EOSINOPHIL FUNCTIONS IN THE DEVELOPMENT OF ALLERGIC ASTHMA

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by
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Eosinophils have been implicated as playing a major role in allergic airway responses. However, the importance of these cells to the development of this disease has remained ambiguous despite many studies, partly due to lack of appropriate model systems. Here, using transgenic murine models, we clearly delineate a role for eosinophils in asthma. We report that there are strain specific differences in the generation of allergic asthma in eosinophil-deficient ΔdblGATA mice, and that eosinophils are required for development of this disease on the C57Bl/6 background, but not the Balb/c background. This defect was due to reduced chemokine production in the lungs, defective T cell recruitment to lungs, and subsequent Th2 cytokine production. We found that adding WT eosinophils or chemokines back to the lungs of these mice could restore all of these processes, suggesting that eosinophils provide a signal integral to the initiation of the allergic response.

The signal that eosinophils provide to induce the recruitment of T cells to the lung during an allergic response is unclear, but other studies and this research show that eosinophils contain preformed message for the cytokine IL-13. Thus, we wanted to determine how eosinophil production of this cytokine contributes to allergic asthma. We were able to rescue symptoms of allergic asthma, such as airway hyperresponsiveness (AHR) and Th2 cytokine production when we administered IL-13 back to the lungs of C57Bl/6 ΔdblGATA mice. We also found that eosinophils deficient in this cytokine were unable to rescue AHR, T cell recruitment to the lungs, and Th2 cytokine/chemokine production in ΔdblGATA mice, even if Th2 cells were added back with IL-13⁻/⁻ eosinophils. However, eosinophil-derived IL-13 was not able to rescue an allergic asthma response in the absence of IL-13-competent T cells in IL-13⁻/⁻ mice, although increased chemokine production and CD4⁺ T cell recruitment were found in the lungs of mice reconstituted with WT eosinophils. This suggested that although eosinophil production of IL-13
is required for T cell recruitment to the lungs, it is not sufficient for the perpetuation of an allergic asthma response. These data indicate that eosinophils and T cells have an interdependent relationship in the development of allergic asthma.

Targeting T cells and eosinophils simultaneously in allergic asthma is an immense challenge. Using small molecule inhibitors against a common protein component in the T cell and eosinophil signaling cascade would be a good way to counteract the effects of these cells during the course of an allergic response. ITK is a non-receptor tyrosine kinase that is expressed in eosinophils and T cells. It is known that this kinase is important in the development of Th2 T cells and that T cells lacking ITK are unable to generate an allergic response. We hypothesized that ITK would also be integral to the function of eosinophils in allergic asthma development. We found that compared to ΔdblGATA reconstituted with WT eosinophils, eosinophils lacking ITK were unable to induce AHR, T cell recruitment to the lung, and TH2 cytokine production when transferred to ΔdblGATA mice and challenged with ovalbumin (OVA). This may partially be due to reduced MAPK signaling in these cells downstream of the CCR3 receptor, as stimulation with Eotaxin-1 led to lower and delayed ERK1/2 activation in these cells. These data suggest that developing drugs against ITK could be one potential therapy to combat allergic asthma.
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List of Abbreviations

AHR- Airways hyperresponsiveness
APC- Antigen presenting cell
BALF- Bronchoalveolar lavage fluid
CCL11- Chemokine ligand 11
CCL24- Chemokine ligand 24
CCL7- Chemokine ligand 7
CCR3- Chemokine receptor
CFSE- Carboxyfluorescein succinimidyl ester
CMP- Carbon myeloid progenitor
DC- Dendritic cell
ECM- Extracellular matrix
ECP- Eosinophil cationic protein
EDN- Eosinophil-derived neurotoxin
EPO- Eosinophil peroxidase
GI- gastrointestinal
i.n.- intranasal
i.o.- intraorbital
i.p.- intraperitoneal
i.v.- intravenous
ICAM- Intracellular adhesion molecule
ITK- IL-2 inducible T cell kinase
MadCAM-1- Mucosal addressin cell adhesion molecule
MAPK- Mitogen-activated protein kinase
MBP- Major basic protein
MMP- Matrix metalloproteinase
NGF- Nerve growth factor
OVA- Ovalbumin
PLC-gamma- Phospholipase C-gamma
PSGL-1- P-selectin glycoprotein ligand-1
SCF- Stem cell factor
SNP- Single nucleotide polymorphism
TARC- Thymus and activation-regulated chemokine
TH1- T helper 1
TH2- T helper 2
TSLP- Thymic stromal lymphopoietin
VCAM- Vascular cell adhesion molecule
VLA-4- Very late antigen-4
WT- Wild-type
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Chapter 1

Eosinophils: development, function, and contribution to allergic asthma

Paul Ehrlich, a pioneer of allergic asthma research, announced his discovery of eosinophils, large granular leukocytes with bi-lobed nuclei that comprise 1-6% of white blood cells in the body, in 1879. He determined that these unique cells could be stained by acidic dyes, and the methods he developed allowed researchers and medical doctors the ability to enumerate the large multi-functional leukocytes in bodily fluids and tissues. This important discovery enabled clinicians to observe the connection between the presence of eosinophils in the lung fluid with the prevalence of allergic asthma in 1889. Since then, researchers and medical doctors alike have been attempting to determine whether there is a causative link between the infiltration of eosinophils into the lung with the development of allergic asthma. It is a complex controversy over which opposing researchers have donned the battle raiment for over a century without resolution, and continues to be a surprising crusade of discovery. In this thesis, I present definitive pieces of the puzzle that help determine that eosinophils are integral to the development of allergic asthma, and provide insight into the potential mechanisms whereby eosinophils could influence the development of this disease.

Overview of allergic asthma

Allergic asthma is a disease of the respiratory tract that is gaining prevalence worldwide in urbanized populations. It is characterized by loss of breath, wheezing, airway constriction, and mucus production in the lung in response to environmental allergens. In chronic asthmatics, remodeling of the lung is also observed. This process results in thickening of the basement
membranes in the lung and airways, smooth muscle proliferation, increased fibrosis, and
decreased lung capacity. The prevalence of asthma continues to rise, sometimes attributed to
increasing urbanization and pollution; in the United States alone, over 11% of the population is
afflicted with this disease, often times affecting inner city minority populations at
disproportionately high rates (up to 3 times higher than the number of Caucasian cases). These
populations are generally ill equipped to handle the continuous high cost of healthcare associated
with asthma, as the disease is treatable but not curable, and thus their quality of life is severely
compromised and asthma-associated death can occur. Development of treatments that offer
continuous protection or prevention of this disease is a high priority among researchers and
medical professionals.

It is not known why individuals develop allergic responses but there are several
hypotheses that are currently under investigation. One school of thought is that the complex
interplay between genetic and environmental factors becomes unbalanced and predisposes
individuals to hypersensitive reactions. There have been a number of genes identified that are
associated with asthma development. For example, individuals who have higher gene expression
of a chemokine called Eotaxin-1 are more likely to develop asthma than individuals who express
lower levels of this gene (1). Other genes that are associated with higher prevalence of asthma
are IL-5, matrix metalloproteinases (MMPs), and certain polymorphisms in IL-4 and IL-13 (2).

Along with the genetic component, another hypothesis that has been gaining support is
the “hygiene hypothesis”. This concept is based on the observation that historically people were
exposed to more harmful and benign microorganisms and parasites, which maintained a balance
in the immune system. With increasing levels of cleanliness and hygienic practices, our exposure
to these environmental factors has become minimized, allowing our immune system to become
more likely to be hypersensitive to other environmental stimulus, such as protein allergens.
Although it is not known exactly why this disease develops, researchers have been studying asthma for over a century and much has been discovered regarding its pathogenesis. The immune response can be divided into two phases, a mast cell dependent early phase, and a Th2 T cell driven late phase. Prior to both of these phases, allergen sensitization takes place, wherein inhaled allergen is taken up by dendritic cells (DCs) and presented to T cells. This sensitization process allows for the generation of clonal selection of allergen-specific T cells, integral to the late phase of allergic asthma, and development of allergen-specific antibodies by B cells, which are important in the early stage of disease. These two phases are detailed below.

**Early phase of allergic asthma**

The early phase of an allergic asthma response occurs within minutes of allergen exposure and is characterized by sudden inability to breathe, constriction of the airways, coughing, wheezing, and mucus production (3, 4). The response begins when allergen cross-links surface bound IgE (an allergen-specific immunoglobulin produced by B cells that recognizes Fc-epsilon (Fce) receptor expressed on mast cells) on the mast cell surface and induces downstream signaling events that lead to secretion of inflammatory mediators (4). Mast cells are able to secrete a variety of products that induce an allergic response, cytoplasmic granules, lipid mediators such as leukotrienes, and cytokines and chemokines (Fig. 1-1) (4). Release of cytoplasmic granules that contain histamine causes vasodilation of vascular arteries, allowing for the influx of other inflammatory cells, and also acts on nerve cells to induce bronchoconstriction, and goblet cells to induce mucus production (4). Cytokine and chemokine secretion, such as IL-13 and Eotaxin-1, lead to mucus hypersecretion and recruitment of leukocytes such as T cells and eosinophils that perpetuate and exacerbate the response (5). Leukotriene and histamine secretion
induce vascular permeability that allows for influx of T cells and other cells that are integral to the late phase of allergic asthma responses (5).

Figure 1-1: Mast cell function in the early phase of allergic asthma. IgE molecules bind to the FcεR on the mast cell surface and causes aggregation to activate mast cells to secrete preformed mediators from granules and lipid mediators. This also increases the synthesis of many cytokines, chemokines, and growth factors. These different secreted factors induce bronchoconstriction (lower left), vasodilation, increased mucus production, and vascular permeability that allows for influx of inflammatory cells. *Nature, Vol 454, 445-455, 2008.*

**Late phase of allergic asthma**

The late phase of an allergic asthma response takes place a few hours after the initial allergen challenge and usually resolves within 1 to 2 days (Fig. 1-2) (3). Airway narrowing,
mucus hypersecretion, and tissue eosinophilia are the main features of this stage of the response and chronic persistence of this phase of the disease leads to long term remodeling of the lung, which will be discussed later (6).

While mast cells are able to prime the environment for influx of inflammatory cells, the initial event of the late phase response is activation of T cells by antigen presenting cells (APCs), often times dendritic cells, located in the airway epithelium and submucosa of the lung (7, 8). Dendritic cells are stimulated to secrete chemokines, and possibly Th2-polarizing cytokines for T cell and eosinophil recruitment to the lung by epithelial cell production of thymic stromal lymphopoietin (TSLP), a factor produced in asthma-like conditions that polarizes T cells to the Th2 phenotype (9). Allergen internalization is enhanced by IgE bound to high-affinity receptors on dendritic cells (10), and once processed, allergen peptides are presented to CD4\(^+\) T cells in the context of MHC Class II (6). Some allergen-loaded APCs migrate to the mediastinal (lung draining) lymph nodes where they can stimulate activation and clonal expansion of T cells specific to the allergen (11).

Once activated, T cells differentiate into Th2 cells under the stimulus of polarizing cytokines, primarily IL-4, that can be produced by a variety of cells, such as DCs, eosinophils, and other granulocytes. These differentiated Th2 cells migrate to the lung under the direction of chemokines CCL11, CCL24, and CCL7, where they can drive the long-term response by the production of Th2 cytokines that lead to AHR, mucus secretion, and epithelial injury and lung remodeling in chronic cases (5). These cytokines have a variety of functions in the course of disease progression, one of which is recruiting inflammatory cells, such as eosinophils, mast cells, and basophils, to the lung, where they can also produce cytokines and chemotactic factors to perpetuate and exacerbate disease (6). Th2 cytokines can also have direct effects on the lung environment, causing mucus hypersecretion, proliferation of smooth muscle cells, and
bronchoconstriction (6). Many of the cytokines that T cells produce have been studied extensively and their functions in this disease are well characterized.

