The Role of IL-25 in Innate Cytokine Production and Pulmonary Immunopathology

by

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It seemed like every day you told me, “Be a leader, not a follower,” and so I dedicate this work to you Dad, for teaching me that some actions matter more than outcomes. That advice (and occasional good fortune) has made a whole lot of difference.

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ABSTRACT

The mucosal surfaces of the lung and intestinal tract are areas at which heterogeneous immune populations must function in concert to maintain barrier integrity and coordinate appropriate responses to both pathogenic and innocuous antigens. The Interleukin-17 (IL-17) family member IL-25 is a cytokine associated with allergy and asthma which functions to promote type 2 and suppress alternative inflammatory responses in epithelial tissues. This study identifies the IL-25 receptor, IL-17RB, as an important mediator of both innate and adaptive type 2 immune responses in the context of allergic airways disease and respiratory viral infection. Through a series of animal models, genetic manipulations, adoptive transfers, and a comprehensive characterization of IL-25 responsive myeloid populations, we have identified a novel granulocytic population of innate IL-25 responsive cells involved in the pathogenesis of allergic asthma. This population, termed Type 2 Myeloid (T2M) cells, produces the type 2 cytokines IL-4 and IL-13, is recruited in large numbers to the lung in response to IL-25 secretion, and induces IL-25 associated immunopathology in the airway. Our findings also indicate that T2M cells may be of significant clinical importance. High dose dexamethasone administration, the front line therapy for severe allergic asthma exacerbations, did not reduce cytokine production in, or total numbers of, T2M cells. Finally, a clinical study using peripheral blood drawn from atopic asthmatic volunteers identified a similar IL-4 and IL-13 producing granulocytic population that was not

observed in non-atopic volunteers. The identification of a novel, pathologically relevant granulocytic subset offers an important new avenue for research investigation with the potential for significant translational impact.

CHAPTER I

Introduction

OVERVIEW

The generation of an effective adaptive immune response requires different strategies to cope with intracellular (type 1) versus extracellular (type 2) pathogens. Atopic asthma is characterized by a heterogeneous influx of inflammatory cells in which the type 2 inflammatory response has been co-opted to attack otherwise innocuous environmental allergens[1]. The hallmarks of chronic allergic asthma; airway hyperresponsiveness, bronchiole smooth muscle hypertrophy, and mucus hypersecretion, are caused by excessive type 2 cytokine production[2,3].

Interleukin (IL)-25 is a cytokine associated with a variety of type 2 diseases which functions to promote type 2 and suppress alternative inflammatory responses[4-10]. IL-25 is produced by the respiratory epithelium in response to noxious stimuli[7], as well as by inflammatory cells recruited to the airway[11-14]. It has been linked to asthma and asthma-like sequelae[4,5,7,10,15], and represents a potential therapeutic target for the treatment of allergic airways disease[16]. The cellular targets of IL-25 have been the focus of several recent investigations, the results of which have begun to illustrate an integral relationship between IL-25 and the development of both innate and adaptive immune

responses[4,5,10,11,17-19]. The search for IL-25 responsive cells has also led to the identification of several previously unrecognized innate immune populations, known collectively as innate lymphoid cells[20-23]. These discoveries offer some insight into the complex regulatory environment associated with type 2 inflammation, and highlight the importance of innate populations in the establishment and maintenance of type 2 immunity.

The composition of IL-25 responsive cells in the lung, and their relative contributions to type 2 diseases such as allergic asthma, remains an area of active study. The investigation of IL-25's role in the pathogenesis of type 2 lung pathologies, including allergic asthma and viral exacerbations of underlying asthmatic disease, may enable the identification of previously unrecognized components of the inflammatory cascade. Refining our understanding of IL-25–mediated biology will provide greater insight into mechanisms of allergy that mediate disease phenotypes which present challenges in both clinical stratification and effective treatment of asthma, a disease currently without prevention or cure.

ASTHMA

Epidemiology

Asthma is a chronic pulmonary disease characterized by episodic symptoms of airway obstruction, including shortness of breath, wheezing, and cough[24,25]. It represents a complex, multifactorial syndrome influenced by a variety of intrinsic and extrinsic factors with variable clinical presentations. The symptoms of asthma are intimately linked to physiologic changes associated with its pathogenesis, including airway

hyperreactivity, mucus hypersecretion, hypertrophy of bronchiole smooth muscle, and the recruitment of inflammatory cells to affected airways[1]. The most common triggers of asthma symptoms are environmental allergens, with atopic asthma accounting for approximately 75% of all asthma diagnoses[26,27]. Chronic exposures to allergens or other noxious stimuli serve to induce inflammatory responses that manifest clinically as an asthma attack.

Asthma and its associated sequelae represent a significant disease burden that affects 9% of all Americans and up to 300 million people worldwide[26,28,29], making asthma the most common chronic lung disease. From 2005–2009, annual asthma-related hospitalizations and deaths in the United States alone were approximately 500,000 and 3,500, respectively[25], with over 250,000 annual deaths worldwide[26,30]. The Centers for Disease Control (CDC) reports that almost half of the 25 million Americans with asthma have experienced an attack in the past year, and the prevalence of asthma has increased in children from 3.6% in 1980[29] to almost 10% in 2009[25]. The total healthcare cost associated with the disease is also rising, reaching $56 billion in 2007[24], a 16% increase over just 5 years[26].

Multiple factors influence asthma's increasing prevalence, and it is clear that effective management strategies must be adopted in order to lower both the incidence of asthma and the frequency at which asthmatic individuals suffer exacerbations of their disease. The most common therapeutic approach to asthma employs a combination of pharmacotherapy and the use of an Asthma Action Plan[24,31]. In general, basic medications include a daily inhaled corticosteroid and a short acting beta-2 agonist, a "rescue" medication for bronchospasm that relaxes airway smooth muscle[24]. An Asthma

Action Plan is a strategy developed between a patient and his or her physician to address how the patient should react when symptoms occur, in order to minimize the incidence of full-blow asthma attacks and to identify when medical intervention is necessary.

While a combination of medications and education offers an effective approach to the management of most asthmatics' symptoms, a subset of individuals (in some studies up to 10% of all asthmatics) with severe disease have symptoms refractory to oral glucocorticoid treatment[32-35]. Effective treatment options for these patients, known as steroid resistant asthmatics, remain very limited[34,36]. A better understanding of the biology underlying steroid-resistance is needed to develop new therapies to help patients cope with disease, including a more complete understanding of the pathogenesis of the pulmonary inflammation that leads to the symptoms of asthma.

Etiology

Factors influencing the severity of asthma may be associated with its mode of development, frequency of exacerbation, or both[24]. The multifactorial nature of the disease, and the complex interplay between genetic and environmental factors in its pathogenesis, make the identification of a single "causative" factor impossible[29]. However, investigations of heritable and environmental risk factors associated with asthma have identified several contributing elements. These include genetic susceptibility loci, the adoption of "Western" diets, the "hygiene hypothesis," increased exposure to indoor allergens, air pollution and tobacco smoke, the incidence of respiratory viral infections in infancy, and the alteration of gut microbiota following antibiotic use.

Genetics

Monozygotic twin and familial studies offer evidence for the heritability of asthma and suggest that between 40–60% of asthma-associated symptoms have some genetic basis[37-39]. Owing to asthma's multifactorial nature, any predisposition to asthma is likely the result of epigenetic mechanisms[40], polygenic inheritance, and locus heterogeneity[27,41] (i.e. the sum effects of many different genes in different individuals may result in similar clinical phenotypes). Furthermore, although candidate gene analyses have identified more than 100 genes associated with asthma, few of these studies have been successfully replicated[42]. Unbiased asthma-associated GWAS analyses of almost 20,000 individuals[43] have identified several asthma-associated loci, however the predictive value of these data are low and there is currently little data linking genotypes identified by GWAS and variance of clinical phenotype[42]. Roughly 40% of all children with asthmatic parents will go on to develop asthma[26], and maternal asthma significantly increases those odds relative to paternal asthma[44]. However, genetics alone cannot explain the rapid rise in asthma's prevalence in some populations over the past few decades, and further investigation is required to determine the relative contributions of various genetic and epigenetic factors[45].

Diet and exercise:

The increasing incidence of asthma has marched in parallel with an increase in obesity for at least 30 years. Although the correlation is not absolute, obese individuals exhibit higher rates of asthma than the general population[46,47], and sedentary lifestyles (associated with both obesity and asthma), have increased as rates of physical activity

have decreased. Changes in dietary composition, specifically increases in highly processed foodstuffs with n-6 polyunsaturated fatty acids (omega-6 PUFA) and decreased consumption of antioxidants and n-3 PUFA (also known as omega-3 fatty acids), have been associated in cross-sectional studies with an increase in asthma rates[48,49]. Overall, data linking this type of "fast food" diet to asthma suggest an association but are not definitive. Connections have also been made between maternal diet, breast-feeding, and the development of childhood asthma[50-54]. Breast–fed children of mothers who consume diets high in antioxidants, including vitamin E and zinc, are less likely to develop asthma than mothers with low antioxidant consumption, whereas children whose mothers consumed diets high in n-6 PUFA while the child was *in utero* have an increased incidence of allergic rhinitis at 5 years of age[50,51,55]. These data support the hypothesis that diet can be either positively or negatively associated with atopic risk.

The Hygiene Hypothesis

Few prospective studies to date have focused on the natural history of asthma, however worldwide prevalence rates indicate that asthma is largely a disease of the developed world[26]. As developing nations in Latin America, Eastern Europe, and Asia improve public health infrastructure, for example by creating reliable supplies of drinking water and improved sanitation, rates of asthma (especially among children) have invariably increased[28,29].

These data beg the question as to why allergic diseases have increased in industrialized societies, while their prevalence has remained relatively constant in the developing world. More than 20 years ago, Strachen proposed what has come to be

known as the "Hygiene Hypothesis" to describe the inverse relationship between the number of siblings in a family, birth order, and the incidence of hay fever[56]. His original work concludes:

> Over the past century declining family size, improvements in household amenities, and higher standards of personal cleanliness have reduced the opportunity for cross infection in young families. This may have resulted in more widespread clinical expression of atopic disease, emerging earlier in wealthier people, as seems to have occurred with hay fever.

While a direct link between Strachen's initial hypothesis and the development of allergic disease has not been established, evidence that exposure to potential allergens is an important component in the development of immunologic tolerance has been born out in multiple studies by several researchers[57-59]. Taken together, these studies indicate that tolerance to environmental antigens is improved by increasing exposure to a diverse range of stimuli, and that decreasing childhood exposures to potential allergens may limit the development of tolerance and increase the odds of allergen sensitization among individuals with other risk factors for atopy.

Indoor allergens and environmental irritants

The most common triggers for allergic asthma are indoor allergens, including house dust mite (HDM), cockroach allergen (CRA), pet dander, and mold[60-62]. Early exposure to these and other allergens is associated with the development of childhood asthma[63]. For example, increased serum levels of HDM–specific IgE as early as age 2 are predictive of persistent wheezing by age five[64,65]. Allergens with protease activity such as HDM and CRA are particularly potent because they can also stimulate inflammatory cells directly by binding to protease-activated receptor 2 (PAR-2), further

exacerbating the allergic response[66]. Several studies have reported a correlation between the increased exposure to indoor allergens and the development of asthma[63]. Others have found that while the risk of sensitization to specific allergens increases with increasing exposure, the overall incidence of atopy is not significantly increased[67]. Thus, individuals predisposed to an atopic phenotype are at greater risk of becoming sensitized to commonly encountered environmental allergens, however increased exposure does not necessarily represent a nidus for the development of allergic asthma in the general population. Therefore, while indoor allergens are the most common triggers for atopic individuals and the pathogenesis of allergic asthma is associated with these stimuli, allergens in and of themselves are not causative agents. It is more likely that a combination of genetic and environmental factors foreshadows the likelihood of sensitization to one or more allergens, and atopic individuals develop sensitivities to those allergens to which they experience the most consistent exposure[67].

Another common set of asthmatic triggers is air pollutants; including tobacco smoke and diesel exhaust particles (DEP)[29,68-70]. Exposure to these chemical irritants in infancy, early childhood, and even *in utero*, is associated with an increased risk of asthma[58,71-73]. Biomass fuels (BMFs), the most common source of fuel for cooking and heating in much of the developing world, also represent a significant source of indoor air pollution and have been linked to asthma, however they are more commonly associated with lower respiratory infections in children and chronic obstructive pulmonary disease (COPD) in adults[74]. In atopic individuals, irritation of the respiratory tract by particulates can exacerbate underlying disease. Particulates are therefore an important cause of clinical symptoms, and exposure to multiple inflammatory stimuli such as chemical

irritants and respiratory infections represent a common scenario requiring medical intervention or hospitalization in affected individuals[75].

Viral exacerbations and respiratory syncytial virus (RSV)

The exacerbation of underlying airways disease by viral infections is a common cause of significant respiratory pathology in asthmatics[1,76,77]. Infants and children are especially at risk, and several studies have linked severe and/or repeated early childhood viral infections to the development of allergic asthma[78-81]. Upper and lower respiratory tract infections not only increase the likelihood of an asthmatic phenotype, they also worsen existing disease and represent the most common precipitant for hospitalization among children under two years of age[82]. In 2003, viral respiratory tract infections accounted for approximately 1 in 5 hospital admissions in the United States among infants under one year old[83]. Common viruses include influenza, parainfluenza, rhinovirus (RV), and respiratory syncytial virus (RSV)[83,84]. Influenza, particularly the H1N1 strain, has been a source of significant morbidity and mortality among pediatric and adult asthmatics[85,86]. The effects of H1N1 infection have also been particularly severe in obese individuals with underlying airway dysfunction[86,87]. RV represents the most common viral cause of asthma exacerbations, however its clinical symptoms are generally less consequential than either influenza or RSV infection, in part due to variation in the pathogenicity of the more than 100 RV strains[88,89]. The virus most associated with type 2 immunopathology (and the most common cause of severe exacerbations) is RSV[79,90,91].

As with each of the risk factors associated with allergic asthma, the number of positive predictive factors an individual has alters his or her odds of developing disease. Early life viral infections appear to be critically important, and have multiplicative effects on the risk for an eventual asthma diagnosis[65,92]. For example, individuals with sensitivities to HDM have an up to seven-fold higher risk for developing asthma if also they experience a severe respiratory infection with either RSV or RV[65]. Genetic predisposition also plays an important role. Children hospitalized with RSV bronchiolitis before their first birthday are at a five-fold increased risk of asthma if a parent also has a history of allergic disease[78].

RSV is a ubiquitous pathogen responsible for the majority of lower respiratory tract infections in children[93]. Upwards of 95% of children have been exposed by age two[94,95], and although subsequent infections are generally less severe, RSV represents a difficult target for the adaptive immune system in that T and B cell memory does not prevent reinfection from occurring throughout life[96-98]. Opportunistic infections with RSV can have significant impacts on elderly and immunocompromised populations[99-101], and present with variable symptoms ranging from asymptomatic illness to bronchiolitis requiring hospitalization and, in the most severe cases, death due to respiratory failure. Initial attempts to generate a vaccine against RSV ended in failure when individuals inoculated with formalin–inactivated virus became hyper-responsive to subsequent RSV infection, causing profound type 2 inflammation, mucus hypersecretion, and pulmonary eosinophilia[102,103]. RSV's clinical importance, and the absence of an effective vaccine, therefore makes understanding immune regulation of RSV infection an important area of research.

RSV is a single stranded RNA virus of the family Paramyxoviridae[104] with a nonsegmented 10 gene genome[105]. The attachment (G) and fusion (F) surface glycoproteins permit virion infection of host epithelial cells, and are the most important determinants of viral pathogenicity[106,107]. The characteristic multinucleated epithelial cells, or syncytia, of RSV are the result of F protein interactions, which mediate fusion of the viral envelope to the cell membrane but also permit cell-to-cell fusion[106,107]. RSV is classified according to G protein type as belonging to one of two subgroups (A or B)[108-110]. As with other respiratory viral infections, the prevalence of each subgroup varies by region and from season to season, with rates of viral infection and hospitalization peaking in mid winter[111,112].

The pathology of severe RSV infection includes significant airway epithelial damage, mucus hypersecretion, and increased type 2 cytokines in the lung[113,114], similar in many ways to the pathology associated with prolonged allergic asthma. As with asthma, several investigators have linked this clinically relevant pathology not with direct effects of the viral infection, but with consequences of the host immune response[115,116]. RSV infection can generate a mixed inflammatory response in addition to type 2 inflammation[117]. This is the result of an induction of neutrophilic Th17 inflammation in addition to type 1 anti-viral responses. Therefore, the dramatic surge in pathology that can accompany viral infection in asthmatic patients is related to the additive effects of mixed Th1, Th2 and Th17 inflammation, topics discussed in the following section.

ACQUIRED IMMUNITY AND THE PATHOGENESIS OF ALLERGIC ASTHMA

Specificity in T helper subsets

The adaptive immune system combats pathogens (viruses, bacteria, fungi, or parasites) by focusing a coordinated inflammatory response on a perceived threat. T helper (Th) cells differentiate into discrete subtypes, characterized by production of specific cytokines, for effective host clearance of the pathogen. For example, while T helper-type 1 (Th1) cells are specialized to battle intracellular threats, T helper-type 2 (Th2) cells respond to parasitic infections[2]. T helper-type 17 (Th17) inflammation represents a third arm of the adaptive immune response associated with anti-microbial immunity[118]. The pro-inflammatory actions of these subsets are tempered by anti-inflammatory signals from T regulatory (Treg) cells, a subset with developmental ties to Th17 cells[119]. Each of the Th subsets can function to alter the severity of allergic airway disease. The following is a concise review of Th1, Th2, and Th17 development, their functions in regulating immunity and inflammation, and the contribution of key cytokines to each subset's function.

Regardless of the pathogen in question, in order for a naive helper T (Th0) cell to become a participant in the adaptive immune response, it must be activated by an antigen presenting cell (APC) via stimulation with both the Th cell's cognate antigen and one of the co-stimulatory B7 family proteins[120]. APCs, most commonly dendritic cells (DCs), sample the external environment by taking up antigen, and detect pathogens with pattern recognition receptors (PRRs) such as the toll-like receptors (TLRs)[121,122]. Pathogen associated molecular patterns (PAMPs) can trigger PRR activation directly on an APC, or can be recognized by cells of the innate immune system via TLRs, which are activated

upon PAMP binding and can then provide an indirect mechanism for APC stimulation[121,122]. APCs then migrate to secondary lymphoid tissues (i.e. lymph nodes and spleen), where the processed antigen is presented and a T cell with a matching T cell receptor (TCR) is activated[120]. This initiates the process that culminates in T helper cell polarization and the generation of an adaptive immune response.

Following activation, T helper cells undergo clonal expansion and mature into effector phenotypes characterized by discrete profiles of cytokine production[2,118-120]. The specificity of these cytokine profiles serves as the basis for classifying effector cells as Th1, Th2, Th17, or Treg populations. The regulatory processes by which this occurs are complex. In short, cytokine expression in the extracellular environment plays a key role in activating subset-specific genes, suppressing competing differentiation profiles, and stabilizing an established T helper phenotype. Th1 and Th2 subsets were initially distinguished by their unique expression patterns of Interferon (IFN)-γ and Interleukin (IL)-4[123]. Subsequent reports indicated important functional differences between subsets, related directly to cytokine secretion from these populations[124]. Th1 cells respond to intracellular viruses and bacteria by producing IFN-γ, Tumor Necrosis Factor (TNF)-α, and TNF-β[125]. Th2 cells secrete IL-4, IL-5, IL-9, IL-10, and IL-13 in response to helminth infection[2], and Th17 inflammation, defined by the secretion of IL-6, IL-17A, IL-17F, IL-21, and IL-22, plays a critical role in inducing the inflammatory response to extracellular bacteria[118]. In contrast to their pro-inflammatory counterparts, Tregs function to suppress effector responses via the secretion of IL-10 and TGF-β, thereby preventing excessive inflammatory responses[126].

Interactions between Janus kinases and signal transducers and activators of transcription (JAK-STATs) are required for the vast majority of cytokine-induced signaling cascades in target cell populations[127]. T helper subtypes achieve specificity of function, in part, by secreting cytokines that induce the activation of distinct STATs, which are phosphorylated, dimerize, and translocate to the nucleus to induce additional T helper lineage-specific transcription factors, which function as master regulators of gene expression for each Th subtype (summarized in **Table 1.1**). Th1 differentiation requires signaling via IL-12/STAT4 or IFN-γ/STAT1 for the induction of the transcription factor T-bet[128]. Th2 differentiation uses IL-4/STAT6 for GATA-3 upregulation[129], and Th17 relies upon IL-23/STAT3 for RORγT activation and the maintenance of RORγT expression[130]. Tregs require IL-2/STAT5 for the activation of forkhead box P3 (Foxp3)[131], however once they develop, Tregs do not require TCR activation or co-stimulation to suppress pro-inflammatory responses[126]. The utilization of unique JAK/STAT combinations for the induction of specific downstream transcription factors

Pathogen or perceived threat	T helper type response	Activating cytokine(s)	JAK pair	STAT signal	Th-specific transcription factor	Secreted cytokines (s)
Intracellular (Virus or Bacteria)	Th1	IL-12	JAK2/tyk2	STAT4	*Tbet*	IFNγ, TNF-β
		IFNγ	JAK1/JAK2	STAT1		
Extracellular (Parasite, Fungus, Allergen)	Th2	IL-4	JAK1/JAK3	STAT6	*Gata3*	IL-4, IL-5, IL-9, IL-10, IL-13
Extracellular (Bacteria)	Th17	IL-23	JAK2/tyk2	STAT3	*Rorγt*	IL-6, IL-17A, IL-17F, IL-21, IL-22
Excess inflammation	Treg	IL-2	JAK1/JAK3	STAT5	*Foxp3*	IL-10

Table 1.1: JAK:STAT signals required for activation of $CD4^+$ T-helper subsets.[127-133]

allows for tight regulation of complex inflammatory processes that involve multiple cell types, tissue-specific responses, and temporal regulation, thereby enabling the immune system to effectively target invading pathogens.

The allergic cascade

Th2 lymphocytes represent critical components of type 2 inflammatory responses in patients with symptoms of what is known clinically as the "Atopic Triad": asthma, allergic rhinitis, and atopic dermatitis[134]. Non-atopic individuals maintain tolerance to environmental allergens because $CD4^+$ T cells with TCRs matching allergen-specific epitopes are either anergic (i.e. they do not respond to TCR stimulation) or are Tregs[135,136]. In atopic disease, when tolerance fails and an individual becomes sensitized to an allergen, the host utilizes a stereotyped inflammatory response to remove the inciting antigen. The symptoms of asthma are the result of collateral damage incurred as immunologic machinery designed to expel invading helminths works to "destroy" the otherwise innocuous allergen[1]. The type 2 cytokines IL-4, IL-5, and IL-13 are most closely associated with allergic asthma, and promote its pathogenesis. Both innate and adaptive populations are sources of these cytokines, a topic that will be discussed at length in subsequent sections and throughout this thesis.

IL-4 (formerly known as B cell stimulating factor 1 (BSF1) or B cell growth factor (BCGF))[137] stimulates the type 2 immune response by inducing B cell proliferation, class switching, and the secretion of allergen-specific IgE, which binds to high affinity IgE receptors (FcεRI) on mast cells and basophils[138,139]. Upon subsequent allergen exposure, allergen activates and crosslinks FcεRI on these cells and triggers a

pro-inflammatory cascade characterized by the release of secretory granules and the synthesis of pro-inflammatory compounds[140]. Mast cell degranulation stimulates a variety of changes in the airway, including increased vascular permeability, constriction of the airway smooth muscle, and mucus hypersecretion from goblet cells[139,141]. Secreted compounds include pre-formed mediators such as histamine, various proteases (tryptase, chymase, and carboxypeptidase), lipid mediators such as the prostaglandin PDG2 and the leukotriene LTC4, cytokines (including TNF-α, IL-4, and IL-13), and chemokines (including MCP-1 (CCL2), CCL5, and Eotaxin (CCL11))[30,141]. Mast cell degranulation marks the early phase of the allergic response, and lasts for 3-4 hours following allergen exposure. The inflammatory cytokines and chemokines released during the early phase allergic response induce the recruitment and activation of a variety of innate immune populations responsible for mediating the so called "late phase" of allergic inflammation.

During the late phase allergic response, chemokines released during the early phase recruit inflammatory populations including eosinophils, macrophages and Th2 cells to the airway[141]. Th2 cells express the chemokine receptors CCR3 and CCR4, and are recruited by several ligands that can interact with these receptors[142]. Once at the site of inflammation, they produce type 2 cytokines that promote additional inflammatory influx. Eosinophils are the primary granulocytic population recruited by a type 2 inflammatory response, and represent a terminally differentiated granulocytic subset that causes much of the pathology associated with allergic disease[139,143].

IL-5 is essential for eosinophilic inflammation, and promotes eosinophil maturation, activation, and migration from the bone marrow[144,145]. IL-5 provides an anti-apoptotic, pro-survival signal to eosinophils and triggers upregulation of the chemokine

receptor CCR3[146]. Ligands for CCR3, such as CCL2 and CCL11, recruit eosinophils to sites of allergic inflammation and are produced by multiple populations including mast cells, Th2 cells, macrophages, and airway epithelial cells in response to stimulation with IL-13[147]. Eosinophils attack perceived pathogens with a variety of enzymes including major basic protein (MBP), eosinophil peroxidase (EP), and eosinophil cationic protein (ECP)[30,148,149]. Eosinophil numbers correlate directly with the severity of asthma, exhibiting a linear relationship between increased eosinophil numbers in bronchoalveolar lavage (BAL) fluid and worsening asthma symptoms[149,150]. They are also important contributors to chronic inflammatory disease and are involved in airway remodeling and fibrosis[143].

IL-13 has several effects on cells of the respiratory tract. IL-13 induces goblet cell hypertrophy and mucus hypersecretion[151-155], and elicits adhesion molecule expression on endothelial cells, enabling the recruitment of additional leukocyte populations to areas of inflammation[153]. IL-13 is also an important mediator of airway remodeling, a critical consequence of chronic allergen exposure whereby the structure of the airways is altered[153]. In individuals with allergic asthma, chronic inflammation results in smooth muscle hypertrophy and the deposition of excess collagen matrix, making the airway more bronchoconstrictive and less elastic. IL-13 acts directly on fibroblasts to induce production of the pro-fibrotic cytokine transforming growth factor–β (TGF-β), which in turn stimulates deposition of type I collagen[152,156]. It also drives alternatively activated macrophages (AAMs) towards a pro-fibrotic phenotype. This process, coupled with excess mucus production, reduces the luminal diameter of affected bronchioles and leads to worsening obstructive lung disease in asthmatics. The neutralization of IL-13 reduces

airway hyperreactivity (AHR), mucus hypersecretion, and the airway remodeling associated with chronic allergen exposure[155]. Similarly IL-13$^{-/-}$ mice are protected from these pathologically important, allergen-induced sequelae, despite maintaining a significant amount of airway eosinophilia and high levels of IL-4 and IL-5[151,155,157].

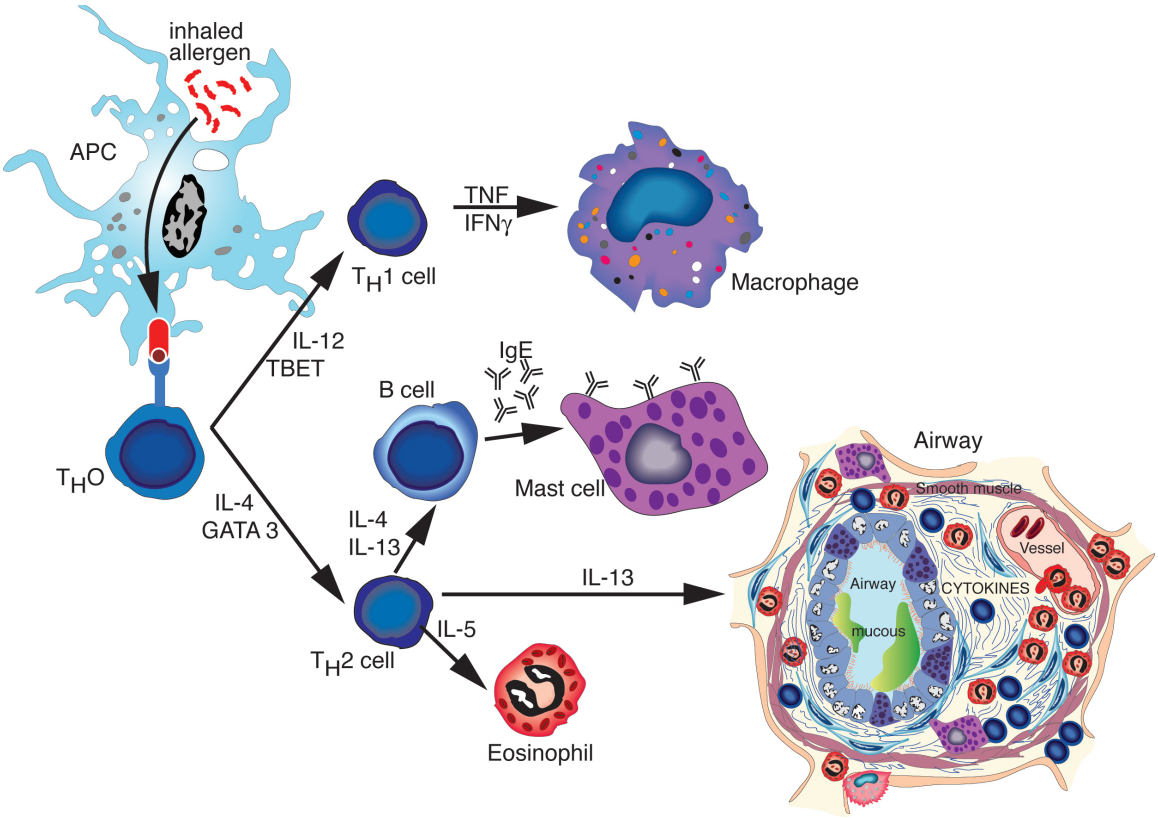


Figure 1.1 Type 2 cytokines regulate the pathogenesis of allergic asthma. Upon encountering environmental allergens in the lung, APCs phagocytose, process, and present allergen epitopes on the cell surface. APCs traffic to draining LNs where naive T cells with TCRs complimentary to the expressed epitopes are activated, differentiate into an effector phenotype, and traffic to the site of inflammation. In type 2 diseases such as allergic asthma, activated T cells are skewed towards a Th2 response by IL-4 and the activation of the transcription factor GATA3, and secrete IL-4, IL-5, and IL-13. These cytokines function to promote the inflammatory response characteristic of allergic asthma by promoting B cell class switching, the recruitment of eosinophils, and goblet cell mucus production/smooth muscle hypertrophy, respectively.

While the most likely inflammatory response to an allergen is an allergic cascade associated with type 2 inflammation and eosinophilia, individuals with severe asthma (including steroid-resistant asthmatics) can exhibit a mixed inflammatory environment with components of Th17 or Th1 inflammation in addition to the typical Th2 response[33,34,36,158-161]. In such cases the inflammatory milieu may include a neutrophilic influx, driven in part by high levels of IL-17A expression[160,162,163]. In contrast to eosinophilic infiltrates associated with IL-5 production during Th2 inflammation, neutrophils represent the primary granulocytic population recruited by Th17-mediated responses. Neutrophil chemotaxis requires interactions between the chemokine receptors CXCR1 and CXCR2 (mice only express CXCR2) and one of several CXC family ligands, including CXCL1 and CXCL8 (also known as IL-8)[147,164]. These are produced by airway epithelial cells (AECs) in response to IL-17A, and may represent a primary mechanism for the severity of some asthma patients' symptoms[165]. Activated neutrophils use several enzymes to produce reactive oxygen species, and neutrophil recruitment can cause widespread nonspecific damage to the airways[164,166]. Some severe asthma patients also exhibit increased levels of IFN-γ, which can induce activation of both eosinophils and neutrophils[162].

Allergic inflammation is typically a reversible process in which infiltrates are cleared in the aftermath of allergen exposure, thus allowing for a resolution of symptoms and a return to normal lung physiology[3]. However, chronic exposure, resulting in IL-13-mediated remodeling, makes the complete resolution of symptoms unlikely and predisposes an individual to future pathology, as the pulmonary environment is primed to respond forcefully to antigenic stimuli[152]. An emerging area of research has focused on

the role of epithelial cell biology in mediating the pro-inflammatory processes that mark asthmatics as prone to allergen exacerbations. The respiratory epithelium is a dynamic actor in the immune response to "non-self," and has been increasingly recognized for its importance in the pathogenesis of asthma.

COORDINATING INFLAMMATORY RESPONSES AND CYTOKINE PRODUCTION AT MUCOSAL SURFACES

Epithelial cells: mediators of mucosal immunity

The mucosal surfaces of the lungs and gastrointestinal (GI) tract represent critical areas at which cells of the innate and adaptive immune system interact to coordinate responses against invading pathogens[167]. As the sites where APCs and other immune cells receive constant stimulation from innocuous environmental antigens, these epithelial barriers are necessary for the maintenance of a homeostatic steady state in which inflammatory cells respond only to those stimuli that represent legitimate threats to the health of the host[168]. In the last decade, it has become increasingly apparent that the mucosal epithelium serves as more than a barrier to potential pathogens. It is often "ground zero" in the initiation of an inflammatory response to invading pathogens, and can secrete a variety of pro-inflammatory mediators to initiate a response when necessary[167-171]. Although these responses are tightly controlled, the disruption of normal epithelial function contributes to inflammatory diseases such as asthma, and represents a mechanism by which allergic individuals may experience flares of inflammation following exposure to atopic triggers. Three epithelial-derived cytokines in particular are associated with antigen-independent activation of type 2 inflammatory responses. These

cytokines, thymic stromal lymphopoietin (TSLP), IL-33, and IL-25 (IL-17E), have been identified as promoters of innate and adaptive immune responses[2,171,172].

Briefly, each of these three epithelial cell-derived cytokines is expressed in a variety of tissues including the skin[173], lung[174], and GI tract[22], and all are associated with type 2 inflammation. Each signals through NFκB, however the receptors and adapter proteins associated with each cytokine differ greatly[171,175-177]. With regards to asthma and atopy, all three cytokines may be produced following allergen exposure and regulate type 2 cytokine production on both T lymphocytes as well as cells of the innate immune system. TSLP appears to play an important role in the genesis of the inflammatory response, primarily by activating APCs and Basophils[18,178]. IL-33, an IL-1 family member, exhibits multiple effects on cells of the innate immune system and also appears to be involved in the initiation of type 2 responses[175]. Like other IL-1 cytokines, IL-33 is secreted as an inactive precursor; however unlike IL-1 or IL-18, IL-33 requires activation via neutrophil elastase and cathepsin G, and is inactivated by caspase 1-mediated proteolytic cleavage[179]. The third epithelial cell-associated cytokine, IL-25, has been established as a regulator of type 2 inflammation and multiple reports have described its ability to exacerbate inflammatory responses at mucosal epithelia, including those in allergic asthma. IL-25 and other IL-17 family members will be discussed in detail in the following sections.

The IL-17 family

IL-17 family cytokines have been identified as critical components in the induction and regulation of Th2 and Th17 inflammatory processes. The IL-17 family consists of 6 homologues, classified A-F[180-182]. Each IL-17 homologue has distinct tissue and cell-specific expression patterns and interacts with a variety of cell types; key aspects of IL-17 family biology are summarized in **Table 1.2**. The prototypical IL-17 family member, IL-17A, promotes what is classically described as Th17-mediated pathology[180,182]. The well-established link between IL-17A and autoimmunity is an area of active study for many investigators, however the following discussion will focus on IL-17A's role as a critical component of host defense against extracellular pathogens. IL-17A's pro-inflammatory effects are the result of multiple downstream cytokines, including GM-CSF, IL-6, IL1β, TNF-α and TGF-β[180,183]. At mucosal surfaces (lung and gut) the Th17 response is characterized by excess mucus production and neutrophilic influx following the IL-17-mediated induction of the neutrophil chemokine IL-8[159,183,184]. IL-17F has similar pro-inflammatory effects, however (perhaps because its binding affinity is 30-40 times lower than IL-17A)[176] IL-17F does not appear to drive as robust an inflammatory response and has been associated with asthma but not autoimmunity[185].

IL-17 family members adopt a unique conformational structure, employing a "cysteine knot" fold similar to members of the neurotrophin family[186,187]. Like the neurotrophins, IL-17 members assemble as non-covalently bound homodimers[182,186], which interact with specific receptor homo or heterodimers (although IL-17A-IL-17F heterodimers have been produced by human monocytes and $CD4^+$ T cells *in vitro*, and exhibit an intermediate binding affinity compared to pure A or F isoforms)[182,188,189]. IL-17 family homologs A

and F signal through a heterodimer of IL-17 Receptor A (IL-17RA) and IL-17RC[176], whereas IL-17C, which has been shown to induce similar inflammatory effects in vivo, requires a heterodimer of IL-17RA and IL-17RE[190-192]. IL-17E, also known as IL-25, signals through an IL-17RA/IL-17RB heterodimer[193]. **Table 1.2** synopsizes known ligand-receptor interactions among IL-17 family members, as well as the signaling cascades shared by IL-17A, F and IL-25[194].

