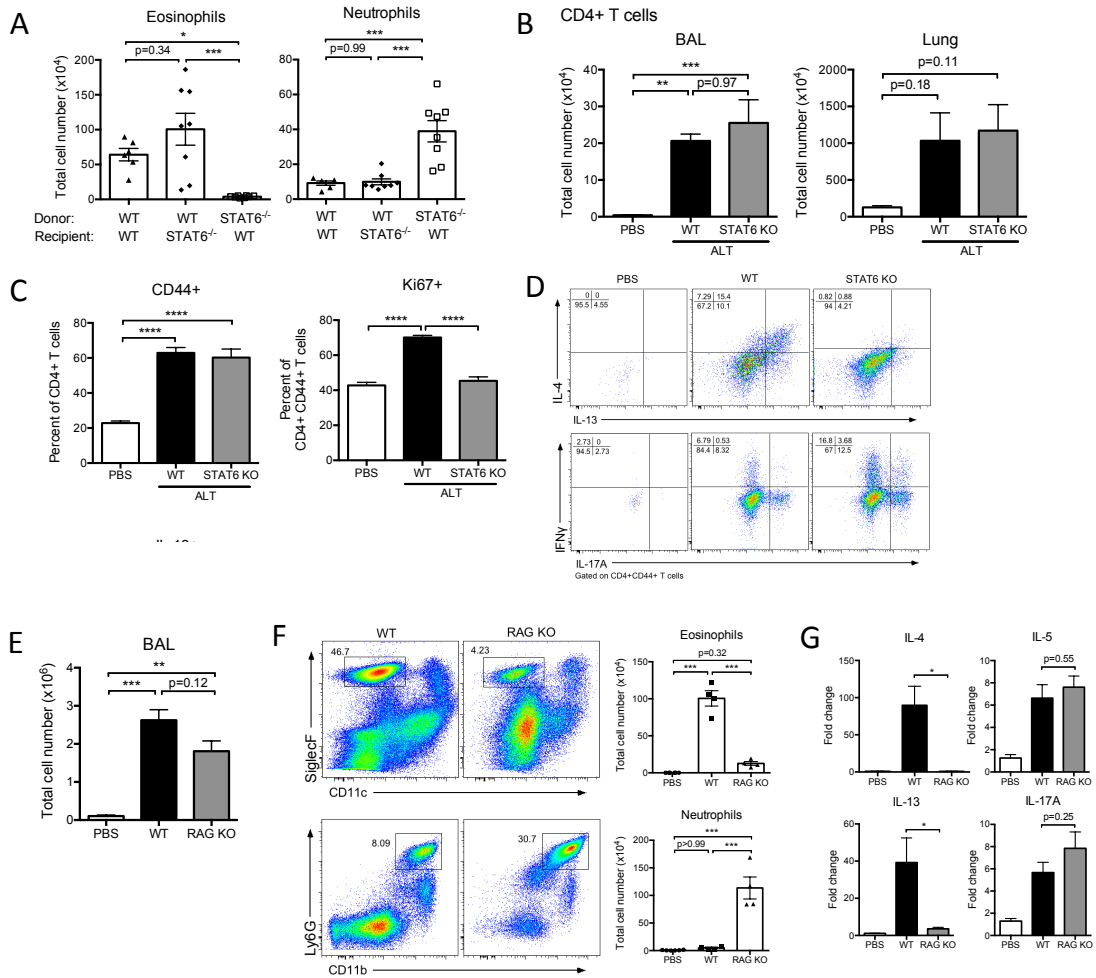


Figure 2-2. T cells are required for persistent airway eosinophilia but not neutrophilia in response to *A. alternata* exposure. Total number of eosinophils and neutrophils in BAL of WT and STAT6^{-/-} chimeric mice 24 hours after last challenge (A). T cell profile in WT and STAT6^{-/-} mice after ALT challenge: Total cell number in BAL and Lung (B); Activation and proliferation of lung CD4+ TCR β + cells (C); Intracellular cytokine staining of lung CD4+ T cells (D). WT and RAG^{-/-} mice airway response after ALT challenge. Total cell number in BAL (E). Percent BAL eosinophils and neutrophils by FACS (left) and total numbers (right) (F). Lung mRNA expressed as fold increase over gapdh (G). All values are expressed as the means \pm SEM (* p<0.05, ** p<0.01, ***p<0.001). Graphs show combined data from two independent experiments (A) and representative data from three independent experiments (n=3-5 mice/group) (B-G).