**IL-4 in allergic asthma**

IL-4 was one of the earliest cytokines identified as having a role in allergic asthma. Mast cell or eosinophil-derived IL-4 may be integral to the differentiation of T cells into the Th2 cells that drive allergic asthma responses (12). It is also important for B cell growth and class switching to IgG1 and IgE antibodies that facilitate the activation and survival of mast cells (13). Goblet cell hypersecretion of mucus is also partially, although not wholly dependent on IL-4, and this cytokine also contributes to epithelial hyperplasia (14). Because IL-4 is involved in B cell class switching, mast cell activation, and Th2 cell differentiation, it is thought that IL-4 is most important during sensitization to allergen, while other cytokines can compensate for later functions. For instance, antibody blockade of IL-4 after sensitization of mice yields only a minor reduction in AHR and has no effect on tissue eosinophilia (15). IL-4−/− mice are also still susceptible to generation of AHR, although they have reduced mucus in the lungs (14). Thus, although important, IL-4 is not wholly responsible for asthma-associated pathology.

**IL-13 in allergic asthma**

IL-13 is one of the most potent cytokines produced by Th2 cells and has a myriad of effects in allergic asthma. IL-13 induces the production of Eotaxin, a chemokine that can synergize with IL-5 to selectively recruit eosinophils to the lung (16, 17). It also promotes B cell proliferation and induction of class switch to IgG4 and IgE (18). IL-13 can activate mast cells as well as aid in their survival. Antibody blockade of IL-13 abolishes AHR in mouse models as well
as alleviating many of the symptoms associated with the disease, including eosinophilia, mucus secretion, and airway inflammation (19). Murine knockout models of this cytokine are protected from allergic asthma altogether (20). It is clear that this cytokine is an integral part of the development of allergic asthma, and it will be discussed more thoroughly later in the context of eosinophils in disease.

**IL-5 in allergic asthma**

IL-5 is the main cytokine associated with eosinophil development, trafficking, and activation. Th2 T cells produce this cytokine in large quantities to generate development and mobilization of eosinophils from the bone marrow (14). Eosinophils are then recruited to the lung to take part in cytokine and chemokine production, as well as secretion of cytoplasmic granule mediators, such as major basic protein (MBP), a protein mediator that activates degranulation of mast cells (2). Their role in allergic asthma is discussed in depth later. Much like IL-4, blockade of IL-5 or gene deletion in mice results in partial alleviation of asthma symptoms, but not prevention (21). Anti-IL-5 clinical studies have shown that while eosinophils are reduced in the bloodstream, there is only a 50% reduction in tissue eosinophils (22). There was no change in AHR in patients in these studies, however, lung remodeling was reduced, and thus this drug and the targeting of eosinophils in allergic asthma may still have clinical significance (22).

The contribution of eosinophils to the development of allergic asthma is still under investigation. There is much known about their abilities as end stage effector cells, however, new evidence suggests that eosinophils may have significance early on in the course of this disease. Because they are such incredibly multifunctional cells, the possibilities are endless, and
there is much yet to be explored. The following sections detail the development, activation, and known functions of these cells in allergic asthma.
Figure 1-2: Early and late phase dependent models of allergic asthma. The early phase of allergic asthma occurs within minutes of allergen challenge and is mediated by mast cell secretion of inflammatory factors as well as degranulation. Histamine secretion induces mucus production, vasodilation of the arteries for influx of inflammatory cells, and bronchoconstriction. The late phase of allergic asthma is initiated by allergen antigen presentation to T cells by an APC. This induces T cells to secrete Th2-type cytokines such as IL-4, IL-5, and IL-13. This induces recruitment of inflammatory cells to the lungs such as eosinophils and neutrophils, which can also secrete cytokines and lead to the long-term effects of epithelial injury and thickening of the basement membrane as well as bronchoconstriction *Kuby Immunology, 5th edition, 2002.*
Eosinophil development

Eosinophils develop in the bone marrow from pluripotent stem cells that differentiate into the common myeloid progenitor (CMP) and then share an intermediate precursor with basophils before diverging into their own lineage. Most of the factors that determine eosinophil lineage commitment are redundant with other cell types; such as GATA-1 and C/EBP expression as well as IL-3 and GM-CSF production, but the unique nature in which these signals are combined result in the differentiation of eosinophils.

Transcription factors necessary for eosinophil production

Three transcription factors drive the development of eosinophils from multipotent CD34⁺ progenitors, PU-1, C/EBP alpha and beta isoforms, and GATA-1 (23). Other cells of the myeloid lineage also express these transcription factors, but PU-1, C/EBP, and GATA-1 coordinate uniquely along with specific cytokine signals to drive eosinophil development (23). Immature eosinophil progenitors, like other cells that share the myeloid lineage, express high levels of PU-1 early on, PU-1 is then downregulated upon low-level induction of GATA-1 (23). Mouse models have also demonstrated the necessity of C/EBP-α expression for eosinophil and neutrophil development, as multipotent progenitors in C/EBP-α⁻/⁻ mice have an inability to respond to granulocyte colony stimulating factor (G-CSF), a cytokine essential to early eosinophil and neutrophil development (24). C/EBP-β expression has been demonstrated to be essential more specifically to eosinophil development. It is thought that C/EBP-β inhibits FOG (friend of GATA) RNA expression, a repressor of GATA-1, enabling expression of GATA-1 and thus development of eosinophils (25).
GATA-1 is the transcription factor that may be most integral to specifying eosinophil lineage differentiation. Deletion of a high affinity binding site for GATA-1 in the enhancer region of the downstream GATA-1 promoter causes eosinophil-specific ablation in mice, and suppression of GATA-1 in \textit{in vitro} differentiation experiments prevents eosinophil development (Fig. 1-3) (26). In addition to being found in the mouse GATA-1 promoter, this double palindromic site is conserved in regulatory regions of several eosinophil-specific human genes, such as IL-5 receptor and major basic protein (MBP) (2). Research in several human eosinophil cell lines has shown that this site is necessary for transcription of MBP-2, an eosinophil-specific secreted factor that causes much pathology in allergic asthma (2).

**Figure 1-3: Conservation of the double palindromic GATA-1 binding site in human and mouse genes.** Identified in a hypersensitivity region of the murine GATA-1 regulatory locus, this sequence is found in the promoters of three human eosinophil lineage-selective genes, including the human IL-5R\(\alpha\) gene promoter 1, human MBP promoter 2, and the human CCR3 regulatory exon-1. \textit{Annu. Rev. Immunol., 24, 147-74.} 2006.

**Cytokine survival factors necessary for eosinophil development**

It is thought that the above transcription factors also have directive roles in production of eosinophil survival and differentiation factors. There are three cytokines that are integral to eosinophil development and differentiation, IL-3, IL-5, and GM-CSF. All three cytokines are encoded on human chromosome 5q31, and signal through a common beta chain, but have unique alpha chains (27). Because they all signal through a common beta subunit, all three are able to
influence eosinophil development. Incubation of CD34+ progenitor cells with IL-3, IL-5, and GM-CSF results in a synergistic upregulation of IL-5 receptor alpha on the cell surface, the receptor specific to eosinophil and basophil development, as well as increased levels of IL-5 mRNA (28). However, while IL-3 and GM-CSF can contribute to eosinophil development, they do not promote efficient eosinophil development on their own (29). Continual culture of progenitor cells with IL-3 alone results in basophil and mast cell development (30), while GM-CSF alone promotes neutrophil and macrophage development (31). Thus, although IL-3 and GM-CSF promote survival and early differentiation of eosinophil progenitor populations and play a role in regulation of mature eosinophils that will be discussed later, only IL-5 is an eosinophil-lineage specific cytokine.

Upregulation of IL-5 receptor alpha on the cell surface of CD34+CD45+ progenitors is one of the earliest signs of commitment to the eosinophil lineage, and this receptor is expressed to varying degrees on the cell surface of mature eosinophils as well, depending on tissue location and activation state (32). Bone marrow cells continually cultured with IL-5 develop into eosinophils, and IL-5 transgenic mice that overproduce this cytokine have profound eosinophilia in blood and lymph tissues that leads to severe pathology later in life (33). High levels of this cytokine are also found in the blood and bronchoalveolar lavage fluid (BALF) of patients with eosinophilic esophagitis and allergic asthma, two diseases associated with eosinophilia. Atopic asthmatics also harbor large numbers of eosinophil progenitors expressing IL-5 receptor alpha in the bone marrow, that when cultured with IL-5 \textit{ex vivo}, develop into eosinophils (22). Mice subjected to models of allergic airway inflammation also show high numbers of these progenitors in blood and bone marrow, suggesting that a pool of these progenitors is made available for rapid maturation and trafficking upon IL-5 stimulation (34). Mice lacking IL-5 have a severe reduction in the number of eosinophils present in tissues and the bloodstream, although they are not completely ablated (35). This partial block in eosinophil development used to be attributed to
residual signaling through the βc receptor by IL-3 and GM-CSF, but even mice that are deficient in the βc receptor still have a small number of eosinophils residing in tissues (28). These studies suggest that there are eosinophil-specific developmental factors that have yet to be discovered.

**Eosinophil Trafficking**

After maturation in the bone marrow under homeostatic conditions, eosinophils enter the bloodstream under the influence of IL-5 and other chemotactic factors, such as eotaxins. The generation of these migratory signals in combination with the subset of adhesion molecules eosinophils express will influence trafficking of eosinophils. Under basal conditions, eosinophils are found primarily in the gastrointestinal (GI) tract, but also home to the thymus, mammary glands, and uterus. In certain diseases, such as allergic asthma, the production of eosinophils and eosinophil progenitors from the bone marrow is increased, and using IL-5 and other signals, the trafficking of eosinophils is changed. The delicate balance between these and other factors influencing eosinophil migration is discussed in the following sections and depicted in Fig. 1-4.
IL-5 as an eosinophil trafficking signal

Several cytokines, such as IL-4, IL-5, IL-13, and TGF-beta have the ability to influence recruitment of eosinophils to inflamed or damaged tissue, however, only IL-5 is an eosinophil-specific recruitment signal. This cytokine, discussed above as a developmental and survival factor, is also involved in directing migration of eosinophils (Fig. 1-4). It has the ability to act alone or in synergy with Eotaxin-1 (36). Mice that overexpress IL-5 have profound eosinophilia of blood and tissues (33), while mice that lack IL-5 have very few numbers of circulating...
eosinophils (14). IL-5 is also able to upregulate integrins on the eosinophil cell surface that aid in migration into tissues (37). IL-5 has the ability to prime eosinophils to induce maturity and mobilize them from the bone marrow, while other chemotactic factors can only attract cells that are already in tissues or are circulating in the bloodstream (33). Systemic administration of IL-5 increases blood and tissue eosinophilia (33), and antibody depletion of this cytokine drastically reduces the number of circulating eosinophils (22), although it has less of an effect on tissue resident eosinophils. Administration of both IL-5 and Eotaxin-2 results in enhanced maturation and mobilization of eosinophils from the bone marrow (38), while simultaneously knocking out IL-5 and Eotaxin-1 results in almost no development of eosinophils (39).

**Chemokine signals integral to eosinophil migration**

The primary receptor, other than the IL-5 receptor, through which eosinophils receive migratory signals, is CCR3, a seven-transmembrane spanning G protein-coupled chemokine receptor. This receptor is expressed constitutively on eosinophils, but can also be expressed transiently on activated mast cells, Th2 cells, and basophils. Compared to other chemokine receptors, which tend to be promiscuous in the number of ligands they will bind, CCR3 has very few ligands. They are limited to Eotaxin-1, Eotaxin-2, and in humans, Eotaxin-3 (40, 41). A few other chemokines; such as, RANTES, MIP-1 alpha, MCP-2, 3, and 4, can also bind to CCR3 with lower affinity than Eotaxins; but as these chemokines can also bind to chemokine receptors expressed on other cell types and appear to have a lesser role in eosinophil migration in allergic disease, they will not be discussed here.