Family member	Homology		Receptor	Tissue distribution	Effects
	Mouse / human	IL-17A			
IL-17A	62%	100%	IL-17RA / IL-17RC	T_h17 cells, $CD8^+$ T cells, γδT cells, NK cells, NKT cells, and LTi cells	A, A/F: Autoimmune pathology
IL-17F	77%	55%			A, A/F, F: Neutrophil recruitment, immunity to extracellular bacteria/fungi
IL-17A / F heterodimer	---	---		Observed in monocytes/ Th17 cells *in vitro*	
IL-17B	88%	29%	IL-17RB	Cells of the GI tract, pancreas, and neurons	Unknown: Activates TNF-α and IL-1β in THP-1 cells
IL-17D	78%	25%	Unknown	Cells of the muscles, brain, heart, lungs, pancreas, and adipose tissue	Inflammatory gene expression in endothelial cells
IL-17C	83%	23%	IL-17RA / IL-17RE	Keratinocytes, GI epithelium, prostate, fetal kidney	Stimulates autocrine cytokine/chemokine production in epithelial cells, mediates immunity against extracellular bacteria
IL-17E / IL-25	81%	17%	IL-17RA / IL-17RB	Airway epithelial cells, eosinophils, alveolar macrophages, mast cells, basophils, Th2 cells, cells of the uterus and GI tract	Promotes type 2 immune responses to parasitic infection, chemical irritants, environmental allergens; suppresses type 1 and Th17 inflammation

Table 1.2: IL-17 family ligands. Ligands are ordered according to sequence homology with IL-17A. Ligands assemble as noncovalently bound homodimers and interact with ligand-specific receptor dimers.[19,130,176,180-183,185,186,188-201]

Unlike the JAK-STAT dependent signaling necessary for cytokine mediated induction of type 1 and type 2 inflammation (summarized in **Table 1.1**) IL-17 family members utilize signaling cascades more commonly associated with innate immunity. These signals are similar to those used by the MyD88 dependent Toll-like Receptors (TLRs) and IL-1 family cytokines[182,186,200]. In fact, a similar binding motif sequence has been identified on both IL-17RA and Toll/IL-1-receptors, both of which also induce NFκB and P38 MAPK following receptor activation. For example, despite their differing biological functions, signaling by IL-17A, F, and IL-25 each require ACT1 and TNFR-associated factor 6 (TRAF6) for the NFκB-dependent activation of downstream target genes[202-205]. In addition, both IL-17A and IL-25 phosphorylate P38-dependent MAPK to achieve cytokine-specific effects: mRNA stabilization in the case of IL-17A[206], and Suppressor of Cytokine Signaling 3 (SOCS3) activation in the case IL-25[198]. The downstream pathways and biological relevance of IL-17B and IL-17D have not yet been determined.

Khader et al. summarized the unique effects of Th17 cells as, "a bridge between innate and adaptive immunity against infectious disease at the mucosa[207]." Although in this case the authors were referring to IL-17A and IL-17F-induced Th17 inflammation, a major component of this thesis involves investigations of the biological activity of IL-25. IL-25 shares the least structural homology with other IL-17 family members[176,182,186] and this structural difference imparts significant differences in its biological effects as well. A linking of innate and adaptive type 2 immunity, mediated by IL-25-responsive populations, represents an apt description of this thesis' theme and will be discussed presently.

IL-25

Unlike the Th17 inflammation and neutrophilia induced by other IL-17 family members, IL-25 regulates mucosal immunity by promoting a type 2 inflammatory response. Both exogenous administration and overexpression of IL-25 induce the production of IL-4, IL-5, and IL-13[4,5,7,10]. Investigators examining IL-25's role in the mucosal biology of the small intestine have identified it as a critical mediator of type 2 inflammation, required for clearance of the murine intestinal parasite *Trichuris muris*[6]. IL-25$^{-/-}$ mice infected with *T. muris* were unable to mount an effective type 2 inflammatory response and instead exhibited an upregulation of both IFNγ and IL-17A. These mice could not clear the parasite, and suffered much greater disease burden as a result. The same group also identified a critical role for IL-25 as a regulator of Th17 inflammation, and determined that commensal intestinal bacteria were required for IL-25 production by the intestinal mucosal epithelia[208]. In the gut, IL-25 functions to maintain the normal immune environment by inhibiting excess Th17 inflammation[6,208-210].

At the pulmonary mucosa, IL-25 induces an inflammatory pathology with many of the hallmarks of allergic asthma, including airway hyperreactivity, mucus hypersecretion, and eosinophilia[4,5,7,15,193]. Pulmonary sources of IL-25 include eosinophils[11,14], mast cells[12], alveolar macrophages[13], and the airway epithelium itself[7]. Like other type 2-associated cytokines, IL-25 is produced by the respiratory epithelium in response to noxious stimuli[2], including environmental allergens, especially those with protease activity[66,211,212]. One report also indicates that it may be induced by viral respiratory infection[213].

The induction of IL-25-mediated responses requires binding of a noncovalently

bound IL-25 homodimer to the IL-17RA/IL-17RB heterodimer[176,182,186,193] (**Figure 1.2**), of which IL-17RB represents an IL-25 specific moiety in the lung[195]. IL-25 is known to enhance Th2 effector functions via Act1 and TRAF6 dependent NFκB activation[18,203,205,214]. Multiple cell types present in the lung, including memory[18] and

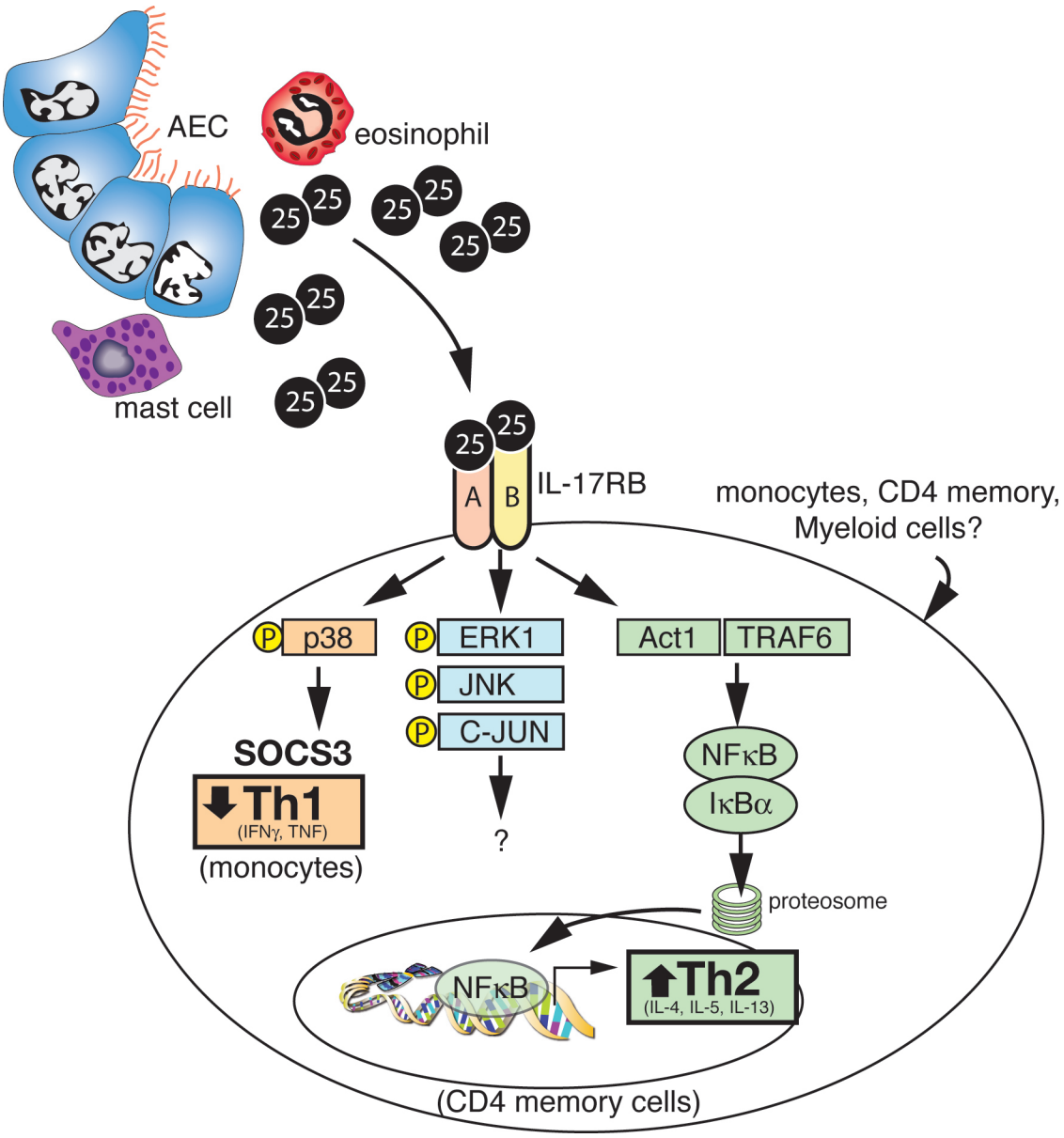


Figure 1.2: Signaling cascades induced by IL-25-IL-25 receptor interaction. IL-25 promotes type 2 cytokine production and an asthma-like inflammatory response. The cell types listed reflect those cells in which aspects of IL-25 signaling were first identified.

effector[18,215] T cells, invariant NKT cells[19,216], APCs[17], and airway smooth muscle[217], have been characterized as expressing IL-17RB. The effects mediated by IL-25 in these populations differ, and to date, data regarding IL-25's role in atopic asthma are by no means definitive[10,11,15,16,18,201,209,218]. Several recent studies have also identified additional IL-25 responsive, type 2 cytokine producing, innate lymphoid populations[20-23] which will be discussed shortly.

Since its identification in 2001[195], IL-25's role as an inducer of type 2 inflammation has been investigated in a variety of clinical contexts. IL-25 and IL-17RB expression are increased in skin biopsies from patients with Atopic Dermatitis[219,220]. Autoimmune hypereosinophilia, (also known as Churg-Strauss syndrome) is a small to medium vessel vasculitis associated with asthma and eosinophilia. Biopsies of CS patients identified IL-25 production in eosinophils, and lymphocyte populations at lesion sites demonstrated almost ubiquitous expression of IL-17RB, suggesting the importance of IL-25-associated inflammation in mediating aspects of the disease[14]. With regard to pulmonary pathology, a recent clinical study found that allergen provocation increases IL-25 and IL-17RB expression in peripheral blood mononuclear cells (PBMCs) from patients with seasonal allergic rhinitis[221]. A separate study compared lung biopsies from atopic individuals before and after allergen challenge, and correlated the allergen-induced expression of IL-25/IL-17RB in atopic asthmatics with severity of disease symptoms[222]. Lastly, there is genetic evidence for the importance of both IL-17RB and IL-25 in human disease. Polymorphisms in IL-17RB have been associated with an increased risk for severe asthma[223], and investigators have also published a case report describing a primary immunodeficiency associated with IL-25 overexpression[224]. In the affected individual,

the overexpression of IL-25 polarized the immune system towards type 2 inflammation, making the patient prone to pathogens requiring a type 1 immune response for clearance. Taken together, these data demonstrate the importance of IL-25 in the normal functioning of type 2 immune responses.

Initial experiments to determine the cellular targets of IL-25 produced a somewhat starling discovery. IL-25 induced type 2 inflammation, and although cytokine production in Th2 effector and memory cells was undoubtedly increased by IL-25, the exogenous administration of IL-25 into $Rag2^{-/-}$ mice (which lack both T and B cells) still induced type 2 cytokine production and recapitulated type 2 mediated pathology[4]. These findings were the first to suggest the existence of an IL-25 responsive non-T cell, non-B cell (NBNT) population as a source of type 2 cytokines. Since this discovery several groups have sought to identify what had been characterized initially as a NBNT myeloid source of type 2 cytokines.

Innate sources of type 2 cytokines

Th2 development requires IL-4 to polarize Th0 cells towards a Th2 phenotype. While the adaptive immune response ultimately contributes IL-4 and IL-13 (two cytokines required for parasite expulsion) the innate immune system is responsible for type 2 cytokine production in the initial stages of infection. A 2004 study by Voehringer et al. described NBNT IL-4 producing populations induced by infection with the hook worm–like parasite *Nippostrongylous brasiliensis*, and described eosinophils, basophils, and mast cells as innate sources of IL-4[225]. In a follow-up study, the same investigators reported that type 2 cytokines from eosinophils were not required for Th2 cell

development, but were critically important for the maintenance of type 2 immune responses[226]. As further evidence of the critical importance of bone marrow derived innate populations, it was shown that innate IL-4/IL-13 production was required for *N. brasiliensis* expulsion, while Th2-derived IL-4/IL-13 was not necessary for effective parasite clearance. In 2009, three groups reported that basophils were key to the development of type 2 immunity in the gut, functioning as IL-4 producing APCs with the capacity to migrate to draining lymph nodes, thereby rendering them both necessary and sufficient for the induction of adaptive immune responses and polarization to Th2[227-229]. Subsequent investigations, however, have questioned the role of basophils in the pathogenesis of allergic asthma[230,231]. These include the finding that certain inflammatory DC subsets express the high affinity IgE receptor I (FcεRI), the same cell surface mark used for both discrimination and depletion of basophil populations in the previous studies, and that it is these FcεRI^{+} DCs, not basophils, that are required for Th2 polarization[232]. What is not in dispute is the critical importance of innate type 2 cytokine production in inflammatory responses. While these investigations shed light on innate cytokine producing populations, they do not address the role of IL-25 as a mediator of type 2 responses.

Our lab sought to further explore the connection between IL-25 and innate cytokine producing cells, as published in 2009[11]. We identified a non-eosinophilic, non-basophilic, IL-4 producing myeloid population that was recruited to the airway in the context of repeated allergen exposure. This population expressed IL-17RB and was stem cell factor (SCF) dependent, indicating that it required activation of the receptor tyrosine kinase c-kit to promote activation, differentiation, or both in order for the cell to be

recruited from the bone marrow. These results merited further investigation into the biology of IL-25-reponsive myeloid populations, and this thesis is the product of those pursuits. Recent findings have identified additional, previously unrecognized sources of type 2 cytokines at mucosal sites in both the lung and GI tract that are IL-25 and IL-33 responsive and associated with mucosal inflammation.

Innate IL-25/IL-33 responsive type 2 cytokine producing populations have been reported by four groups, and have been named Multipotent progenitor type 2 (MPP^{type2}) [22] cells, Nuocytes[21], Innate type 2 helper (Ih2) cells[23], and Natural helper cells (NHCs)[20], respectively. These innate lymphoid populations share a common lymphoid morphology and distinct cell surface receptor expression. All four populations lack mature Lineage markers (CD4, CD8, CD45R (B220), CD11b, Gr-1 (Ly6C/G), and Terr-119), and so by definition are not T cells, B cells, monocyte-macrophages, granulocytes, or erythrocytes, and are c-kit^+ and $Sca1^+$. MPP^{type2}, Nuocytes, and Ih2 cells are present in spleen and mesenteric lymph nodes (mLN), are important components of type 2 immunity in response to helminth infection, and function to mediate helminth expulsion via the production of IL-5 and IL-13. NHCs are found in structures known as Fat-associated lymphoid clusters (FALC), and although they produce IL-5 and IL-13, their role in type 2 immunity remains undefined. Each research group identified functional or phenotypic differences between these cell types, including differences in cell surface receptor expression, transcription factor expression, and degree of responsiveness to IL-25 and/or IL-33. Questions remain as to whether these populations are in fact the same cells characterized in slightly different inflammatory contexts, or whether each represents a discrete population, although it seems likely that these cells share a common

progenitor[233]. The most pronounced difference with regard to innate lymphoid populations involves their plasticity. Whereas Nuocytes, Ih2, and NHCs appear to be terminally differentiated[20,23,234], MPP^{type2} cells can give rise to macrophages, basophils, and mast cells[22].

The identification of innate lymphoid cells has sparked a great deal of interest among researchers focused on type 2 inflammation and mucosal immunology. One of the investigative groups credited with the discovery of innate lymphoid cells has subsequently described nuocytes in the context of allergic airways disease[235], indicating that these cells may play a role in type 2 immunity at multiple mucosal sites. Innate lymphoid cells, similar to nuocytes and Ih2 cells, were also identified as playing a significant role in the resolution (not exacerbation) of inflammation following influenza A infection, specifically via upregulation of the epidermal growth factor receptor (EGFR) amphiregulin[236]. Amphiregulin production by this population played a protective role during influenza infection. This is an interesting finding, as it indicates that innate lymphoid populations can exhibit both pro– and anti–inflammatory functions depending on immunologic context. Their pro–inflammatory potential was further reinforced by the identification of IL-13 and IL-22 producing, IL-25/IL-33 responsive innate lymphoid cells in humans[237]. These cells were enriched in chronic rhinosinusitus polyps (a type 2 inflammatory condition) and characterized as Lin^{-} $CD161^{+}$ $CRTH2^{+}$.

Innate lymphoid populations represent an intriguing component of the innate immune system, and appear to provide an important source of type 2 cytokines produced in the interval between the induction of inflammation and the establishment of Th2 immunity. Despite convincing data of the importance of these populations in the

clearance of helminth infection, as well as additional reports identifying these cells in the lung, the relevance of innate cytokine producing populations in allergic asthma requires further investigation. For example, each of these innate lymphoid populations has been shown to be effective producers of IL-5 and IL-13, however no studies to date have identified IL-4 production either *in vitro* or *in vivo* from these populations.

SUMMARY

A primary goal of this dissertation has been to further investigate type 2 inflammation in the context of allergic airways disease and viral infection. The relationship between IL-25, innate cytokine producing cells, and the pathogenesis of allergic asthma forms the basis of this thesis. The initial hypothesis for this work was that an IL-25-responsive myeloid population contributed to type 2 immunopathology in the lung. This was based in large part on previous findings that bone marrow-derived type 2 cytokine producing populations were important contributors to type 2 inflammatory responses. Through a series of animal models, genetic manipulations, adoptive transfers, and a comprehensive characterization of IL-25-responsive myeloid populations, the studies presented in this thesis have identified a novel granulocytic population of innate IL-25 responsive cells involved in the pathogenesis of allergic asthma. This population, termed Type 2 Myeloid (T2M) cells, produces IL-4 and IL-13, is recruited in large numbers to the lung in response to IL-25 secretion, and induces IL-25-associated immunopathology in the airway. Our findings also indicate that T2M cells may be of significant clinical importance. High dose dexamethasone administration, the front line therapy for severe allergic asthma exacerbations, did not reduce cytokine

production in, or total numbers of, T2M cells. Finally, a clinical study using peripheral blood drawn from atopic asthmatic volunteers identified a similar IL-4 and IL-13 producing granulocytic population. The identification of a novel, pathologically relevant, granulocytic subset offers an important new avenue of research investigation with the potential for significant translational impact.

CHAPTER II

Materials and Methods

Animals and allergen model:

Female C57BL/6J, BALBc, 4get, and *Rag2*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Il17rb*$^{-/-}$ mice were provided by A.L. Budelsky (Amgen). All mice were used at 6–8 wk of age. For allergen sensitization, we used clinical skin test grade, low endotoxin contamination (<20 ng/ml) cockroach allergen (CRA) (HollisterStier Laboratories). Our chronic model was performed as previously described[11,238]. Animals were immunized systemically (day 0) via intra-peritoneal and subcutaneous injections of allergen emulsified with Incomplete Freund's Adjuvant (IFA). After 14 days, mice were given 4 intranasal allergen challenges (1.5 μg in 15 μl PBS on days 14, 18, 22, and 26), followed by two intra-tracheal (IT) administrations (5 μg in 50 μl PBS on days 30 and 32), for a total of 6 pulmonary allergen exposures. Tissues were analyzed 24 hours post final allergen challenge. The left lung was used for histologic analysis, upper right lobe for RNA analysis, middle right lobe for protein quantification, and lower right lobe for flow cytometric analysis. All animal experiments were reviewed and approved by the University of Michigan University Committee on Care.

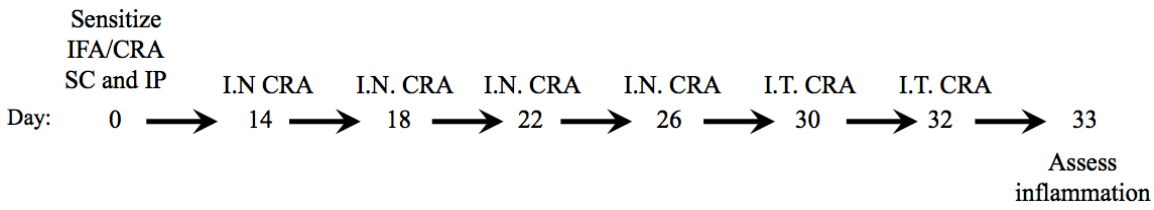


Figure 2.1: Protocol for allergen-induced model of allergic airways disease.

Intra-tracheal IL-25 administration and dexamethasone treatments:

6–8 week old female C57BL/6J, BALBc, 4get, or *Il17rb*$^{-/-}$ mice received daily intra-tracheal injections (0.5 µg recombinant IL-25 (R&D) in 50 µL PBS) for 4 days[193]. Dexamethasone (Sigma) was administered in 2 doses (3 mg per kg per day) via IP injection, 1 hour prior the first and third IL-25 injections. Tissues were analyzed 24 hours after final IL-25 administration.

Respiratory Syncytial Virus:

Our laboratory utilized two clinical isolates of the antigenic subgroup A strain of RSV, referred to as Line 19[239] and A2-20[240], respectively. Both have been demonstrated in animal models to mimic human infection by stimulating mucus production, promoting airway hyperreactivity, and increasing IL-13 production[113,240]. Line 19 virus was used for experiments in BALBc mice, the A2-20 strain was used for all experiments with C57BL/6J mice, including *Il17rb*$^{-/-}$ animals. All mice were infected with $5x10^4$ PFU of RSV per mouse via IT injection.

Antibody Administration:

Neutralization of IL-25 was conducted using a polyclonal rabbit anti-mouse IL-25 antibody developed in our laboratory. Protein A column (Pierce) purified anti-IL-25 or

control IgG was administered IP (200 µg/mouse) every other day, starting at the time of RSV inoculation. Animals received five total doses of antibody, on days 0, 2, 4, 6, and 8 post infection.

Viral Exacerbation Model:

To simulate the effects of RSV infection in the context of allergic airways disease, C57BL/6J and *Il-17rb*$^{-/-}$ mice were sensitized to allergen, and then inoculated with RSV. Mice received 3 total allergen challenges, 2 of which occurred post RSV inoculation, and the inflammatory response was assessed on day 6 of RSV infection, as depicted in **Figure 2.2**.

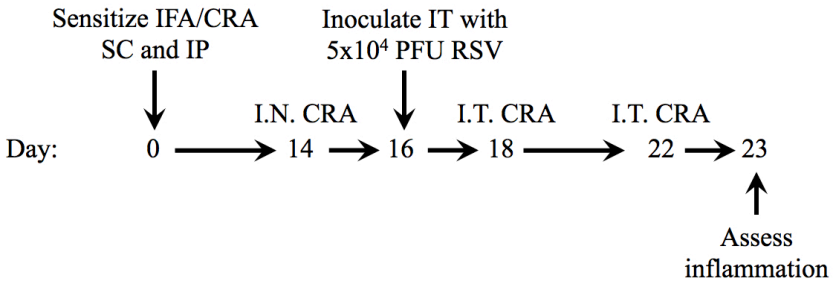


Figure 2.2 Protocol for RSV-induced exacerbation of allergic airways disease.

Measurement of airway hyperreactivity:

Airway hyperreactivity was measured using mouse plethysmography, which is specifically designed for the low tidal volumes (Buxco Research Systems), as previously described. Briefly, the mouse to be tested was anesthetized with sodium pentobarbital and intubated via cannulation of the trachea with an 18-gauge metal tube. The intubated mice were ventilated at a volume of 200 µl at a rate of 120 breaths/min. Airway resistance was measured in the closed plethysmograph by directly assessing tracheal

pressure and comparing the level to corresponding box pressure changes. These values were monitored and immediately transformed into resistance measurements using computer-assisted calculations. Once baseline levels had stabilized and initial readings were taken, a methacholine challenge was given via the cannulated tail vein. After determining a dose-response curve (0.001–0.5 mg), an optimal dose was chosen (0.250 mg of methacholine). This dose was used throughout the rest of the experiments in these studies. After the methacholine challenge, the response was monitored and the peak airway resistance was recorded as a measure of airway hyperreactivity.

Lung histology:

Mouse lungs were perfused with 10% formalin (Sigma-Aldrich) instilled directly into the trachea and then immersed in 10% formalin solution overnight at RT. Serial 6 μM sections were obtained from paraffin embedded, formalin-fixed left lungs stained with hematoxylin and eosin (H+E) or periodic acid-Schiff (PAS).

Primary cell isolation:

Lung tissue was processed via enzymatic digestion with 1 mg/ml collagenase A (Roche) in RPMI 1640 (Invitrogen) with 5% FCS (Atlas Biologicals) and 2 U DNase (Sigma-Aldrich) at 37°C for 1 hour then dispersed to a single-cell population by flushing through an 18-gauge cannula. Draining mediastinal lymph nodes (LN) and spleen cells were dispersed by mechanical disruption of the LN or splenic capsule, followed by washing through a 40 μM filter. Bone marrow was isolated by flushing femurs and tibias

with 1x PBS and 1% FCS through a 40 μM filter. Total cell numbers were counted after RBC lysis.

LN restimulation:

Draining mediastinal lymph nodes from allergen-sensitized mice were isolated, and single cell suspensions were obtained as described above for *in vitro* experiments. LN cells ($4x10^5$ per well, plated in triplicate) were re-stimulated with 10 μL mL^{-1} allergen, 10 ng mL^{-1} IL-25, or both. RNA was isolated after 2 hours in culture; supernatants were analyzed for protein production after 48 hours in culture.

Isolation and co-culture of dendritic cells and T cells:

Antigen presenting cells were isolated by culturing a single cell suspension of whole bone marrow from Naïve C57BL6 or *Il17rb*$^{-/-}$ mice in T150 flasks with complete media (10 % FCS, 1% Penicillin/Streptomycin, 1% glutamate (Invitrogen), 1% Na Pyruvate (Invitrogen), 1% Non-essential amino acids (Invitrogen), 50 μM β-mercaptoethanol) and 20 ng mL^{-1} murine GM-CSF (R&D) at 10% CO_2, 37º C for 10 days. Media and GM-CSF were changed every other day, cells passaged at 70% confluency. $CD4^+$ T cells were isolated from spleens of Naïve or chronically allergen challenged C57BL6 or *Il17rb*$^{-/-}$ mice using MACS CD4 negative magnetic selection kit (Miltenyl Biotech).

Co-culture experiments were performed in 96 well plates with complete media. $2x10^4$ C57BL6 or *Il17rb*$^{-/-}$ DCs per well were pulsed with 10 uL mL^{-1} CRA for 4 hours, after which cells were washed and $2x10^5$ C57BL6 or *Il17rb*$^{-/-}$ $CD4^+$ T cells added. RNA

was isolated using TRIzol (Invitrogen) after 2 hours in culture, culture supernatants were analyzed for protein production by Bioplex (Applied Biosystems) after 48 hours in culture.

$CD4^+$ T cell adoptive transfer:

$6x10^6$ $CD4^+$ T cells from allergen sensitized C57BL6 or *Il17rb*$^{-/-}$ mice, isolated as described above, were suspended in 100 μL PBS and injected into the tail veins of *Rag*$^{-/-}$ recipients. Recipient mice received 2 intra-tracheal challenges of allergen, the first immediately after T cell transfer and the second 4 days later. Lungs were harvested 24 hours after the second allergen challenge, and analyzed with the same methodology used in chronic allergen experiments.

mRNA and Protein Quantification:

RNA was isolated from the upper right lobes of lung, lymph nodes, and bone marrow using TRIzol (Invitrogen). RNA from cells sorted by FACS analysis was isolated with a microprep kit (Qiagen), DNase treated (Invitrogen), and reverse transcribed with Superscript III (Invitrogen). Levels of mRNA were assessed using quantitative PCR analysis (TaqMan) with predeveloped primers and probe sets from Applied Biosystems. Quantification of genes of interest was normalized to GAPDH, expressed as fold increases over the negative control. Protein levels of cytokines were quantified using a Bio-Plex bead-based (Luminex) cytokine assay purchased from Bio-Rad Laboratories.

Flow cytometry:

Analyses of pulmonary and bone marrow cell populations were assessed using standard techniques. Samples for intracellular staining were treated with 0.5 μL/mL brefeldin A, 0.5 μL/mL monensin, 0.5 ng/mL PMA, and 500 ng/mL ionomycin and incubated for 6 hours at 37ºC, 5% CO_2. Cells were stained according to manufacturer's instructions (BD Biosciences Fix/Perm Kit). Data were collected on a BD Biosciences LSR II flow cytometer and on a BD Biosciences FACS Aria, and analyzed using FlowJo software (Tree Star). The cellular surface markers of spleen cell controls were not altered by the collagenase treatment as compared to untreated cells consistent with our previous published studies. The following monoclonal antibodies were obtained for mouse flow cytometry and T2M cell characterization (**Table 2.1**):

Antibody	Conjugate	Company	Catalogue #	Dilution
CD11b	PerCP-Cy5.5	BD Biosciences	101228	1/400
CD11c	PE	BD Biosciences	553802	1/400
CD125	PE	BD Biosciences	558488	1/600
CD4	FITC	BD Biosciences	553055	1/400
CD80	PE	BD Biosciences	553769	1/400
CD86	PE	BD Biosciences	553692	1/400
CD8a	FITC	BD Biosciences	553031	1/400
IL-4	AF488	BD Biosciences	557728	1/100
IL-4	PE	BD Biosciences	554435	1/100
Ly-6G	PeCy7	BD Biosciences	560601	1/400
Nk1.1	PE	BD Biosciences	553165	1/400
B220	PE	Biolegend	553380	1/400
c-kit	PE	Biolegend	105808	1/400
CCR3	AF647	Biolegend	129401	1/200
CD3	PeCy7	Biolegend	100220	1/400
CD3	Pacific Blue	Biolegend	100214	1/400
CD8a	PE	Biolegend	100707	1/400
Gr-1	AF700	Biolegend	108422	1/400
IL-7ra	Biotin	Biolegend	121104	1/500
Ly-6C	PE	Biolegend	128008	1/400
Sca1	PeCy7	Biolegend	108114	1/600
Streptavidin	APC-Cy7	Biolegend	405208	1/200
c-kit	APC	eBioscience	17-1171-82	1/400
CD11c	eFluor780	eBioscience	47-0114-82	1/400
CD4	PeCy7	eBioscience	25-0041-82	1/400
CD49b	PE	eBioscience	12-5971-81	1/400
CD69	PE	eBioscience	12-0691-83	1/400
F4/80	PE	eBioscience	12-4801-82	1/200
FcγR1	PE	eBioscience	101308	1/200
FcεR1a	AF647	eBioscience	51-5898-80	1/400
Gr-1	eFluor450	eBioscience	48-5931-82	1/400
IL-13	AF647	eBioscience	51-7133-80	1/100
MHC II	APC	eBioscience	17-5321-82	1/1000
CXCR2	PE	R&D	FAB2164P	1/100
ST2	PE	R&D	FAB10041P	1/100
IL-17RB	Biotin	Gift of A.L.B.		1/200

Table 2.1: Antibodies for mouse flow cytometry and T2M cell characterization.

Isolation of T2M Cells for Adoptive Transfer:

$CD11b^{+}$ $Gr\text{-}1^{mid}$ $Fc\gamma R^{+}$ $CD4^{-}$ $CD8^{-}$ $B220^{-}$ $IL\text{-}7R\alpha^{-}$ $Sca1^{-}$ $c\text{-}kit^{-}$ cells were isolated by FACS from the lungs of wild type C57BL6 mice treated with intra-tracheal IL-25, as described above. Isolated cells ($2.0x10^{5}$ per recipient) were adoptively transferred into the airways of $IL\text{-}17RB^{-/-}$ mice via intra-tracheal injection of PBS with 0.5 µg recombinant IL-25. Mice received four total transfers, each at 24 hour intervals; IL-25 alone, T2M cells alone, or T2M cells and IL-25. Lung tissue was harvested 24 hours after the final adoptive transfer and processed for histological and biochemical analysis.

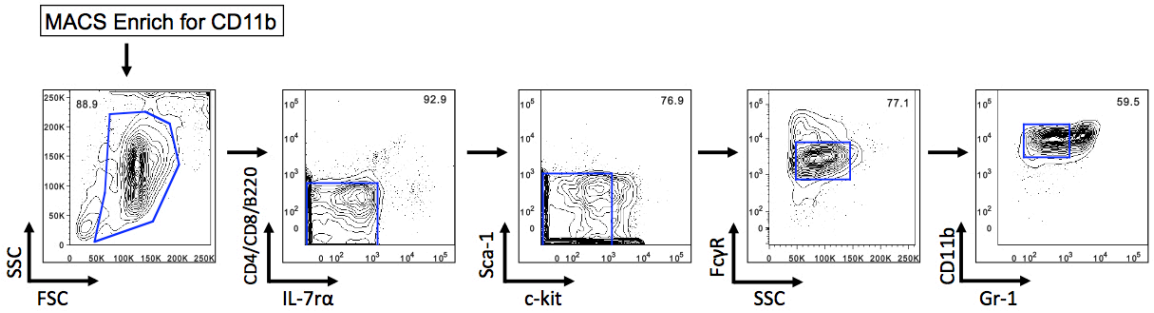


Figure 2.3: Gating strategy for the isolation of T2M cells. We did not use IL-17RB as a marker to enrich for T2M cells used in adoptive transfers because this mouse anti-mouse monoclonal was originally developed as a blocking antibody and we wanted to assess the affect of IL-25 administration on these cells. Type 2 cytokine producing cells were initially characterized by their level of Gr-1 expression (see **Fig 3.2c**), therefore this marker was used to enrich for $IL\text{-}17RB^{+}$ IL-25-responsive cells. Cells isolated were $CD11b^{+}$ $Gr\text{-}1^{mid}$ $Fc\gamma R^{+}$ $CD4^{-}$ $CD8^{-}$ $B220^{-}$ $IL\text{-}7R\alpha^{-}$ $Sca1^{-}$ $c\text{-}kit^{-}$.

Microarray Analysis:

Cells from IL-25 treated 4get mice (n = 4 mice per sample) were pooled and sorted by FACS, and RNA was isolated for microarray analysis. Affymetrix Mouse 430 2.0 microarrays were processed in the University of Michigan DNA Sequencing Core using the WT-Pico V.2 kit.