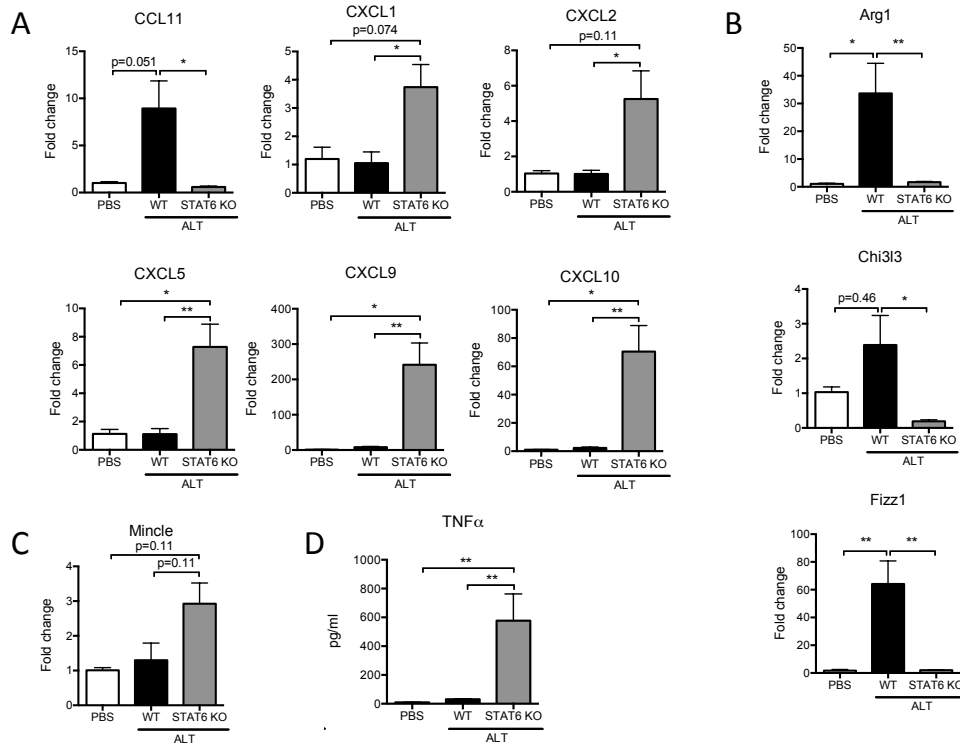


Figure 2-3. Differential gene expression profile in lungs of WT and STAT6 KO mice after *A. alternata* challenge. Lung mRNA expressed as fold increase over gapdh 24 hours after one ALT challenge: chemokines (A), macrophage-activating genes (B), and Mincle (C). Concentration of TNF- α protein in BAL fluid 24 hours after one ALT challenge (D). All values are expressed as the means \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs show combined data from two independent experiments ($n = 4$ mice/group).