Eotaxin was first discovered in the bronchoalveolar lavage fluid (BALF) of allergic guinea pigs (42). Subsequently, two other isoforms, Eotaxin-2, and Eotaxin-3 were cloned and identified; however, they only share 30% sequence homology with Eotaxin-1 and are located on
different chromosomes (40, 41). Eotaxins are upregulated in the lung as quickly as six hours after antigen challenge in models of airway inflammation (42, 43). Eotaxin-1 is the initial chemokine that is induced rapidly upon allergen challenge, but later in asthmatic responses, Eotaxin-2 becomes more prevalent (43). They are produced primarily by epithelial cells in response to IL-4 and IL-13, but can also be made by Th2 cells, macrophages, eosinophils, mast cells, vascular endothelial cells, and airway smooth muscle cells (44). Eotaxin binding to CCR3 induces receptor internalization, calcium mobilization, actin polymerization, shape change in the cell, and chemotaxis (45).

The evidence for Eotaxins involved in eosinophil trafficking is extensive in both the laboratory as well as in human patients. Research on murine knockout models of Eotaxin-1 and Eotaxin-2 has yielded much insight into the role of these chemokines in eosinophil trafficking. Eotaxin-1 knockout mice have reduced numbers of eosinophils in the lamina propria of the GI tract, the basal resting place for eosinophils, which normally has constitutive expression of Eotaxin-1 (46). CCR3 deficient mice have reduced numbers of eosinophils in this location as well (47). Eotaxin-1 is also necessary for the migration of eosinophils to the thymus, mammary gland, and uterus under homeostatic conditions (2, 45, 46, 48).

Besides controlling steady state eosinophil migration, Eotaxin-1 can act as an important chemoattractant during allergic and parasitic responses, and cooperates synergistically with IL-5 and Eotaxin-2 to promote eosinophil recruitment to sites of allergic inflammation (17, 39, 49). Analysis of a naturally occurring single nucleotide polymorphism (SNP) in the last amino acid of the signal peptide sequence (alanine to threonine) in the human Eotaxin-1 gene also lends evidence to the importance of this chemokine in human asthma (1). This mutation is associated with reduced ability for cellular secretion of Eotaxin-1, lower levels of circulating Eotaxin-1 and eosinophils, and increased lung function (1).
Eotaxin-1 is not the sole chemokine that is able to induce eosinophil migration during allergic responses. Single knockout models of this chemokine show a reduction in the number of eosinophils able to be recruited to allergen-challenged lungs, but there are still sufficient numbers to cause disease (39). Antibody neutralization studies against Eotaxin-1 show the same trend, decreased numbers of eosinophils in the bloodstream and lung of allergen-challenged mice, but no change in the generation of airway inflammation (50). This indicates that the functions of Eotaxin-1 may be somewhat redundant with another family member or chemotactic factor. Eotaxin-2, the other primary chemokine that signals through CCR3, appears to share many functions with Eotaxin-1. Although it is induced later than Eotaxin-1 in the allergic lung, around 24 hours, in the absence of Eotaxin-1 it is still able to mobilize eosinophils and vice versa (50). Eotaxin-2 knockout murine models look very similar to Eotaxin-1 single knockout models in that they have reduced numbers of eosinophils recruited to sites of allergic inflammation, but still sufficient numbers to aid in disease development (39). Eotaxin-2 antibody depletion studies also correlate with this trend (50). However, when a double knockout mouse of Eotaxin-1 and Eotaxin-2 was analyzed, there was a severe reduction in the ability of eosinophils to migrate to sites of inflammation, indicating that while Eotaxin-1 and Eotaxin-2 may be able to compensate for or still function in the absence of the other, other chemokines that signal through CCR3 had non-redundant roles with the Eotaxins (51). Interestingly, these mice also had problems generating Th2 T cells, suggesting that eosinophils may have a role in Th2 T cell maturation (51).

While Eotaxin-1 or Eotaxin-2 alone are sufficient to recruit eosinophils to a site of allergic inflammation, they do have some non-redundant functions. For example, although Eotaxin-1 is important for basal homing of eosinophils into tissues, Eotaxin-2 appears to play no role in this process (46, 48). Also, Eotaxin-1 but not Eotaxin-2 synergizes with IL-5 to promote eosinophilopoiesis and migration of eosinophil progenitors from the bone marrow into the bloodstream (6, 39). During allergic airway inflammation, while both Eotaxin-1 and Eotaxin-2
are induced by IL-13, infiltration of eosinophils into the airway lumen is dependent solely on Eotaxin-2 (17, 52), although Eotaxin-1 is important for early homing of eosinophils into the lung tissue (36). Overexpression of IL-5 by T cells combined with overproduction of Eotaxin-2 by airway epithelial cells leads to severe pathology in the lung and airway remodeling (38, 53). Clearly, there is an intricate temporal and spatial balance between these two chemokines, and regardless of whether their functions are overlapping or not, it is clear that both Eotaxins are integral to the trafficking of eosinophils in the body during disease development.

**Adhesion molecules in Eosinophil Trafficking**

There is a wealth of knowledge available on the role of adhesion molecules in eosinophil homing during the course of allergic disease, however how eosinophils home to their normal resting places in tissues is still a mystery. The only knowledge available is that gleaned from negative data. For instance, β7 gene-targeted mice have reduced eosinophil trafficking to the peritoneum during *Trichinella spiralis* infection, but baseline homing to the gut under noninfectious conditions is intact (54). Because there is very little information available on baseline homing of eosinophils, adhesion molecules involved in eosinophil migration during disease pathogenesis will be the primary topic of discussion.

Eosinophils express many adhesion molecules, but most research has focused on their highly expressed integrins of the CD18 and very late antigen-4 family (VLA-4). These integrin molecules interact with adhesion molecules on the endothelial cell surface to initiate rolling, adhesion, and transendothelial migration of eosinophils into sites of inflammation (53). Eosinophils are known to express seven heterodimeric integrins on their cell surfaces: α4β1 (CD49d/29), α6β1 (CD49f/29), αMβ2 (CD11b/18), αLβ2 (CD11a/18), αXβ2 (CD11c/18), αDβ2 (CD11d/18), and α4β7 (CD49d/β7) (53). Each heterodimer can interact with its own set of
ligands, although these ligands often overlap between the different integrins. A schematic representation of the different integrin to ligand binding combinations for eosinophils can be found in Figure 1-5.

![Integrin heterodimer expression on the cell surface of eosinophils](image)

Figure 1-5: Integrin heterodimer expression on the cell surface of eosinophils. Eosinophils express seven integrin heterodimers. Each heterodimer is involved in a variety of different functions. *Journal of Leukocyte Biology, 83, 1-12, 2008.*

Two of these integrin heterodimers, α4β1 (also called VLA-4) and αMβ2 (also called Mac-1), are likely the most important integrins to eosinophil trafficking and migration during asthmatic responses as antibody blockade studies of both these molecules prevents the generation of allergic responses in the lungs (55). Both of these molecules are present on purified blood eosinophils, however α4β1 is generally present in a more activated form than αMβ2 integrin (53). α4β1 integrin interacts with VCAM-1, which can be upregulated on lung endothelial cells by Eotaxin-1 and IL-5 during an allergic asthma response in order to induce eosinophil homing to the lung (Fig. 1-4) (56). Attachment to VCAM-1 by α4β1 integrin supports rolling, firm adhesion, and transendothelial migration of eosinophils in steady state as well as activation conditions (53). In contrast, αMβ2 integrin attaches to V-CAM-1 only for low-level static adhesion under homeostatic conditions and does not promote rolling or transendothelial migration (53). However, when an eosinophil becomes activated by IL-5, the conformation change of
αMβ2 integrin on the eosinophil surface allows this integrin to then play a role in eosinophil migration to the tissues by interacting more extensively with VCAM-1 and ICAM-1 (53).

Some adhesion molecules can also play a role in cell survival, in the case of the eosinophil, α4β7 interacts with MadCAM-1 under steady state conditions for static adhesion (53). It is found that cross-linking β7 results in increased viability of eosinophils, so this weak steady state interaction with MadCAM-1 may be important for eosinophil longevity in tissues (53). With flow, such as in the bloodstream, α4β7 interacts with MadCAM-1 and VCAM-1 to mediate rolling (53).

P-selectin glycoprotein ligand-1 (PSGL-1) is a non-integrin eosinophil molecule expressed exclusively on eosinophils that could also be important for eosinophil trafficking. PSGL-1 interacts with P-selectin on endothelial and epithelial cells (57). P-selectin knockout mice have reduced pulmonary eosinophil infiltration, indicating that this molecule may also be playing a role in eosinophil adhesion and migration into tissues (58). Another study using P-selectin deficient mice showed that eosinophils were defective in their ability to migrate to the peritoneum in response to ragweed allergen inoculation at that site, and more specifically were impaired in rolling and firm adhesion (59). Further research showed that P-selectin is integral to eosinophil recruitment in the early responses of pulmonary allergen challenge (53, 60).

**Eosinophil Functions In Allergy and Asthma**

Eosinophils are veritable chameleons with regard to their function in disease. They are able to present antigen to T cells, polarize T cells, activate mast cells, and secrete cytokines, chemokines, leukotrienes, granule proteins, and nerve mediators. They can respond to a variety of stimuli ranging from tissue injury to allergen inhalation and influence any number of other cells during the course of a response by secretion of inflammatory mediators. Eosinophils can
also play a role in tissue repair and remodeling. The eosinophil function in the context of allergic diseases is discussed below, but they are also capable of acting in many other types of diseases (2).

**Eosinophil granule proteins in allergy and asthma**

Eosinophils can act as primary effector cells inducing tissue damage and dysfunction by releasing toxic granule proteins (60). Eosinophils secrete several cytotoxic granule cationic proteins; major basic protein (MBP-1 and MBP-2), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN), that induce tissue inflammation, damage, and necrosis (61). Eosinophil granules contain a crystallloid core composed of MBP-1 (and MBP-2) and a matrix composed of ECP, EDN, and EPO (61). They secrete their granule proteins mainly by regulated exocytosis and piecemeal degranulation, a process by which eosinophils selectively release components of their specific granules (61). Small secretory vesicles densely packed in the cytoplasm of eosinophils also contain some of the same components as granules, and can be selectively released (61). Many of these secreted proteins are involved in allergic responses through various mechanisms of action outlined below.

**MBP**

MBP is one of the most highly cationic proteins synthesized by eosinophils, and is expressed as two different homologs, MBP-1 and MBP-2. MBP is a small protein that consists of single polypeptide chain of 117 amino acids and is among the most abundant proteins in eosinophils, making up as much as 50% of the cell’s mass (61). Many of the eosinophil’s classical roles in responding to parasitic diseases, such as helminth worm infestation, is due to the
release of extremely toxic MBP that causes tissue damage and necrosis in these organisms (62). However, MBP can also have damaging effects to human tissues in allergic responses as well (60, 62). Direct installation of MBP and EPO intratracheally causes both bronchoconstriction and AHR in the primate airway (61). MBP directly alters smooth muscle contraction during asthmatic responses by dysregulating vagal muscarinic M2, and causing increased acetylcholine release, subsequent M3 receptor activation, and bronchoconstriction (61). This ties into data from human asthmatic lung tissue samples that has shown eosinophils and extracellular MBP associated with nerve fibers in the airway smooth muscle. MBP has been shown to be cytotoxic to lung tissue, perturbing the cell-surface lipid bilayer by increasing membrane permeability through surface charge interactions, and may be partly to blame for tissue damage associated with eosinophil infiltration in the bronchial mucosa in asthma (63). MBP also causes degranulation of mast cells, basophils, and neutrophils, resulting in the propagation of the inflammatory response (64).