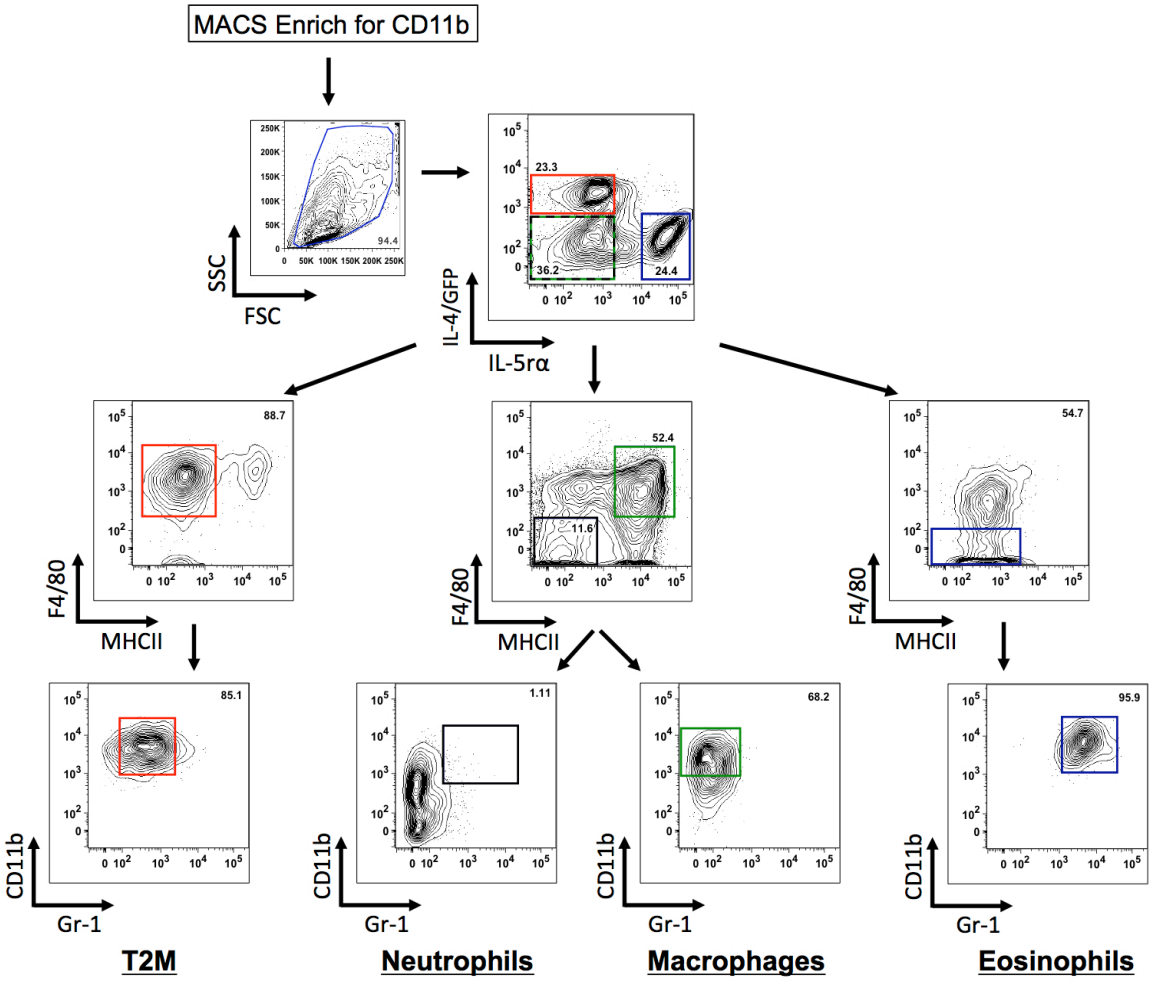


Figure 2.4: Gating strategy for microarray analyses. T2M Cells (IL-4/GFP^{+} IL-5rα^{-} F4/80^{+} MHCII^{lo} CD11b^{+} Gr-1^{mid}), Neutrophils (IL-4/GFP^{-} IL-5rα^{-} F4/80^{-} MHCII^{lo} CD11b^{+} Gr-1^{+}), Macrophages (IL-4/GFP^{-} IL-5rα^{-} F4/80^{+} MHCII^{+} CD11b^{+} Gr-1^{-}), and Eosinophils (IL-4/GFP^{-} IL-5rα^{+} F4/80^{-} MHCII^{lo} CD11b^{+} Gr-1^{hi}) were isolated for microarray analysis from the lungs of 4Get mice challenged with intra-tracheal IL-25. 400,000 cells were collected per sample, pooled from 4 mice/sample.

Clinical Studies:

All human studies were performed in accordance with an approved University of Michigan IRB protocol after legal consent from adult volunteers. All subjects were recruited from the U of Michigan Asthma Clinic and had a physician diagnosis of asthma based on a clinical picture and pulmonary testing consistent with the 2007 NIH/NAEPP guidelines. All patients (n = 9) were persistent asthmatics and were on daily controller medication. Healthy control subjects (n = 8) had not previously been diagnosed with asthma.

Flow cytometric analysis of human peripheral blood:

Granulocytes and PBMCs were isolated from heparinized peripheral blood (20 ml) following separation by Ficoll-Paque (GE Healthcare). PBMCs were isolated as per manufacturer's instructions; granulocytes were isolated from the erythrocyte layer following erythrocyte sedimentation with 5% dextran (Sigma) at room temperature for 30', followed by RBC lysis with 0.2% NaCl. For *in vitro* restimulation and intracellular cytokine staining, samples were treated with 0.5 μL mL^{-1} brefeldin A, 0.5 μL mL^{-1} monensin, and cultured for 2 h in RPMI 1640 ± 50 ng mL^{-1} recombinant IL-25 (R&D). Cell subsets were analyzed using specific antibodies for flow cytometry using standard techniques. The following monoclonal antibodies were obtained for human flow cytometry (**Table 2.2**):

Antibody	Conjugate	Company	Catalogue #	Dilution
CD11b	Pacific Blue	Biolegend	301315	1/200
CD14	AF700	Biolegend	325614	1/200
CD177	FITC	Biolegend	315803	1/200
CD33	PerCP Cy5.5	Biolegend	303414	1/200
CD56 (NCAM)	AF700	Biolegend	318315	1/200
IL-13	APC	Biolegend	501907	3/100
IL-4	PeCy7	Biolegend	500824	2/100
CD117 (c-Kit)	PerCP Cy5.5	eBioscience	46-1178-41	1/200
CD4	PerCP Cy5.5	eBioscience	45-0048-42	1/150
CD45R	AF488	eBioscience	53-0452-80	1/100
CD8	AF700	eBioscience	56-0086-41	1/200
HLA-DR	APC efluor 780	eBioscience	47-9956-42	1/400
IL-17RB	PE	R&D	FAB1207P	1/100

Table 2.2: Antibodies for human flow cytometry.

Statistical analysis:

Data were evaluated by one-way ANOVA and, where appropriate, further evaluated with the parametric Student-Newman-Keuls test for multiple comparisons or the nonparametric Mann-Whitney rank-sum test. For microarray analysis, expression values for each gene were assessed using a robust multi-array average (RMA). The Affymetrix package of bio-conductor implemented in the R statistical language was used to analyze probesets.

CHAPTER III

Type 2 Inflammation in Allergic Asthma is Exacerbated by the Recruitment of IL-25 Responsive Cells

Abstract

The pathogenesis of allergic asthma involves the induction of type 2 inflammation via IL-4, IL-5, and IL-13, cytokines whose production is increased by IL-25. We utilized a model of CRA induced allergic asthma to identify IL-25–responsive immune populations following allergen challenge. Our findings indicate that type 2 inflammation in allergic asthma, including the expression of *Il25* and *Il17rb*, is a dose–dependent phenomenon. The majority of IL-17RB^{+} cells in our model are of myeloid origin, and are capable of producing levels of IL-4 and IL-13 similar to those observed in activated CD4^{+} T lymphocytes. Furthermore, the production of pro–inflammatory cytokines in both myeloid and lymphoid subsets is dependent, at least in part, on IL-25–IL-17RB interactions. *Il17rb*$^{-/-}$ mice are protected from allergen–induced inflammation, and adoptive transfer experiments indicate that the type 2 response in *Il17rb*$^{-/-}$ CD4^{+} T cells is blunted compared to their WT counterparts. Additionally, the production of type 2 cytokine is almost entirely abrogated in *Il17rb*$^{-/-}$ myeloid cells. These finding represent the first report of the inflammatory response to allergen in *Il17rb*$^{-/-}$ animals. They

demonstrate the importance of IL-25 in chronic allergic inflammation, and indicate contributions of both classical Th2 driven inflammation, as well as IL-17RB^{+} myeloid cells, to an allergic response in which IL-25 acts as a pro-inflammatory mediator of type 2 responses.

Introduction

The inflammatory response in allergic asthma is a dynamic process, in which a heterogeneous population of immune cells, with functions dependent upon both frequency and duration of pro–inflammatory chemical queues, is recruited to the airway following allergen exposure[3,30]. IL-25 inhibits type 1[9,198] and Th17[6,201] and promotes type 2 inflammatory responses at the pulmonary mucosa[4-7,10,15,16,18,241,242], thereby enhancing the inflammatory response in allergic airways disease. The aim of this study was to characterize the role of IL-25 responsive cells in the pathophysiology of allergic asthma, in order to determine those inflammatory subsets involved in IL-25-mediated pathology.

Recent studies have emphasized the importance of IL-25 as a mediator of type 2 responses, and marked it as a critical component in the clearance of intestinal helminthes[6,241]. Investigators have reported on the sufficiency of IL-25 to induce marked type 2 responses in the lung, and both transgenic models as well as exogenous IL-25 administration induce inflammatory responses similar to those observed in the context of allergic airways disease[4,7,15,193]. However, no studies to date have addressed how the pathophysiology of IL-25-associated inflammation, or cytokine production in IL-25 responsive populations, is affected by the loss of IL-17RB. To that end, we examined

allergen-induced inflammatory responses in WT and *Il17rb*$^{-/-}$ mice. Our investigation focused on systemic, allergen–mediated pathology, as well as on cell–specific effects related to the absence of IL-17RB. Our lab has previously reported that type 2 cytokine producing myeloid cells are induced following chronic allergen exposure, and that these cells express IL-17RB[11]. Other recent studies have stressed the importance of innate, IL-25 responsive lymphoid populations as mediators of type 2 inflammation[233]. Given these findings, we investigated IL-17RB^{+} populations and focused on the effects the loss of IL-17RB had on these subsets.

Results

Allergen exposure induces dose-dependent type 2 inflammation and upregulates *Il25*/*Il17rb*

Several reports have linked IL-25 expression to the severity of allergic asthma[7,10,11,15,16]. We employed a well–established murine model of allergen–induced type 2 inflammation to recapitulate the pathology of allergic airways disease[238], and assessed pathologically relevant responses following allergen exposure. The pulmonary instillation of allergen promoted both airway inflammation and the induction of type 2 cytokine expression (**Fig. 3.1**), and was accompanied by increased mRNA expression of *Il25* and *Il17rb* (**Fig. 3.1c**). Increased cytokine transcripts tracked with disease severity (as depicted by histology) (**Fig. 3.1a**) in a dose–dependent manner. Neither *Ifng*, other IL-17 family members, *Il33*, nor its receptor *Il1rl1* were upregulated with our model of cockroach allergen challenge (**Fig. 3.1b,c**), indicating that type 2 inflammation represents the dominant response induced by this model.

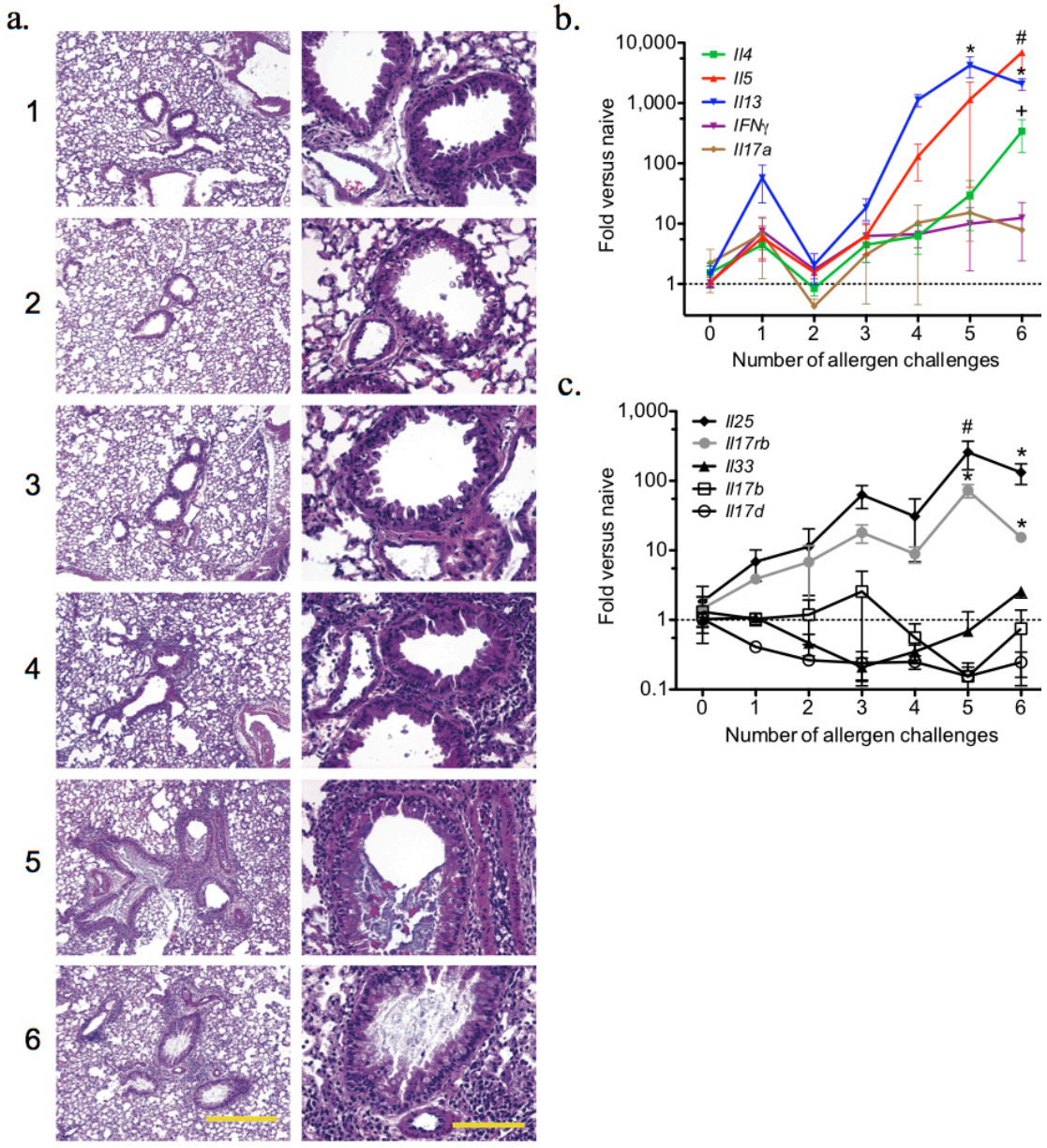


Figure 3.1: Allergen exposure promotes dose-dependent type 2 inflammation. C57BL6 mice (n = 3 per group) were sensitized by intraperitoneal and subcutaneous injection of allergen in IFA, and an allergic response was localized to the lung by a series of 6 allergen challenges. (**a**) Time course of representative Periodic Acid Schiff (PAS) staining, taken 6 h post indicated allergen challenge. Scale bar for left column, 400 μm; right column, 100 μm. (**b**) Time course of pulmonary IL-4, IL-5, IL-13, IFN-γ and IL-17A mRNA expression 6 h post allergen challenge, analyzed by QPCR. Data represent the mean ± s.e.m. for each time point versus naive control (*$P < 8.44E^{-05}$, #$P = 0.0008$, +$P = 0.001$). (**c**) Time course of allergen induced IL-25, IL-17RB, IL-33, IL-17B, and IL17D mRNA expression 6 h post indicated allergen challenge. ST2 and IL-22 transcripts were not detectable. Data represent the mean ± s.e.m. for each time point versus naive control (*$P < 0.007$).

Allergic airways inflammation promotes recruitment of IL-17RB$^+$ type 2 cytokine producing myeloid cells

We have previously identified a pathologically relevant population of IL-17RB$^+$ myeloid cells with the capacity to produce IL-4 during chronic allergic airway disease[11]. Our data clearly demonstrate that type 2 cytokine production following repeated allergen exposure is associated with increased IL-17RB transcripts, however the composition of IL-17RB$^+$ inflammatory cells within this inflammatory milieu has not been previously characterized. An analysis of type 2 cytokine production in IL-17RB$^+$ inflammatory subsets determined that while CD4$^+$ T cells are present following antigen sensitization, the most numerous IL-17RB$^+$ IL-4 and IL-13 producing population in the lung are CD11b$^+$ myeloid cells (**Fig. 3.2a-c**). Because IL-25 responsive innate lymphoid populations have been identified as important contributors to type 2 inflammatory responses, we examined the capacity of innate Lin$^-$ c-kit$^+$ Sca1$^+$ IL-17RB$^+$ NBNT cells to produce type 2 cytokines. Lin$^-$ c-kit$^+$ Sca1$^+$ IL-17RB$^+$ cells comprised a relatively rare population in the lungs of both naive and allergen-challenged mice (range 250-1000 per lobe), and were not significantly increased following allergen sensitization (**Fig. 3.2a,b**). Few myeloid IL-4 and IL-13 producing cells naive were present in the lungs of naive mice, and the IL-17RB$^+$ cells preferentially produced type 2 cytokine. Myeloid IL-17RB$^+$ cells represented the major NBNT IL-4 and IL-13 cytokine producing population in the lung, producing comparable amounts of IL-13 and outnumbering cytokine producing CD4$^+$ T cells 68:1 (**Fig 3.3**).

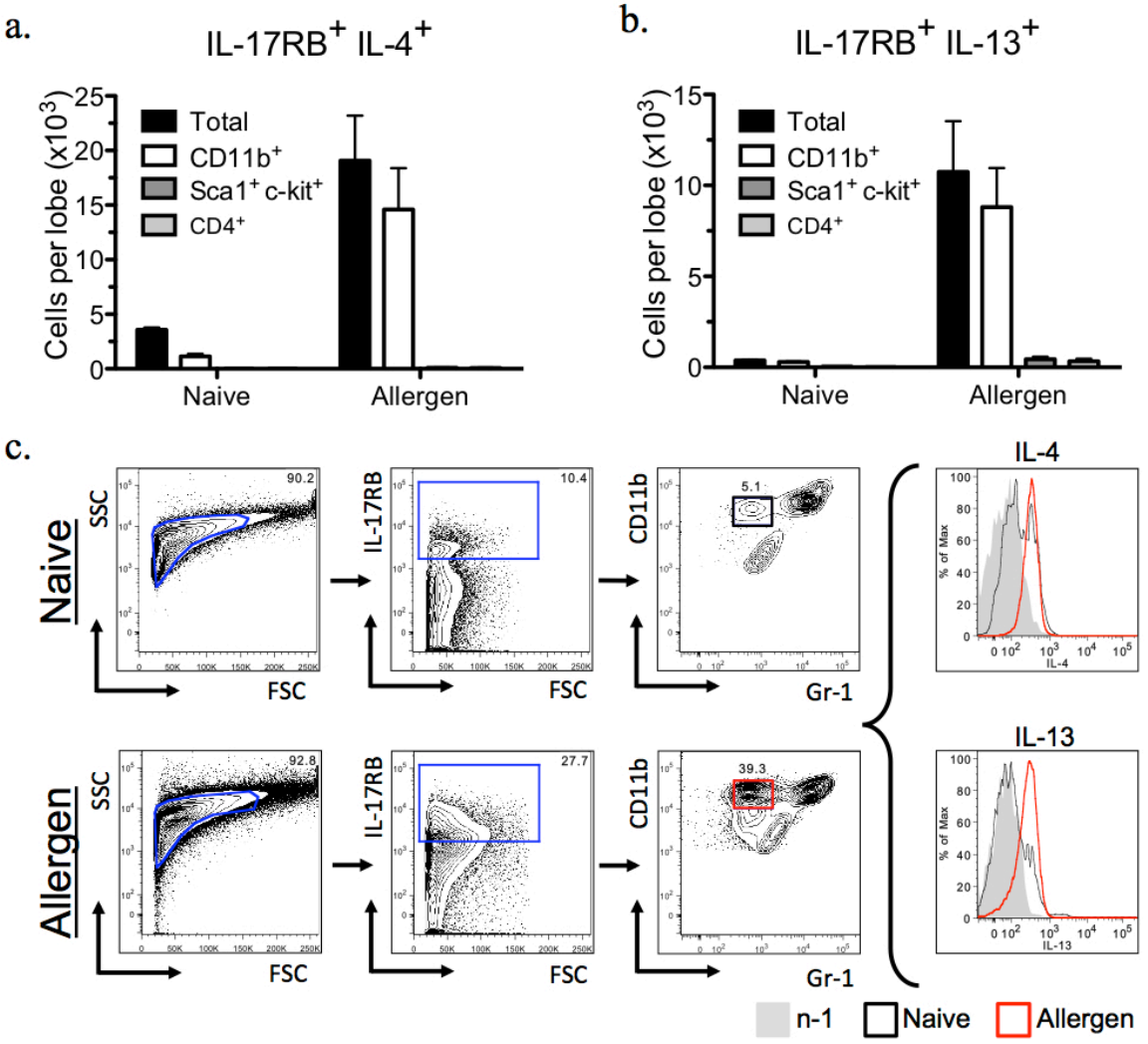


Figure 3.2: Allergen exposure recruits bone marrow-derived IL-17RB$^+$ IL-4 and IL-13 producing myeloid cells to the lung. (**a** and **b**) IL-17RB$^+$ lung subsets from naive and allergen sensitized C57BL/6J mice (n = 5 per group) were assessed by flow cytometry for IL-4 and IL-13 production. (**c**) Representative flow plots of intracellular cytokine staining in IL-17RB$^+$ CD11b$^+$ Gr-1^{mid} cells from naive and allergen sensitized C57BL/6J mice. Gray: n-1 staining, black: naive IL-17RB$^+$ CD11b$^+$ Gr-1^{mid}, red: allergen challenged IL-17RB$^+$ CD11b$^+$Gr-1^{mid}.

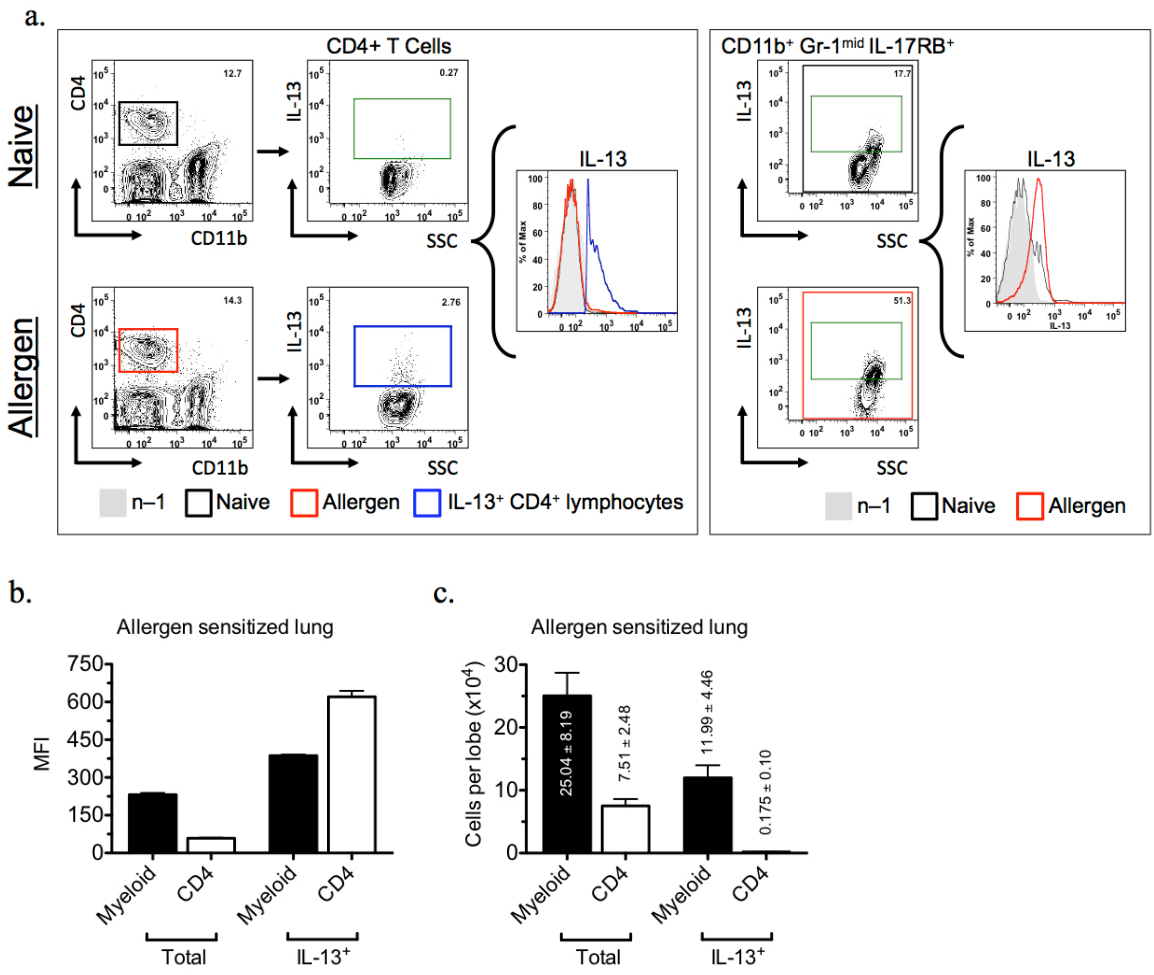


Figure 3.3: Comparison of IL-13 production in CD4$^+$ T cells versus myeloid cells. WT C57BL/6J mice were sensitized to allergen and pulmonary populations were assessed by flow cytometry. Myeloid cells were identified by gating on the CD11b$^+$ Gr1^{mid} IL-17RB$^+$ population (as in **Fig. 3.2c**), and IL-13 production was assessed in both myeloid and CD4$^+$ T cells. (**a**) Representative dot plots of pulmonary IL-13 staining for CD4$^+$ T cells and myeloid cells. Analysis of IL-13 production in CD4$^+$ T cells versus myeloid cells demonstrates a higher percentage of IL-13$^+$ cells among the myeloid population. Histograms were derived from total CD4$^+$ or myeloid populations, respectively. For histograms: gray shaded area: isotype, black line: cells from naive animals, red line; cells from allergen sensitized animals, blue line: IL-13$^+$ CD4$^+$ T cells, shown to illustrate the relative intensity of positive IL-13 staining. Data are representative of 2 independent experiments (n = 5 mice per group). (**b**) Mean fluorescence intensity (MFI) of IL-13 staining in pulmonary derived cells. The MFI of total Myeloid (CD11b$^+$ Gr-1^{mid} IL-17RB$^+$) cells, total CD4$^+$ T cells and the IL-13$^+$ subsets of these populations was assessed. (**c**) Total number of cells per lobe in allergen sensitized mice. While average IL-13 production was higher in CD4$^+$ T cells (panel **b**), following allergen challenges there were, on average, 68.5 IL-13$^+$ myeloid cells for each IL-13$^+$ CD4$^+$ T cell. Bars represent the mean ± s.e.m. of values recorded from 5 mice, data are representative of 2 independent experiments.

Despite significant increases in IL-4 and IL-13 producing myeloid cells in environments with elevated levels of IL-25, not all pulmonary IL-17RB^{+} myeloid populations appeared to produce these cytokines. Analysis of IL-4 and IL-13 production in IL-17RB^{+} myeloid subsets, based on levels of Gr-1 expression, allowed for the identification of two distinct IL-17RB^{+} myeloid populations (**Fig. 3.2c, 3.4a**). A comparison of total cell numbers between naive and allergic animals identified significant increases in both Gr-1^{mid} and Gr-1^{hi} subsets in the lungs of allergic mice (**Fig. 3.4a**), however the IL-17RB^{+} CD11b^{+} Gr-1^{mid} population produced IL-4 and IL-13 while the Gr-1^{hi} population did not (**data not shown**). Isolation of the Gr-1^{mid} subset by FACS identified a granulocytic population with a circular or partially segmented nuclei and relatively high nucleus to cytoplasm ratios (**Fig. 3.4b**). These data inspired our next set of experiments, an investigation of IL-17RB's involvement in the production of type 2 cytokines in this granulocytic IL-17RB^{+} population.

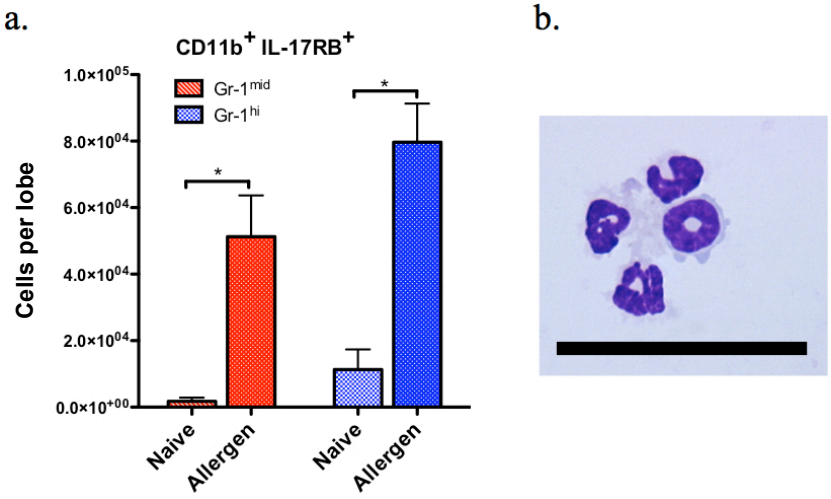


Figure 3.4: IL-17RB^{+} myeloid subsets are significantly increased in allergic airways disease. (**a**) (**g**) Pulmonary IL-17RB^{+} CD11b^{+} Gr-1^{mid} populations are significantly increased following allergen sensitization. (*P = 0.0026). Bars represent the mean ± s.e.m. for each group of 5 animals. (*$P < 0.0026$ versus naive). Results are representative of two independent experiments. (**b**) Morphology of myeloid cells isolated from the lungs of allergen sensitized mice. Cells were sorted as CD11b^{+} Gr-1^{mid} FcγR^{+} IL-17RB^{+} CD4^{-}

$CD8^-$ $B220^-$ IL-7Rα^- $Sca1^-$ $c\text{-}kit^-$ (see **Fig 2.1**) and stained with Hematoxylin and Eosin (H+E). Scale bar 50 μm.

Allergen sensitized *Il17rb*$^{-/-}$ mice exhibit decreased pathophysiology and type 2 inflammation

To further explore the overall role of IL-17RB in allergic asthma, *Il17rb*$^{-/-}$ mice were sensitized to allergen. The loss of the IL-25–specific receptor protected *Il17rb*$^{-/-}$ mice from allergen-induced inflammation (**Fig. 3.5**). *Il17rb*$^{-/-}$ allergic mice exhibited a dramatic reduction in peribronchial and perivascular inflammation, eosinophilic infiltrates, and mucus production (**Fig. 3.5a**). Pulmonary expression of type 2 cytokines as well as the eosinophil–associated chemokine eotaxin were significantly decreased in lungs of *Il17rb*$^{-/-}$ mice (**Fig. 3.5b**). RNA transcripts from bone marrow cultures demonstrated a similar reduction in type 2 responses, indicating a systemic reduction in type 2 cytokines (**Fig 3.5c**). Furthermore, *Il17rb*$^{-/-}$ draining lymph node cells re-stimulated with antigen produced significantly less type 2 cytokines than WT lymph node cells (**Fig. 3.5d**). Interestingly, a short–term model of allergen sensitization that utilized fewer allergen challenges revealed no phenotypic differences in *Il17rb*$^{-/-}$ mice (although relative levels of cytokine mRNA in whole lung isolates trended as had been observed previously) (**Fig 3.6**) suggesting that IL-17RB is most relevant during more chronic allergic responses when IL-25 production is significantly increased.

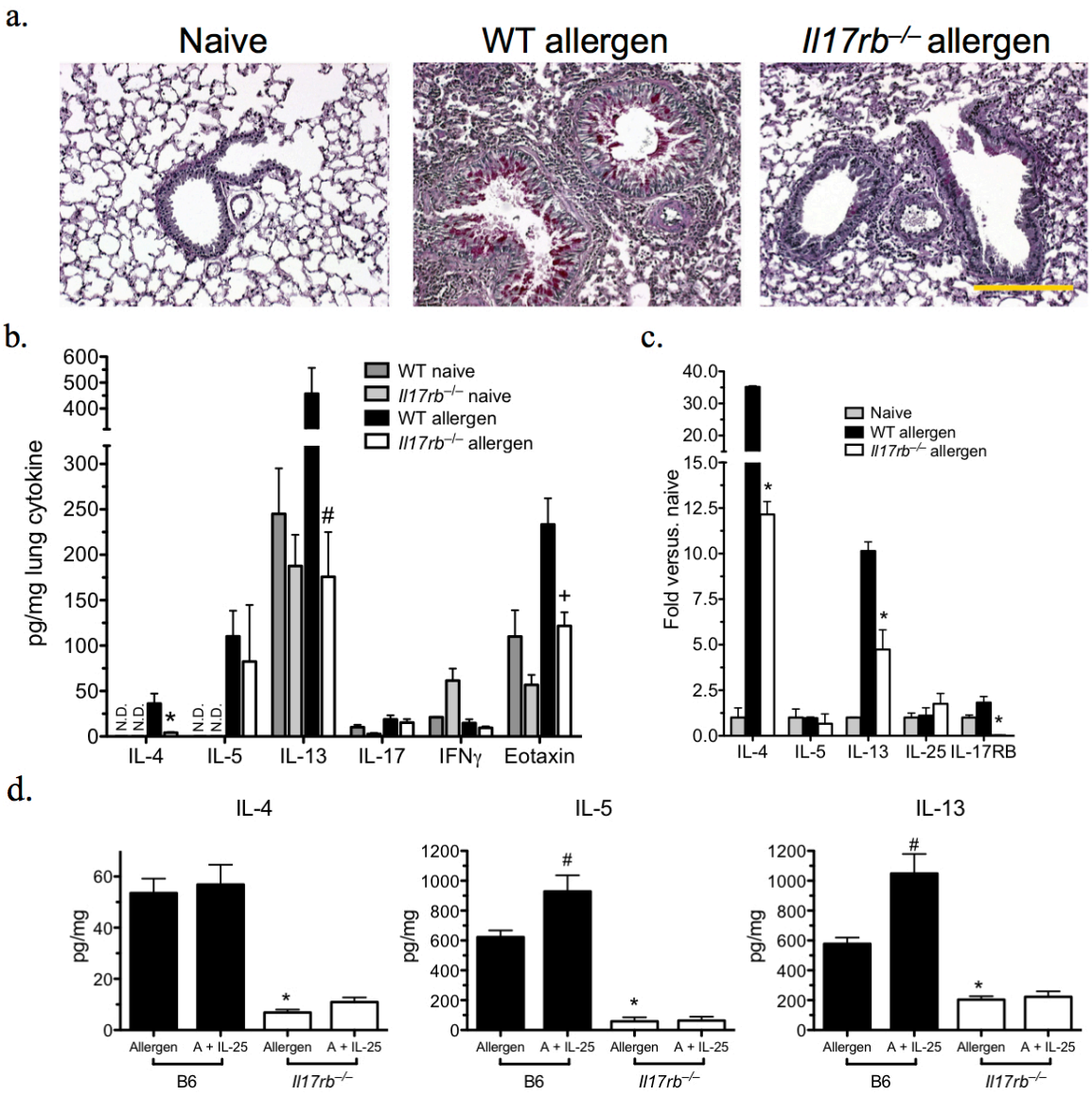


Figure 3.5: *Il17rb*$^{-/-}$ mice are protected from allergen induced type 2 inflammation. (**a**) PAS staining of lungs from WT and *Il17rb*$^{-/-}$ mice following chronic allergen sensitization. Scale bar 200 μm. (**b**) Lungs from allergic mice (n = 5 animals per group) were harvested 24 h post final allergen challenge and whole lung homogenates were analyzed for cytokine production by bioplex. Bars represent the mean ± s.e.m. for each group (*P = 0.001, #P = 0.048, +P < 0.05 versus WT allergen). (**c**) QPCR analysis of cytokines in whole bone marrow (*P < 0.05) (**d**) Draining lymph nodes from allergic mice (n = 5 animals per group) were harvested and pooled 24 h post final antigen challenge. Cells were cultured in media alone or restimulated with 10 μL per mL allergen ± 10 ng per mL IL-25. Supernatants were collected after 48 h and analyzed for cytokine production by bioplex. Bars represent the mean ± s.e.m. from triplicate wells. (*P < 0.01 versus WT allergen, #P < 0.05 versus WT allergen + IL-25).

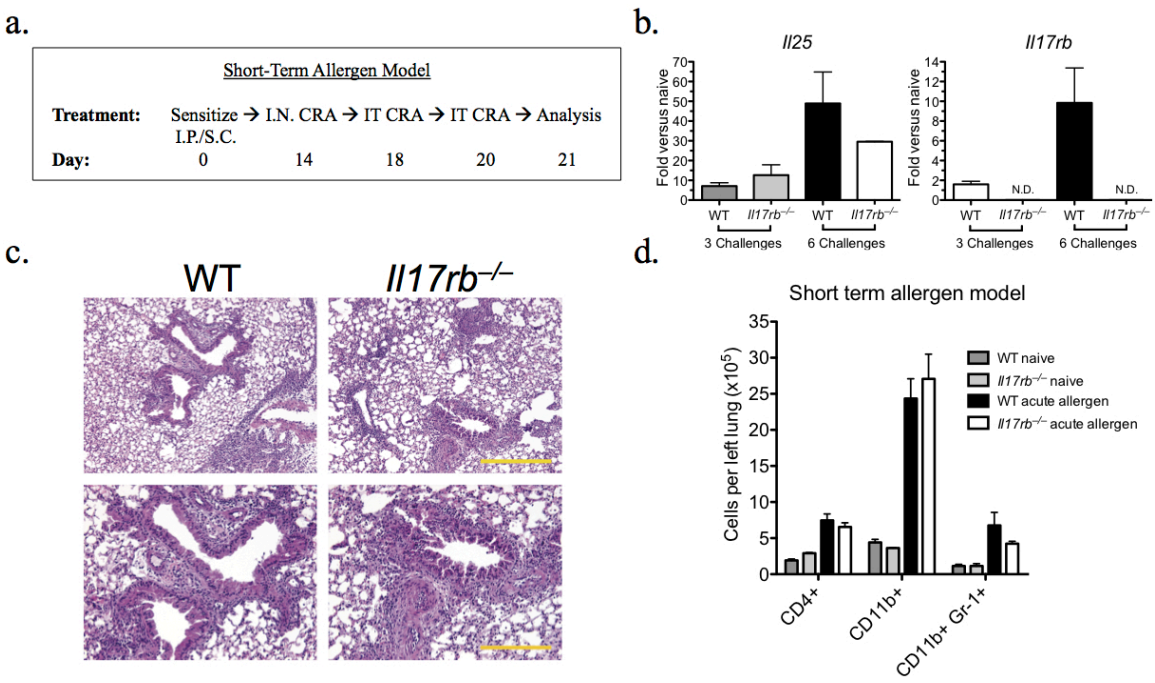


Figure 3.6: Repeated allergen challenges are necessary for pulmonary recruitment of IL-25 responsive CD11b$^+$ Gr-1$^+$ myeloid cells. (**a**) Model used in a short-term model of allergen-induced asthma. Mice received 3 challenges (as opposed to 6) and were assessed for recruitment of inflammatory cells to the lung by histology and flow cytometry. (**b**) *Il25* and *Il17rb* expression following short-term versus chronic allergen models. (**c**) H+E staining indicates that in a more acute setting, *Il17rb*$^{-/-}$ mice generate similar amounts of peribronchial inflammation as WT allergen sensitized mice. Scale bar for upper row, 400 μm; lower row, 200 μm. (**d**) Flow cytometric analysis for CD4$^+$ T cells and myeloid populations showed no differences between WT and *Il17rb*$^{-/-}$ allergen treated groups (n = 5 animals per group) after a shortened model of allergen sensitization.