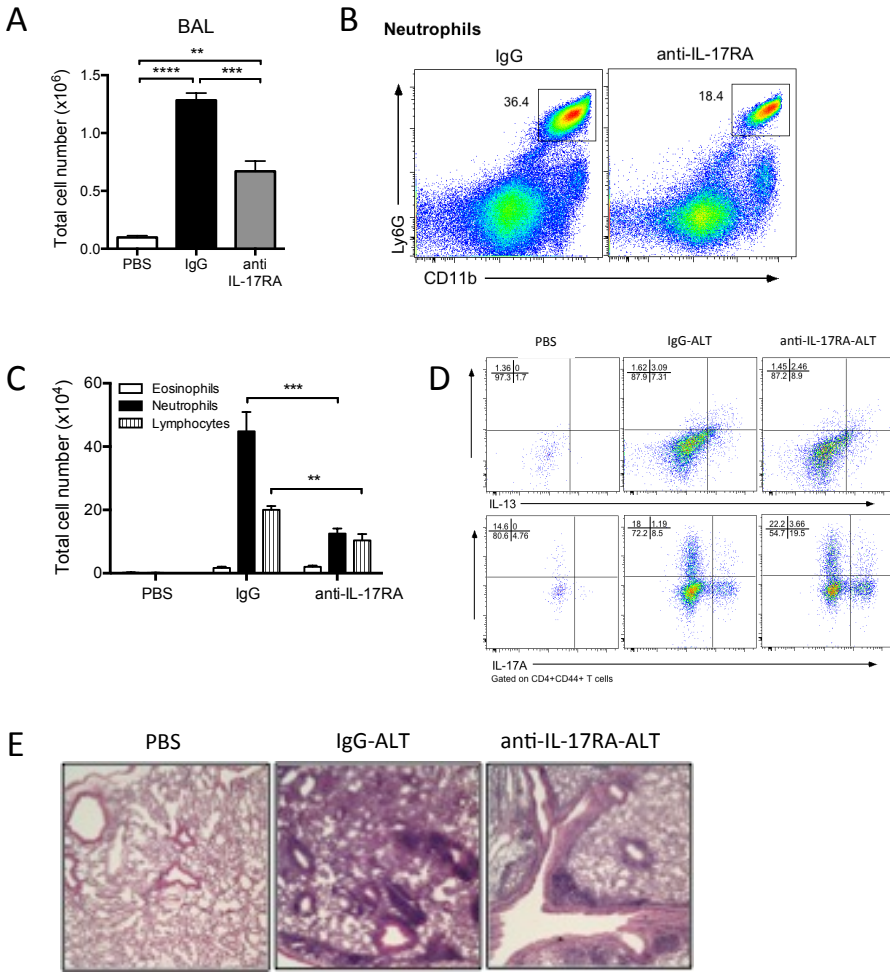


Figure 2-4. Neutrophilic asthma in *STAT6*^{-/-} mice is partially dependent on IL-17A. Airway responses of *STAT6*^{-/-} mice treated with anti-IL-17RA or IgG control antibody. Total cell number in BAL (A). Percent BAL neutrophils by FACS (B). Total numbers of eosinophils, neutrophils, and lymphocytes in BAL (C). Intracellular cytokine staining in lung CD4⁺ T cells (D). Representative histology images of H&E stained lung sections (E). All values are expressed as the means \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs show combined data from two independent experiments ($n = 3-5$ mice/group).