**EDN**

A second member of the RNase A multigene family, EDN, is expressed in eosinophils, although not exclusively as it can also be produced by mononuclear cells and neutrophils. It is less basic than ECP or MBP, and present in much lower quantity (about 10 picograms/cell compared to hundreds of picograms for MBP) (61). One purported function of EDN is mast cell survival near the nerve fiber endings in the lungs (65). It is possible that eosinophil secretion of EDN can induce chymase production by mast cells, which then stimulates production of the mast cell survival factor, SCF, by eosinophils and endothelial cells (65). Another function of EDN is that it may induce DC maturation during Th2 responses (66). Yang, et al, discovered that EDN is a potent chemoattractant for DCs, and that it also induces DC maturation and activation in a TLR-2/MyD88 signal dependent manner (66). DCs activated in this fashion can then stimulate
increased Th2 cytokine production (IL-5, IL-6, IL10, and IL-13) in response to airway allergen challenge (66).

**ECP**

ECP is another member of a subfamily of ribonuclease A multigenes expressed in eosinophils, with approximately 15–25 pg synthesized per cell in human eosinophils (61, 67). The amino acid sequence is 66% homologous to EDN, but its ribonuclease activity is 100 times less potent than EDN (67). ECP can participate in early and late stages of the allergic lung response in several ways. It can activate immunoglobulin synthesis by B cells, induce mast cell degranulation, and stimulate goblet cell mucus secretion in the earlier stages of allergic asthma, but it also has a role in the lung remodeling process (61). ECP has been shown to be involved in an intricate process by which growth factors for fibroblasts are released and signal to activate downstream remodeling processes (61).

**EPO**

EPO is a heme-containing haloperoxidase that has 68% sequence identity to neutrophil myeloperoxidase (61). EPO comprises about 25% of an eosinophil’s granule load. EPO seems to play less of a role in allergic asthma propagation, being more involved in bactericidal functions. However, this primary activity in which EPO catalyzes the peroxidative oxidation of halides (such as bromide, chloride, and iodide) can have secondary effects during the course of allergic response by causing generation of reactive oxygen intermediates that can increases lung tissue damage (61). In fact, eosinophils express high levels of the enzyme complex that generates superoxide (NADPH oxidase) (61), making an interesting corollary to research showing that prior
infection with some species of bacteria predisposes an individual to development of allergic asthma.

**Eosinophils as antigen-presenting cells**

One perspective that has been gathering momentum in recent studies in mouse models of allergic inflammation is that eosinophils play a larger and more pivotal role in the development of allergic disease than has been previously suggested. Surprisingly, many of these studies suggest that the actions of eosinophils propel T cell responses rather than being solely driven by them. Clinical evidence suggests that eosinophils are able to process and present a myriad of viral, parasitic, and microbial antigens, and adding antigen to T cell/eosinophil co-cultures stimulates enhanced T cell proliferation (68). Mature eosinophils stimulated with GM-CSF express MHC Class II on their cell surfaces, and they are also able to present soluble antigens to CD4+ T cells to promote proliferation and differentiation (68). Antigen presentation by eosinophils is an important event that is often overlooked, but recently has become of greater interest to researchers in allergy and asthma.

Eosinophils are becoming more recognized for their ability to present antigen in Th2 responses. Clinical evidence suggests that eosinophils are able to transiently express MHC Class II on their cell surfaces, as sputum eosinophils from asthmatics express this marker but peripheral blood cells from the same patients do not (68). Clinical and mouse research also found that eosinophils upregulate MHC Class II, CD40, and CD80/CD86 upon allergen exposure in the lung, as well as process antigen and migrate to lung draining lymph nodes where they presumably present antigenic peptides to T cells (69). In a model of *Strongyloides stercoralis* infection, eosinophils have been demonstrated to present antigen and promote Th2 differentiation of T cells in both primary and secondary T cell responses (70). Studies with *S. stercoralis* have also shown
that antigen-primed mouse eosinophils express high levels of MHC Class II and CD86, and induce both naïve and antigen-experienced CD4+ T cells to produce IL-5 in co-culture (70). In other co-culture experiments, eosinophils from an OVA airways challenge model cultured with *in vitro* polarized Th2 cells promote IL-4, IL-5, and IL-13 secretion and promote IL-5 production in co-culture with antigen-specific CD4+ T cells (68). These studies were dependent on CD80/CD86 upregulation on the eosinophil cell surface as monoclonal antibodies against these markers inhibited T cell cytokine production (68).

An important aspect of antigen presentation is carrying antigen to regional lymph nodes to activate T cells to amplify the immune response. Several lines of evidence suggest that eosinophils are capable of performing this function. Lung resident eosinophils exposed to antigen as well as eosinophils that have been antigen-loaded and instilled intratracheally are both able to traffic to draining thoracic lymph nodes as well as mediastinal nodes (71). This process is independent of eotaxin as both CCR3+ and CCR3− cells are capable of migrating to regional nodes, indicating that the signals that cause immature eosinophils to migrate into tissue are separate from those required to attract the activated antigen-activated cells (68). These migrating activated eosinophils also express much higher levels of MHC Class II and CD80/86 in the lymph nodes compared to the lung (72). Fluorescence microscopy has revealed that once in the draining lymph node, eosinophils are able to interact directly with antigen-specific CD4+ T cells (68). Most recently, Wang, *et al.*, has demonstrated that OVA-loaded eosinophils introduced intratracheally to the airways of mice adoptively transferred with naïve D011 OVA-specific T cells, traffic to the paratracheal lymph nodes to present antigen, induce activation and proliferation of naïve T cells, and also promote IL-4 cytokine production by these cells (72). All of these data provide evidence to contest the old argument made by van Rijt, *et al.*, who proposed that eosinophils play no role in antigen presentation and activation of naïve T cells in the draining lymph nodes despite trafficking there during an allergic response in the lung (73). These two
contrasting reports likely have much to do with the different isolation techniques used to procure the eosinophils. It has been shown that the common practice of treating cells with ammonium chloride to induce erythrocyte lysis, as was performed by van Rijt, et al, prevents lysosomal acidification and thereby antigen processing in antigen presenting cells (74).

Taken together, a new trend is emerging, which indicates that eosinophils have been previously underestimated in their capability of antigen processing and presentation, and that this function can be added to the ever-growing list of mechanisms by which eosinophils regulate the immune system.

**Mast cell activation by Eosinophils**

Eosinophils are able to secrete a variety of functional mediators that act on mast cells and induce them to degranulate during allergic responses. Major basic protein (MBP), a protein factor produced by eosinophils can promote histamine secretion by rat peritoneal mast cells, as well as a number of inflammatory cytokines, such as GM-CSF and TNF-alpha (75). Mast cells previously activated in an IgE-dependent manner can be reactivated to release histamine in an IgE-independent manner by exposure to eosinophil sonicate or to purified MBP, a component of eosinophil granules (64). Eosinophils can also be stimulated by mast cells to produce stem cell factor (SCF), an important mast cell growth and survival cytokine (64). Other granule proteins produced by eosinophils, ECP and EPO, have been shown to induce dose-dependent release of histamine from rat peritoneal mast cells (75). NGF secreted by eosinophils cannot only promote the health and survival of sympathetic neurons, but also aids in mast cell survival and activation (4).
Eosinophilia of the lung and airways has been observed in concurrence with other symptoms in humans as well as in animal models of allergic asthma, and has been regarded as a cardinal feature of asthmatic response. During an allergic asthma response, eosinophils are recruited to the lung by chemokines produced by the airway epithelium, such as, CCL11/CCL24, as well as cytokines such as IL-13 (36, 42, 51). Once in the lung, eosinophils can have a variety
of functions such as: antigen presentation, cytokine production, chemokine production, secretion of granule mediators, as well as leukotriene secretion (2). Eosinophils have the ability to secrete the same Th2-type cytokines as T cells, in addition to others. They can also produce histamine, much like mast cells, as well as their own unique products, for example, EPO. The absence of these cells has been shown to prevent long term remodeling of the lung in chronic mouse models of allergic asthma (76), but evidence has suggested an even more involved role in the initiation of allergic asthma.

Classically, eosinophils have been considered to be terminal effector cells in allergic diseases, mobilized en mass by T cell IL-5 production to sites of inflammation where they produce such products as cytokines (IL-4, IL-13), chemokines (CCL11, CCL24), and leukotrienes (LTC4, LTB4) (2). There have been hints that eosinophils have the potential to play a more centralized role in Th2 responses. Lee, et al, showed a defect in the development of airway inflammation in C57Bl/6 PHIL−/− (eosinophil deficient mice), indicating that eosinophils may potentially be more integral to the development of allergic asthma than previously thought (77). At the same time, Humbles, et al, in Balb/c ∆dblGATA mice showed that there was no effect at the level of AHR, but that eosinophils were required for long term remodeling in a chronic model of airway inflammation (76). Both groups used the classical model of OVA sensitization to induce allergic airway inflammation. Closer analyses of these strains and other eosinophil-impaired mice have revealed defects in different aspects of generation of Th2-type responses. Fulkerson, et al, used the same Balb/c ∆dblGATA mice as Humbles, et al, and in an Aspergillus fumigatus-induced model of allergic airway inflammation showed that although AHR is not reduced, there is a significant reduction in the production of IL-4 and IL-13, as well as mucus hypersecretion in the lungs of these mice (51). They also reported reduced IL-4, IL-5, and IL-13 production in ex vivo splenocyte restimulation, again suggesting that eosinophils may provide some sort of activation stimulus to T cells for Th2 cytokine production (51). These
results are similar to findings in IL-5/Eotaxin-1 deficient mice, whose Th2 cells lack the ability to produce IL-13 (39). This suggests that either eosinophils are stimulated through IL-5 or Eotaxin-1 to produce a factor that then acts on T cells to produce cytokines, or perhaps that these factors act directly on T cells to promote Th2 cytokine production.

More recently, striking new evidence supporting these findings has been reported. Studies in mice deficient in βc IL-3R, the common beta receptor for IL-3, IL-5, and GM-CSF that is required for development of eosinophils, basophils, and mast cells, show that inability to signal through this receptor results in reduction in airway hyperresponsiveness in these mice (28). These mice also fail to develop OVA-specific IgE responses and do not proliferate in antigen-specific ex vivo restimulation, suggesting a defect in T cell activation (28). Along with this, T cells from the peribronchial lymph nodes of allergic βc−/− mice have reduced production of Th2-type cytokines IL-4, IL-5, and IL-13, and these T cells are attenuated in their ability to migrate to the lung upon allergic provocation, as well as having reduced activation as denoted by CD69 expression (28). The caveat is that this lack of T cell function cannot definitively be attributed to the absence of signal-competent granulocytes as βc signaling is also important in myeloid dendritic cell development.

Of course, one must consider that the different backgrounds used in these mouse models would certainly contribute to the differences in the development of characteristics of allergic disease. There have been many discrepancies between mouse models and strains in the generation of allergic disease. For instance, IL-5−/− mice on the C57Bl/6 background, which have drastically reduced numbers of eosinophils, show abolition of many of the symptoms allergic airway disease in an OVA-induced model, whereas blockade of IL-5 in Balb/c mice indicates that this cytokine does not appear to have a regulatory role in this disease (35). Other examples of the reported difference in the importance of genes in disparate mouse strains are shown in studies of the contributions of IL-4 and IL-13 to development of this disease (14, 17, 78). Regardless, many
of these studies collectively suggest that eosinophils may regulate T cell generation of Th2 cytokines during allergic responses in the lung, playing a larger role in this process than was previously suspected.