CD4$^+$ T cell function is altered by loss of IL-17RB

To address the relative importance of IL-17RB on cell types involved in the adaptive immune response to allergic inflammation, we focused our assessment on the function of CD4$^+$ T cells and dendritic cells (DCs). Using *in vitro* co–culture experiments, mature allergen pulsed bone marrow–derived WT and *Il17rb*$^{-/-}$ DCs were cultured with CD4$^+$ T lymphocytes isolated from allergen sensitized WT or *Il17rb*$^{-/-}$ mice. As **Figure 3.7** indicates, both WT and *Il17rb*$^{-/-}$ DCs generated an equivalent response in WT CD4$^+$ T cells, indicating that DCs from *Il17rb*$^{-/-}$ mice can function

properly to activate Th2 cytokine production. This result is consistent with flow cytometric data indicating that IL-17RB is either not expressed, or expressed at low levels, on DCs (**data not shown**). Cytokine production in *Il17rb*$^{-/-}$ T cells, however, was significantly diminished compared to WT T cells, irrespective of DC source, WT or *Il17rb*$^{-/-}$. These data suggest that a component of the diminished inflammatory response observed in *Il17rb*$^{-/-}$ mice is related to a deficit in Th2 cell but not DC function.

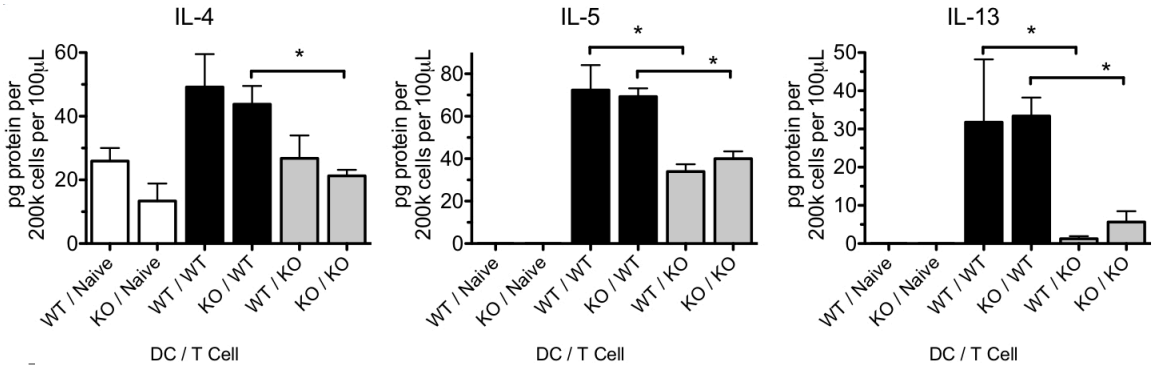


Figure 3.7: *In vitro*: Loss of IL-17RB alters T cell but not dendritic cell function. *Il17rb*$^{-/-}$ CD4^{+} T cells produce fewer type 2 cytokines than WT CD4^{+} T cells, irrespective of DC origin. Mature bone marrow derived dendritic cells (BMDCs) cultured from 3 WT or 3 *Il17rb*$^{-/-}$ mice were pulsed with 10 μL per mL allergen for four h, and then co-cultured with WT or *Il17rb*$^{-/-}$ CD4^{+} T cells isolated from the pooled spleens of allergic mice (n = 5 mice per group), at 2x10^{4} BMDCs and 2x10^{5} CD4^{+} T cells per well. Supernatants were collected after 48 h in culture and analyzed for protein by bioplex. Bars represent the mean ± s.e.m. from triplicate wells (*P < 0.05). These data indicate that IL-17RB is not necessary for the antigen-induced activation of previously activated T cells by DC.

To address T cell function *in vivo*, we adoptively transferred CD4^{+} T lymphocytes (6 x 10^{6} per mouse) from allergen-sensitized mice into *Rag2*$^{-/-}$ recipients. Our *in vivo* results paralleled our *in vitro* experimental findings. Transfer of T cells from WT mice significantly upregulated mRNA expression of both *Il25* and *Tslp*, as well as *Ccl11* (Eotaxin), *Ccl17* (TARC), and its receptor *Ccr4*, whereas *Il17rb*$^{-/-}$ T cell transfer showed

significantly lower transcript levels (**Figure 3.8a**). While both WT and *Il17rb*$^{-/-}$ T cell recipients mounted a Th2 response to allergen, Th2 cytokine production in *Il17rb*$^{-/-}$ T cell recipients was significantly lower than that seen in WT T cell recipients (**Figure 3.8b**). Similar to our findings with allergen studies in WT and *Il17rb*$^{-/-}$ mice, recipients of *Il17rb*$^{-/-}$ T cells also exhibited a significant reduction in numbers of myeloid cell infiltrates following allergen challenge (**Figure 3.8c**). Taken together, the results of these *in vitro* re-stimulation experiments, as well as *in vivo* adoptive transfer studies with *Rag2*$^{-/-}$ mice, demonstrated that CD4^{+} T cell responses were altered in *Il17rb*$^{-/-}$ mice and could not efficiently transfer a type 2 immune response to *Rag2*$^{-/-}$ mice. These data were similar to findings published by several investigators establishing a role for IL-25 in promoting T cell mediated type 2 responses.

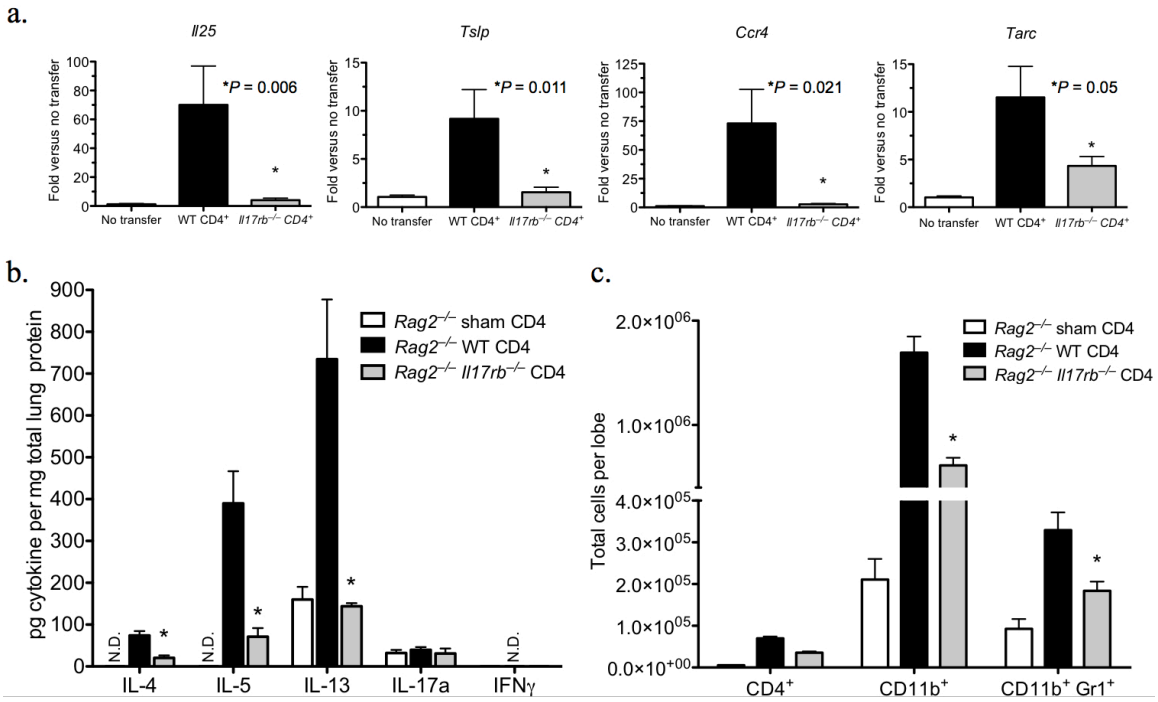


Figure 3.8: *In vivo*: Loss of IL-17RB alters T cell but not dendritic cell function. 6x10^{6} WT or *Il17rb*$^{-/-}$ CD4^{+} T cells isolated from the pooled spleens of allergic mice (n = 5 mice per group) were adoptively transferred by tail vein injection into naive *Rag2*$^{-/-}$ mice. Recipient mice received two IT allergen challenges, the first immediately following T cell transfer and the second 4 days later. (**a**) mRNA analysis was performed

by QPCR on whole lung tissue from recipient mice (n = 4 mice per group) 24 h post final allergen challenge. Bars represent the mean ± s.e.m. for each group, *P < 0.05. (**b**) Lungs of *Rag2*$^{-/-}$ recipient mice (n = 4 animals per group) were harvested 24 h post final antigen challenge and whole lung homogenates were analyzed for cytokine production by bioplex. Bars represent the mean ± s.e.m. for each group, *P < 0.01. (**c**) Flow cytometric analysis of *Rag2*$^{-/-}$ mice following adoptive transfer of CD4^{+} T cells from allergic donors. Bars represent the mean ± s.e.m. for each group, *P < 0.03. Despite the ability of both WT and *Il17rb*$^{-/-}$ T cells to induce a type 2 inflammatory response, a significant reduction in overall pulmonary inflammation, and subsequent response to allergen, was observed in recipients of *Il17rb*$^{-/-}$ T cells compared to WT. This altered response affected the recruitment of other cells via a reduction in transcripts of allergen-associated chemokines like *Ccl17* (TARC). It also led to a significant decrease in myeloid infiltrates following allergen challenge, indicating that the absence of IL-17RB in CD4^{+} T cells led to a systemic deficiency in inducing a type 2 cytokine mediated inflammatory environment.

IL-4 and IL-13 production in myeloid cells is abrogated by the absence of IL-17RB

Flow cytometric analysis of inflammatory populations demonstrated no difference in numbers of CD4^{+} and CD8^{+} T lymphocyte subsets between the lungs of allergen sensitized *Il17rb*$^{-/-}$ or WT mice. However, *Il17rb*$^{-/-}$ mice exhibited significant reductions in the CD11b^{+} Gr-1^{mid} myeloid cells previously identified as a source of type 2 cytokines in allergic animals (**Fig. 3.9a**). Intracellular cytokine staining of the CD11b^{+} Gr-1^{mid} population verified that *Il17rb*$^{-/-}$ mice had a significantly reduction in type 2 cytokine producing cells relative to WT mice (**Fig. 3.9b**). Reduced lymph node cytokine production, altered CD4^{+} T cell function, the reduction in type 2 cytokines, and a dramatic decrease in pulmonary myeloid cell infiltrates indicated that multiple cell types were affected by the absence of IL-17RB.

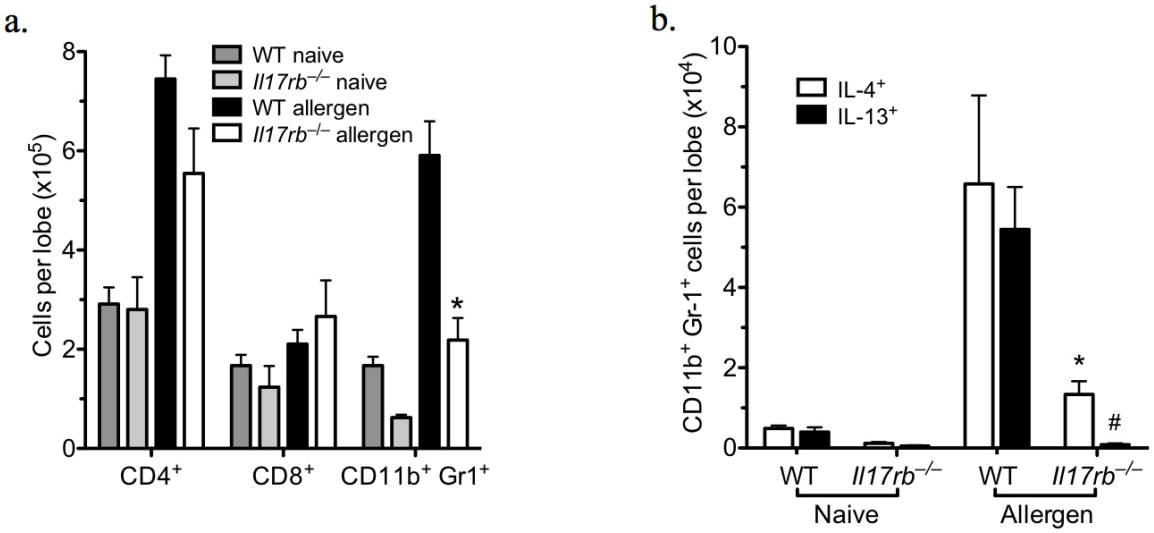


Figure 3.9: Type 2 cytokine production in CD11b+ Gr-1$^+$ myeloid cells is IL-17RB dependent. (**a**) Flow cytometric analysis of lungs from allergic WT and *Il17rb*$^{-/-}$ mice (n = 5 animals per group). Bars represent the mean ± s.e.m. for each group (*P < 0.03). (**b**) Intracellular cytokine staining for IL-4 and IL-13 producing cells was performed in allergic WT and *Il17rb*$^{-/-}$ mice (n = 4 animals per group). Results are gated on CD11b$^+$ Gr-1^{mid} cells. Bars represent the mean ± s.e.m. for each group (*P < 0.05, #P < 0.0009). Data are representative of two independent experiments.

Discussion

The present study used a mouse model of chronic allergic asthma to identify both T and non–T IL-25 responsive cells involved in pulmonary inflammation. While previous studies have established that targeting IL-25 leads to the reduction of type 2 responses[16,193,201], this study is the first to characterize how deficiency in IL-17RB reduces the pathology of allergic asthma induced by a common environmental allergen. Other reports, including one from our laboratory, have demonstrated that eosinophils produce IL-25[11,14], thus linking IL-25 production to eosinophilia induced by the allergic response. These data are consistent with clinical studies, as peripheral blood mononuclear cells from patients with severe allergic rhinitis exhibit increased IL-17RB[221], and

polymorphisms in IL-17RB have been associated with increased risk for severe asthma[223]. Furthermore, a recent study has identified that allergen–induced expression of IL-25 and its receptor in atopic asthmatics correlates with disease severity[222]. *Il17rb*$^{-/-}$ mice had reduced allergen–induced pathology, including a significant reduction in type 2 cytokines primarily associated with the CD11b^{+} Gr1^{mid} cells that were most prominent during persistent allergen–induced disease.

Because IL-17RB^{+} subsets could be distinguished based upon their intensity of Gr-1 expression, and in light of the differing capacity for IL-4 and IL-13 production between Gr-1^{mid} and Gr-1^{hi} populations, IL-17RB^{+} Gr-1^{hi} cells may have functions that overlap with other CD11b^{+} Gr-1^{+} populations, such as myeloid suppressor cells[243,244]. A recent study reported that both IL-17RA and IL-17RB can be expressed on the surface of human neutrophils[245].

As evidenced by alterations in cytokine expression in lung, LN, and bone marrow populations, signaling through IL-17RB plays an important role in the biology of both innate and adaptive immune populations. The adoptive transfer of CD4^{+} T cells demonstrated the systemic effects of a global absence of IL-25 signaling. *Il17rb*$^{-/-}$ T cells not only exhibited reduced cytokine production when presented with antigen, the absence of an IL-25 receptor created a domino effect, whereby reduced cytokine production led to reduced inflammation, and diminished chemokine production from a less inflamed airway epithelium. As flow cytometric data demonstrated, without IL-17RB on T cells, chemokine transcripts were lowered in the lung and there was a significant reduction in the influx of type 2 cytokine producing myeloid cells identified in

our model of chronic allergic inflammation. This same population is absent in *Il17rb*$^{-/-}$ mice following allergen exposure as well.

The recent identification of IL-25 and IL-33 responsive innate lymphoid cells offers evidence supporting the critical role innate immunity plays in the development and type 2 inflammatory responses, however these cells were not increased following allergen administration[20-23]. Given our findings that IL-25 expression is upregulated during chronic, but not short–term allergen exposure, the IL-17RB^{+} myeloid population identified in these experiments may represent an arm of the innate immune system required for maintenance of the chronic inflammatory environment. However, the resolution provided by these experiments does not enable comment on IL-25–dependent events that may occur in the absence of allergen. These represent the topic of Chapter 5.

The present study identifies a role for IL-25 in the development of allergic asthma. One question raised by these results relates to the purity of the induced inflammatory response, specifically whether this type of "pure" type 2 inflammation is similar to the types of inflammatory stimuli commonly encountered in the environment. The most severe symptoms of respiratory syncytial virus (RSV), for example, are associated with excess mucus production, largely driven by the overproduction of IL-13[113]. In the case of RSV, increased IL-13 represents one component of a mixed inflammatory response that involves an upregulation of both IFNγ and IL-17A. IL-25's role in the mixed inflammatory environment created in response to RSV infection is the topic of our next chapter.

CHAPTER IV

RSV Infection Induces IL-25–Dependent Type 2 Inflammation

Abstract

Individuals who suffer severe respiratory viral infections in infancy and early childhood are at significantly increased risk for the development of childhood asthma and pulmonary disease, and respiratory syncytial virus (RSV) is a well–established cause of this phenomena. The mechanisms by which RSV infection promotes the development of the asthma are not well understood, however reducing the incidence and severity of RSV infections reduces the likelihood that an individual will develop asthma. The most severe pathology of RSV infection is associated with overproduction of type 2 cytokines, leading to mucus hypersecretion. This study investigated the role of IL-25 in the development of RSV-associated immunopathology. IL-25 was increased following RSV infection, and IL-25 blockade reduced RSV associated pathology and type 2 cytokine production. *Il17rb*$^{-/-}$ mice demonstrated a dysregulated inflammatory response characterized by decreased Th2 but increased Th17 cytokine production. The inhibition of IL-25 signaling also significantly reduced inflammation and cytokine production in a model of RSV driven asthma exacerbation. These results indicate that IL-25 regulates

the inflammatory response to RSV infection and that its inhibition may enable a reduction in the severity of RSV–associated pulmonary inflammation.

Introduction

RSV is a ubiquitous pathogen and the most common cause of hospitalization in the first year of life[82]. Upwards of 95% of children have been exposed to the virus by age two[94,95], and although subsequent infections are generally less severe, RSV represents a difficult target for the adaptive immune system because T and B cell memory is not fully protective, and does not prevent reinfection from occurring throughout life[96-98]. Opportunistic infections with RSV can have significant impacts on elderly and immunocompromised populations[99-101], and present with variable symptoms ranging from asymptomatic illness to bronchiolitis requiring hospitalization and, in the most severe cases, death due to respiratory failure. Initial attempts to generate a vaccine against RSV ended in failure when individuals inoculated with an inactivated virus became hyper-responsive to subsequent RSV infection, causing profound type 2 inflammation, mucus hypersecretion, and pulmonary eosinophilia[102,103]. RSV's clinical importance, and the absence of an effective vaccine, therefore makes understanding immune regulation of RSV infection an important are of research.

The pathology of RSV infection includes significant airway epithelial damage, mucus hypersecretion, and increased type 2 cytokines in the lung[113,114], similar in many ways to pathology associated with allergic asthma. As with asthma, several investigators have linked this clinically relevant pathology not with direct effects of the viral infection, but with consequences of the host immune response[115,116]. RSV infection can generate a

mixed inflammatory response in addition to type 2 inflammation. This is the result of an induction of neutrophilic Th17 inflammation along with type 1 anti–viral responses[117]. Therefore, the dramatic surge in pathology that can accompany this viral infection in asthmatic patients is related to the additive effects of mixed Th1, Th2 and Th17 inflammation.

IL-25 is an IL-17 family member that regulates multiple aspects of mucosal immunity by promoting type 2 inflammation and production of IL-4, IL-5, and IL-13[4]. We investigated the role of IL-25 in the pathogenesis of RSV; specifically how inhibition of IL-25–mediated signals may alter the development of RSV-associated immunopathology. Here we report that IL-25 is upregulated following RSV infection, and that peak cytokine expression corresponds with peak inflammation. Our findings indicate that IL-25 plays an important role in the establishment of type 2 immunopathology in RSV infection. RSV–induced pathology, including inflammatory infiltrates, airway hyperreactivity, and lymph node cytokine production were attenuated by the inhibition of IL-25 signaling through the use of a blocking antibody and in *Il17rb*$^{-/-}$ mice, indicating that Th2-associated pathology in RSV infection is IL-25–dependent. Using a model of allergic asthma, we further report that RSV–associated exacerbation of allergic airways disease is reduced in the absence of *Il-17rb*, and associated with a reduction in type 2 cytokine production. Previous studies have identified an inverse relationship between the expression of IL-25 and IL-17A, and our findings support these assertions[201,213,246]. Taken together, our data suggest that mucus hypersecretion, the most significant pathology associated with RSV infection, may be effectively managed through the tandem targeting of IL-25 and IL-17A.

Results

Clinically, the most severe sequela of both allergic asthma and RSV infection results from airway obstruction and subsequent ventilation-perfusion mismatch[153,157,247]. Type 2 cytokines, IL-13 in particular, act as primary mediators of mucus hypersecretion in both asthma and RSV infection, and IL-25's ability to promote their production was therefore of great interest because the overproduction of type 2 cytokines has the highest correlation with poor clinical outcomes. The mechanism by which viruses such as RSV induce type 2 inflammatory responses remains unclear. In our initial experiments, we sought to determine whether IL-25 plays a role in the inflammatory response to RSV infection by utilizing a time course to measure *Il-25* and *Il17rb* lung transcripts (**Figure 4.1**). Transcripts of both genes increased following infection, peaking between days 4 and 8 and returning to baseline levels by day 12. This pattern of expression corresponded to previous findings of peak inflammatory responses at day 6 post infection, and indicated that IL-25 was playing an active role in the host inflammatory response to viral infection.

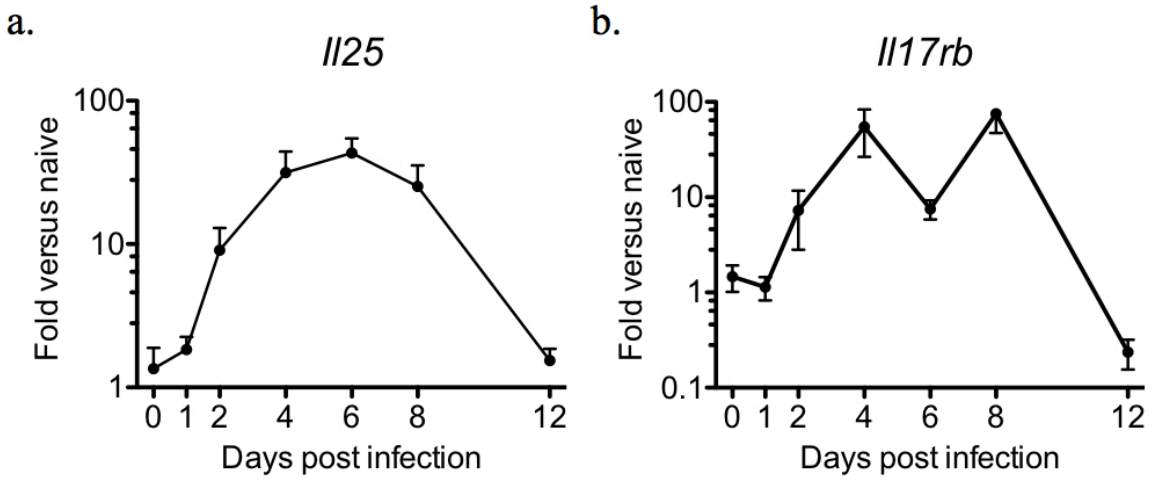


Figure 4.1: RSV infection induces upregulation of *Il25* and *Il17rb*. BALBc mice were infected with RSV and transcripts of Il-25 and Il-17rb were assessed at days 1, 2, 4, 6, 8, and 12 post infection. (**a** and **b**) Increasing expression of pulmonary IL-25 and IL-17RB transcripts mirror the inflammatory response induced by RSV infection. RSV induces peak levels of inflammatory cytokines on day 6 post-infection. Values represent the mean ± s.e.m., *n*= 4 per group.

Anti-IL-25 administration reduces RSV-associated pathology and LN cytokine production

To further investigate the role of IL-25 in the inflammatory response to RSV infection, mice were infected with RSV and treated with a polyclonal antibody to IL-25. The effects of anti–IL-25 therapy in these mice were assessed on day 10 post infection, corresponding to peak pathophysiology in our model. RSV infected mice treated with anti-IL-25 antibody exhibited a significant reduction in airway hyperreactivity (**Figure 4.2a**), as well as a significant reduction in the mucus-specific gene *Gob5* (**Figure 4.2b**). Histologic analysis confirmed that inhibiting IL-25 reduced airway inflammation and mucus (**Figure 4.3a**), however it did not affect total numbers of inflammatory cells in the lung (**Figure 4.3b**), nor did it alter levels of lung cytokine (**Figure 4.3c**).

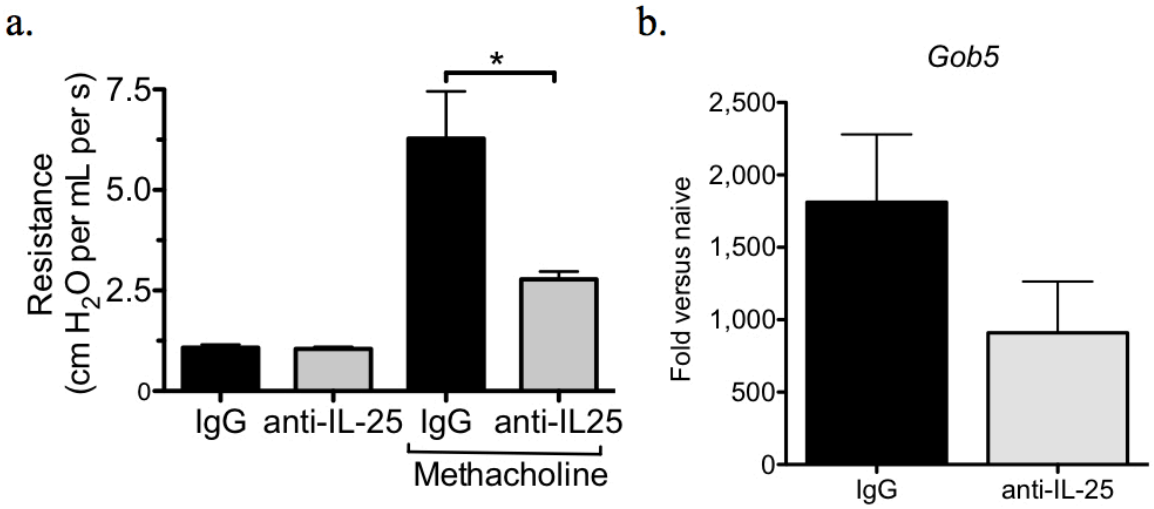


Figure 4.2: IL-25 blockade has significant effects on airway function. (**a**) Airway resistance following methacholine challenge was assessed in RSV infected mice at day 10 post-infection. Values represent the mean ± s.e.m., $n = 5$ mice per group * $P < 0.05$. Data are representative of 2 independent experiments. **B)** QPCR of the mucus specific gene *Gob5*.

In our next set of experiments, the draining LNs of RSV infected animals were collected and restimulated *in vitro* to assess the effects of IL-25 blockade on cytokine production (**Figure 4.4**). RSV–infected LN cells from mice treated with control IgG produced type 2 cytokines in response to both RSV and recombinant IL-25, while LN cells from animals that had been treated with anti-IL-25 antibody showed dramatic reductions in production of both type 2 cytokines (**Figure 4.4a-c**), as well as IL-17A (**Figure 4.4d**) and IFNγ (**Figure 4.4e**). Whereas culture with IL-25 further increased type 2 cytokine production in RSV restimulated LN cells from IgG treated mice, anti–IL-25 treated LN cells exhibited blunted or absent responses to stimulation with both RSV and IL-25 stimulation. We have previously shown that loss of IL-25–IL-17RB interaction does not affect antigen presentation (see **Fig 3.7**). Therefore, these results indicate that IL-25 inhibition prevented the activation of RSV–specific T lymphocytes, as

cytokine expression from anti-IL-25 treated animals could not be restored by exogenous IL-25 or RSV restimulation.

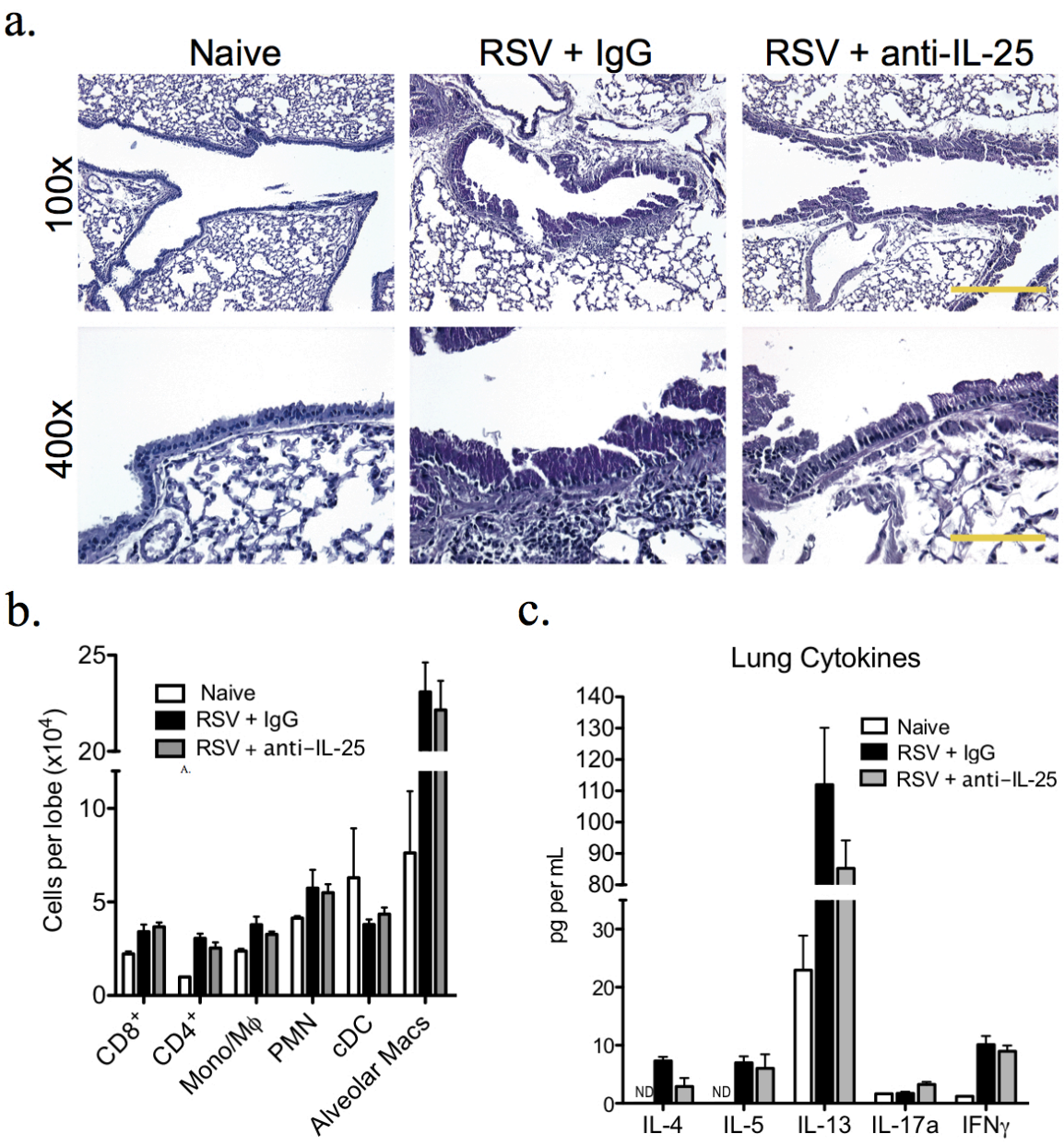
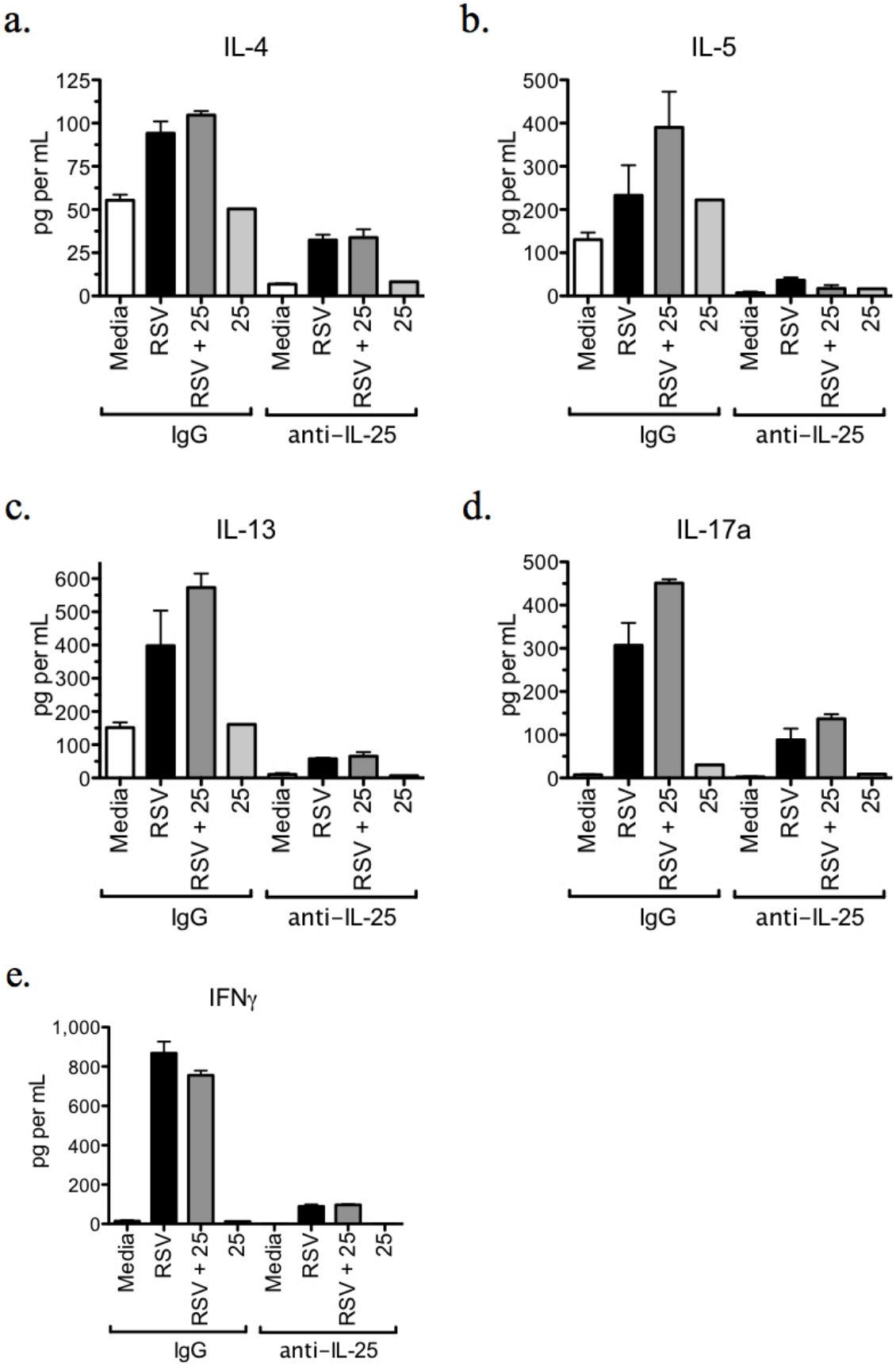


Figure 4.3: IL-25 blockade reduces airway mucus but not inflammatory infiltrates. (**a**) Representative lung histology from RSV infected mice treated with either control antibody or αIL-25, showing inflammation and mucus in the airway at day 10 post infection. Upper row scale bar 400 μM, lower row scale bar 100 μM. (**b**) Quantification of leukocyte populations in the lung on D8 post-infection by flow cytometry. (**c**) Protein quantification of lung cytokines by Bioplex. Anti-IL-25 treatment significantly reduced levels of IL-4. Values represent the mean ± s.e.m., n = 5 mice per group. Data are representative of 2 independent experiments. *P = 0.05.


a.
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anti-IL-25

Figure 4.4: Anti-IL-25 treatment inhibits cytokine production in draining lymph nodes. (**a–e**) Draining LN cells were isolated on day 8 post infection and cultured for 48 h *in vitro* ± RSV, ± IL-25. Cytokine production was analyzed by Bioplex. Values represent the mean ± s.e.m. of wells run in triplicate, with 400,000 cells per well. Data are representative of 2 independent experiments.