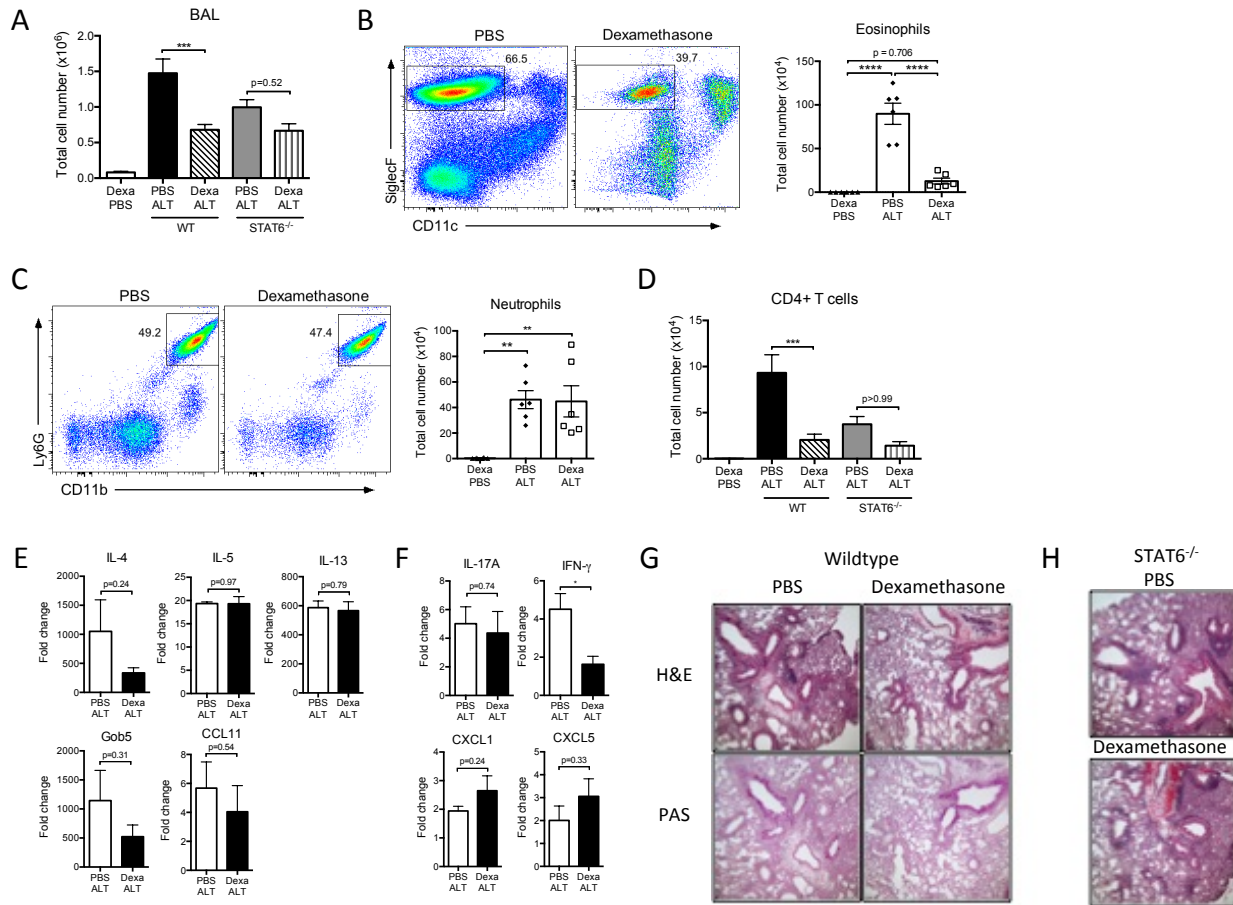


Figure 2-5. Airway neutrophilia in response to *A. alternata* is steroid-resistant. Airway responses in WT and STAT6^{-/-} mice treated with dexamethasone prior to ALT challenge. Total cell number in BAL (A). Percent of BAL eosinophils in WT mice (B) and neutrophils in STAT6^{-/-} mice (C) by FACS (left) and total number (right). CD4+ T cell numbers in BAL of WT and STAT6^{-/-} mice (D). Lung mRNA expression of cytokines and chemokines in WT (E) and STAT6^{-/-} (F) mice. Representative H&E & PAS stains in WT lungs (G) and PAS stains in STAT6^{-/-} lungs (H). All values are expressed as the means \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs are representative of four independent experiments ($n = 3-6$ mice/group).