**Eosinophils in lung remodeling during chronic asthma**

With the continued persistence of airway inflammation in chronic asthmatics, one symptom that occurs frequently is airway remodeling (79). It is not known why this occurs, but it is suggested that it is a defense mechanism in response to continuous inflammation of the lung. Airway remodeling consists of extracellular matrix deposition (ECM), which involves formation of new collagen fibers and proliferation of microvessels, and production of proteoglycan molecules such as tenascin, and lumican that can cause thickening, fibrosis, and heightened vasculature of the airways (80). The amount of airway smooth muscle also increases due to hypertrophy and hyperplasia, however, no cellular or molecular abnormalities in these cells have been identified (81). The end result of these events is decreased lung capacity and increased baseline airway resistance, decreasing the overall ability of an individual to breathe.

Eosinophils have a variety of functions in long term lung remodeling. They are a potent source of cytokines, such as TGF-β1, that are known to stimulate fibroblast transformation into myofibroblasts, an intermediary cell in smooth muscle differentiation, and are associated with ECM expression (80, 82, 83). In one *in vitro* model, eosinophils expressing TGF-β1 were temporally associated with fibroblast transformation to myofibroblasts and ECM expression (83). Neutralizing antibodies against TGF-β1 inhibited tenascin and procollagen I production (83). Eosinophils can also induce other cells, such as airway epithelial cells to produce TGF-β1 through release of cytotoxic mediators, such as MBP, that have been shown *in vitro* to induce epithelial detachment and ciliary dysfunction (83, 84). When this occurs, the epithelial cells
detect airway injury, and secrete TGF-β1 to stimulate tissue repair, but this also results in collagen and ECM production (84). Eosinophil granules, specifically ECP, have been shown to inhibit the degradation of proteoglycans by fibroblasts, causing a buildup of these factors that promote smooth muscle cell proliferation and production of collagen fibers (85). Other eosinophil-secreted cytokines can contribute to airway remodeling as well. IL-13 can induce fibroblast differentiation into myofibroblasts with activation of collagen production (86). IL-13 also promotes goblet cell hyperplasia and mucus secretion, as well inducing the production of TGF-β (86).

Studies performed in eosinophil deficient mouse strains have demonstrated the importance of eosinophils to lung remodeling in vivo. The ΔdblGATA mutant mice on a Balb/c background were protected from airway collagen deposition and increases in airway smooth muscle (76). IL-5 gene deletion also reduced some of the characteristics of remodeling; peribronchial fibrosis, collagen III, collagen V, and total lung collagen content (87). As in the ΔdblGATA mutant mice, peribronchial smooth muscle thickness was also reduced, as was mucus hypersecretion(87). In contrast, hypereosinophilic IL-5 transgenic mice developed increased airway fibrosis after challenging the lungs with allergen (38). Studies in humans using anti-IL-5 antibodies also support a role for eosinophils airway remodeling (88). Administration of this antibody resulted in 90% reduction in blood and BALF eosinophils, while only reducing tissue eosinophils by half (22). However, several of the mediators associated with ECM deposition and fibrosis, tenascin, lumican, and procollagen III, were drastically reduced compared with placebo (22). The number of eosinophils expressing TGF-β1 RNA was reduced, as was overall protein TGF-β1 in the BALF (22). These clinical and laboratory research studies indicate an integral role for eosinophils in long term lung remodeling in asthmatic patients and remain a primary target for drug development.
As stated previously, most of the functions of eosinophils outlined above are end stage effector functions. However, it is possible that eosinophils may be important earlier in the generation of allergic asthma. Some clues outlined above suggest that this may be the case, either in the role of antigen presentation, or perhaps they are able to secrete a factor that acts as a differentiation or migration signal for T cells. There is some evidence to suggest that eosinophils may produce or induce production of IL-13, a very potent inducer of allergic asthma, that could activate or recruit T cells in the initial development of the late phase of allergic asthma.

**Interplay between eosinophils, IL-13, and T cells in allergic asthma**

Eosinophils and IL-13 are components of allergic asthma pathogenesis that have been targeted for decades by clinical and laboratory researchers in an effort to produce therapeutic agents to counteract damaging effects to the lung. However, current clinical antibody therapies targeting inhibition or neutralization of some of these factors individually have met with minimal success. It could be that the relationship between eosinophils and T cells as well as the cytokines they secrete may be more complex than originally thought, and that targeting just one of these components is not enough to counteract the symptoms of allergic asthma. Both eosinophils and T cells are able to produce IL-13, a cytokine whose effects on the lung are wide-ranging and severe. It is certainly a cytokine that bears further scrutiny, especially in how it affects initiation of allergic asthma and the interplay between eosinophils and T cells.
**IL-13 in asthma pathogenesis**

IL-13 is a cytokine that was originally cloned from activated T cells. The gene is located on the human chromosome 5q31, the same chromosome that encodes genes for IL-4, IL-5, and GM-CSF (89). The IL-13 receptor is a heterodimer composed of an IL-4 receptor alpha chain (also utilized by IL-4 receptors), as well as an IL-13 receptor alpha chain (89). Because their receptors share signaling components, IL-13 and IL-4 cytokines have similar functional properties as well. However, IL-13 does have some non-overlapping functions with IL-4 and seems to be more integral to sustaining allergic asthma pathogenesis in the long term (89).

Numerous studies of bronchial biopsy specimens and BALF from allergic individuals have shown that both message and protein levels of IL-13 are elevated when compared with those of control subjects, although IL-4 upregulation seems to be more transient (90). Conversely, patients on steroid treatment or receiving injections for allergen desensitization have reduced levels of IL-13 (91).

IL-13 is capable of stimulating many different gene families (i.e. cytokines, chemokines, adhesion molecules, and MMPs) in order to orchestrate Th2-mediated inflammatory responses (Fig. 1-7) (92). The combinatorial effects that result from the induction of these different factors facilitate the selective recruitment, homing, and activation of a number of inflammatory cells, such as eosinophils and T cells, from the bloodstream into the airway spaces by inducing the expression of VCAM-1 (6). Secondly, IL-13 induces a multitude of chemokines, including, but not limited to: MDC, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, MCP-5, Eotaxin-1, Eotaxin-2, macrophage inhibitory protein-1α (MIP-1α), and thymus- and activation-regulated chemokine (TARC) (89). These chemoattractants recruit various cell types from the bloodstream into the airways, some perhaps synergistically or redundantly, and can also establish gradients in the tissue for movement of cells between different lung compartments.
Another class of mediators induced by IL-13 that has become increasingly associated with cellular inflammation in allergic asthma is the MMP family of proteases. IL-13 is able to induce the expression of several MMPs in murine lungs, including MMP9 and MMP2 (93). MMPs have mainly been studied for their extracellular matrix degrading activity, but, although still considered controversial, research in allergic diseases has shown that they may also modulate inflammation by contributing to the establishment of transepithelial chemokine gradients through proteolytic activity on cytokines, chemokines, and growth factors (93).

Whatever its function in allergic asthma, it seems that IL-13 can induce many mediators that are integral to the development and exacerbation of this disease. It, as well as the cells that produce it, continue to be important clinical targets. However, as it is not clear whether IL-13 facilitates different aspects of the disease depending on which cell is producing it at any given time, further study is needed to see how best to approach a clinical strategy for ameliorating the effects of this potent cytokine.
Figure 1-7: IL-13 functions in allergic asthma. IL-13 is involved in several processes during the generation of allergic asthma. It can induce class-switch in B cells to the IgE isotype, induce high expression of FceRII on the mast cell surface, as well as promote mucus hypersecretion from goblet cells and eotaxin release from airway epithelium. IL-13 is also able to induce steroid resistance, but can be blocked by antibodies to IL-13R2 or by inhibitors against STAT-6. *Nature Reviews Drug Discovery*, 3, 831-844, 2004.
IL-13, eosinophils, and T cells

IL-13 is involved in a tangled web of many different components of the allergic asthma immune response. Besides being involved in immune cell recruitment and chemokine induction, IL-13 can also induce production of other cytokines, such as IL-4, and act directly on smooth muscle cells to induce bronchoconstriction (94). It seems that perhaps IL-13 is involved in many complex mini-networks that involve disease induction, perpetuation, and modulation depending on when it is being produced and what cell type is producing it. It is known that eosinophils are able to produce IL-13, but not what the specific contribution of eosinophil-produced IL-13 is to allergic asthma. However, there is evidence to suggest that eosinophils either stimulate secretion of this cytokine or produce it themselves (2).

Much research has been conducted that supports the hypothesis that IL-13 recruits eosinophils to the lungs during asthma pathogenesis. IL-13 is a potent inducer of chemokines Eotaxin-1 and Eotaxin-2 in endothelial tissues, which promote recruitment of eosinophils (16, 17). Transgenic mice that over-express IL-13 in the lung produce elevated levels of Eotaxin-1 (86), and IL-13 can also synergize with IL-5 to selectively induce eosinophil chemotaxis (45). Besides recruiting eosinophils, IL-13 can also promote survival and activation of eosinophils directly by interacting with IL-13R-alpha on the eosinophil cell surface, and also indirectly by stimulating T cells to produce IL-5 (95).

There may also be a role for eosinophil-derived IL-13 in allergic asthma. Eosinophils contain preformed message for IL-13 and are capable of rapidly releasing IL-13 from secondary granules upon stimulus with CCR3 ligands Eotaxin-1 and Eotaxin-2 (20, 96). Simultaneous delivery of Eotaxin-2 and IL-5 to the lung induces significant production of IL-13 in the BALF (49). Knockout mouse models also lend integrity to the proposal that eosinophils influence IL-13 production in the lungs. IL-5/Eotaxin-1 double deficient mice have synergistic impairment of
eosinophil and T cell recruitment as well as a drastic reduction in IL-13 production, even when immunized under Th2-polarizing conditions (39). Impairment in AHR can be rescued by administration of recombinant IL-13 to the lung, but not by IL-13+/− T cells (39). Adoptive transfer of wildtype eosinophils to IL-5/Eotaxin-1+/− mice restores Th2 IL-13 production, indicating that eosinophils are able to either induce T cells to produce IL-13 by secreting IL-13 or another stimulatory factor (39). Other knockout models also suggest that eosinophils are integral directly or indirectly to IL-13 production. Fulkerson, et al, observed a reduction in IL-13 levels in the BALF of ΔdblGATA mutant mice, which lack eosinophils, in a model of A. fumigatus-induced asthma (51). IL-4 receptor alpha/IL-5+/− mice have impaired production of IL-13, reduced AHR, and very little recruitment of eosinophils to lungs (20), whereas IL-4 receptor alpha−/− mice alone have only partial impairment in these characteristics of disease (97). As IL-5 activates eosinophils and promotes their survival, it is possible that these cells are partially responsible for IL-13 production and development of AHR in the IL-4 receptor alpha/IL-5−/− double knockout mice (98). Collectively, these studies suggest that eosinophils could be a potent source of IL-13 early on in disease, before large scale T cell recruitment, as well as a source of IL-13 later in pathogenesis, making the eosinophil, along with IL-13, an attractive target for clinical therapy.

Targeting the effects of multiple cells in allergic disease as a clinical strategy can prove to be quite complex. Combining antibody therapies to target IL-4, IL-5, and IL-13 could be costly and not guaranteed to be efficacious on their own without also depleting stores of Th2 cells, a daunting task as these cells would be extremely difficult to target without compromising T cell responses in other diseases. Thus, other strategies must also be explored, such as creating a small molecule inhibitor that could target eosinophil and Th2 cell function. T cells and eosinophils express many of the same proteins that are used as components of their intracellular signaling cascades during activation. One potential molecule that Th2 cells express that
eosinophils also express is IL-2 inducible T cell kinase (ITK). It has been shown that mice lacking this kinase have T cells that are unable to differentiate into Th2 cells, while other immune responses involving T helper 1 (Th1) responses remain intact. If ITK is also important to eosinophil activation and cytokine production during the course of an allergic response, ITK may be a good clinical target that could simultaneously wipe out the undesirable effects of both eosinophils and T cells.