Loss of IL-17RB reduces RSV-associated immunopathology and dysregulation of LN cytokine production

To confirm our findings, *Il17rb*$^{-/-}$ mice were inoculated with RSV and their inflammatory response to viral infection was assessed. *Il17rb*$^{-/-}$ mice exhibited diminished inflammation following RSV infection, including a reduction in airway inflammation (**Figure 4.5a**) and significantly reduced pulmonary type 2 cytokine transcripts (**Figure 4.5b**). As was the case with anti–IL-25 therapy, cell numbers in WT and *Il17rb*$^{-/-}$ did not change following infection (**data not shown**). However, cytokine production in draining LN cultures demonstrated the importance of IL-25 in regulating the inflammatory response to RSV infection (**Figure 4.6**). Whereas production of the type 2 cytokines IL-4 and IL-5 were significantly decreased, the absence of IL-17RB was associated with a dramatic increase in IL-17A, as well as a more moderate surge in IFNγ production. These findings are consistent with a previous study, in which an inverse relationship was reported between IL-17A and IL-25 expression in an OVA model of allergic asthma.

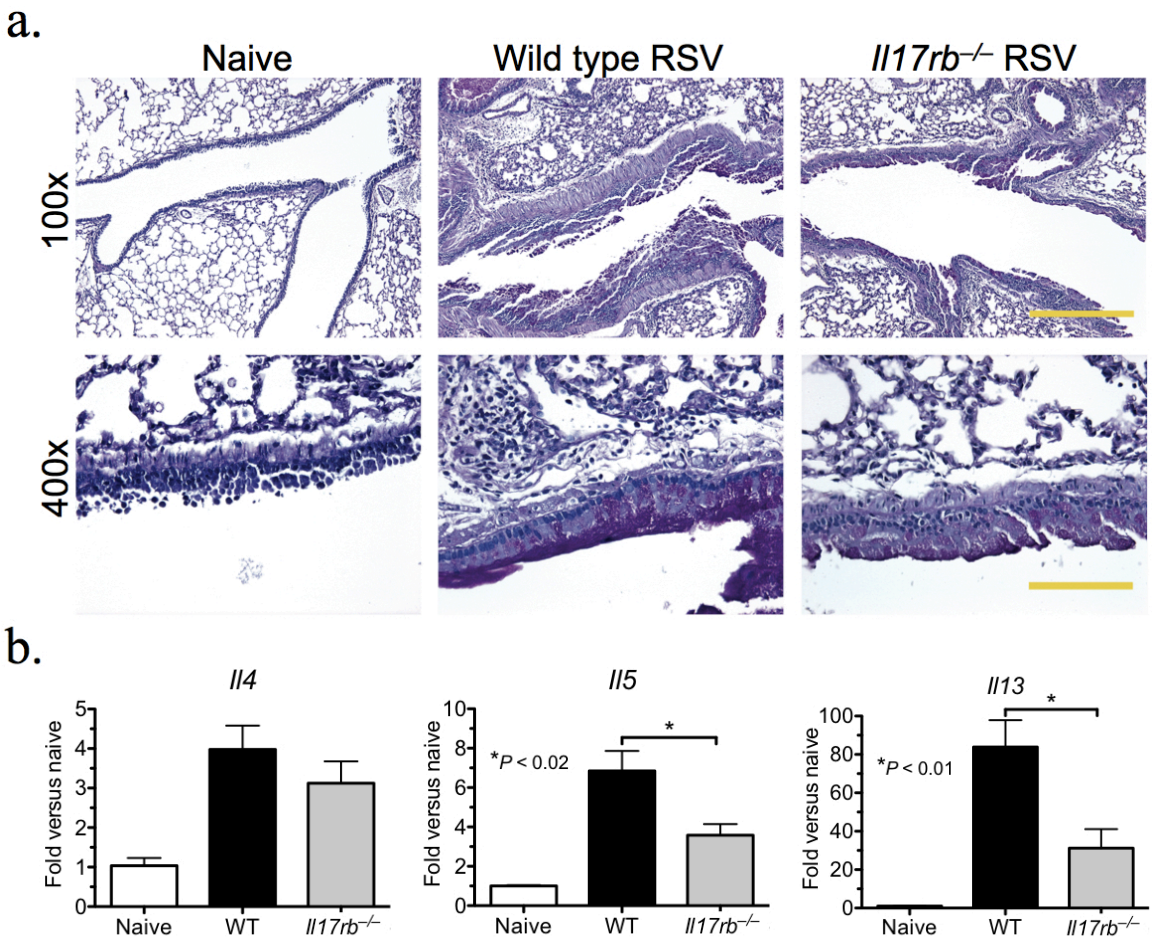
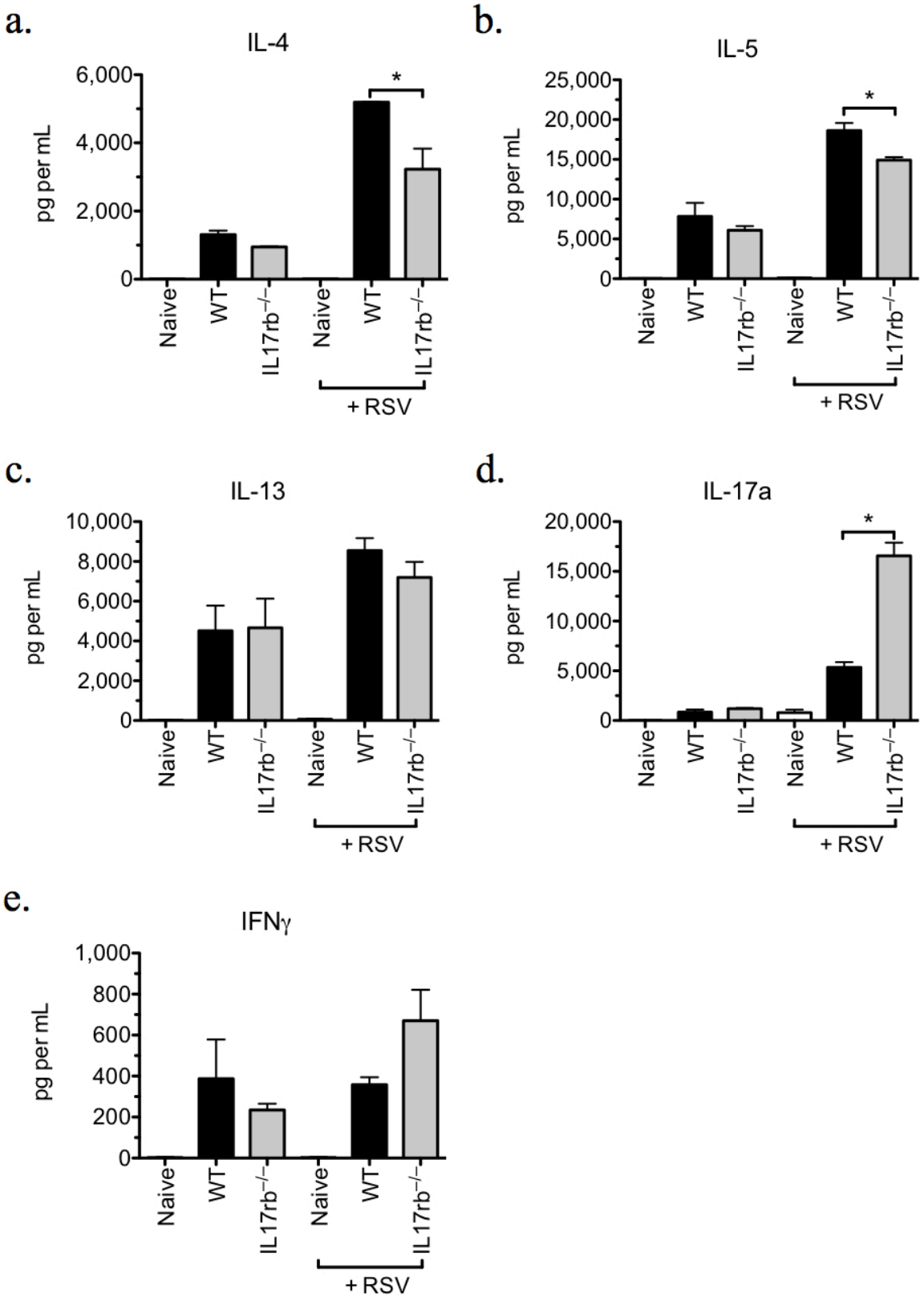


Figure 4.5: RSV-induced pulmonary inflammation is attenuated in the absence of IL-17RB. WT (C57BL6) or *Il17rb*$^{-/-}$ mice (n = 5 per group) were infected with RSV, and the inflammatory response was assessed at day 6 post infection. (**a**) Representative lung histology, stained with PAS and demonstrating reduced inflammation and mucus in RSV infected *Il17rb*$^{-/-}$ mice. Upper row scale bar 400 μM, lower row scale bar 100 μM. (**b**) Lung QPCR of *Il4*, *Il5*, and *Il13*. Transcripts of *Ifng* and *Il17a* were not significantly different between wild type and *Il17rb*$^{-/-}$ animals. Values represent the mean ± s.e.m.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c.
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WT
IL17rb−/−
+ RSV

Figure 4.6: *Il17rb*$^{-/-}$ LNs exhibit dysregulated cytokine production following RSV infection. Draining LNs from WT and *Il17rb*$^{-/-}$ mice (n = 5 per group) were harvested and restimulated with RSV. Supernatants were collected after 48 h *in vitro* and analyzed by Bioplex. Values represent the mean ± s.e.m. of wells run in triplicate. **P* < 0.05.

Viral exacerbation of allergic asthma is attenuated in the absence of IL-25 signaling

In our final set of experiments, we employed a shortened model of allergic asthma to study viral exacerbation with RSV (see **Figure 2.2**). As we have shown previously, IL-25 plays an important role in the inflammatory response to environmental allergens (**Chapter 3**), however the involvement of IL-25 in the context of allergy appears to be dose-dependent and requires chronic exposure to manifest significant effects (see **Figure 3.6**). While we did not observe the type 2 cytokine producing myeloid population identified in Chapter 3 following RSV infection alone, we hypothesized that the viral exacerbation of pre-existing allergic disease may induce sufficient IL-25 production to trigger recruitment of myeloid cells. As has been discussed in Chapter 1, viral exacerbations of allergic asthma are of great concern to susceptible populations such as children, the elderly, and immunosuppressed individuals[90,91,99-101]. The single most pathogenic component of RSV infection is the overproduction of mucus, which can block airways and require medical intervention or respiratory support in severe cases[113,114]. Underlying airway inflammation, such as that seen in asthma, necessarily reduces an individual's ability to tolerate added airway obstruction from mucus production.

We utilized a short–term, three challenge model of allergic disease (as described in **Figure 3.6**) to induce a moderate degree of pulmonary inflammation. The use of a less severe allergen model enabled us to assess the additive effects of RSV infection. WT and *Il-17rb*$^{-/-}$ mice were sensitized to allergen as previously described, inoculated with RSV,

and then challenged with allergen during the course of RSV infection. To further simulate an exacerbation of underlying airways disease, we administered two of three allergen challenges following inoculation with RSV (see **Figure 2.2**). The exacerbation of underlying allergic inflammation with RSV infection produced a patchy, heterogeneous inflammatory response (**Figure 4.7**), with both mononuclear and granulocytic infiltrates. The absence of IL-17RB had modest effects on reducing the overall inflammatory response. Compared to WT animals, *Il17rb*$^{-/-}$ mice exhibited a reduction in airway inflammation following RSV exacerbation, however the most apparent difference between groups was the reduction in mucus production in the RSV exacerbated *Il17rb*$^{-/-}$ group (**Figure 4.7**). Flow cytometric analysis of inflammatory subsets in the lung identified significant differences in only one population following viral exacerbation. The myeloid population (here labeled as T2M cells) identified in Chapter 3 was significantly increased in WT mice inoculated with virus and absent in *Il17rb*$^{-/-}$ mice (**Figure 4.8a**). The pathologic relevance of T2M cells are explored and discussed at length in Chapters 5 and 6, however it is important to note that mucus hypersecretion is associated with T2M IL-13 production, and a reduction in mucus is consistent with the absence of this population (see **Figures 5.3, 6.3 6.4,** and **6.5**).

As in previous experiments, the draining LN of WT and *Il17rb*$^{-/-}$ mice sensitized to allergen and inoculated with RSV were isolated and restimulated in vitro with allergen and RSV. An analysis of cytokine levels from the supernatants of these cultures showed no differences in cytokine production between WT and *Il17rb*$^{-/-}$ mice upon restimulation with allergen and RSV (data not shown). These data require further investigation to

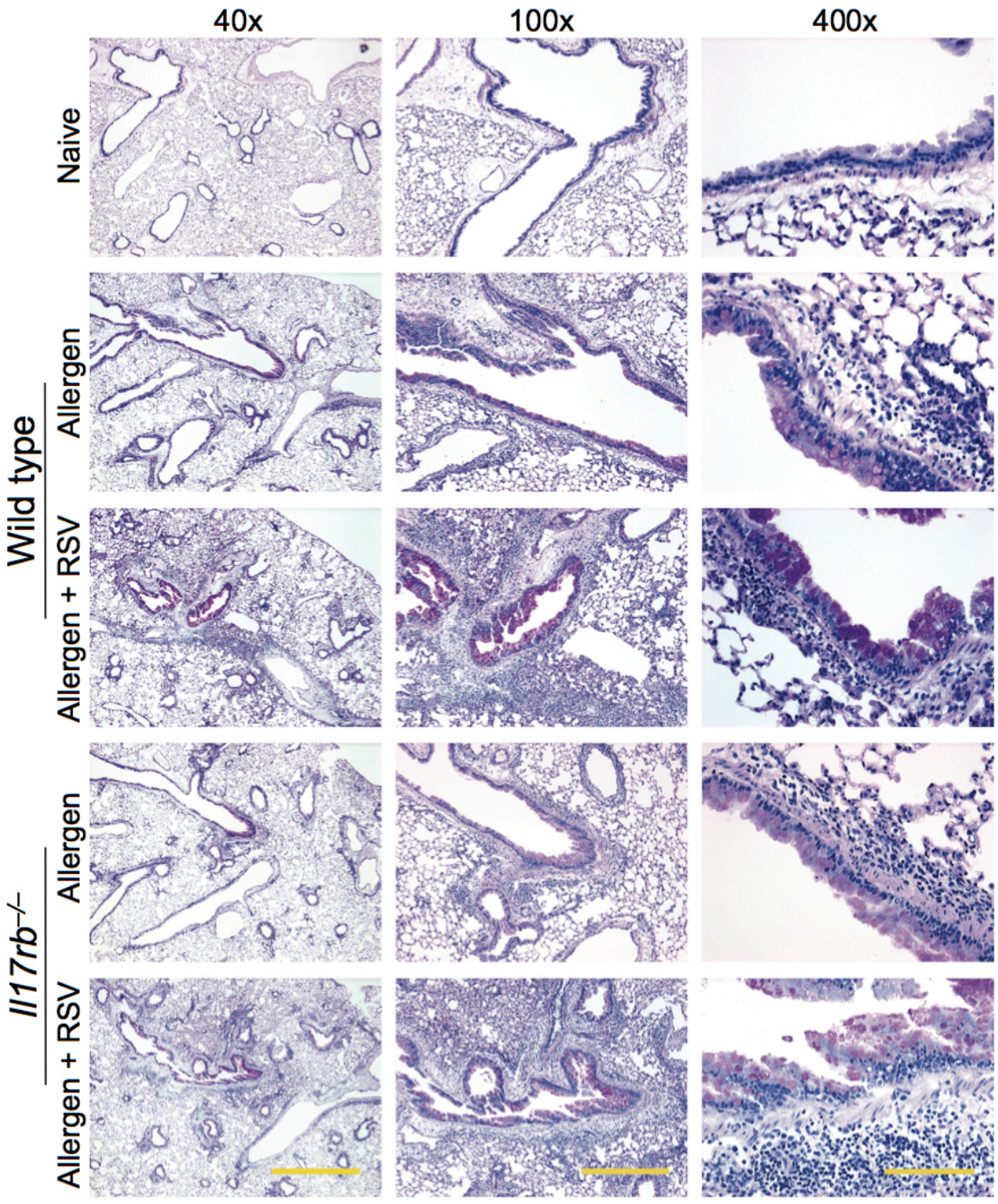
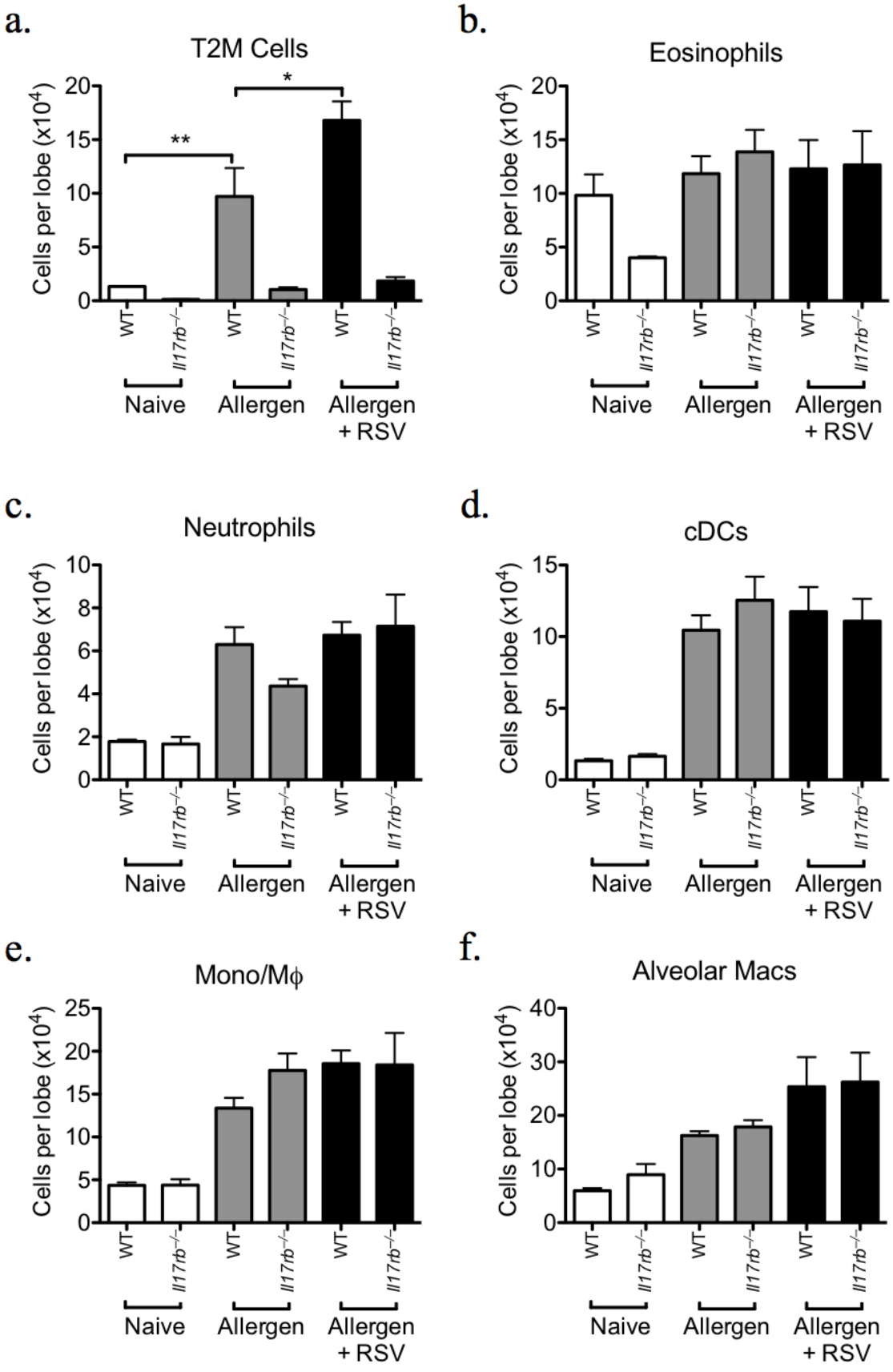


Figure 4.7: Exacerbation of allergic airways disease by RSV infection. To model viral exacerbation in the context of allergic airways disease, WT and *Il17rb*$^{-/-}$ mice (n = 5 per group) were sensitized to allergen and infected with RSV. Mice received 3 total allergen challenges, and inflammation was assessed on day 6 post RSV infection. Representative PAS stained slides illustrating the inflammatory response. Left column scale bar 1,000 μM, center column scale bar 400 μM, right column scale bar 100 μM.


a.
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Il17rb−/−
WT
Il17rb−/−
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Il17rb−/−
Naive
Allergen
Allergen + RSV
b.
Eosinophils
Cells per lobe (x10^4)
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WT
Il17rb−/−
WT
Il17rb−/−
WT
Il17rb−/−
Naive
Allergen
Allergen + RSV
c.
Neutrophils
Cells per lobe (x10^4)
10
8
6
4
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WT
Il17rb−/−
WT
Il17rb−/−
WT
Il17rb−/−
Naive
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Il17rb−/−
Naive
Allergen
Allergen + RSV
e.
Mono/Mϕ
Cells per lobe (x10^4)
25
20
15
10
5
0
WT
Il17rb−/−
WT
Il17rb−/−
WT
Il17rb−/−
Naive
Allergen
Allergen + RSV
f.
Alveolar Macs
Cells per lobe (x10^4)
40
30
20
10
0
WT
Il17rb−/−
WT
Il17rb−/−
WT
Il17rb−/−
Naive
Allergen
Allergen + RSV

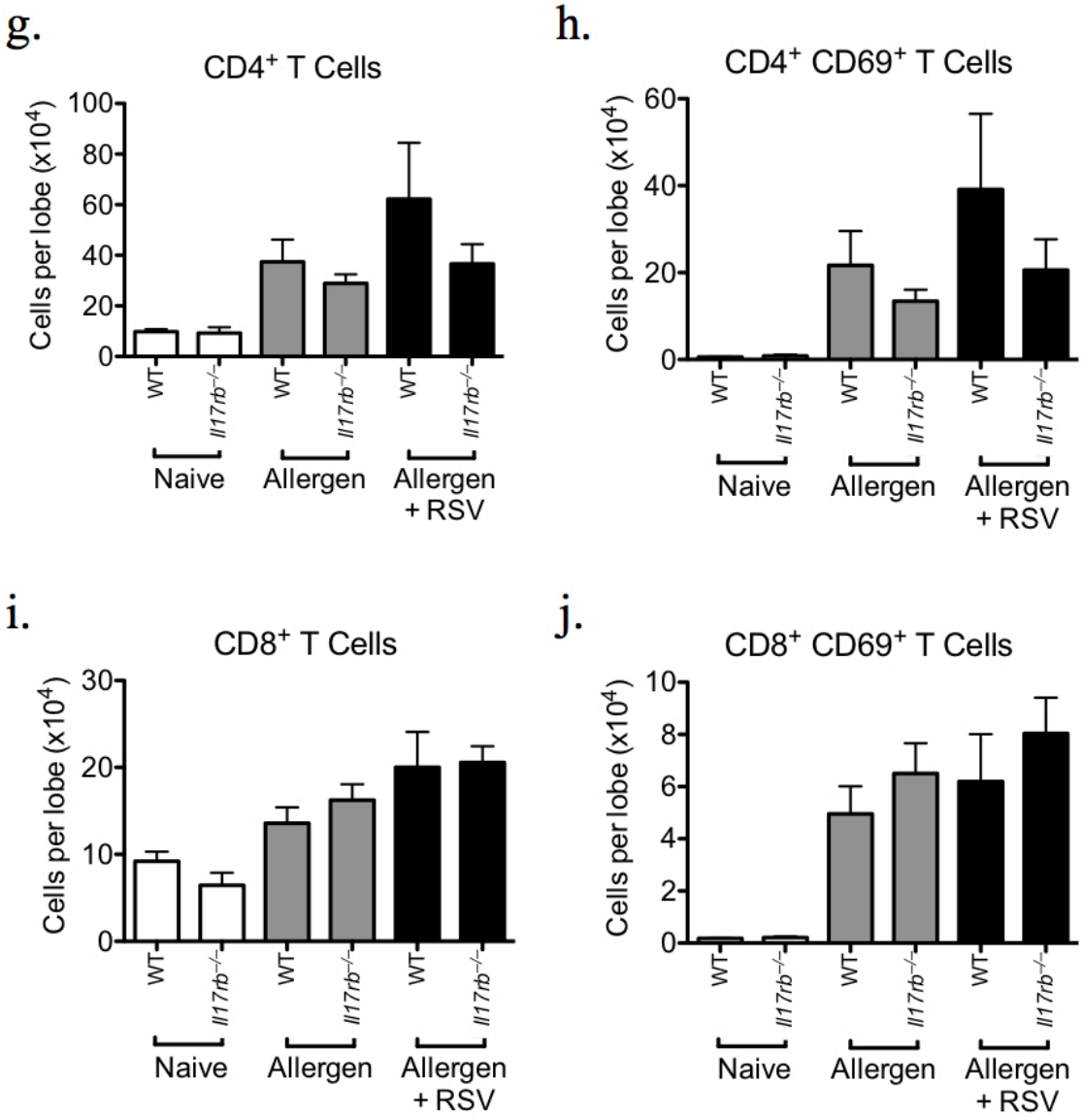


Figure 4.8: Quantification of pulmonary immune populations following the exacerbation of allergic asthma by RSV. WT C57BL6 and Il17rb$^{-/-}$ mice were sensitized to allergen and inoculated with RSV to model viral exacerbation of underlying airways inflammation. (**a-j**) Quantification of lung inflammatory populations by flow cytometry, characterized as follows: T2M cells (CD11b^{+} Gr1^{mid} F4/80^{+} IL-17RB^{+}, CD11c^{-}, MHCII^{-}, IL-5rα^{-}, discussed at length in Chapters 5 and 6), eosinophils (CD11b^{+} Gr1^{hi} F4/80^{-} IL-17RB^{-}, CD11c^{-}, MHCII^{-}, IL-5rα^{+}) neutrophils (CD11b^{+} Gr1^{mid} F4/80^{-} IL-17RB^{-}, CD11c^{-}, MHCII^{-}, IL-5rα^{-}), conventional DCs (CD11b^{+} Gr1^{lo} F4/80^{-} IL-17RB^{-}, CD11c^{mid}, MHCII^{mid}, IL-5rα^{-}), Monocyte/Macrophages (CD11b^{+} Gr1^{lo} F4/80^{+} IL-17RB^{-}, CD11c^{-}, MHCII^{+}, IL-5rα^{-}), and Alveolar Macrophages (CD11b^{lo} Gr1^{lo} F4/80^{-} IL-17RB^{-}, CD11c^{hi}, MHCII^{mid}, IL-5rα^{-}). Lymphocytes were gated on CD3^{+} CD11b^{-} cells, and included CD4^{+} T cells, activated CD4^{+} CD69^{+} T cells, CD8^{+} T cells, and activated CD8^{+} CD69^{+} T cells. (n = 5 mice per group, *P = 0.05, ** P = 0.013)

determine the effects of simultaneous stimulation with RSV and allergen. Whereas previous experiments with *Il17rb*$^{-/-}$ mice demonstrated significant differences in LN cytokine production (see **Figures 3.5, 3.7**, and **4.6)** between *Il17rb*$^{-/-}$ and WT mice, in this case the effect of simultaneous antigen stimulation did not have a similar effect. This may be related to the mechanisms by which allergen and RSV are taken up and processed by APCs[248-250], or to APC maturation following RSV stimulation (leading to decreased uptake of additional antigen), or it could be related to counterregulatory mechanisms associated with increased IL-17A production in RSV–specific T cells and type 2 cytokine production in allergen-specific cells[201,251]. Future experiments will be necessary to resolve this discrepancy, however on the whole it appears that *Il17rb*$^{-/-}$ mice suffered a more mild form of asthma exacerbation than WT mice.

Discussion

The results of this study indicate that IL-25 may represent a target to inhibit the pathogenesis of severe lower respiratory tract infections, thereby decreasing the risk of developing airway disease by reducing the severity of the inflammatory response. Individuals predisposed to the development of recurrent wheeze, whether due to family history or pre–existing atopy, may benefit from reduced airway damage and pulmonary dysfunction in early life. Childhood viral infections have multiplicative effects on the risk for an eventual asthma diagnosis[64,92]. For example, individuals with sensitivities to house dust mite (HDM) are at an up to seven–fold higher risk for asthma if they experience a severe respiratory infection with either RSV or RV[64]. Genetic predisposition also plays an important role. Children hospitalized with RSV bronchiolitis

before their first birthday are at a five–fold increased risk of asthma if one parent has a history of allergic disease[78].

Based on diminished T cell responses to antigen specific stimuli, as well as blunted responses to IL-25, the results of this study indicate that anti–IL-25 administration may inhibit T cell activation. In Chapter 3, similar effects were observed in experiments assessing type 2 cytokine secretion by *Il17rb*$^{-/-}$ T cells. However, it is clear that *Il17rb*$^{-/-}$ T cells are capable of responding to antigen and of producing large amounts of cytokine (as evidenced by their overproduction of IL-17A in response to RSV restimulation). Taken together, these findings indicate that T cells may require activation prior to becoming IL-25 responsive, but that activation itself can occur in an IL-25 independent manner. Therefore IL-25 may act as an additional polarizing signal for antigen-dependent responses; enabling T cells to differentiate and mount a Th2 response in situations where IL-25 is being produced, such as allergic asthma and RSV infection[18]. An evaluation of pulmonary populations with the capacity to respond to IL-25 in an antigen-independent manner represents the topic of our next chapter.

Palivizumab, a monoclonal RSV-specific antibody targeting the RSV F (fusion) protein, has been shown to offer passive immunization against RSV infection. In light of the absence of an effective vaccine, it currently represents the most effective RSV prophylaxis in individuals with high risk of developing complications from RSV and its long-term sequelae[252]. A prospective cohort study of palivizumab prophylaxis among 2-5 year old patients identified significant protective effects associated with the prevention of early life lower respiratory tract infections[79]. These included a reduction in hospitalizations, and an 80% reduction in the relative risk of developing recurrent

asthma-associated wheeze among patients with no family history of atopy. However, atopic children, or children with at least 1 atopic parent, were not similarly protected from wheeze onset. Based on these findings, the authors conclude that early life RSV infection and the later development of recurrent wheeze are linked, but that the mechanism by which RSV leads to later airway dysfunction is atopy–independent[79,253].

Our data suggest that mucus hypersecretion, the most significant pathologic component associated with RSV infection and asthma exacerbation, may be effectively managed through the tandem targeting of IL-25 and IL-17A. The cytokine specific receptors of these molecules require common adapter proteins for signal transduction[176,203-205], and a recent report identified a peptidomimetic–based strategy that targeted the shared adapter molecule Act1[254]. A CC loop mimetic of the Act1 SEFIR domain effectively blocked interactions between Act1 and the IL-17A and IL-25 receptors, thus inhibiting both cytokines with one compound. In the context of RSV infection, where a dysregulated inflammatory response drives disease pathology, the ability to regulate oppositional inflammatory signals could represent a powerful tool for preventing unwanted consequences of viral infection.

The fact that the myeloid population first described in Chapter 3 is significantly increased in the context of viral asthma exacerbation represents an interesting finding that links the type 2 inflammation of viral pulmonary infections to that seen in allergic asthma. This suggests that some synergy between allergic and viral disease, likely associated with IL-25 expression, drives their recruitment to the airway. The identification of myeloid cells provides further evidence for their potential pathologic relevance, and supports the hypothesis that IL-25 is a critical mediator of pulmonary type

2 inflammatory responses. These findings are especially intriguing given that numbers of other inflammatory populations did not differ between virally exacerbated allergic WT and *Il17rb*$^{-/-}$ mice, yet *Il17rb*$^{-/-}$ mice had reduced mucus in their airways. Our next set of experiments sought to evaluate the role of IL-25 in promoting antigen-independent inflammatory responses. As the results in Chapters 5 and 6 will indicate, the IL-25 dependent promotion of a myeloid population in the context of viral exacerbation may have clinical relevance and should be further investigated.

CHAPTER V

T2M Cells are a Distinct Granulocytic Subset

Abstract

Using an antigen–independent model of IL-25 induced inflammation, we identified IL-17RB^{+} CD11b^{+} Gr-1^{mid} cells, previously observed in the context of chronic allergen exposure, as the primary IL-25–responsive population in the lung. Subsequent experiments verified these cells as a source of IL-4 and IL-13 in response to IL-25 administration, and established a unique combination of cell surface antigens that mark them as distinct from other granulocytic subsets. Based on the production of type 2 cytokines, a combination of distinct surface antigens, and characteristic granulocytic morphology, we termed this population Type 2 Myeloid (T2M) cells. Microarray analysis identified T2M cells as readily distinguishable from other myeloid populations, and most closely related to eosinophils. Taken together, these findings mark T2M cells as a distinct IL-4 and IL-13 producing IL-25 responsive granulocytic subset.

Introduction

While the three granulocytic subsets, neutrophils, eosinophils, and basophils, are derived from a common progenitor, the stage in development at which these populations

diverge is a matter of some debate. Reports have identified discrete progenitor populations for eosinophils and basophils that cast doubt upon the functional utility of describing these populations as sharing a common granulocytic-monocyte progenitor (GMP). In 2009, Mori et al. reported a discrete eosinophil lineage progenitor in humans, characterized as IL-5rα^{+}, but sharing other progenitor cell surface markers, which gave rise exclusively to eosinophils[255]. Based on these findings, the authors concluded that eosinophils should be excluded from the human CMP lineage and recognized as an independent subset. These findings somewhat mirrored those of a separate group, which identified eosinophil-committed progenitors in murine bone marrow as also expressing IL-5rα[144]. Basophil-committed progenitors were subsequently identified as originating from GMPs in response to IL-3, and highly expressing FcεR1[256]. The identification of receptor expression associated with cell fate implies that granulocytic subsets may diverge from a common ancestor either contemporaneous to or soon after the CMP stage. This early divergence may necessarily limit the functional and/or phenotypic plasticity between granulocytic subsets.

Given our findings that IL-25 expression is upregulated by repeated but not short-term allergen exposure, the IL-17RB^{+} myeloid population we have identified in the allergic lung may represent an innate immune population involved in the maintenance of a chronic inflammatory environment. This population has a granulocytic morphology and produces IL-4 and IL-13, however it does not readily align itself morphologically or functionally with neutrophils, eosinophils, or basophils. Our next set of experiments sought to characterize IL-25 dependent pulmonary inflammation, specifically role of IL-25 in the recruitment of type 2 cytokine producing myeloid cells to the airway, in order to

better understand the origin and phenotype of these cells as well as other IL-25 responsive cell types.

The production of type 2 cytokine(s) by a myeloid population is by no means unprecedented. Several populations have been associated with IL-4 production, including basophils, eosinophils, mast cells, and NK cells[19,226,257-260]. However, IL-25 responsive myeloid cells have remained an elusive target since they was first identified as a pathologically relevant population in T and B cell deficient *Rag2*$^{-/-}$ mice[4,5]. Our effort to characterize T2M cells was greatly aided by the use of an IL-17RB–specific mouse anti–mouse antibody, which was not available to previous investigators. Here we report that pulmonary IL-25 administration increases T2M cells in multiple tissues, including lung, bone marrow, spleen, and peripheral blood, and promotes the recruitment of T2M but not other IL-17RB^{+} cell types to the lung. Our findings indicate that T2M cells are readily distinguishable from other type 2 associated inflammatory cells, and represent a novel IL-25–responsive population.