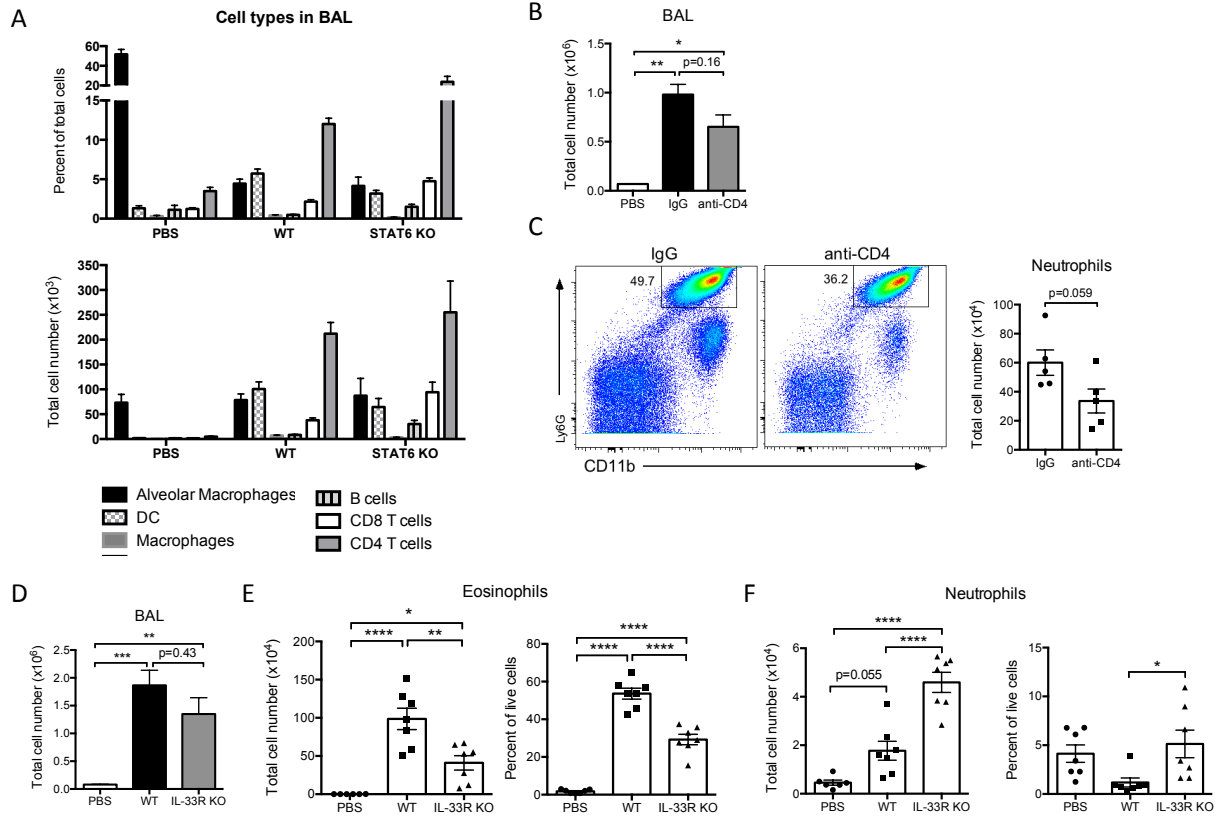


Figure 2-6: Examination of cellular infiltrate in response to *A. alternata*. Cell types in BAL of WT and STAT6^{-/-} mice after ALT challenge (A). Airway responses of STAT6^{-/-} mice treated with anti-CD4 or IgG control antibody. Total cell number in BAL (B). Percent BAL neutrophils by FACS (left) and total numbers (right) (C). Airway responses of WT and IL-33R^{-/-} mice treated ALT. Total cell number in BAL (D). Total cell number and percent of BAL eosinophils (E) and neutrophils (F). All values are expressed as the means ± SEM (* p<0.05, ** p<0.01, ***p<0.001). Graphs are representative of three independent experiments (n=3-6 mice/group) (A-C) and combined data from two independent experiments (D-F).

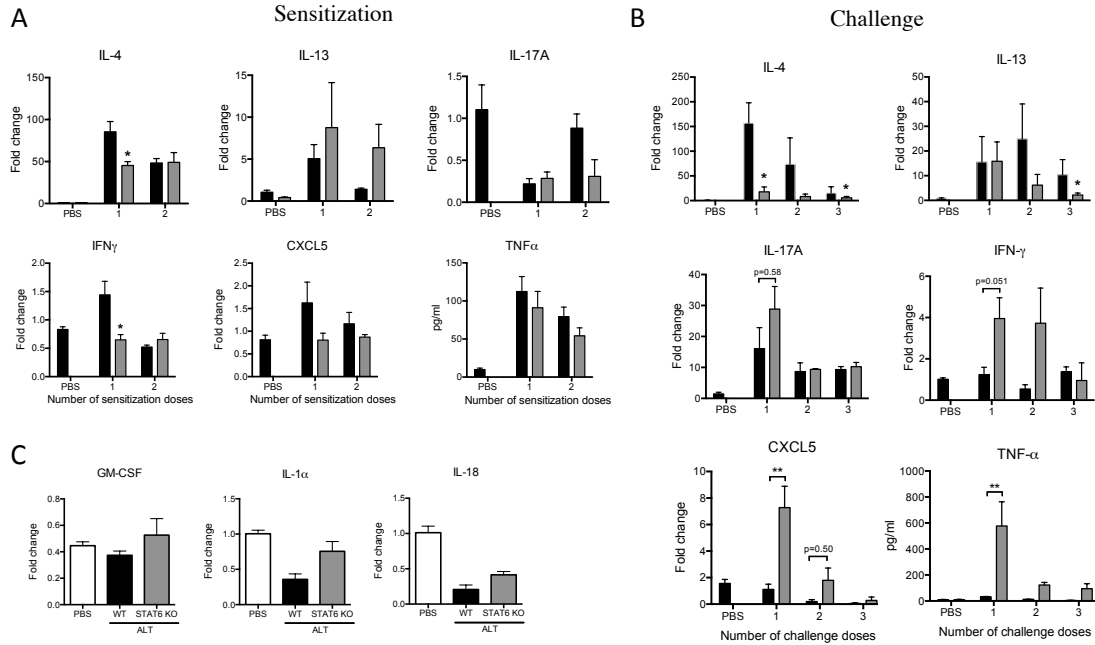
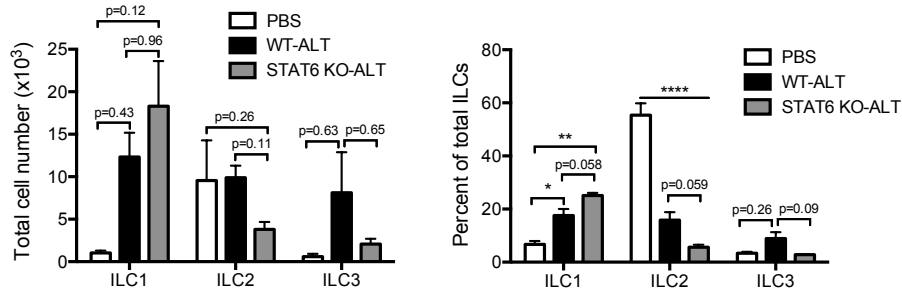