**ITK in allergic diseases**

**ITK structure and function**

ITK is a seventy-two kilodalton nonreceptor tyrosine kinase that was first discovered in T cells, but it is now known that it is also expressed in mast cells, eosinophils, NKT cells, NK cells, and macrophages (99, 100). It is a member of the Tec Kinase family (other members are Btk, Txk, BMX and Tec), a family characterized by presence of a Tec kinase domain, SH2 and SH3 domains, as well as a variable TH domain (Fig. 1-8) (100). ITK is best characterized in CD4+ T cells where its expression can differentially regulate Th1 and Th2 differentiation (100). Upon TCR stimulation, ITK can phosphorylate phospholipase c gamma-1 (PLCγ–1), which leads to intracellular calcium mobilization, actin reorganization, activation of the mitogen-activated protein kinase (MAPK) cascade, and nuclear translocation of NFAT (101-103). In the absence of ITK all of these functions are impaired, as well as reduced activation of AP-1 and NFκB (101-103). The ITK signaling cascade is depicted in Figure 1-9.
Itk is also activated downstream of other receptors on the T cell surface that play a role in a variety of functions. It is known that ITK is activated downstream of CXCR4, a receptor which is important in chemotaxis of T cells (104). ITK<sup>−/−</sup> T cells have reduced chemotactic abilities in response to CXCL12 (the ligand for CXCR4) as well as a number of other chemokines, indicating that CXCR4 probably isn’t the only chemokine for which ITK signaling is integral (99, 104, 105). It has also been shown that ITK signals downstream of CD2, IL-2 receptor, and CD28, and some adhesion molecules (99, 106, 107).

Figure 1-8: Structure of Tec Kinase family members. ITK contains a kinase domain, Src Homology 2 and Src Homology 3 domains that facilitate interaction of ITK with other signaling molecules, a Tec homology domain, and a Pleckstrin Homology domain that is responsible for recruitment of ITK to the cell membrane. *Nature Reviews Immunology, 5, 284-295, 2005.*
One of the most critical functions of ITK takes place early on in the generation of the T cell. It has been shown that ITK is expressed at high levels in the thymus, the site of T cell selection. In the absence of ITK, the number of CD4$^+$ T cells generated in the thymus is drastically reduced to a 1:1 ratio of CD4:CD8 T cells rather than the normal 2:1 ratio. This defect is attributed to ITK being involved in the positive selection of T cells (108).

Figure 1-9: ITK forms a complex with several signaling molecules on the scaffolding protein LAT and SLP76. ITK activates PLC-γ results in downstream release of intracellular Ca$^{++}$ stores and activation of MAPKs. This leads to activation of gene transcription that controls T cell polarization, activation, and migration. *Nature Reviews Immunol.*, 5, 284-295, 2005.
ITK is also important in the differentiation of mature CD4+ T cells after they leave the thymus. Normally, upon antigen stimulus CD4+ T cells can differentiate into Th1 T cells capable of secreting high quantities of IFN-γ for fighting many bacterial and viral infections, or Th2 T cells which secrete IL-4, IL-5, and IL-13 important in parasitic and some bacterial infections. Cytokines produced by dendritic cells as well as other cells can skew CD4+ T cells toward Th1 T cells (IL-12 and IFN-γ) or Th2 T cells (IL-4). However, in the absence of ITK CD4+ T cells have a profound defect in the generation of Th2 T cells, even under polarizing conditions, and effector cytokine production (103, 108, 109). This inability of T cells lacking ITK to differentiate into Th2 T cells impairs the immune response to parasitic and some bacterial infections and exhibits a protective effect in the generation of allergic asthma. These effects and others are discussed in the next section.

**ITK in immune responses to Th2-type disease**

There is some clinical evidence to associate ITK with allergic responses. Patients with severe cases of atopic dermatitis display high numbers of circulating Th2 T cells in their bloodstream. T cells purified from the peripheral blood of these patients displayed elevated ITK mRNA levels compared to healthy controls (110). Expression levels of ITK are also correlated with disease severity as patients with higher levels of ITK also exhibited greater disease severity (110). Another clinical study examined the effects of SNPs in ITK on development of atopy (allergic hypersensitivity), eczema, and asthma. While the study found no linkage between SNPs in ITK with eczema or asthma in their patient clusters, there was a significant role for a SNP in the 5-prime region of ITK in atopy (111). This does not necessarily exclude the possibility that ITK is associated with other forms of allergic disease as there are many other genes, such as SLP-76, ZAP-70, or cyclophilin A that could cause deregulation of ITK. In fact, a murine model of
cyclophilin A deletion shows upregulation of ITK activity and overproduction of Th2 cytokines (112).

Most data connecting ITK with allergic disease has been generated from research using murine models. ITK−/− mice have a significant defect in their ability to mount protective Th2 responses against extracellular parasites. For instance, in a *Schistosoma mansoni* model of infection, ITK−/− mice exhibit low levels of T cell and eosinophil migration to the lung and reduced production of Th2 cytokines IL-4, IL-13, and IL-5 (113). ITK−/− mice also have reduced Th2 responses to *N. brasiliensis* infection (103).

There are several lines of evidence to indicate that ITK has a multi-varied role in the pathogenesis of allergic asthma. ITK−/− mice have ablated symptoms of the disease, including reduced AHR, chemokine and Th2 cytokine production, T cell infiltration of the airways, reduced mucus hypersecretion, eosinophilia, and airway inflammation (109). These reduced disease symptoms are due partially to the lack of infiltrating Th2 T cells into the lungs but may also be attributed to other factors. T cells from sensitized mice that are restimulated with antigen *ex vivo* have reduced proliferation and production of Th2 cytokines, indicating that if T cells were able to get into the lungs, they still may not be able to mount a response (Fig. 1-10)(109).
Figure 1-10: ITK−/− T cell function in murine models of allergic asthma. Th2 effector responses in lung-allergy models in wild-type and Itk−/− mice. (a) In wild-type mice, allergens or pathogens enter the lungs through the airways (i), where they encounter dendritic cells (light blue) (ii). Dendritic cells migrate to the lymph nodes (LN) or spleen (SP) and activate CD4+ T cells (green) (iii). Activated CD4+ T cells proliferate (iv) and induce rapid Th2 gene transcription (Txn) (v). Th2-committed CD4+ T cells then migrate to the B-cell area of the secondary lymphoid organ, where they produce IL-4 and other Th2 cytokines to induce B-cell activation, proliferation, and Ig class switching (vi). Additionally, activated CD4+ T cells migrate to the affected site (vii), where they produce IL-4 and other Th2 cytokines (viii) and induce the recruitment of eosinophils (dark blue) (ix), resulting in the symptoms of asthma (including lung inflammation, mucus production, etc.). (b) In Itk−/− mice the initial steps of presentation (i–iii) are similar, but proliferation of the activated CD4+ T cells is impaired (iv). Rapid Th2 gene transcription occurs (v) but, following migration to the B-cell area of the secondary lymphoid organ (vi), activated CD4+ T cells are unable to produce Th2 cytokines efficiently. However, B-cell activation, proliferation and Ig class switching still occur, indicating another source of Th2 cytokines in Itk−/− mice. Activated Itk−/− CD4+ T cells migrate to the affected site (vii), but in reduced numbers, owing to impaired proliferation or a partial defect in migration. CD4+ T cells at the affected site express Th2 cytokine mRNA but lack the ability to produce protein (viii), causing decreased eosinophil recruitment (ix) and a negligible asthma response (reduced lung inflammation, reduced mucus production, etc.).

T cells are not the only cells affected in the allergic asthma response. ITK−/− mast cells have reduced allergen/IgE-induced histamine release in vivo (114). This is due to induced inactivity of mast cells by saturation of FceR on the cell surface with IgE, as these mice have extremely elevated levels of this immunoglobulin (114). ITK−/− mast cells cultured in vitro are able to degranulate and secrete histamine similar to WT mast cells, and when these cultured ITK−/− mast cells are transferred to mast cell deficient mice they are able to degranulate and induce AHR (114). Finally, a study showed that ITK−/− mice have reduced tracheal responses to cholinergic challenge in vitro before as well as after allergen challenge (115). This study also confirmed that these mice have reduced AHR and showed that this defect could be rescued by transferring wildtype splenocytes or purified wildtype CD4+ T cells back into these mice (115).

No one has yet explored the role of ITK in eosinophil signaling and function, although these cells have numerous functions in Th2 responses. Eosinophil-produced IL-4 is important in parasitic responses, and it is possible that one reason ITK−/− mice were unable to clear these types of infections is due to lack of eosinophil degranulation and IL-4 production. It is also possible that attenuation of eosinophil action in allergic asthma responses could be another factor in protection of ITK−/− mice from development of this disease. These questions and others remain to be answered regarding the role of ITK and eosinophils in pathogenesis of allergic asthma.
Chapter 2

Materials and Methods

Mice

Wild-type C57BL/6 and Balb/c (The Jackson Laboratory, Bar Harbor, ME), Balb/c and C57BL/6 ΔdblGata1 (backcrossed to the C57BL/6 background for 7 generations, kind gift of Drs. Stuart Orkin, Craig Gerard and Allison Humbles, Harvard Medical School)(26), and C57Bl/6 and Balb/c IL-5 transgenic mice ((34), kind gift of Drs. Jamie and Nancy Lee, Mayo Clinic, Arizona, and Allison Humbles, Medimmune) as well as C57Bl/6 IL-13−/− and ITK−/− IL-5 transgenic mice and C57Bl/6 IL-13−/− mice. Mice were kept in microisolater cages in the animal facilities at Pennsylvania State University, and provided with food and water ad libitum. All experiments were approved by the Office of Research Protection’s IACUC at The Pennsylvania State University.

Ovalbumin-induced allergic asthma model

Groups of mice (WT or ΔdblGATA) were primed with OVA (Sigma-Aldrich, St. Louis, MO) or vehicle control (PBS) as follows: Days 0 and 5, mice were primed with 220 ul of 50ug/ml OVA complexed to alum. Days 12-15, mice were challenged intranasally with 40 μl of 2 mg/ml OVA in PBS. In experiments where ΔdblGATA mice received eosinophil transfers, ΔdblGATA mice received 1.5X10^6 eosinophils i.v.on day 11 or 12, and were then challenged with OVA on day 12 at least 6 hours after eosinophil transfer. In some experiments, ΔdblGATA
or WT mice received 0.75 µg CCL11/Eotaxin-1 or 2 µg of recombinant IL-13 combined with the normal dose of 40 µl OVA on days 12-15. WT and ΔdblGATA mice primed with OVA/alum and intranasally challenged with PBS were used as controls in these experiments.