Results

IL-25 induces type 2 inflammation with allergic asthma–like pathology

Our investigation of IL-25–dependent allergic responses suggested the importance of an innate myeloid population as a source of type 2 cytokines during chronic, allergen–induced inflammation. In order to disentangle the relative contributions T cell mediated responses from those of the myeloid population, we adapted a model of antigen–independent IL-25–induced inflammation to directly assess the effects of IL-25 on type 2 cytokine producing cells *in vivo*, thereby avoiding the confounding pro–inflammatory

effects of antigen–specific activation[193]. Recombinant murine IL-25 was instilled into the airways of IL-4–IRES–eGFP (4get) mice. 4get mice express GFP in cells in which the IL-4 promoter is transcriptionally active, thereby enabling the identification of cells poised to produce type 2 cytokines[261]. As has been reported previously, the intra-tracheal administration of IL-25 induced a type 2 type inflammatory response, characterized by airway hyperreactivity, eosinophil infiltrates, mucus production, and the upregulation of inflammatory genes including *Il25* and *Il17rb* (**Fig. 5.1**).

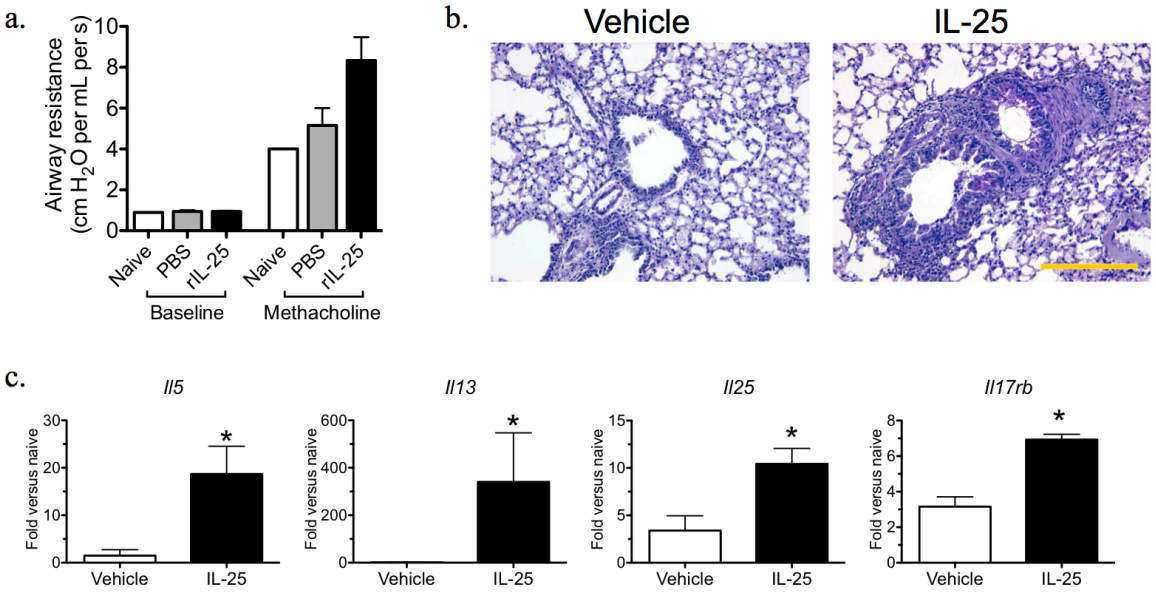


Figure 5.1: Intra-tracheal administration of IL-25 induces antigen-independent type 2 inflammation. 4get mice (n = 4 animals per group) were IT dosed with vehicle or 0.5 µg IL-25 for 4 days, and the inflammatory response was investigated 24 h post final IT. (**a**) Airway hyperreactivity was assessed by plethysmography, reported as baseline pressure versus peak pressure following methacholine injection. (n = 3 mice per group, *$P < 0.05$). (**b**) Lungs were harvested for histological analysis and paraffin sections were stained with PAS. Scale bar 200 µm. (**c**) mRNA analysis of lungs from 4get mice for *Il25*, *Il17rb*, and the type 2 cytokines *Il5* and *Il13*, analyzed by QPCR (*$P < 0.05$). All data are presented as the mean ± s.e.m. for each group, and are representative of 3 independent experiments.

IL-25–induced inflammation identifies Type 2 Myeloid (T2M) cells as a source of type 2 cytokines

The analysis of cell–specific GFP/IL-4 expression in 4get animals suggests that $CD11b^{+}$ $Gr\text{-}1^{mid}$ $IL\text{-}17RB^{+}$ myeloid (T2M) cells are the primary IL-25 responsive population in the lung (**Fig. 5.2**). $CD4^{+}$ T cells, a source of IL-4 during the antigen–specific inflammatory response, do not respond to IL-25 without prior activation. Likewise, while innate IL-25 responsive lymphoid populations have been identified in the gut using 4get reporter mice[22], these populations do not increase in number or demonstrate an induction of GFP/IL-4 expression following pulmonary IL-25 instillation. The myeloid population exhibited a dramatic increase of GFP/IL-4 in response to IL-25 (**Fig. 5.2a**), with approximately 80% of GFP/$IL\text{-}4^{+}$ cells being $CD11b^{+}$. Of these myeloid cells, the $CD11b^{+}$ $Gr\text{-}1^{mid}$ $IL\text{-}17RB^{+}$ subset, termed T2M cells to describe their propensity for type 2 cytokine production, demonstrated particularly dramatic enrichment for GFP/IL-4 expression. While IL-25 treated mice showed no increase numbers of other GFP/$IL\text{-}4^{+}$ populations, there was a significant increase in $CD11b^{+}$ $Gr\text{-}1^{mid}$ infiltrates (**Fig. 5.2b**). **Figure 5.2c** illustrates that among the $CD11b^{+}$ $Gr\text{-}1^{mid}$ population, all GFP^{+} cells were also $IL\text{-}17RB^{+}$, indicating that IL-25 acts on IL-25 responsive myeloid cells in part by activating transcription at the IL-4 promoter. Flow cytometric analysis further confirmed that the predominant pulmonary source of IL-13 following IL-25 administration were also T2M cells (**Fig. 5.2d**). Innate lymphoid Lin^{-} $c\text{-}kit^{+}$ $Sca\text{-}1^{+}$ $IL\text{-}17RB^{+}$ cells, a source of IL-25-induced IL-13 in the gut during helminth infection, were not altered by pulmonary IL-25 administration (**Fig. 5.2d**).

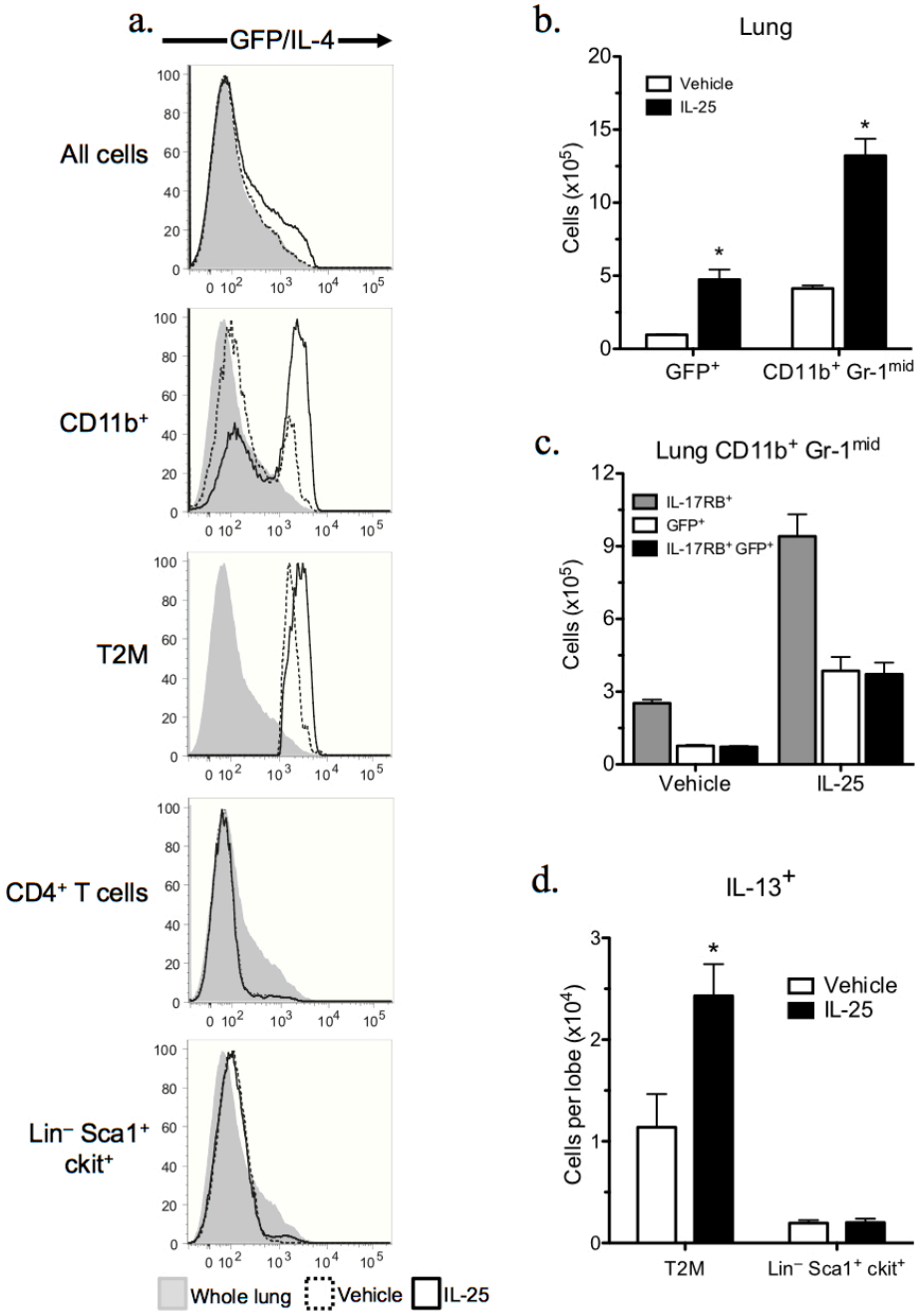


Figure 5.2: T2M cells represent the primary source of type 2 cytokines following pulmonary IL-25 administration. 4get mice (n = 4 animals per group) were IT dosed with vehicle or 0.5 μg IL-25 for 4 days, and the inflammatory response was investigated 24 h post final IT. (**a**) Histograms of lung tissue from 4get mice treated with vehicle or IL-25, gated on total lung, CD11b$^+$, T2M, CD4$^+$ Lymphocytes, and Lin$^-$ Sca$^+$ c-kit$^+$ cells respectively. (**b**) GFP$^+$ and CD11b$^+$ Gr-1^{mid} populations in the lung were assessed by flow cytometry, (*$P < 0.026$). (**c**) Pulmonary IL-17RB$^+$ CD11b$^+$ GFP/IL-4$^+$ cell numbers following IL-25 administration. Data are representative of two independent experiments. (**d**) Pulmonary IL-13$^+$ populations following IL-25 treatment (n = 5 animals per group, *$P = 0.038$).

To further verify that myeloid cells were producing type 2 cytokines in response to IL-25 administration, we isolated T2M cells from the lungs of IL-25 treated mice (see **Fig. 2.3**) and assessed type 2 transcripts in this population. T2M cells exposed to IL-25 *in vivo* exhibited dramatic increases in *Il4* and *Il13* transcripts, whereas *Il5* was derived from a CD11b^{-} cell population that remains ill-defined in our studies (**Fig. 5.3a-c**). In addition to the pulmonary effects of intra–tracheal IL-25 administration, T2M cells were also identified in the bone marrow of 4get mice following IL-25 treatments (**Fig. 5.4**), as well as in spleen and peripheral blood. Thus, IL-25 had both local and systemic effects linked to development of T2M cells.

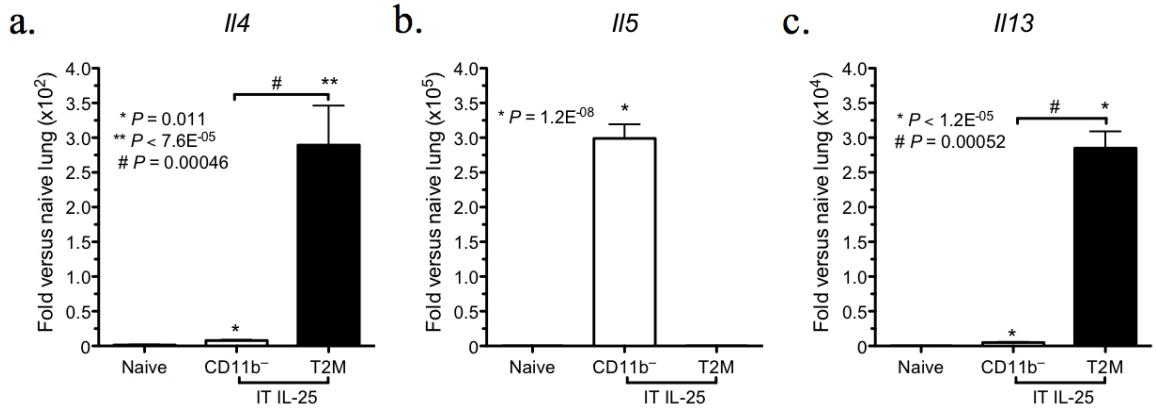


Figure 5.3: IL-25 induces T2M expression of *Il4* and *Il13* but not *Il5*. (**a–c**) QPCR analysis of *Il4*, *Il5*, and *Il13* transcripts in T2M cells. Cells were isolated from C57BL/6J mice dosed with 0.5 µg IL-25 for 4 days (n = 5 animals per group), and plated in triplicate. T2M cells were isolated using MACS magnetic bead enrichment followed by FACS. mRNA was isolated from naive C57BL/6J mice, CD11b depleted lung from IL-25 treated mice, and T2M cells isolated from IL-25 treated mice. All data are presented as mean ± s.e.m.

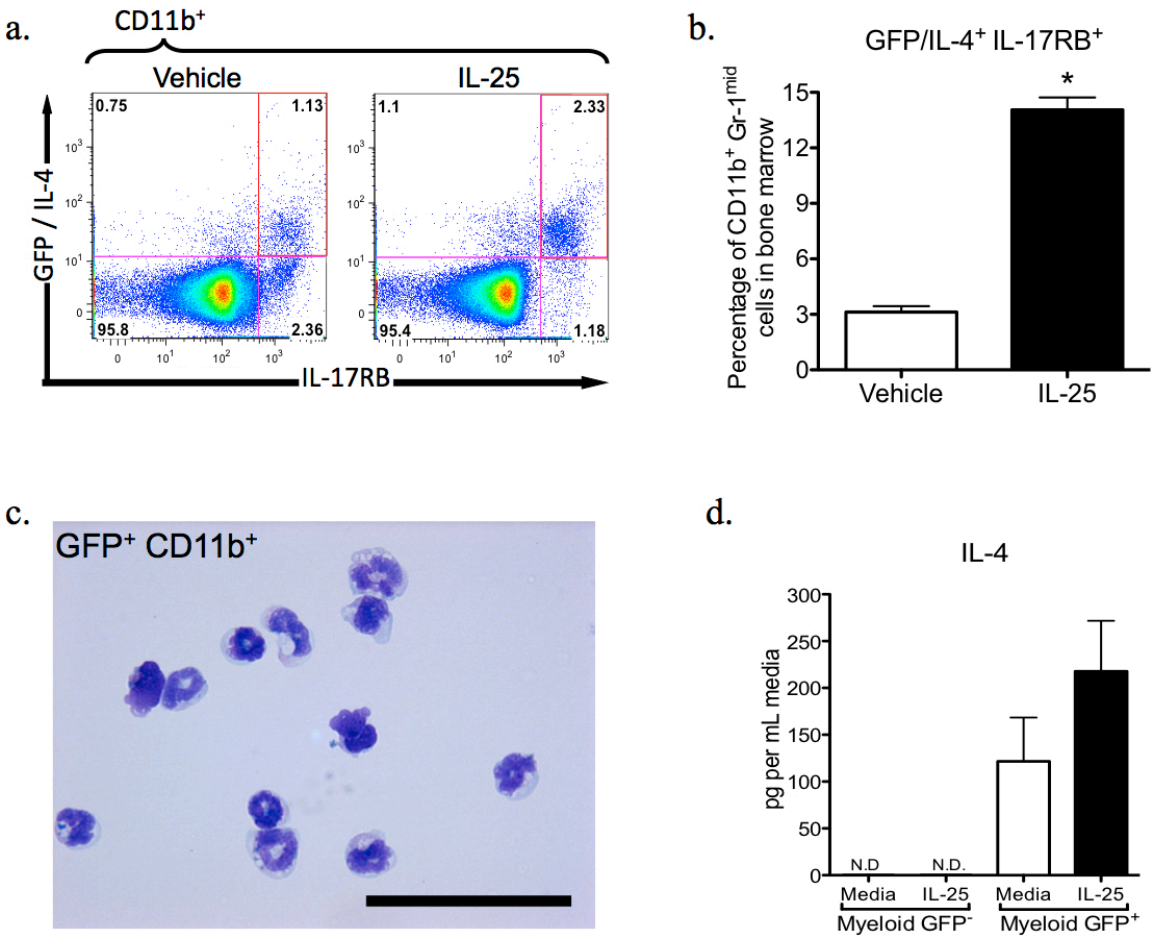


Figure 5.4: Pulmonary IL-25 administration induces IL-4 expression in myeloid bone marrow cells. 4get mice (n = 4 mice per group) were IT dosed with vehicle or 0.5 µg IL-25 for 4 days. The bone marrow response to pulmonary IL-25 administration was assessed. (**a**) Pulmonary IL-25 administration induces a type 2 inflammatory response in bone marrow CD11b$^+$ IL-17RB$^+$ cells. Representative flow plot of CD11b$^+$ bone marrow from 4get mice treated with vehicle or IL-25. (**b**) The percentage of CD11b$^+$ Gr-1$^+$ IL-4/GFP$^+$ IL-17RB$^+$ bone marrow cells is increased by pulmonary IL-25 administration. Bars represent the mean ± s.e.m. for each group of mice, *P = 0.0008). Data are representative of two independent experiments. (**c**) GFP$^+$ CD11b$^+$ CD4$^-$ bone marrow cells were isolated by FACS and H+E stained. IL-4/GFP$^+$ myeloid cells from IL-25 treated 4get mice have similar morphology to T2M cells isolated from lungs of allergic mice. Scale bar 50 µm. (**d**) IL-4/GFP$^+$ myeloid cells produce IL-4. Whole bone marrow from IL-25 treated 4get mice (n = 4 mice per group) was pooled, isolated by FACS for GFP$^+$ CD11b$^+$ CD4$^-$ cells, and cultured *in vitro* with 10 ng per mL IL-25 for 48 h. Supernatants were analyzed by bioplex. Bars represent the mean ± s.e.m. for CD11b$^+$ GFP$^-$ and CD11b$^+$ GFP$^+$ cells, plated in triplicate.

T2M cells exhibit a distinct pattern of cell surface antigen expression

Our data thus far suggest that T2M cells represent a distinct, IL-25 responsive granulocytic subset. To confirm these findings, we assessed T2M cells for expression of cell surface antigens associated with cell-type specific distribution, as well as immunologic functions including activation, costimulation, antigen presentation, migration, and adhesion. The resulting data confirm that pulmonary T2M cells are defined by a distinct combination of cell surface antigens, and can be characterized as $CD11b^{+}$ Gr-1(Ly6C/G)^{mid}, IL-17RB^{+}, FcγR (CD16/CD32)$^{+}$, F4/80^{mid}, $CD80^{+}$ (**Fig. 5.5**). Similar expression patterns were observed in T2M cells derived from other tissues, including spleen, bone marrow, and peripheral blood (**data not shown**).

Because T2M cells have a granulocytic morphology, we evaluated the expression of surface antigens considered to be specific for each of the three granulocytic populations: neutrophils, eosinophils, and basophils (**Fig. 5.5**). T2M cells did not express the neutrophil–specific receptor CXCR2, nor did they express basophil markers including CD49b or FcεR1a. T2M's did not express the eosinophil marker CCR3, however they did exhibit low expression of IL-5rα (although it is important to note that the eosinophils present in these analyses demonstrated approximately three logs more intense expression for this marker, at ~10^5 versus ~10^2). FACS isolated T2Ms did not produce detectable transcripts of neutrophil or eosinophil–associated enzymes, including myeloperoxidase, major basic protein, or eosinophil peroxidase (**data not shown**), further supporting them as a separate granulocytic population. Finally, T2M cells did not express antigens associated with innate lymphoid subsets, including c-kit, Sca-1, and the IL-33 receptor ST2, indicating that they are a separate innate type 2 cytokine producing population.

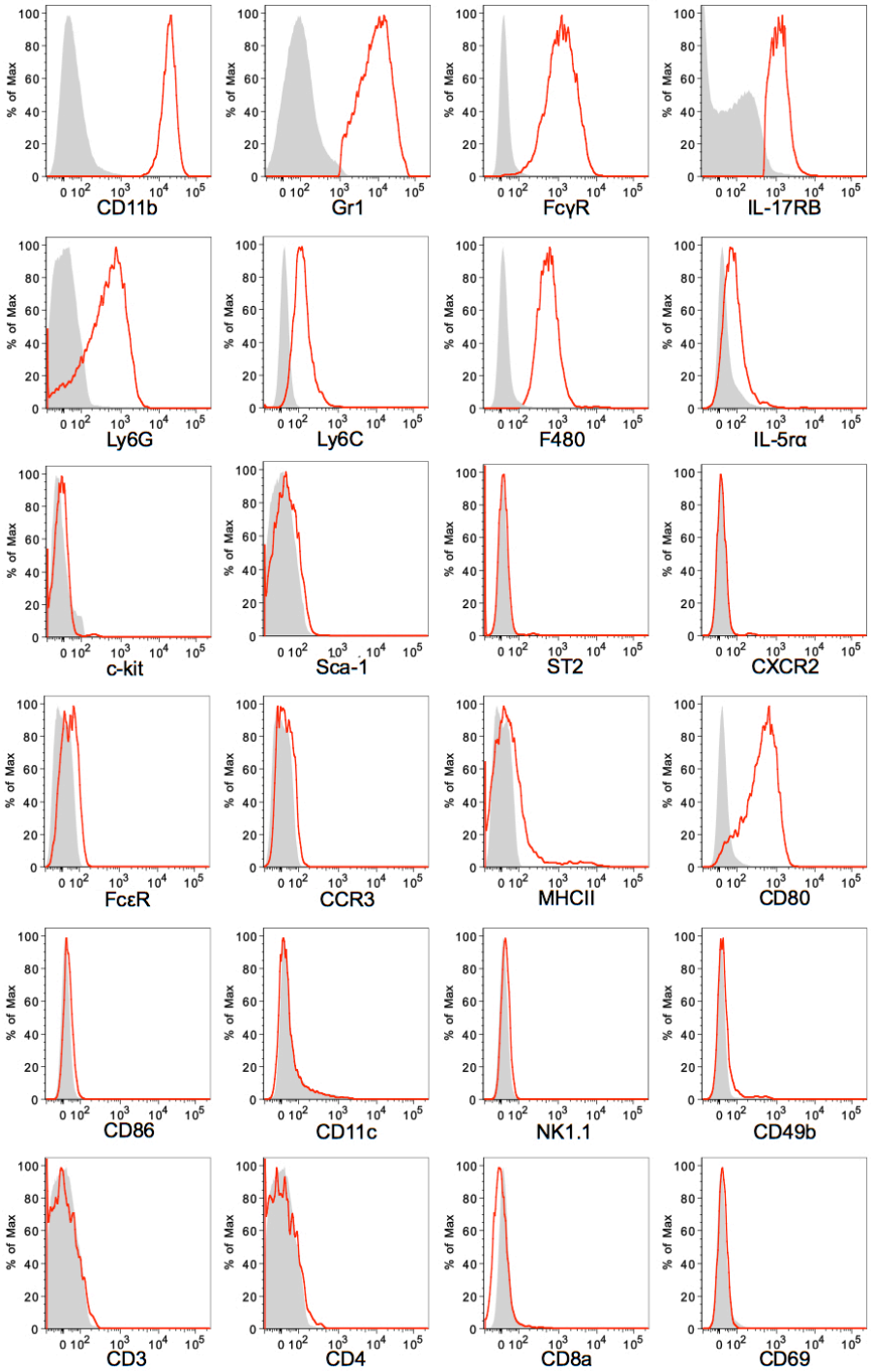


Figure 5.5: Patterns of surface antigen expression define T2M cells as a distinct granulocytic subset. Characterization of pulmonary T2M cells by cell surface marker expression. 4get mice were challenged with intra-tracheal IL-25 to induce recruitment of T2M cells to the lung. T2M cells were identified by gating on $CD11b^{+}$ $Gr1^{mid}$ $IL\text{-}17RB^{+}$ $GFP/IL\text{-}4^{+}$ cells, and expression of various surface markers was then assessed. Gray shaded area: isotype, red line; T2M cells. Data are representative of 2 independent experiments.

Microarray analysis identifies T2M cells as a distinct granulocytic subset

In an effort to fully characterize T2M cells, and to assess whether they are truly a distinct granulocytic subset, we employed our antigen–independent model of IL-25–induced type 2 inflammation to induce T2M cells to the lung. We isolated four myeloid populations from whole lung tissue; T2M cells, eosinophils, neutrophils, and monocytes (see **Fig. 2.4**). Microarray analysis of total RNA from T2M cells compared to other myeloid populations (**Fig. 5.6a-c**) confirm that T2M cells represent a distinct granulocytic subset with a gene expression profile most closely related to eosinophils. Based on the large amount of variation observed between T2M cells and other assessed populations, we classified differences in gene expression as a three–fold or greater change in expression profile, with the added constraint the expression level of at least one cell subset was at least 2^6 or more (these parameters were suggested by the University of Michigan Microarray Core, and are more strict than their usual two-fold change cutoff).

Based on these criteria, the T2M population had differential expression at 2299 loci when compared to eosinophils and 2057 loci when compared to neutrophils (**Fig. 5.6b**). As one might predict given their differing morphologies and cell surface receptor expression patterns, the T2M population was most different from macrophages, with 4324 differentially expressed loci. Hierarchical analysis of T2M cells versus other examined cell types indicates that they are most closely related to, but still readily discernable from, eosinophils in terms of their expression pattern (**Fig. 5.6a**). In addition, principal components analysis of each array sample indicated that T2M cells are a discrete subset, which share the highest degree of relatedness (in this case expressed along the x-axis) to eosinophils (**Fig. 5.6c**).

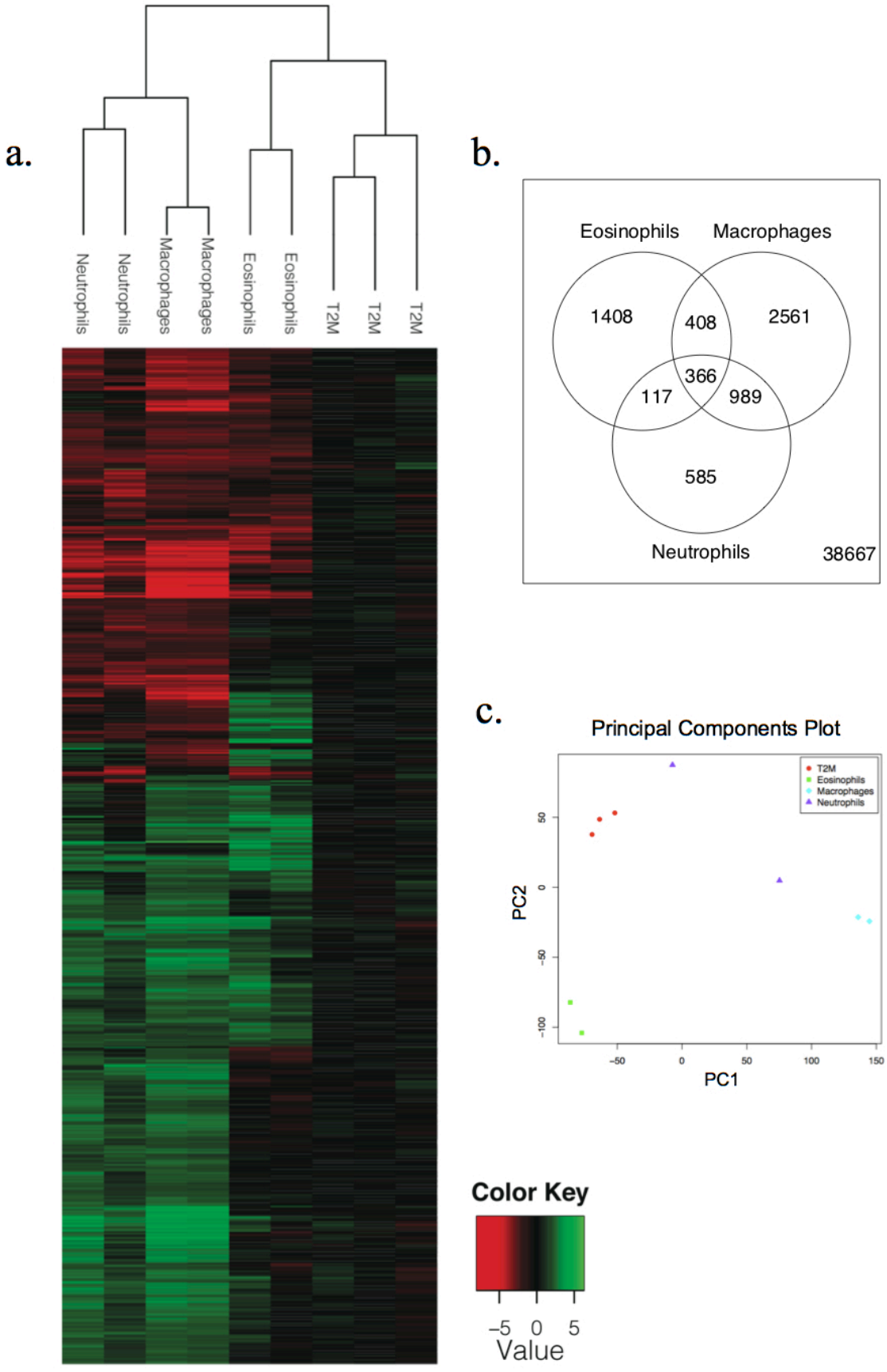

a.
Neutrophils
Neutrophils
Macrophages
Macrophages
Eosinophils
Eosinophils
T2M
T2M
T2M
b.
Eosinophils
Macrophages
1408
408
2561
366
117
989
585
Neutrophils
38667
c.
Principal Components Plot
T2M
Eosinophils
Macrophages
Neutrophils
PC2
PC1
Color Key
−5 0 5
Value

Figure 5.6: Microarray expression profiles define T2M cells as a distinct granulocytic subset. (**a**) Hierarchical clustering analysis and heat map generated from microarray analysis of pulmonary T2M cells compared to eosinophils, neutrophils, and macrophages. Colors illustrate fold changes among 1,880 probes for which T2M cells exhibited a minimum 3 fold difference in expression from 2 or more cell types, and an average expression value of at least 2^6, normalized to average T2M expression levels. (**b**) Venn diagram illustrating differences in locus expression between T2M cells and other myeloid populations. 366 probes were differentially expressed between T2M cells and all cell types analyzed, 1,880 probes differed between T2M cells and at least 2 of the comparison populations. (**c**) Principal components plot illustrating relative degrees of relatedness between populations analyzed by microarray demonstrates that the subset most closely associated with T2M cells are eosinophils. Red dots: T2M cells, light blue: eosinophils, green: macrophages, purple: neutrophils.

T2M cells in the lung are non-proliferative

The identification of a novel granulocytic subset raises many questions related to how T2M cells may be incorporated into the hierarchy of myeloid subsets or progenitor populations. In an effort to determine whether the T2M cells recruited to the lung in the context of high IL-25 are actively proliferating, and therefore may represent a phenotypically plastic population capable of differentiation or expansion into a separate granulocytic subset, 4get mice were treated (as in previous experiments) with intra–tracheal IL-25. On the final day of IL-25 administration, recipient mice were given an intra–peritoneal injection of EDU to track proliferating cells. EDU is a thymidine analogue that is incorporated into replicating DNA, therefore EDU offers a means to identify proliferating cells via the detection of newly synthesized DNA[262]. Flow cytometric analysis of whole lung cells 18 hours after final IL-25 administration indicated that approximately 99% of T2M cells in the lung had not undergone proliferation in the time since EDU administration (**Figure 5.7**). Therefore we conclude that high levels of IL-25 in an end organ site do not induce T2M cell expansion at that site, and T2M cells do not proliferate in the lung.

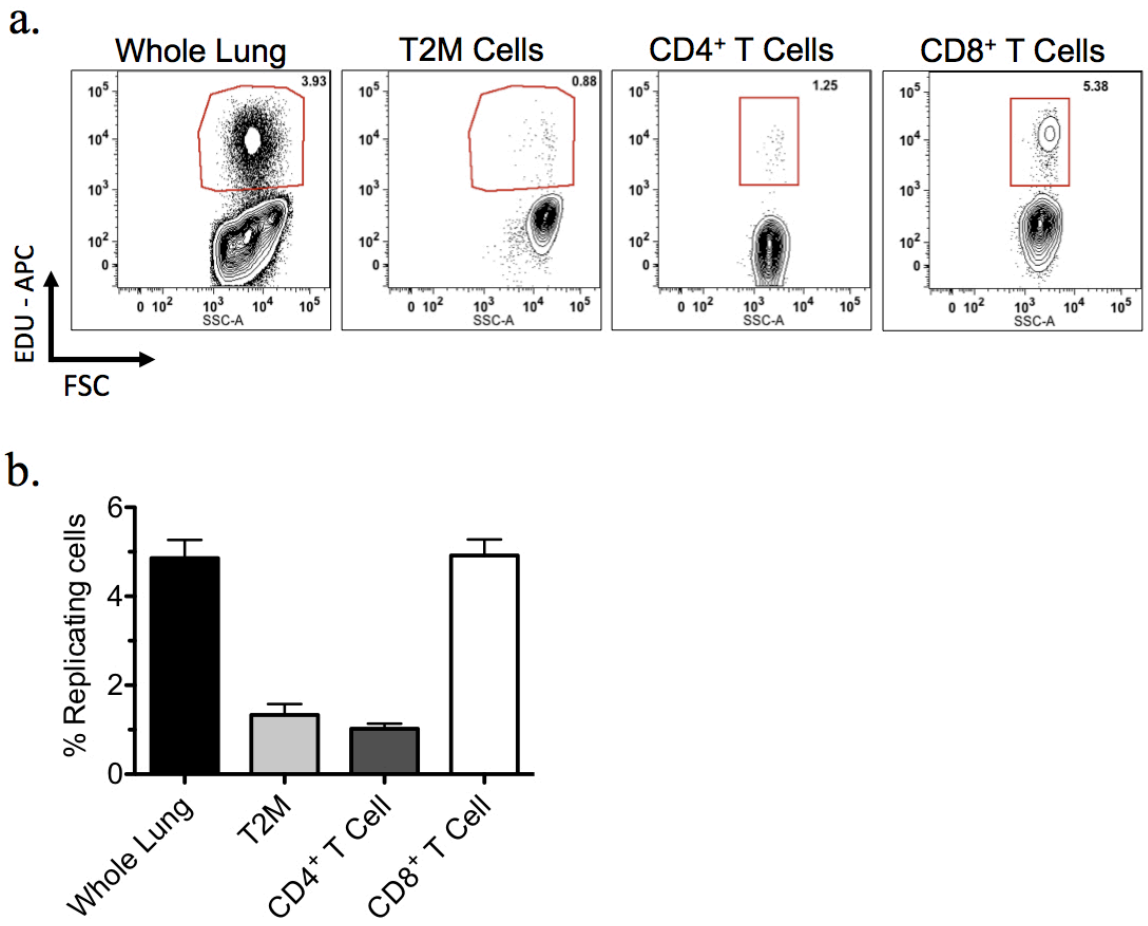


Figure 5.7: Pulmonary T2M cells are non–proliferative. IL-25 treated 4get mice (*n* = 6) were injected IP with EDU following their fourth and final IL-25 challenge, and proliferation among lung cells was assessed 18 h later (a) Representative histograms of whole lung, T2M, $CD4^+$ and $CD8^+$ T cells following EDU administration. Gates indicate the percentage of each population in which cells have replicated DNA, indicating active proliferation. (**b**) Percentage of proliferating cells in the lung following IL-25 administration. Bars represent mean ± s.e.m. for each population.

Discussion

This study is the first to identify the T2M cell, an IL-25 responsive IL-4 and IL-13 producing granulocyte. This population, first observed in the context of chronic allergic lung disease, is the primary IL-17RB$^+$ population recruited during conditions characterized by increased levels of IL-25. While these cells have been studied most extensively in the lung, we have also identified them in all tissues assessed to date,

including spleen, bone marrow, and peripheral blood. The systemic circulation of bone marrow derived T2M cells implies that, in addition to allergic asthma, several other diseases associated with elevated IL-25 levels and type 2 inflammation may involve an influx of T2M cells. These include diseases of epithelial tissues such as atopic dermatitis[173,220], hypereosinophilic syndromes such as Eosinophilic Esophagitis[263], and autoimmune disorders such as Churg-Strauss syndrome[14,264].