Figure 2-7: Lung mRNA expression in response to *A. alternata* exposure. WT and STAT6^{-/-} lung mRNA (IL-4, IL-13, IL-17A, IFN- γ) expressed as fold increase over gapdh and BAL TNF- α protein concentration 24 hours after one and two ALT sensitization doses (A) and one, two, and three ALT challenge doses (B). Lung mRNA (GM-CSF, IL-1 α , and IL-18) expressed as fold increase over gapdh 24 hours after the first ALT challenge dose. All values are expressed as the means \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs show combined data from two independent experiments ($n = 4$ mice/group).

A



B

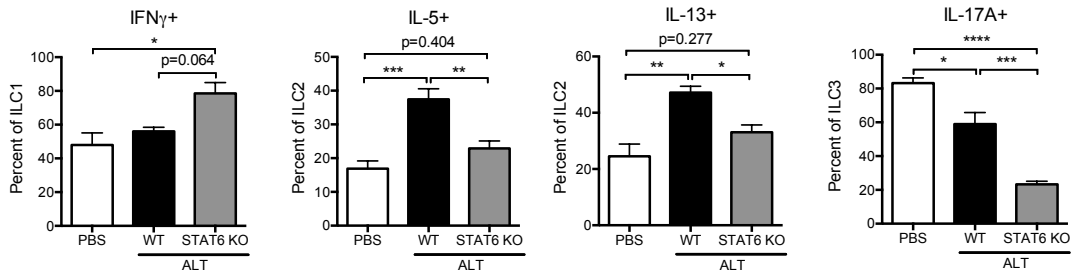


Figure 2-8: Examination of ILC numbers and cytokine expression in lungs of WT and STAT6 KO mice. Total number and percent of ILC subsets (ILC1: CD45+CD90.2+ST2-ROR γ t-NKp46+CD11b⁻), ILC2: CD45+CD90.2+ST2+ROR γ t⁻, ILC3: CD45+CD90.2+ST2-ROR γ t⁺) in lungs of WT and STAT6^{-/-} mice after ALT challenge (A). Intracellular cytokine staining by the different lung ILC subsets. All values are expressed as the means \pm SEM (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Graphs show data from one experiment (n=4 mice/group).

Chapter 3

Concluding Remarks

Alternaria alternata is one of the most potent aeroallergens associated with severe asthma. The innate immune mechanisms orchestrating airway responses to *A. alternata* have been extensively studied over the last several years. *A. alternata* is recognized by airway epithelial cells via PAR-2 dependent and independent mechanisms. Activation of epithelial cells leads to the production of a variety of pro-inflammatory factors, the most important of which is IL-33. IL-33, a critical mediator of airway responses to *A. alternata*, induces the proliferation and activation of type 2 innate lymphoid cells to produce IL-5 and IL-13. This cascade of events results in eosinophil infiltration to the airways, mucus production, epithelial thickening, and airway hyperresponsiveness. Increased lung levels of the Th2 cytokines IL-4 and IL-13 are also observed after *A. alternata* sensitization. However, unlike innate immune responses, the precise role of the adaptive immune system and the IL-4/IL-13 signaling pathway in the airway responses to *A. alternata* are still not fully understood.

Here we used a number of experimental approaches to determine the role of the STAT6 signaling pathway and adaptive immune system in the development of *A. alternata*-induced asthma. We found that persistent Th2 eosinophilic responses after exposure to *A. alternata* are dependent on the presence of CD4⁺ T cells and STAT6 signaling in hematopoietic cells. Most interestingly though, STAT6-signaling deficient mice still had substantial lung inflammation at levels similar to those observed in wildtype mice. However, wildtype mice, STAT6-deficient mice had a predominantly neutrophilic, non-Th2 phenotype characterized by increased levels of IL-17A, IFN- γ , TNF- α , and neutrophil attracting chemokines including CXCL2 and CXCL5.

The change from an eosinophilic to neutrophilic phenotype is similar to that observed by Choy *et al.* in response to house dust mite exposure. Our findings have important clinical implications as we show that blockade of one inflammatory pathway is not a successful therapeutic strategy. Rather, patients need to be stratified based on their allergen sensitivities and therapies carefully planned to target all the pathways mediating a specific individual's airway inflammation.

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