**Determination of AHR and analysis of airway inflammation**

Airway responsiveness was assessed using a Buxco whole body plethysmograph (Buxco Electronics, Inc., Wilmington NC.). Conscious mice were placed into a chamber of the plethysmograph (Buxco Electronics, Sharon, CT), and respiratory parameters in response to methylcholine (0-100 mg/ml in PBS, Sigma-Aldrich, St. Louis, MO) as previously described (41). Airflow obstruction was expressed as enhanced pause (Penh). To determine airway inflammation mice were euthanized and lungs collected, fixed in paraformaldehyde, and sectioned and stained with H&E to detect cellular infiltration or PAS to detect mucous (performed by the Animal Diagnostic Laboratories, The Pennsylvania State University). Alternatively, airway hyperresponsiveness was determined using a custom made mechanical ventilator (42) Mice were anesthetized with a 50-100 µl intraperitoneal injection of sodium pentobarbital, then a canula was inserted into the trachea, tied in place, and the mouse was placed on a platform attached to a mechanical ventilator. Mice were ventilated at 120 breaths/min, VT=0.2ml, flow rate 1.5 ml/sec at 2-3 cm H2O PEEP, and airway pressure in response to methylcholine (0-100 mg/ml in PBS, Sigma-Aldrich, St. Louis, MO) was determined using a differential pressure transducer. Results were recorded using a Powerlab 4/SP data acquisition system and accompanying software, and were reported as respiratory system resistance (Rrs, cmH2O/ml/sec). Alternatively, the animals were anesthetized with pentobarbital sodium (70 mg/kg ip, 65 mg/ml diluted 1:2 in saline. Tracheostomy was then performed, and a metal cannula tube (20-gauge needle, 0.050 in., 4 mm long; Small Parts, Miami Lakes, FL) was inserted into the
trachea. The cannula was attached to a tube leading to a three-way connector (Small Parts), with one port leading to a PEEP trap and Flexivent sensor and the other port leading to a nebulizer (Porta-Sonic model 8500C, DeVillbiss Health Care, Somerset, PA) for methylcholine challenge. The animals were mechanically ventilated with a tidal volume of 8 ml/kg and a frequency of 2.5 Hz. Positive end-expiratory pressure (PEEP) of 2-4 cmH2O was applied by placing the expiratory line of the ventilator under water (116).

**Adoptive transfer of eosinophils**

Peritoneal eosinophils were obtained from IL-5 transgenic mice by peritoneal lavage with RPMI media, washed 2 times in 1XPBS and sorted by negative MACS magnetic bead selection with antibodies against CD3, CD4, CD8, CD25, B220, Fcε, CD11c, and F4/80. Cells were washed two times with 1XPBS + 2% FBS at 250 X G. 1.5X10^6 cells were then resuspended in 100 µl 1X PBS and injected intravenously through tail vein or eye vein into ΔdblGATA mice. Typical purity was 85% as determined by CCR3 and Gr-1 double positive flow cytometric analysis.

**Determination of T cell recruitment into the lungs and BALF**

BALF was collected from lungs of mice in 1 ml 1X PBS using a flexible canula and syringe. Cells were spun down at 250 X G and supernatant collected and frozen for cytokine analysis, and cells either analyzed on an Advia Blood Analyzer for cell differentials or stained with antibodies against CD4 and CCR3 for detection of T cells and eosinophils. In other mice, whole lungs were collected, chopped with a razor blade, and dissociated for 40 minutes at 37 degrees Celsius with collagenase or Liberase (Roche), using 1 ml of a 10 mg/ml solution or 100
µl of a 10 mg/ml solution respectively in 5 mls of HBSS w/ Ca\(^{++}\) and Mg\(^{++}\). Immediately after incubations, tubes were filled to the top with 1XPBS + 2% FBS and spun down at 300 X G, and then washed two more times. Remaining lung pieces were filtered through nylon mesh, resuspended in 1 ml of 1XPBS + 2% FBS, and either enumerated on the Advia Blood Analyzer or hemocytometer. Cells were then stained for flow cytometry or centrifuged for RNA analysis.

**In vivo eosinophil migration assay**

IL-5 transgenic eosinophils purified from mouse peritoneal cavity were labeled with CFSE as follows. Cells were resuspended at 10 X 10\(^6\) cells/ml in HBSS w/ Ca\(^{++}\) and Mg\(^{++}\), and 1 µl of 5µm CFSE per ml of cells was added to solution. Tubes were immediately inverted several times to mix and incubated in the dark at 37 degrees Celsius for 10 minutes. Cells were then washed 2 times with 10 mls of 1XPBS + 2% FBS for 5 minutes at 300 X G. Cells were resuspended at 10X10\(^6\) cells per 100 µl, and 100 µl of cells were injected intravenously by intraorbital or tail vein injection of ΔdblGATA mice that had been challenged intranasally with 40 µl of 2 mg/ml OVA one day prior to iv injection. Mice were then given another intranasal challenge and allowed to rest for 12 hours before they were sacrificed and tissues analyzed for presence of CCR3\(^{+}\)CFSE\(^{+}\) cells. Again, this dye is light sensitive and care was taken in all steps to minimize exposure to light.

**In vitro differentiation of Th2 OT-II T cells and transfer to ΔdblGATA or WT mice**

OT-II cells from lymph nodes were excised and processed into a single cell suspension under sterile conditions. Cells were cultured at 1 X 10\(^6\) cells/ml in RPMI complete medium with the addition of IL-4, anti-IFN-γ, and 10 µg/ml OVA for 5-6 days, changing the media one time
and adding media as needed to maintain proper concentration of cells. Cells were washed in 1XPBS twice and an aliquot taken for RT-PCR analysis of Th2 cytokine production. Supernatant was kept to analyze protein levels of Th2 cytokine expression. 10 X 10⁶ differentiated T cells were transferred to naïve WT or ΔdblGATA mice in 100 μl of 1X PBS by intraorbital injection. In some experiments, ΔdblGATA mice received OT-II cells and either WT or IL-13⁻/⁻ eosinophils as well. In this case, 5 X 10⁶ eosinophils were injected intravenously by intraorbital injection, and OT-II Th2 cells were injected intraorbitally to the other eye to keep the cells from interacting before they were injected into the mice. The same day, mice were given an intranasal OVA challenge of 40 μl of 2 mg/ml OVA in PBS, followed by three more days of consecutive intranasal OVA challenge. On the day after the last OVA challenge, mice were analyzed by mechanical ventilation to determine AHR as described above, lungs taken for sections, cellular infiltrate analysis, and RNA.

**Analysis of cytokine and IgE levels**

BALF (concentrated using Amicon Centricon filters from Millipore) or supernatants from T cell cultures were analyzed for levels of IL-4, IL-5, and IL-13 by a Luminex multiplex bead system kit (Lincoplex) as per manufacturer’s instructions, and analyzed on a Biorad Bioplex system. For serum analysis, blood was collected from mice, spun down and serum removed. Serum levels of total IgE and OVA-specific IgE were determined by ELISA as previously described (40).
In vitro restimulation of T cells for cytokine production analysis

To determine whether T cells were capable of generating a recall response following secondary restimulation, 2X10^5 spleen cells were cultured in vitro with 100 µg/ml OVA for 36 hours. One hundred ng/ml PMA/0.5 µm Ionomycin was used as a control. Cells were spun down and supernatant collected and frozen at -80 degrees Celsius for later Bioplex analysis of Th2 cytokines.

Quantitative RT-PCR analysis of gene expression

RNA was isolated from lung tissue using Trizol reagent (Invitrogen Life Technologies). Total RNA (5 µg) was reverse transcribed to cDNA with random primers using the Ready-To-Go You-Prime First Strand Beads following manufacturer’s instructions (Amersham Biosciences). PCR was performed in triplicate with commercially available primers and FAM labeled probes (Assays on DemandTM, Applied Biosystems). Relative quantification against the reference gene GAPDH was performed using the Comparative CT value (threshold cycle) method, which uses the arithmetic formula 2^-ΔΔCT value.

Analysis of T cell proliferation

To determine T cell responses following allergic airway induction, 2X10^5 spleen cells were cultured in vitro with 100 µg/ml OVA for 36 hours. One hundred ng/ml PMA/0.5 µm Ionomycin was used as a control. After 2 days in culture, cells were pulsed with 3H-thymidine for 16 hours, harvested, and read on a beta-scintillation counter.
Analysis of ITK signaling downstream of CCR3 receptor in eosinophils

Eosinophils were purified from the peritoneum of IL-5 transgenic mice as described previously. As IL-5 transgenic mice have high levels of IL-5 in circulation that could affect eosinophil signaling, eosinophils were rested for an hour on ice in 1XPBS + 2% FBS to allow for cells to return to baseline. Eosinophils were then stimulated with 10 ng/ml of Eotaxin-1 in RPMI 1640 in a time course of 0, 1, 5, 10, 15, 30, and 60 minutes. Cells were washed immediately after each time point, spun down, and cell pellet frozen at -20 degrees Celcius or immediated lysed for 15 minutes on ice in lysis buffer containing: 50 mM Tris HCl, 150 mM NaCl, 2 mM EDTA, 50 mM Sodium pyrophosphate, .5% Triton X 100, 2 mM sodium vanadate, 50 mM sodium fluoride, 1mM PMSF, 2 µg/ml leupeptin, and 2 µg/ml aprotinin. Cells were spun down at 4 degrees Celsius for 10 minutes at 8,000 X G, supernatant collected, and protein quantified. Twenty micrograms of protein/sample were reduced for 10 minutes at 100 degrees Celsius with 2X SDS-PAGE reducing buffer. Reduced samples were then run on an SDS-PAGE gel and electrophoretically transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA). Membranes were blocked with 2% OVA solution (for detection of phosphorylated proteins) or 5% milk (for non-phosphorylated proteins) in TBS-Tween overnight at 4 degrees, or 1 hour at room temperature, then washed in TBS/Tween, and incubated consecutively with primary antibody and HRP-conjugated secondary antibody, washing between steps. Immunoreactive proteins were visualized by enhanced chemiluminescence reagents (ECL, Amersham) and visualized by Xray film. Antibodies used in these studies were anti-Erk/phospho-ERK, anti-p38/phospho-p38, anti-JNK/phospho-JNK.
Chapter 3

Strain specific requirement for eosinophils in the recruitment of T cells to the lung during the development of allergic asthma

Introduction

Eosinophilia of the lung and airways has been observed in concurrence with other symptoms in models of allergic asthma, as well as in humans, and has been regarded as a cardinal feature of asthmatic responses (117, 118). However, the importance of eosinophils to the generation of allergic asthma has remained ambiguous despite the quantity of research that has been performed on the subject. The cytokine IL-5 has been shown to be important for eosinophil development (88, 117) and levels are elevated during asthmatic responses, but studies in IL-5 deficient mice, which have reduced numbers of eosinophils, have yielded inconsistent results. On a C57/Bl6 background lacking IL-5, airway hyperresponsiveness (AHR) is abolished (2, 14), whereas, on the Balb/c background, AHR is either affected or not, dependent on the model used, perhaps due to residual numbers of eosinophils that may remain in the lungs (2, 21, 119). These studies suggest that in addition to IL-5, other factors may be required for regulation of eosinophils, and that perhaps Eotaxins, including Eotaxin-1, a chemokine that attracts eosinophils to sites of inflammation, may need to be blocked in combination with IL-5 in order to counteract the function of eosinophils in the lungs (39, 88).

T cells, in particular IL-4, IL-5, and IL-13 producing Th2 cells, have been shown to be important in allergic asthma, as introducing antigen specific Th2 cells followed by antigen challenge is sufficient to cause AHR (6, 39). Administration of Th2 cytokines IL-4, IL-5, or IL-13 independently can also induce AHR (17, 19, 20). Evidence from mouse models suggests that IL-13 is necessary for mucous hypersecretion and AHR, and has also been shown to aid in eosinophil induction by Eotaxin-1 and IL-5 dependent mechanisms (16, 19, 39). The relationship
among these three factors is still under investigation, but studies in double transgenic mice lacking IL-5 and Eotaxin-1 have shown a defect in T cell IL-13 production (39).