The data provide evidence for the importance of T2M cells in promoting pathology resulting from IL-25 mediated type 2 inflammation. However, they do not address the sufficiency of these cells to promote inflammatory responses, nor do they address how T2M cells may be involved in chronic allergic asthma. T2Ms are a unique population, but several questions remain regarding their role in the context of innate immunity. Based on the data presented herein, it could be argued that these cells are defined as much by what they lack as by their phenotypic and functional characteristics. The absence of surface antigens related to other myeloid subsets, specifically other granulocyte populations (eosinophils, neutrophils, and basophils), coupled with the lack of detectable cell-specific transcripts by QPCR provides evidence that T2M cells are unlike other defined myeloid subsets.

Likewise, the production of IL-4 and IL-13, but not IL-5, differentiates T2Ms functional from innate lymphoid populations, which are known to produce IL-5 and IL-13, but not IL-4[20-23]. A recent study has identified an innate lymphocytic population in the gut, lung, and nasal polyps of humans, further supporting their potential role in human disease[237]. In our studies, this population was present at low numbers in the lung, and did not increase following IL-25 administration. Previous reports have identified

other non–lymphoid populations as sources of type 2 cytokines that can contribute to the allergic environment, including basophils, mast cells, eosinophils, and macrophages[76,77,227,229,257,258,260,265-268]. We did not detect significant IL-17RB expression in any of these populations in the lung. Thus, it appears there are both Lineage$^+$ and Lineage$^-$ IL-25 responsive cells whose recruitment may depend upon the type of mucosal surface (gut versus lung), both with the capacity to produce type 2 cytokines in an antigen–independent manner.

T2M morphology also offers clues as to their appropriate classification among the other myeloid subsets. T2M cells resemble an immature granulocyte, and appear at first glance to be similar to banded neutrophils (also known as metamyelocytes). It is their inability to be classified with conventional labels that marks T2M cells as a unique population. Microarray analysis adds further weight to the T2M as being different from other myeloid populations. It links T2M most closely to eosinophils, and provides a great deal of information that could offer additional insight into T2M biology. The chemokine receptors important for T2M functions have not yet been determined, and further investigation of these data may yield insight as to chemokine receptors important for T2M cell migration and recruitment to distant inflammatory sites. Interestingly, microarray data indicate that the T2M population is enriched in expression of genes associated with all three comparison populations, as well as transcripts of some proteins (CCR3 and IL-5rα, for example), that were not expressed at the cell surface. This may relate to the maturity of the T2M population, or to post–translational regulatory events that prevent these proteins from being expressed. As we have previously discussed, IL-5rα is expressed early in eosinophil progenitor development, prior to migration from the

bone marrow[144,255]. The potential significance of mRNA expression in the absence of cell surface expression, coupled with the overall similarities between T2M and eosinophilic subsets, is further discussed in Chapter 7.

It is unknown whether T2M cells may differentiate into one of the 3 major granulocytic populations, however our data indicate that these cells are not actively proliferating, suggesting that they are migrating directly from the bone marrow during conditions with elevated IL-25 and not expanding outside the bone marrow. The identification of T2Ms in bone marrow, and the finding that the population of bone marrow derived T2Ms rapidly increases upon pulmonary IL-25 administration, suggests the presence of important and as yet undetermined feedback loops capable of dynamic regulation of T2M granulopoiesis, including dramatic surges in T2M production upon receipt of the appropriate stimuli. The fact that T2M cells in the bone marrow can actively secrete IL-4 upon IL-25 stimulation implies that they are functionally differentiated prior to their exit from the bone marrow niche. This may represent a critical aspect of T2M function; the ability to differentiate quickly, migrate to an area with increased IL-25, and secrete type 2 cytokines upon arrival and IL-25 stimulation. The pathologic relevance of T2M cells, including their potential relevance of human disease, is the topic of the next chapter.

CHAPTER VI

The Pathologic Relevance of T2M Cells: Steroid Resistance, Mucus Production, and the Identification of an IL-25 Responsive T2M Human Homologue

Abstract

This set of experiments investigated the potential pathologic significance of T2M cells. Given data indicating that T2Ms represent a distinct granulocytic population, as well as evidence linking the presence of T2Ms to asthmatic pathology, we hypothesized that T2M cells may represent a previously unrecognized, pathologically relevant IL-25–responsive population. The results of our investigation offer two pieces of data in support of this hypothesis. The first is that IL-25–induced type 2 inflammation is steroid-resistant, and T2M cells are insensitive to high dose dexamethasone administration. The second is that the adoptive transfer of T2M cells is sufficient to induce mucus hypersecretion and *Il-13* in otherwise IL-25–insensitive *Il17rb*$^{-/-}$ recipients. In a separate clinical study, an IL-17RB^{+} granulocytic population analogous to T2M cells was identified in human peripheral blood. This population produced IL-4 and IL-13, and was significantly increased in allergic asthmatics compared to non-asthmatic volunteers. These findings support T2M cells as a pathologically relevant granulocytic population

and suggest a link between the sequelae of asthma and a previously unrecognized steroid resistant population.

Introduction

In the past 5 years several groundbreaking discoveries have been made regarding the importance of type 2 cytokine producing innate immune cells and regulation of the inflammatory response in mucosal tissues. Initially these centered on NBNT type 2 cytokine producing populations[5,226,241]. More recently investigations have focused on the importance of innate lymphoid cells in the clearance of intestinal helminth infections[20-23]. The importance of innate lymphoid populations in mediating intestinal type 2 pathologies, when compared to with our findings that an entirely separate population of T2M cells are recruited in the context of IL-25–associated pulmonary pathologies such as allergic asthma, naturally led us to question to what extent T2M cells may contribute to the pathogenesis of asthma.

While a juxtaposition of T2M and innate lymphoid biology is not necessarily an equivalent comparison, reports have indicated that relatively minute numbers of type 2 cytokine producing innate lymphoid populations are sufficient to alter disease phenotypes[235]. For example, a recent study identified an estimated 1,500 lymphoid cells in the lung following influenza infection, and attributed significant disease modifying effects to this population[236]. Our initial characterization of IL-17RB^{+} myeloid subsets (defined as T2M cells in later experiments) indicated that between 50–70 thousand T2M cells are present in a single lobe during chronic allergen-induced airways disease (see **Figs 3.4a** and **3.9b**). FACS isolation of T2M cells from lungs of IL-25 treated mice

consistently yields between 250–300,000 cells per mouse at 75% efficiency (**data not shown**), implying that IL-25–mediated inflammation results recruits a population of ~400,000 pulmonary T2M cells. This represents a dramatic influx of T2M cells compared to the naive pulmonary environment, and this myeloid IL-17RB^{+} population overwhelmingly outnumbers innate lymphoid populations in these same tissues (see **Fig 3.2**). Given their ability to produce IL-4 and IL-13, as well as their predominance in lung tissues in the context of increased IL-25 expression, we hypothesized that T2M cells represent a significant and previously unrecognized component of the type 2 inflammatory response promoted by IL-25.

To test our hypothesis, we evaluated the potential importance of T2M cells through three independent experimental approaches. The first investigated T2M responses to corticosteroids, the front line anti–inflammatory drug in asthma exacerbations[75]. The second focused on the sufficiency of T2M cells to promote type 2 inflammation in the lung. Our third mode of investigation aimed to determine whether a population analogous to T2M cells is present in humans, specifically those individuals with allergic airways disease. The results of these experiments provide strong evidence that T2M cells represent a pathologically relevant component of IL-25–mediated disease with the potential to alter clinical manifestations of allergic asthma.

Results

IL-25-induced inflammation and T2M cell responses are steroid resistant

To examine potential clinical implications of type 2 cytokine production in the T2M population, we examined how steroid treatment affected IL-25–induced pulmonary

inflammation. Granulocytes, neutrophils in particular, are known to be resistant to the pro-apoptotic effects of corticosteroids[32-36,163]. Steroid–resistant neutrophilic infiltrates have been implicated as a major cause of inflammatory pathology in the context of Th17 inflammation, and have also been linked to the severe pathology associated with steroid–resistant asthma. The relevance of T2M cells in promoting type 2 inflammation and severe asthma pathology would be supported if these cells, like neutrophils, were also resistant to steroid administration.

To focus on T2M–mediated inflammatory responses, 4get animals were treated with IL-25 as in previous experiments, with or without dexamethasone. Histologic examination (**Fig. 6.1a**) and measurements of airway hyperreactivity (**Fig. 6.1b**) indicated that IL-25–induced responses were not significantly altered by dexamethasone. QPCR analyses demonstrated significant increases in type 2 cytokines, mucus genes, and *Il17rb* were also unaffected by dexamethasone administration (**Fig. 6.1c**). Flow cytometric analysis measured equivalent numbers of IL-25–induced GFP/IL-4^+ myeloid cells in animals treated with or without the glucocorticoid (**Fig. 6.1d,e**). As was shown in **Fig 5.2**, antigen independent IL-25 administration significantly increased pulmonary myeloid but not lymphoid populations, which are known to be steroid–sensitive. To verify that dexamethasone treatment was effective, we examined splenic cell subsets (**Table 6.1**). Dexamethasone significantly reduced total splenocytes, with a specific reduction in $CD4^+$ and $CD8^+$ T cells as well as eosinophils, but had no effect on splenic T2M cells. Overall these data present a striking finding that the IL-25–induced T2M cells are resistant to high dose glucocorticoid treatment.

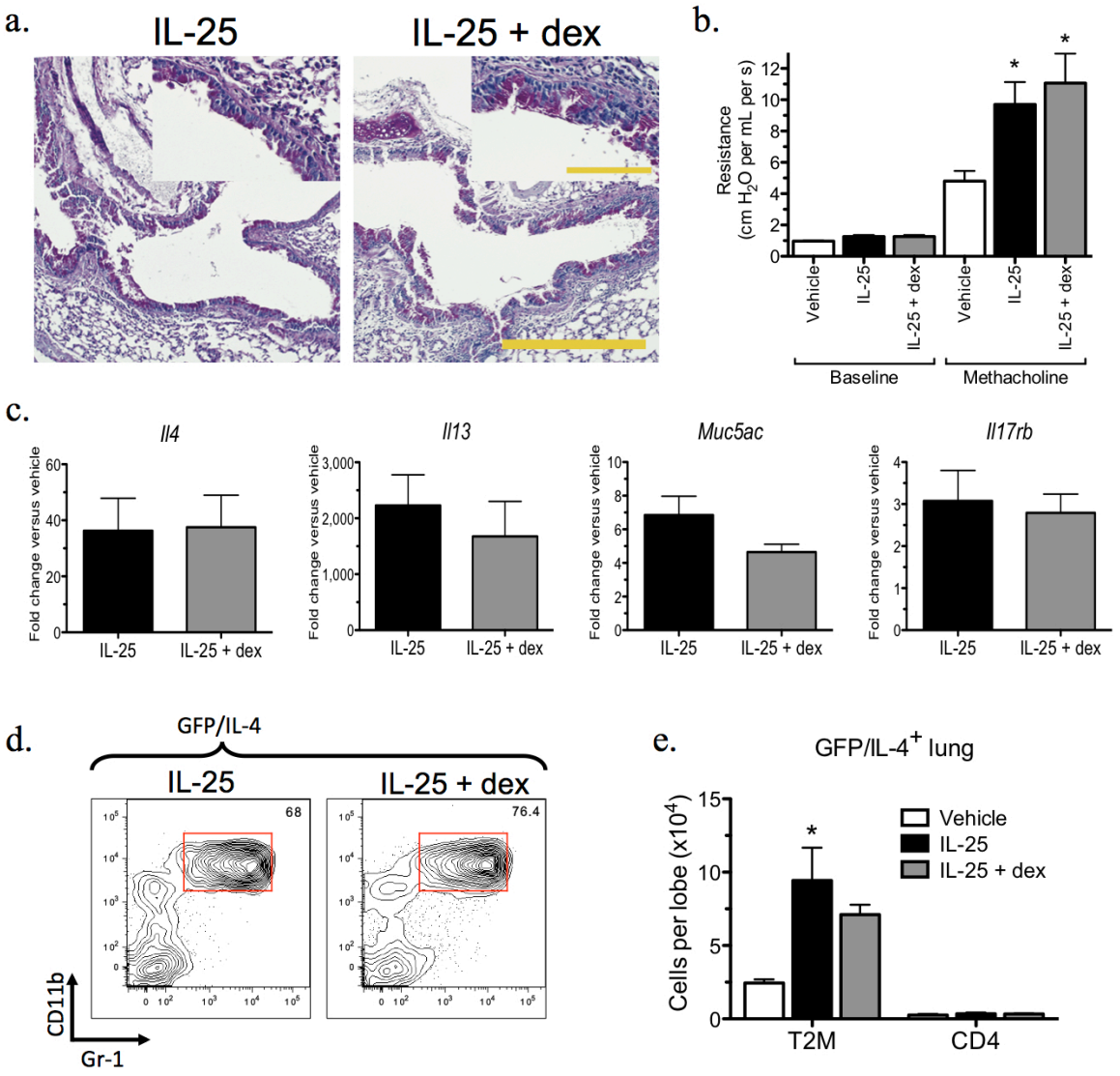


Figure 6.1: T2M cells are steroid resistant. (**a**) Representative PAS staining indicates IL-25-induced mucus production in 4get mice (n = 5 per group) is not altered by dexamethasone administration. Scale bar 400 μm; inset 100 μm. (**b**) Airway hyperreactivity (*$P < 0.01$ versus methacholine treated vehicle). (**c**) QPCR analysis of whole lung following dexamethasone treatment. (**d**) Representative flow plots of GFP/IL-4$^+$ CD11b$^+$ Gr-1^{mid} pulmonary populations. (**e**) Total numbers of pulmonary GFP/IL-4$^+$ cells. Bars represent the mean ± s.e.m. of 4 mice per group. *$P < 0.01$ versus vehicle alone. Data are representative of two independent experiments.

	Cell Numbers (Millions/Spleen)			*P* Value	
	Vehicle	**IL-25**	**IL-25 + Dex**	**Vehicle: IL-25**	**Dex + IL-25: IL-25**
Total	52.99 ± 5.37	52.19 ± 4.69	40.43 ± 3.71	0.825	0.003
T2M	**0.82 ± 0.10**	**2.43 ± 0.73**	**2.31 ± 0.64**	**0.013**	**0.803**
Eosinophils	6.64 ± 0.75	7.27 ± 1.84	5.00 ± 0.65	0.511	0.027
CD4 T Cells	7.05 ± .21	4.75 ± .64	3.62 ± .66	0.001	0.058
GFP$^+$ CD4$^+$ T Cells	**2.46 ± 0.52**	**2.35 ± 0.37**	**1.79 ± 0.14**	**0.782**	**0.023**
CD8$^+$ T Cells	4.51 ± 0.78	5.28 ± 1.03	2.15 ± 0.42	0.450	0.006

Table 6.1: Splenic T2M cells are increased following pulmonary IL-25 administration and are not altered by dexamethasone administration. Flow cytometric analysis of splenic populations illustrates the effect of dexamethasone (n = 4 mice per group). 4get mice were treated with vehicle, IL-25, or IL-25 and 3mg kg^{-1} dexamethasone. IL-25 administration significantly increased numbers of T2M cells but had no effect on other splenic populations. In contrast, treatment with dexamethasone significantly reduced total cell numbers, CD4$^+$ and CD8$^+$ T cells, and eosinophils, but had no effect on T2M cells.

In separate experiments, we utilized the allergen model previously described in Chapter 3 to investigate the effects of high dose dexamethasone administration on antigen–dependent inflammation. To inhibit IL-25–dependent inflammation in this system we also administered a polyclonal anti–IL-25 antibody (**Figure 6.2**). Given our findings that IL-25 mediated inflammation was dexamethasone resistant, we hypothesized that IL-25 itself could be promoting T2M cells' steroid–resistant phenotype. Therefore, the inhibition of IL-25 may sensitize this population to high dose dexamethasone and reduce pulmonary type 2 inflammation. As **Figure 6.2a** demonstrates, neither anti–IL-25 administration nor dexamethasone treatment altered *Il4* transcript expression in allergen–sensitized mice. However, the administration of anti–IL-25 in conjunction with dexamethasone resulted in a significant reduction in whole lung *Il4* transcripts. Given (as we have previously shown) that cytokine production in this model can be attributed to both T cell and T2M populations, these findings do not conclusively tie IL-25–dependent inflammation in this case to T2M cells. Despite the absence of definitive data linking T2M cell–derived *Il4* to changes in cytokine transcripts, the finding that anti-IL-25 administration significantly improved the anti-inflammatory effects of dexamethasone production suggests a role for IL-25 in steroid–resistant disease.

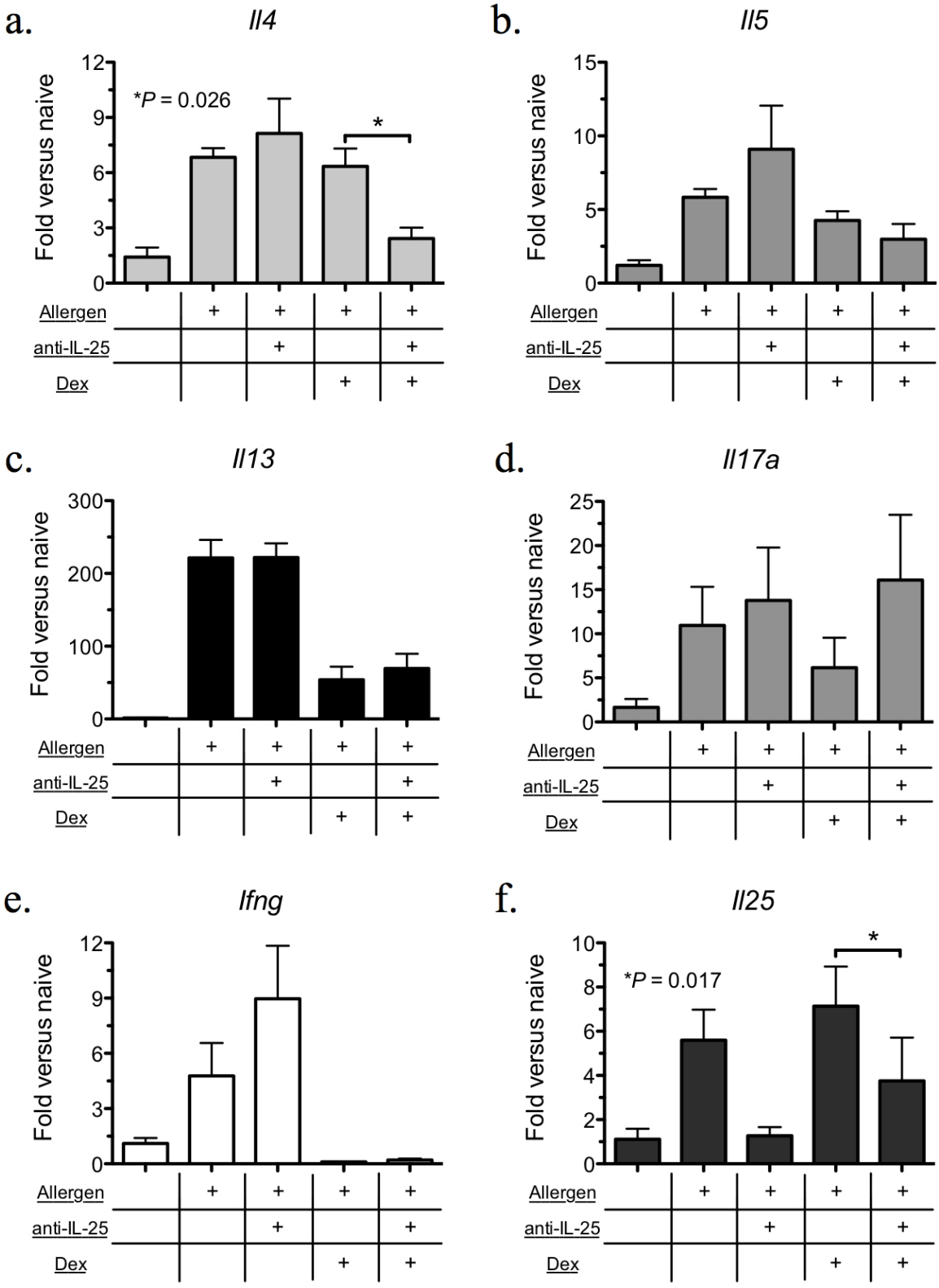

a.
Il4
Fold versus naive
*P = 0.026
*
b.
Il5
Fold versus naive
c.
Il13
Fold versus naive
d.
Il17a
Fold versus naive
e.
Ifng
Fold versus naive
f.
Il25
Fold versus naive
*P = 0.017
*
Allergen
anti-IL-25
Dex

Figure 6.2: Anti–IL-25 administration increases the anti-inflammatory effects of high dose dexamethasone treatment. 4get mice were sensitized to allergen as previously described, ± 0.5 mg anti-IL-25, ± 3 mg per kg dexamethasone. Antibody was administered in 3 doses, on days 28, 30, and 32 post initial sensitization. Dexamethasone was administered in 2 doses, prior to the intra-tracheal allergen challenge on days 30 and 32. (**a-f**) QPCR analysis of whole lung cytokine transcripts for *Il4*, *Il5*, *Il13*, *Il17a*, *Ifng*, and *Il25*, isolated 24 h post final allergen challenge. Bars represent the mean ± s.e.m. of each group ($n = 5$).

Transfer of T2M cells reconstitutes IL-25-induced airway responses

In order to determine if IL-17RB^{+} T2M cells were sufficient to induce pulmonary inflammation, T2M cells from IL-25 treated mice were isolated and instilled into the airways of *Il17rb*$^{-/-}$ mice with recombinant IL-25. The transfer of T2M cells from IL-25 treated WT mice into *Il17rb*$^{-/-}$ recipients, coupled with instillations of IL-25, induced mucus production and inflammation in otherwise IL-25 insensitive *Il17rb*$^{-/-}$ animals (**Fig. 6.3**). Recipients of T2M cells also demonstrated significant increases *Il13* transcripts, and the transfer of T2M cells with IL-25 further upregulated *Il13* expression (**Fig. 6.3b**) as well as the mucus–specific gene *Muc5ac* (**Fig. 6.3c**). In separate experiments, T2M transfer exacerbated the inflammatory response in WT recipients and increased *Muc5ac* transcripts to levels in *Il17rb*$^{-/-}$ recipients to levels comparable with those observed in IL-25 treated WT animals (**Fig. 6.4**). A third set of similar experiments focused on T2M transfer in the context of allergic inflammation. A similar pattern of increased mucus specific gene expression was observed following the adoptive transfer of T2M cells into allergen-sensitized recipients (**Fig. 6.5**). Therefore, in the context of increased pulmonary IL-25 levels, T2M cells are sufficient to induce pulmonary inflammation, IL-13, and mucus production; all hallmarks of allergic asthma.

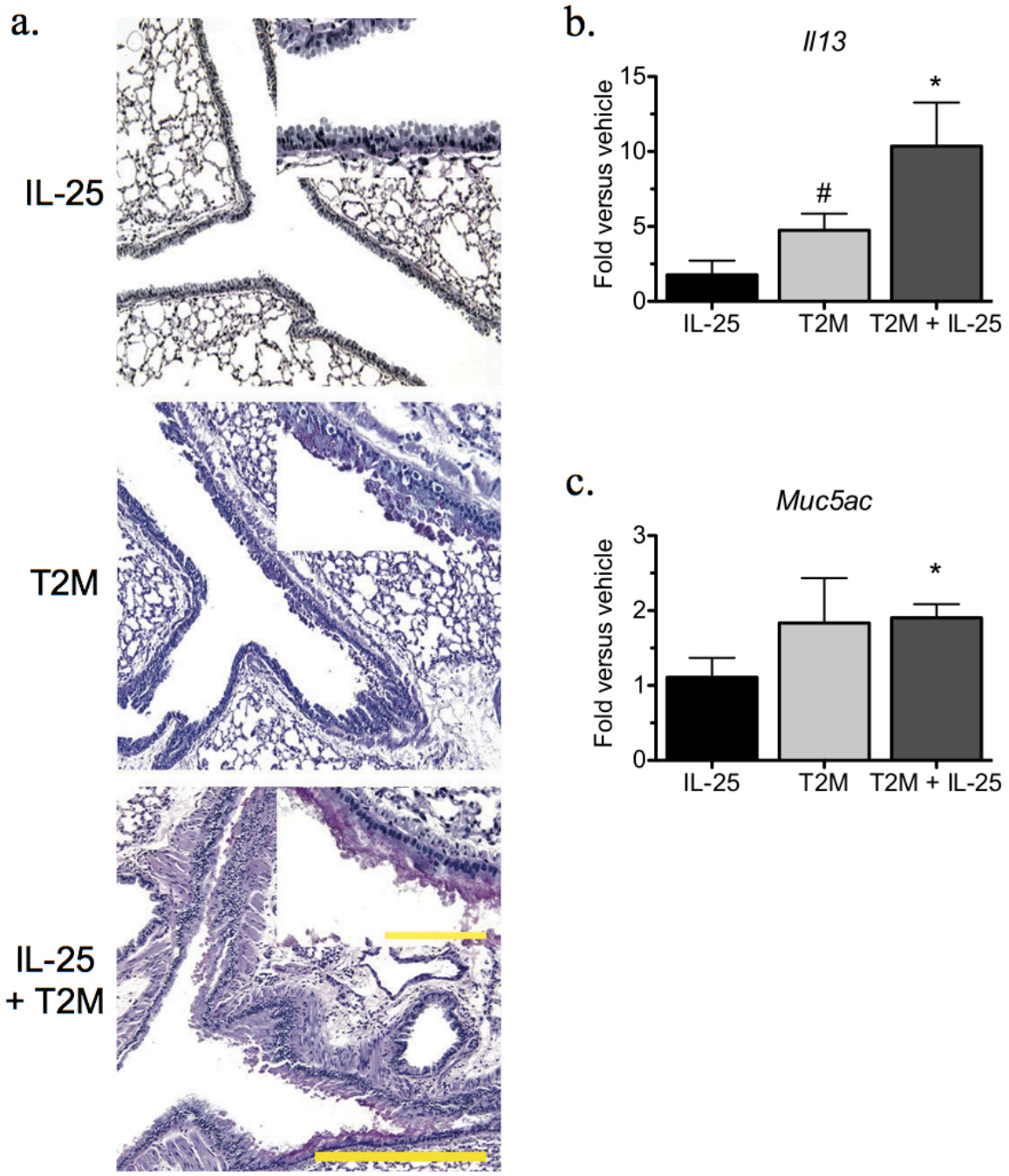


Figure 6.3: T2M cells are sufficient to induce airway pathology in *Il17rb*$^{-/-}$ mice. (**a**) Representative histology from recipients of T2M transfer stained with PAS, 24 h post final IL-25 administration. Scale bar 400 µm; inset 100 µm. T2M cells were isolated by MACS enrichment and FACS, and 2.0 x 10^5 cells were instilled into the airways of *Il17rb*$^{-/-}$ mice. Mice received 4 total treatments, consisting of 0.5 µg IL-25, T2M cells alone, or IL-25 + T2M cells. (**b**) QPCR expression of *Il13* following T2M transfer (*P = 0.017, #P = 0.042). (**c**) QPCR expression for the mucus specific gene *muc5ac* (*P < 0.026). Bars represent the mean ± s.e.m. of 4 mice per group. Results are representative of 3 independent experiments.

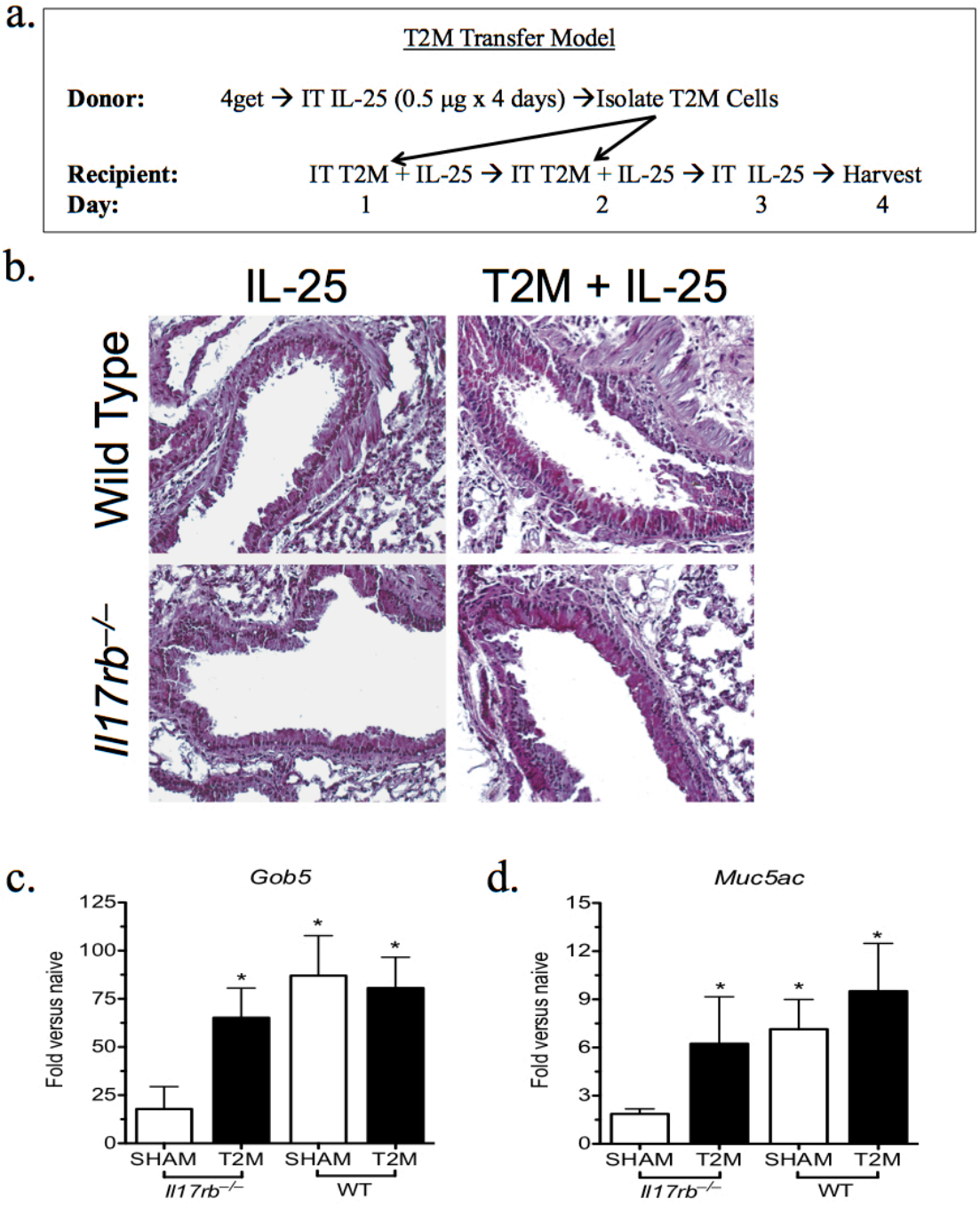
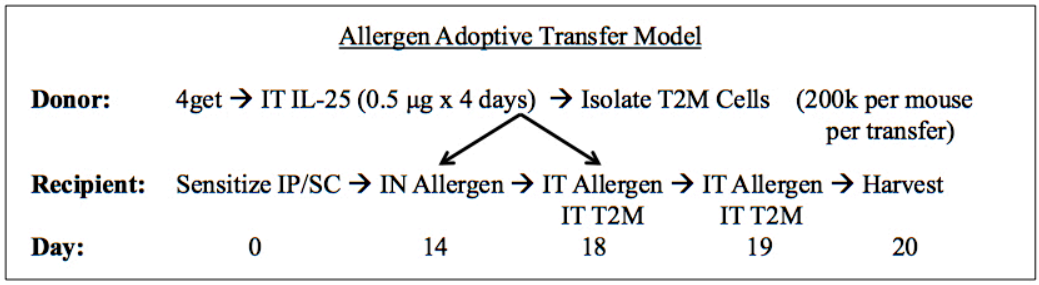
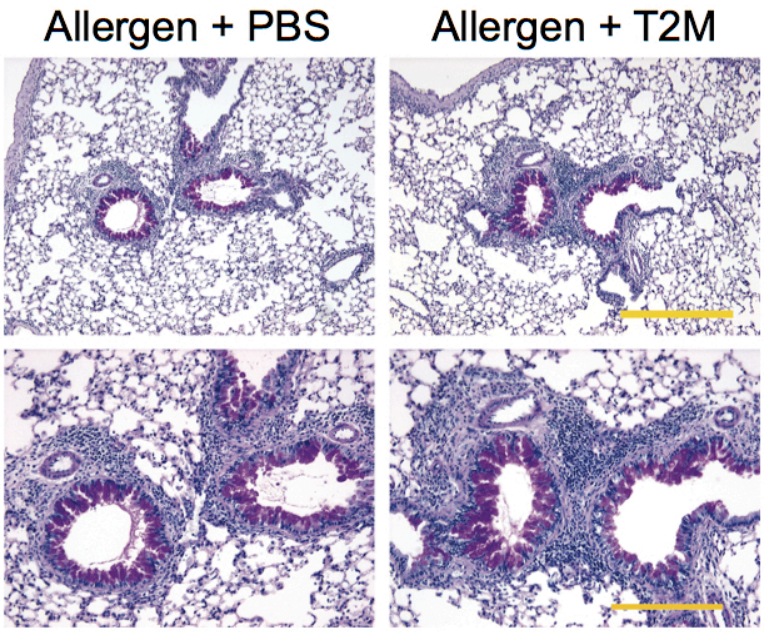


Figure 6.4: T2M adoptive transfer induces mucus production in IL-25 insensitive *Il17rb*$^{-/-}$ mice and exacerbates inflammation in WT recipients. FACS isolated T2M cells from the lungs of donor mice were treated with intra-tracheal IL-25. **(a)** Model used for T2M adoptive transfer ($3.5x10^4$ T2M cells per transfer) into either WT or *Il17rb*$^{-/-}$ recipients (n = 4 per group) . **(b)** Representative PAS stained histology from WT and *Il17rb*$^{-/-}$ mice. **(c)** mRNA analysis of lungs from recipient mice for the mucus gene *Gob5*, analyzed by QPCR. Data represent the mean ± s.e.m. for each group, (*$P < 0.05$). **(d)** mRNA analysis of lungs from recipient mice for the mucus gene *Muc5ac*. Data represent the mean ± s.e.m. for each group, (*$P < 0.05$).

a.



b.



c.

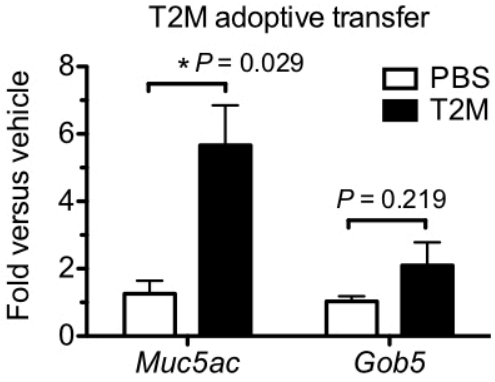


Figure 6.5: T2M adoptive transfer increases mucus production in allergen sensitized recipients. **(a)** Model used for T2M adoptive transfer into allergen-sensitized recipients. Recipient 4get mice received 3 pulmonary allergen challenges and 2 adoptive transfers. **(b)** Representative PAS stained histology from allergen sensitized SHAM transfer and adoptive transfer recipients (scale bar top row 400 µm, bottom row 200 µm). **(c)** mRNA analysis of lungs of allergen sensitized 4get mice show significant increases in *muc5ac* transcripts following T2M adoptive transfer. Data represent the mean ± s.e.m. for each group (n = 4 per group).

T2M–like cells are increased in asthmatics

The above data establish T2M cells as contributors to type 2 inflammation and pulmonary pathology in mice, however they do not shed light on whether these cells are involved, or even present, in the context of human disease. Our third experimental approach sought to address our lack of clinical data directly, through a clinical study of allergic asthmatics. To investigate the potential correlation between T2M cells and clinically relevant disease, we investigated whether a similar population of IL-17RB expression granulocytes was present in humans. Our initial experiments relied upon several suppositions, which could only be addressed through a direct assessment of clinical samples.

The first of these suppositions was that an IL-25 responsive, bone marrow derived myeloid population must at some point traffic to the lung via peripheral circulation, and therefore could be detectable (if present) in a peripheral blood sample. The fact that most asthmatic patients are on daily medications that effectively manage their symptoms and reduce the incidence of asthma attack somewhat complicated this point, as did an inability to know whether a T2M–like population, if even present in humans, would be released from the bone marrow at some detectable basal level or would only be produced upon a noxious stimulus or release of IL-25.

Our second assumption was that a population analogous to T2M cells would express cell surface markers similar to those found in mice. Human myeloid populations do not express markers analogous to either Gr-1 or F4/80, two of the antigens used to differentiate murine T2M cells from other myeloid subsets. To address the lack of resolution resulting from this interspecies variation, we chose to evaluate peripheral

blood samples via flow cytometry by staining for surface antigens that could characterize IL-17RB^{+} subsets via the following parameters:

1) Discriminate between lymphoid and myeloid IL-17RB^{+} populations.

2) Distinguish myeloid cells as either monocytic or granulocytic.

3) Identify type 2 cytokine production in IL-17RB^{+} subsets.

4) Provide confirmation that a T2M–like population, if present, has similar expression patterns of those cell surface antigens shared between humans and mice.

With these caveats in mind, volunteers were recruited from the University of Michigan Asthma Clinic, and the expression of IL-17RB in peripheral blood was compared to non-asthmatic volunteers. While IL-17RB expression was either low or undetectable in non–asthmatic individuals, flow cytometric analysis identified significantly increased numbers of granulocytic IL-17RB^{+} cells in asthmatic patients (**Fig. 6.6a, b**). The granulocytic IL-17RB^{+} population co-expressed CD11b, CD16, and the Ly6 family member/myeloid progenitor/neutrophil marker CD177$^{269\text{-}271}$ (**Fig. 6.6c**). In addition, most IL-17RB^{+} cells were CD33^{+272}, weakly expressed HLA-DR, and did not express the lymphocyte markers CD4 or CD8.

Based on these findings, we focused our analysis upon IL-17RB^{+} CD11b^{+} CD16^{+} CD177^{+} cells and identified a significantly increased percentage of these cells in asthmatics (**Fig. 6.7a**) that was further elevated following *in vitro* stimulation with IL-25 (**Fig. 6.7b**). This IL-17RB^{+} subset produced both IL-4 and IL-13, whereas IL-17RB^{-} cells with otherwise similar cell surface antigen expression did not. Thus, a population with similar cell surface receptor expression and phenotype as murine T2M cells can be

identified in peripheral blood, is significantly elevated in asthmatics, and represents a source of both IL-4 and IL-13.

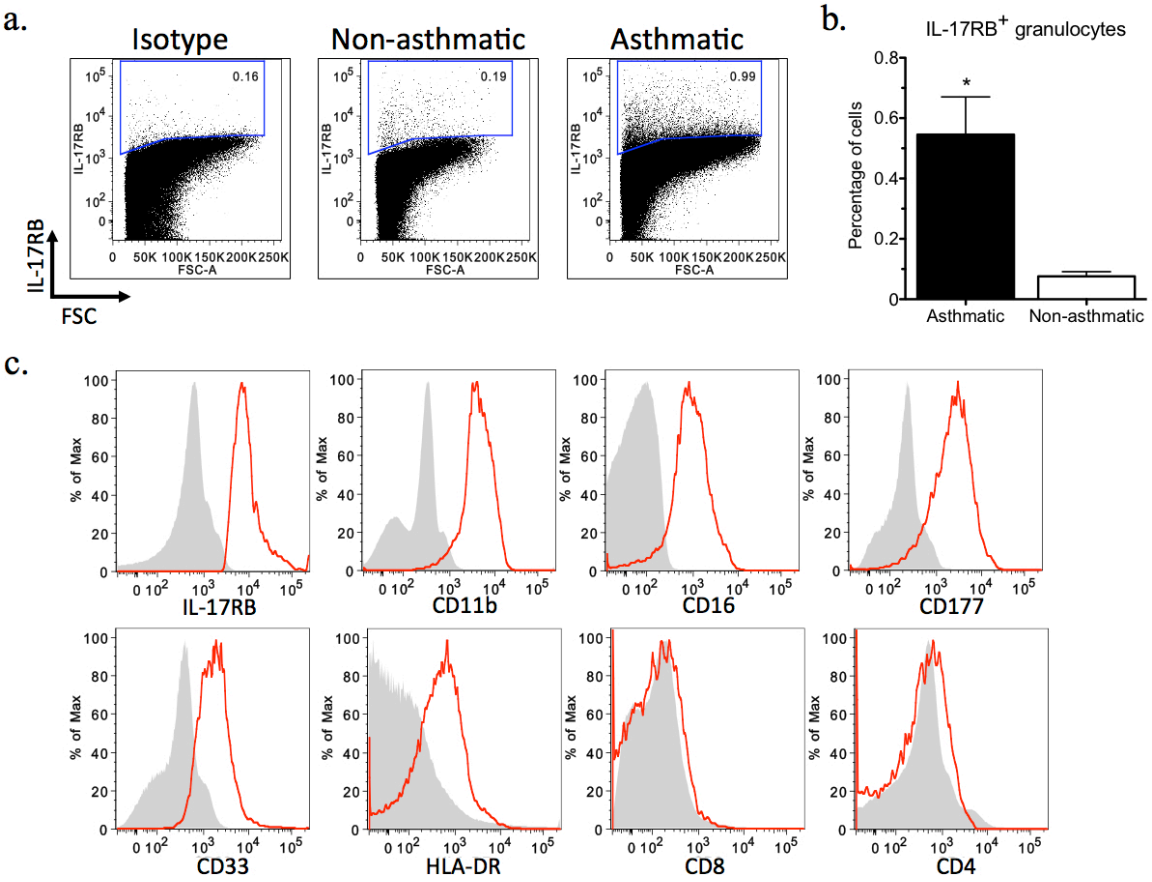


Figure 6.6: Human IL-17RB$^+$ granulocytes analogous to T2M cells are increased in asthmatics. Granulocytes were isolated from peripheral blood samples donated by asthmatic (n = 9) or non-asthmatic (n = 8) volunteers and analyzed by flow cytometry. (**a**) Representative dot plots of granulocytes stained for IL-17RB, normalized to 400k events. (**b**) Percent total IL-17RB$^+$ granulocytes isolated from peripheral blood of volunteer donors. Bars represent the mean ± s.e.m. for each group, (*P = 0.0031). (**c**) Representative histograms of IL-17RB$^+$ granulocytes from an asthmatic donor indicate the majority of IL-17RB$^+$ granulocytes are CD11b$^+$ CD16$^+$ CD177$^+$. Cells were gated on total IL-17RB$^+$ cells and assessed for surface marker expression.

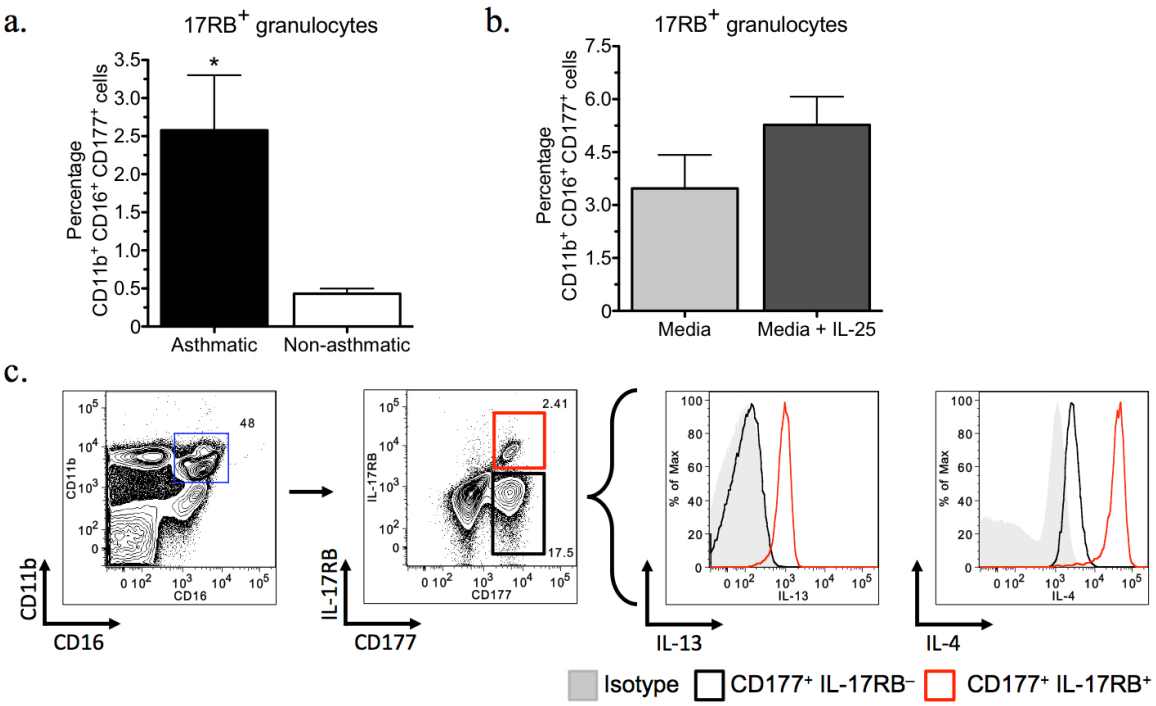


Figure 6.7: Human T2M-like cells are a source of IL-4 and IL-13. (**a**) Percent total $CD11b^+$ $CD16^+$ $CD177^+$ IL-17RB$^+$ granulocytes from volunteer donors. Bars represent the mean ± s.e.m. for each group, (*P = 0.023). (**b**) Percent total $CD11b^+$ $CD16^+$ $CD177^+$ IL-17RB$^+$ cells from whole blood of volunteer asthmatic donors, cultured *in vitro* for 2 h ± 50 ng mL^{-1} IL-25. (**c**) Representative intracellular cytokine staining for IL-4 and IL-13 from whole blood from an asthmatic volunteer, cultured for 2 h with RPMI 1640.

Discussion

While current therapies effectively manage the symptoms of a majority of asthmatic individuals[31], there is a significant subset of patients for whom corticosteroids, the most common medication used to suppress airway inflammation and the frequency of asthma attacks, remains ineffective[34-36,163]. The possible mechanisms for steroid–resistance are diverse and somewhat vague, in part because the exact cause is not fully understood[34]. Therefore the finding that T2M cells are steroid–resistant may be a great clinical relevance.

Our data indicate that, based on the rapid accumulation of myeloid cells in lungs following IL-25 administration, there is a pool of cytokine producing IL-25 responsive cells capable of amplifying a type 2 response. In allergic individuals, T2M cells may play an important role in the immediate response to an environmental allergen, priming the system for a type 2 response by producing cytokines prior to T lymphocyte activation. T2M cells could also contribute to chronic disease, as airway epithelial damage stimulates IL-25 secretion. This concept may be especially relevant in asthmatics, as our studies identified increased numbers of T2M–like cells in the peripheral blood of asthmatics that may be recruited upon an exacerbation and accumulate in the lungs. The induction of IL-25 in airways by pathogens, allergens, or other noxious stimuli may amplify the severity of the response by activating steroid resistant T2M cells, especially in patients with underlying pulmonary disease.

The fact that T2Ms are sufficient to induce mucus production, and that the adoptive transfer of T2Ms in the presence of IL-25 further exacerbates disease, provides strong evidence that these cells are pathologically relevant in environments with increased IL-25. The exact mechanism by which T2M cells mediate their pro–inflammatory effects has not been fully determined, however the significant upregulation of pulmonary *Il13* in conjunction with their transfer offers evidence that T2M related pathology is driven, at least in part, by the secretion of IL-4 and IL-13.

The identification of circulating T2M–like cells in asthmatics represents an extremely exciting finding, as it offers both evidence for the potential role of T2Ms in human disease as well as the potential for a relatively noninvasive measure of asthmatic pathology. Clearly further study is needed, but characterization of the distribution of

T2M–like cells among a clinical population could represent a biomarker with some predictive value related to the quality of asthma symptom and a given patient's likelihood of responding to specific therapies. If IL-25 responsive granulocytes are truly associated with steroid-resistant disease, then management of those patients with high levels of T2M–like cells may be modified in order to reflect their underlying disease pathology.

One could also image the utility of targeting such a population, perhaps by blocking the IL-25 receptor, in order to "sensitize" those IL-25 responsive cells and make them more prone to the effects of steroid. In fact our data indicating that IL-25 can act as a sensitizing agent by reducing type 2 cytokine in conjunction with dexamethasone administration offers intriguing evidence for the potential clinical utility of targeting IL-25 responsive populations in asthma. A complete understanding of the development and function of T2M cells will require further investigation, however our findings identify them as an intriguing and pathologically relevant component in the pathogenesis of allergic asthma.

CHAPTER VII

Conclusions and Discussion

Despite medicine's long pursuit of effective treatment for asthma, to date there are no prophylactic or curative therapies available for what is generally a manageable but lifelong disease. Many investigators have offered novel etiologies to explain the variety of intrinsic and extrinsic factors associated with its pathogenesis, as well is its variable clinical presentations. These hypotheses have passed in and out of favor over time, as have their associated treatments. Following the advent of psychoanalysis, for example, asthma was classified as one of the "holy seven" psychosomatic illnesses and thought by some physicians to reflect more about a patient's emotional labiality than any underlying inflammatory process[273]. For those practitioners who focused on asthma's inflammatory etiology, therapies of the 20th century included "asthma cigarettes" (often laced with belladonna alkaloids, parasympathetic antagonists that inhibit bronchoconstriction), ephedra (later replaced in favor of epinephrine), and in the 1980s by inhaled β2 adrenergic agonists such as albuterol[274].

The effectiveness of glucocorticoids in the treatment of asthma was first recognized in the 1950's[275], and marked a turning point in our understanding of the disease. Subsequently, the "new" conventional wisdom was to view asthma as an

inflammatory condition, best treated by quenching the inflammatory response. While this resulted in positive outcomes for many patients, after more than 50 years of use, medical practice has collectively come to appreciate the limitations of long-term steroid use, as well as the costs of indiscriminate immunosuppression. Furthermore, while long-term corticosteroids reduce the incidence of asthma exacerbations[276], they do not improve lung function or modify underlying disease[277]. The identification of steroid-resistant asthmatics further complicated the paradigm of asthma as a generalized inflammatory response, as certain individuals have severe disease that cannot be effectively managed with these drugs[33-36,163].

Discoveries in the last quarter century have unveiled dynamic regulatory mechanisms governing inflammatory responses, and offered the potential for targeted therapies that provide more effective symptomatic relief and fewer side effects for asthmatic individuals[3]. A diverse array of helper T cell subsets have been identified, each with specific cytokine expression profiles related to responses required for the clearance of unique pathogens[181]. In the past decade, innate immune cells have also been implicated in the production of these cytokines, and are now known to be critical mediators of type 2 inflammation required for the clearance of parasitic infections[4,226,233]. Innate immune populations produce cytokine in IL-25 and/or IL-33 dependent manners, thereby linking innate immunity with chemical signals released by the mucosal epithelia. Taken together, these findings have identified additional variables that must be considered, as part of a systemic response to pro-inflammatory stimuli such as environmental allergens, in order to develop treatments that target these previously unrecognized and pathologically relevant populations.

The initial objective of this research was to characterize the role of IL-25 in the pathogenesis of type 2 inflammatory responses resulting from chronic allergen exposure and RSV infection. This was addressed in Chapters 3 and 4, in which we identified critical roles for IL-25 and its receptor, IL-17RB, in type 2 inflammatory responses induced during chronic allergic airways disease and RSV infection. In the context of chronic allergic inflammation, both Th2 and IL-17RB^{+} myeloid cells acted as sources of type 2 cytokines. Cytokine production in these cells was IL-25 dependent, and significantly reduced or absent in *Il17rb*$^{-/-}$ mice. The establishment of systemic immunity required functional IL-25 receptors on CD4^{+} T cells, without which an effective antigen-specific response was not established.

In Chapter 4, we identified IL-25 dependent responses in the mixed inflammatory reaction resulting from RSV infection. IL-25 played a critical role in the immune response to RSV, particularly with respect to IL-13 mediated mucus hypersecretion. IL-25 blockade, or loss of a functional IL-25 receptor, significantly reduced type 2 cytokine production and RSV–associated pathology. The exacerbation of underlying allergic disease by RSV infection further increased type 2 cytokines in an IL-25 dependent manner; therefore IL-25 plays a role in both RSV pathogenesis and the viral exacerbation of allergic disease. *Il17rb*$^{-/-}$ mice exposed to allergen, RSV infection, or both exhibited a significant reduction in airway pathology and type 2 cytokine production, thereby providing additional evidence for the pathologic relevance of IL-25 in these diseases.

In sum, the data presented in Chapters 3 and 4 emphasize the importance of IL-25 as a regulator of type 2 immunity. It is a cytokine that influences the biology of multiple cell types, enhances antigen-specific responses, and can promote type 2 inflammation *de*

novo. However the biologic function of IL-25 is not solely to promote a type 2 response; it is potent regulator of inflammation at the mucosal epithelia, and insensitivity to IL-25-dependent signals does not simply reduce type 2 responses. IL-25 blockade releases an inhibitory signal, resulting in significant increases in Th1 and Th17 cytokine production in mixed inflammatory environments (**Chapter 4**). While this phenomenon has not been previously reported in the context of pulmonary infection, the importance of IL-25 in the maintenance of intestinal inflammatory homeostasis was recognized by Zaph et al.[208]. The role of IL-25 in the development and maintenance of immune tolerance sheds some light on the important counterregulatory function of type 2 immunity in maintaining balance in immunologically active tissues. Commensal bacteria stimulate IL-25 production in the intestinal mucosa, and in so doing suppress production of IL-17A and IL-23. Conversely, IL-25 is not produced by the intestinal epithelium of germ free mice, resulting in an overproduction of IL-17A and IL-23, which can be suppressed via exogenous IL-25 administration. While these findings do not directly demonstrate the pathologic relevance of IL-25 as an inhibitor of Th17-associated signals, subsequent reports have indicated a counterregulatory role for IL-25 in the inhibition of both type 1[198] and type 17 inflammation[209,246], with recent studies focusing on the inverse relationship between IL-17A and IL-25 mediated pulmonary pathology[201,278]. The counterbalancing effect of IL-17A and IL-25 was certainly one component of the inflammatory response observed in *Il17rb*$^{-/-}$ animals following RSV infection, in which LN restimulation resulted in a dramatic increase in both IFNγ and IL-17A.

The findings presented in Chapter 4 offer important insight into how to therapeutically target IL-25-dependent pathologies. The contribution of multiple cell

subsets to type 2 immunity foreshadows potential difficulties involved in designing therapies to abrogate effects of a system with redundancies in both the function of chemical signals and the cellular sources of those signals. Type 2 immunity requires this level of complexity to thwart multiple mechanisms a pathogen may use to evade immune recognition and/or activation. It is only through an understanding of these regulatory motifs that we will be able to effectively target processes at multiple levels of regulation.

Clinically, the most severe sequela of both allergic asthma and RSV infection results from airway obstruction and subsequent ventilation-perfusion mismatch. Type 2 cytokines, IL-13 in particular, act as primary mediators of mucus hypersecretion in both asthma and RSV infection[153,157,247]. IL-25's ability to promote cytokine production was therefore of great interest because IL-13 has been identified as the type 2 cytokine most closely associated with poor clinical outcomes. The role of IL-25 in other respiratory viral infections such as influenza and rhinovirus remains to be determined. The mixed inflammatory environment associated with viral exacerbations could be modulated in part by IL-25-dependent processes. Any agent, infectious or otherwise, which increases the level of baseline inflammation may bring an asthmatic individual closer to their "tipping point" and an asthma exacerbation. A greater understanding of IL-25's role in various viral infections will provide valuable insight as to how best to treat the symptoms of viral exacerbation.

Upstream regulators of heterogeneous, chemokine-dependent inflammatory processes such as IL-17A and IL-25 offer intriguing targets for the inhibition of signals required for the recruitment of allergen–induced effector populations. Allergen–induced inflammatory responses, and inflammation associated with viral exacerbations of existing

pulmonary disease, are significantly reduced when either the ligands or receptors for both IL-17A and IL-25 are blocked[16,117,193,213]. IL-17A induces CXCL1, CXCL8, CCL20 (MIP-3α), and IL-6[207], and its blockade inhibits the production of these mediators, preventing the influx of lymphocytes and neutrophils, and causing a blunted inflammatory response[279]. Likewise, the blockade of IL-25 decreases Th2 inflammation by reducing IL-4 and IL-13 and thus the production of Th2-induced chemokines CCL11 (Eotaxin) and CCL17 (TARC). By blocking IL-17A and IL-25 in tandem, it may be possible to prevent the counterregulatory swing observed following the inhibition of IL-25 alone.

A recent study has exploited the similarities in IL-17A and IL-25 signaling to engineer a peptide that blocks signal transduction by both cytokines. As has been discussed previously, IL-17A and IL-25 require Act1 and TRAF6 dependent interactions to induce downstream NFκB activation[202-205,214,280]. Liu et al. reported that a CC loop mimetic of the Act1 SEFIR domain effectively blocked interactions between Act1 and the IL-17A and IL-25 receptors[254]. It reduced phosphorylation of downstream kinases, as well as transcripts of cytokine-dependent genes such as *Cxcl1* and *Il13* by 50% and 60%, respectively. These findings offer a peptidomimetic-based strategy to effectively target cytokines with oppositional actions, one that may also enable the development of receptor-specific peptides in cases where the abrogation of IL-17 or IL-25 signals would not be advantageous[254].

In Chapter 5, we employed an antigen-independent model of IL-25-mediated inflammation to identify a novel granulocytic population, termed T2M cells. The initial hypothesis for this work was that an IL-25–responsive myeloid population contributed to

type 2 immunopathology in the lung. This was based in large part on previous findings from our lab and others that bone marrow–derived cytokine producing populations were important contributors to type 2 inflammatory responses, specifically in the context of chronic allergic inflammation (**Chapter 3**)[11,225]. Through comprehensive characterization of IL-25 responsive myeloid populations, T2M cells were identified as a novel granulocytic population of innate IL-25 responsive cells involved in the pathogenesis of allergic asthma. T2Ms produce IL-4 and IL-13, and are recruited in large numbers to the lung in response to IL-25 secretion. They exhibit a distinct morphology, a unique pattern of cell–surface antigen expression, as well as a microarray expression profile distinct from other myeloid subsets.

One question raised by these findings is why are there multiple innate cytokine producing populations when the adaptive immune response functions to produce those same cytokines? Neutrophils, for example, can produce both IL-17A[281] and IFNγ[282] in certain contexts, and we have previously discussed the many cell types capable of IL-4 secretion. The answer may lie in the importance of these signals at multiple stages of the inflammatory response, as well as in the conservation of evolutionarily important inflammatory mechanisms. Cytokine production from innate lymphoid cells[234], eosinophils[268,283], basophils[258], NK cells[216], and, as this thesis describes, T2M cells, plays a critical role in the appropriate function of mucosal immunity. Redundancies in cytokine production serve as reminders of the system's evolutionary history as well as its importance, as innate cytokine production is required for helminth clearance while adaptive immunity is dispensable[226]. Prior to the evolution of adaptive immunity, innate immune populations relied upon similar cytokine signals to coordinate an appropriate

inflammatory response. This concept is supported by phylogenetic analysis of immune populations in a range of animal species. The IL-17 family, for example, is evolutionarily conserved and plays a critical role in agnathan (hagfish and lamprey) immunity[284]. These primitive, jawless vertebrates rely upon a thick layer of mucus as their primary barrier against environmental pathogens, and coordinate inflammatory responses with an IL-17 family member resembling IL-17D[284]. Depending on context, it may be that innate populations produce cytokine to effect a reaction while the adaptive response is being established. As the studies described in this thesis indicate, cytokine production by T2M cells may contribute to the enhancement or maintenance of chronic inflammation by promoting mucus production.

Another question raised is whether T2M cells represent a terminally differentiated subset. Their morphology suggests an immature or progenitor phenotype, yet microarray analysis indicates that they are most closely associated with eosinophils, despite differing in expression from eosinophils at several thousand loci. T2Ms are not mature eosinophils: they do not express CCR3 or IL-5rα, nor did FACS sorted T2Ms express transcripts of eosinophil peroxidase or major basic protein, both of which are linked to mature eosinophil function. Interestingly, both CCR3 and IL-5rα mRNA were enriched in T2M cells upon microarray analysis. Eosinophils and their progenitors express IL-5rα prior to migrating from the bone marrow[144,255]. CCR3 expression is required for chemotaxis to inflammatory sites, and is upregulated following IL-5 exposure[146,149]. T2M differentiation into eosinophils, neutrophils or basophils, would require extramedullary terminal differentiation by an as yet unknown mechanism.

The most straightforward way to test whether T2Ms are capable of differentiation into another cell type would involve culturing T2Ms in a variety of cytokine cocktails that promote differentiation of the various myeloid subsets, and then assessing for altered cell surface marker expression and cell-type specific products. For example, murine eosinophils can be derived by culture of whole bone marrow with SCF and Flt3 ligand, followed by culture with IL-5[285]. The derivation of eosinophils from *in vitro* T2M cultures would demonstrate that T2Ms are not terminally differentiated. **Table 7.1** lists examples of culture conditions that have been used for the *in vitro* generation of eosinophils, basophils, and neutrophils.

Desired cell type	Cytokine combination reported to drive differentiation from progenitors *in vitro*	Cell-surface markers used to define subset	Cellular products
Eosinophil	SCF, Flt3 (days 1-4), IL-5 days 5-8[285]	IL-5rα, CCR3	Eosinophil peroxidase, major basic protein, eosinophil cationic protein
Basophil	GM-CSF, SCF, IL-3, anti-IL-5[256]	IL-3r, FcεR1, CD49b	Histamine
Neutrophil	G-CSF, SCF, Flt3 ligand[286]	CXCR2	Myeloperoxidase

Table 7.1: Potential culture conditions to stimulate granulocyte maturation and differentiation in T2M cells.[256,285,286]

While these experiments appear straightforward, initial attempts to characterize T2M biology *in vitro* have been hampered by their granulocytic nature. Even when cultured in Teflon plates, T2M cells isolated by FACS aggregate en masse, likely as a result of adhesion molecule activation. This presents a somewhat difficult problem, in that the isolation of T2M cells requires the induction of IL-25 associated inflammation, however IL-25 promotes type 2 cytokine production in T2Ms. Therefore IL-25 acts as an

activating signal and may itself be responsible for adhesion molecule activation in these cells. Experiments employing different culture conditions, including changes to plated cell density or perhaps the use of specific media, may be required to enable the effective study of T2Ms *in vitro*.

Our data indicate that T2M cells do not actively proliferate in target tissues such as lung (**Chapter 5**). Therefore, the differentiation of T2M cells into another subset would require a metamorphosis of both phenotype and biological function from T2Ms to a terminally differentiated population. This seems an unlikely scenario, and to date we have no data to suggest that this is the case. It is more likely that T2Ms share a common progenitor with the other granulocytes. However, the absence of IL-5rα[144,255] and FcεR1[256] implies that T2M cells are not members of committed eosinophil or basophil subsets. One possibility is that exposure to an activating signal, perhaps IL-25 itself or IL-25 in concert with SCF, may promote T2M differentiation instead of the fate normally assumed by a common progenitor population, thereby altering the pattern of differentiation those myeloid progenitors would otherwise undergo. This concept is logical, as previous studies from our laboratory have demonstrated that the induction of IL-25 by SCF during chronic allergen challenge is a requirement for the generation of IL-4 producing myeloid cells in the bone marrow[11]. In separate experiments, mice deficient in the SCF receptor c-kit exhibited equivalent inflammatory responses to WT mice when challenged with IL-25 (data not shown). Taken together, these findings suggest that while the production of IL-25 requires c-kit, T2M cell differentiation may be c-kit independent. Although experiments investigating the origins of T2M cells are in their

infancy, identifying the parent population of T2M cells will offer an alternative approach to investigate their differentiation.

Our final set of experiments explored the pathological significance of T2M cells (**Chapter 6**). T2M adoptive transfer generated IL-25–associated immunopathology in the airway. High dose dexamethasone administration, the front line therapy for allergic asthma exacerbations, did not reduce cytokine production in, or total numbers of, T2M cells. These findings indicated that T2Ms are sufficient to induce clinically relevant IL-25–mediated pathology. However, it was the identification of an analogous human population of T2M-like cells in peripheral blood of atopic asthmatics that offered the most powerful evidence in support of T2Ms as a clinically relevant population. T2M–like cells were identified in 9 out of 9 asthmatic subjects, and were either absent or present at significantly lower levels in non–atopic individuals (see **Figure 6.7**). Similar to the population identified in mice, these human T2M-like granulocytes were IL-17RB^{+}, responded to IL-25, and produced IL-4 and IL-13. Based on these data, the identification of a novel and pathologically relevant granulocytic subset offers an important new avenue of research investigation with the potential for significant translational impact.

What are the mechanisms by which T2M cells function as a pathologically relevant population? Specifically, how do T2M cells resist glucocorticoid–mediated apoptosis and promote type 2 inflammation? The mechanisms of steroid resistance in inflammatory populations are diverse and not fully understood[33,34,36]. Glucocorticoid (GC) suppresses inflammation by three main mechanisms. The first involves binding of GC to the glucocorticoid receptor (GR) in the cytosol, which translocates to the nucleus and can bind directly to GC response elements (GREs) to upregulate target genes such as

the anti–inflammatory cytokine IL-10[287]. An activated GR can also bind directly to transcription factors such as NFκB and suppress downstream activation of target cytokines[288]. The third major mechanism of GC action involves a pro-apoptotic response. GCs can induce apoptosis directly through a bax-dependent mechanism, in which cytochrome c is released from the mitochondria and activates caspases in the cytosol[289]. In asthmatics, GC resistance among lymphocyte populations has been linked to increased expression of IL-2 and IL-4[290], and patients with steroid–resistant asthma have significantly increased serum IL-4[32]. These cytokines reduce binding affinity and T cell responsiveness to GCs, however the precise mechanism for this effect is unknown. As Le et al. summarize,

> GC resistance in asthma **cannot** [sic] be explained by malabsorption, pharmacokinetic mechanisms, defects in the binding of steroids to cytosolic glucocorticoid receptors, nor by defective nuclear translocation of this receptor. This implies that the major mechanisms for GC resistance occur distal to the nuclear translocation step[291].

In *in vitro* experiments with $CD4^+$ T cells skewed toward a Th2 or Th17 phenotype, McKinley et al. demonstrated that Th17 cells are resistant to steroid, despite nuclear translocation of the GR[161]. The adoptive transfer of Th17 cells was sufficient to induce GC resistant airway hyperreactivity, supporting the hypothesis that specific cytokines (in this case IL-17A) may play a role in the development of GC resistance. Cytokine-mediated steroid-resistance may represent a mechanism by which T2M cells maintain resistance to GCs, and is supported in part by findings that IL-17A increases production of the GC receptor beta in the airway epithelial cells of asthmatics[292]. GC beta cannot bind glucocorticoids, thus it acts in a dominant-negative manner to inhibit normal GC signaling through GC receptor alpha[292].

An interesting parallel to our finding that T2M cells are resistant to steroids, as well as a potential mechanism to explain T2M steroid-resistance, can be drawn from the findings of a group studying malignant breast cancers[293]. These investigators identified tumors that are exquisitely sensitive to IL-25. IL-17RB binding promotes apoptosis in breast cancer cells but not normal breast tissue, and IL-25 is now being pursued as a possible therapeutic agent for IL-17RB^{+} tumors. As part of the investigation into the mechanism of IL-25-mediated apoptosis, IL-17RB's TRAF6 binding site was mutated to prevent TRAF6 interaction. Surprisingly, TRAF6 mutations made cells even more sensitive to apoptosis, indicating that TRAF6 binding to IL-17RB acts as an anti-apoptotic signal. Although these experiments were conducted in completely different cells and experimental conditions, the results of these experiments indicate that activation of NFκB via TRAF6 (the mechanism by which IL-25 induces the expression of type 2 cytokines) can act as a pro-survival signal. As previously discussed, GCs promote apoptosis via bax-dependent caspase activation[289]. Therefore, in the context of IL-25 mediated inflammation, IL-25-IL-17RB interactions alone may serve to protect T2M cells from apoptotic responses by inhibiting GC mediated caspase activation. Although we have not investigated this hypothesis, our finding that the administration of an anti-IL-25 antibody in allergen-sensitized mice significantly increased the anti-inflammatory effects of dexamethasone offers some circumstantial evidence that may corroborate an IL-25–dependent pro–survival effect (see **Figure 6.2**). Future studies to further characterize T2M cells should include an investigation of the mechanism underlying GC resistance in this population.

Several questions remain regarding the biologic and pathogenic mechanisms of T2M function. The most consistent T2M–associated pathology involves goblet cell hypertrophy and mucus hypersecretion. These actions are most likely driven by IL-13, as it is both produced by T2Ms and intimately associated with increased mucus production[153]. We have not explored other potential mechanisms for T2M–mediated pathology, however several other groups have associated so–called "myeloid suppressor" ($CD11b^+$ $Gr\text{-}1^{mid}$) cells with anti–inflammatory effects dependent upon the production of nitric oxide and superoxide[243,244]. A recent study identified three populations of myeloid suppressor cells in a model of allergic asthma, characterized according to Ly6G and Ly6C expression[294]. These populations mediated cytotoxic effects via NO and superoxide production (depending on the cell type). However, the model used by these investigators did not generate sufficient numbers of these $Ly6C^+$ $Ly6G^+$ $F4/80^+$ cells (similar by surface marker to T2Ms) to investigate their production of reactive oxygen species, so their mechanism of action remains unknown. Based on their anti-inflammatory properties, it may be that myeloid suppression described in these investigators relates to the biological function of the $IL\text{-}17RB^+$ $Gr\text{-}1^{hi}$ myeloid population we identified that did not produce IL-4 or IL-13 (see **Figure 3.4**).

The biological activity of $Gr\text{-}1^{hi}$ myeloid cells has not been investigated beyond their initial characterization as a separate $CD11b^+$ $IL\text{-}17RB^+$ subset. This population is significantly increased in allergic asthma and present in similar numbers to T2M cells, however unlike T2Ms this population does not produce type 2 cytokines (**Chapter 3**). Similar findings were observed in our antigen–independent model of type 2 inflammation, where 50% of the $IL\text{-}17RB^+$ myeloid population also expressed GFP/IL-4

(**Chapter 5**). It would be interesting to determine whether the 50% lacking GFP expression has some immunomodulatory function related to T2M biology, if these cells contribute significantly to disease, and whether they are modulated in some manner by IL-25.

Further investigation of T2M origins, and the cellular signals regulating T2M biology, may also add substantially to our understanding of innate immunity and IL-25 dependent biological processes. For example, the feedback mechanism(s) required for T2M induction in the bone marrow has not been fully determined. While we have previously identified an SCF–dependent mechanism of myeloid cell recruitment to the allergic lung[11], it remains to be seen whether IL-25 acts exclusively on T2M cells through an SCF-dependent mechanism, or if other signals are required for T2M development. The distribution of T2M cells in the lung parenchyma versus the airway has not been fully characterized, nor has how inflammatory stimuli or chemotactic agents may affect the distribution of these cells. It would also be interesting to investigate whether T2M cells are present in the gastrointestinal tract in addition to hematopoietic and lung tissue. Innate lymphoid populations do not appear to increase in the lung, so perhaps certain innate populations respond preferentially to inflammatory signals from intestinal or pulmonary epithelia. One possible explanation is that T2M cells do not express the IL-33 receptor, whereas innate lymphoid populations have been found to be four to six times more sensitive to IL-33 than to IL-25[23,235]. Perhaps innate lymphoid cell recruitment requires a strong IL-33 stimulus that is lacking in both allergic asthma and RSV infection, but present in the context of infection with intestinal helminthes[295].

The identification of a human T2M homologue is the most intriguing, and in time may be the most meaningful, contribution made by this thesis. Our initial findings indicate that the presence or absence of T2M-like cells is related directly to the symptoms of moderate to severe atopic asthma. The identification of these cells in the peripheral circulation of asthmatic individuals is extremely exciting as it offers a minimally invasive test that may have significant predictive value. By contributing an additional parameter through which asthmatic patients could be stratified in the future, the identification of this population presents a variety of questions that will require further investigation.

The clinical impact of T2M cells can be classified according to potential prognostic and therapeutic utility. For example, evaluating a larger patient population for the presence of IL-17RB^{+} granulocytes may make it possible to correlate numbers of circulating cells with demographic data, including age, sex, exposure history to various allergens, urban versus rural habitation, clinical symptoms, family history, medical comorbidities, and drug response profiles. Further study may enable the stratification of an individual's disease severity based on their unique profile of T2M–like cells, and could be particularly useful in individuals with steroid–resistant disease. Further study may also provide some predictive value as to how an individual may react to specific pharmacotherapy, or enable monitoring of the effectiveness of a treatment protocol, particularly related to patients with steroid-resistant asthma.

The ability to readily assess and quantify this circulating population also offers the potential for the use of these cells as a biomarker for disease pathogenesis. For example, measuring numbers of circulating T2M–like cells in an individual when asthma is well–controlled, versus during IL-25 associated events such as a viral exacerbations or

bronchospasm following allergen exposure, could offer insight into how these cells relate to clinical pathology or aid in the prediction of an impending exacerbation. Finally, while we have identified T2M cells and their human homologue in the context of allergic asthma, we have not investigated whether these cells are present in, or contribute to, a myriad of other conditions associated with type 2 immune responses. These may include diseases of epithelial tissues such as atopic dermatitis[173,220], hypereosinophilic syndromes such as Eosinophilic Esophagitis[263], and autoimmune disorders such as Churg-Strauss syndrome[14,264]. Our findings offer a framework for future investigators interested in these and other questions related to IL-25 biology, innate immunity, and novel targets for the therapeutic treatment of asthma.

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