Most recently, two research groups published conflicting data on the importance of eosinophils to the development of this disease. Using a transgenic cell ablation approach on a C57Bl/6 background, Lee and colleagues found that eosinophils are integral to the development of airway inflammation and airway hyperresponsiveness (77). In contrast, Gerard and colleagues used the ΔdblGATA mutant mouse, which lacks eosinophils on a Balb/c background and determined that the absence of eosinophils did not protect mice from AHR development in an acute model of allergic inflammation, but are required for extensive airway remodeling (76). It is possible that different backgrounds have dissimilar responses as has been observed for other genes, such as IL-4 and IL-5, and the development of allergic asthma (14, 20). Here we have performed a more detailed analysis of the ΔdblGATA mice on C57Bl/6 and Balb/c backgrounds. Our results show that the hallmarks of allergic asthma, including T cell infiltration of the lungs, Th2 cytokine production, and chemokine production are reduced in C57Bl/6 ΔdblGATA mice. Also unique to our study, we reconstituted ΔdblGATA mice with eosinophils to determine whether the characteristics observed were indeed due to these mice lacking eosinophils and determined that eosinophils are required for T cell infiltration as well as cytokine production in the lungs during allergic airway responses in C57Bl/6 mice. Finally, we show that intranasal delivery of CCL11/Eotaxin-1 rescued T cell recruitment and the development of AHR in C57Bl/6 ΔdblGATA mice.
Results

**Eosinophils are required for the development of airways hyperresponsiveness and lung inflammation in C57Bl/6 but not Balb/c allergic asthma.**

We used OVA in a standard sensitization protocol in WT and ΔdblGATA mutant mice to induce allergic airway inflammation. Twenty-four hours after the last intranasal challenge, mice were subjected to mechanical ventilation for analysis of airway hyperresponsiveness (AHR). We found that WT mice on both C57Bl/6 and Balb/c backgrounds as well as Balb/c ΔdblGATA mice showed an increase in AHR by mechanical ventilation, whereas C57Bl/6 ΔdblGATA mice did not show a significant increase in this parameter by mechanical ventilation or full body plethysmography (Figs. 3-2A,2B, 3-3A). Analysis of lung sections from these mice stained with H&E showed that both groups of WT mice and Balb/c ΔdblGATA mice exposed to OVA intranasally had elevated airway inflammation and epithelial hyperplasia (Figs. 3-1A, 1B, 3-3B). In contrast, lungs from the C57Bl/6 ΔdblGATA mice had significantly reduced inflammation (Figs. 3-1A, 1B). To determine if inflammation was accompanied by goblet cell mucous production in the airways, we analyzed similar sections stained with PAS. Again, as expected, there was mucous in the airways of WT and Balb/c ΔdblGATA lungs, however, there was little if any mucous detected in the airways of lungs from the C57Bl/6 ΔdblGATA mice (Figs. 3-1A, 1B, 3-3B). These data indicate that on the C57BL/6, but not Balb/c background, eosinophils are critical for the development of allergic airway inflammation, suggesting that different mouse strains have differing requirements for development of AHR.
Figure 3-1: C57Bl/6 ΔdblGATA mice have significantly reduced lung pathology compared to WT mice. (A) Lungs from C57Bl/6 + OVA, ΔdblGATA + OVA, ΔdblGATA + eos + OVA, were fixed, sectioned, and H&E or PAS-stained to determine airway inflammation or mucus production respectively. (B) Fixed and H&E stained sections from C57Bl/6 + OVA, ΔdblGATA + OVA, ΔdblGATA + eos + OVA, or ΔdblGATA + eos + PBS mice were scored blindly on a scale of 0-4, and averaged by a Veterinary Pathologist for severity of inflammation and epithelial hyperplasia of bronchus and bronchioles (n=6/group). *=p<.0001 for WT or ΔdblGATA + eos + OVA vs. ΔdblGATA + OVA or ΔdblGATA + eos + PBS. Fixed and PAS stained sections were treated as above to determine degree of mucus production in lungs of mice from (A) (n=6/group). *'=p<.007 for WT or ΔdblGATA + eos + OVA vs. ΔdblGATA + OVA or ΔdblGATA + eos + PBS.
Reduced CD4+ T cell recruitment and Th2 cytokines in the lungs of C57Bl/6 mice lacking eosinophils following airway challenge

CD4+ T cells are recruited to the lung during chronic asthmatic responses, producing Th2 cytokines such as IL-4, IL-5, and IL-13 that perpetuate and exacerbate the pathology of this disease (6, 20). To determine if the underlying cause of the observed reduced AHR and pathology in the lungs of C57Bl/6 ΔdblGATA mice was reduced recruitment of inflammatory cells to the lungs, we analyzed BALF from these mice for the presence of CD4+ T cells. We found that C57Bl/6 ΔdblGATA mice have significantly reduced numbers of CD4+ T cells in the BALF (Fig. 3-2C). Balb/c ΔdblGATA mice also exhibited reduced T cell numbers in the lungs, however the number of T cell that infiltrated the lungs was still significantly higher than PBS controls, and BALF Th2 cytokine levels of IL-4 and IL-13 were similar to Balb/c WT, indicating that T cell numbers were still sufficient to produce a robust cytokine response (Fig 3-3D, 3E, 3F). In contrast, C57Bl/6 ΔdblGATA lungs had drastically reduced levels of RNA for cytokines IL-4, IL-13, and IFNγ (Fig. 3-2D). Protein levels of Th2 cytokines IL-4, IL-5, and IL-13 were also reduced in the BALF of C57Bl/6 ΔdblGATA mice (Fig. 3-2E). Thus C57Bl/6 ΔdblGATA mice are defective in recruitment of CD4+ T cells and the production of Th2 cytokines required for the induction of the disease.
Normal systemic immune responses in C57Bl/6 ΔdblGATA mice upon OVA immunization and challenge

Reduction in Th2 cytokines in the lung could be the result of inefficient T cell activation or differentiation to Th2 cells. Since eosinophils have been suggested to serve as antigen...
presenting cells under certain conditions (69-71, 120), we determined if splenic T cells from C57Bl/6 ΔdblGATA mice were able to respond to OVA restimulation. We found that splenic T cells from both C57Bl/6 WT and ΔdblGATA mice were able to proliferate in response to OVA stimulation similarly (Fig. 3-4A). We also found that C57Bl/6 ΔdblGATA mice were able to generate a Th2 response by class switching antibodies to the IgE isotype, as they generated similar levels of total IgE as well as OVA specific IgE to WT mice (Fig. 3-4B,C). Thus, T cell populations from these mice are capable of mounting an immune response, but are not able to migrate into the lungs to respond to OVA challenges.
Figure 3-3: Eosinophils are not required for the development of airway hyperresponsiveness, lung inflammation, and mucous production during allergic asthma induction in Balb/c ΔdblGATA mice. (A) Balb/c WT and ΔdblGATA mice were treated as in Fig. 3-1(A), followed by analysis of AHR by mechanical ventilation (n=7-8/group for OVA-challenged mice, n=4 Sham). *p<0.02 for WT, and ΔdblGATA +/- Eos vs. Sham groups. (B) Mouse lungs from WT, ΔdblGATA, or ΔdblGATA + eosinophils treated as in (A), and analyzed by H&E or PAS stain. (C) Lungs from immunized and OVA challenged Balb/c WT and ΔdblGATA +/- eos mice were analyzed for CD4+ T cell percentages by flow cytometry as outlined in Materials and Methods (D) CD4+ T cell numbers were determined from the lungs of mice treated as in (A). (E) 2X10^5 splenocytes from mice treated as in (A) were restimulated in vitro with 100 mg/ml OVA for 48 hours, supernatants harvested, and IL-4 production determined by Luminex multi-bead assay as outlined in Materials and Methods. (F) IL-13 production by splenocytes treated as in (E) was assessed by Luminex multi-bead assay. n=6/group OVA-challenged mice, n=4/group Sham mice.
Reduced expression of CCL7/MCP-3, CCL11/Eotaxin-1, and CCL24/Eotaxin-2 in lungs of C57Bl/6 ΔdblGATA mice after airway challenge

One reason that T cells may not be able to migrate to the lungs during OVA challenge is reduced expression of chemokines critical for their migration into tissues (51, 121). In particular, CCL7/MCP-3 and CCL11/Eotaxin-1 have been shown to be important for recruitment of T cells into the lung during the development of allergic asthma (39, 51, 121). Eosinophils can induce proliferation and cytokine secretion from T cells (5), as well as secrete T cell growth and chemotactic factors themselves, such as CCL11/Eotaxin-1 and CCL24/Eotaxin-2 (2). Analysis of lung RNA shows that C57Bl/6 ΔdblGATA mice had much reduced expression of these three chemokines (Fig. 3-5A), which suggests that T cells are not recruited into the lungs of C57Bl/6 ΔdblGATA mice due to a deficiency in chemokines able to aid migration of these cells to the lung.
Rescue of T cell recruitment and lung inflammation by transfer of eosinophils to C57Bl/6 ΔdblGATA mice

To determine whether eosinophils are indeed required for recruitment of T cells and generation of allergic asthma symptoms on the C57Bl/6 background, we performed intravenous transfers of 1.5X10^6 eosinophils, purified from the peritoneum of IL-5 transgenic mice, into OVA
immunized ΔdblGATA mice, followed by intranasal challenges with OVA. These mice were then analyzed for the development of AHR 24 hours after the last challenge. We found that transfer of eosinophils into the C57Bl/6 ΔdblGATA mice 6 hours prior to the first IN challenge was able to rescue the development of AHR (Fig. 3-2A), as well as lung inflammation and mucous production (Fig. 3-1A, 1B). Because there could be a small contaminating population of T cells as well as neutrophils in our purified eosinophil population, we also transferred some C57Bl/6 ΔdblGATA mice with 1.5 X 10⁶ neutrophils into C57Bl/6 ΔdblGATA mice and then challenged them with OVA, which did not result in increases in AHR or T cell infiltration to lungs (Fig. 3-2A, 6C), demonstrating that increased numbers of inflammatory cells are not sufficient to rescue allergic airway responses. In addition, we transferred IL-5 transgenic T cells into C57Bl/6 ΔdblGATA mice. This transfer did not result in increased airway inflammation or mucous production, indicating that rescue of lung airway inflammation is not due to contaminating populations of T cells (Fig. 3-6A, 6B).

Transfer of eosinophils, but not neutrophils or IL-5 transgenic T cells, followed by OVA challenge was also able to rescue the recruitment of T cells into the BALF and lungs of C57Bl/6 ΔdblGATA mice (Fig. 3-5B, 5C, 6B, 6C). In contrast, C57Bl/6 ΔdblGATA mice that received eosinophils, but were challenged with PBS had significantly fewer CD4+ T cells than those challenged with OVA (Fig. 3-5B,C). Transfer of eosinophils into C57Bl/6 ΔdblGATA mice followed by OVA challenge also rescued the expression of RNA for cytokines IL-4 and IL-13, and these mice actually displayed higher levels of IL-13 than WT mice challenged with OVA (Fig. 3-5D).

Analysis of cytokine protein levels in the BALF of C57Bl/6 ΔdblGATA mice showed that when these mice received eosinophils and were challenged with OVA, they produced similar levels to WT mice of IL-4, IL-5, and IL-13 (Fig. 3-5E). C57Bl/6 ΔdblGATA mice transferred with eosinophils and challenged with PBS had low levels of these cytokines, comparable to
C57Bl/6 ΔdblGATA mice challenged with OVA (Figure 3-5E). Eosinophil transfer-mediated rescue of CD4+ T cell recruitment into the lung was also accompanied by rescue of CCL7/MCP-3, CCL11/Eotaxin-1, and CCL24/Eotaxin 2 expression (Fig. 3-5A). WT C57Bl/6 lung CCL17 levels were significantly higher than ΔdblGATA, but expression of this chemokine was not rescued by eosinophil transfer into these mice; indicating that eosinophils may not directly regulate CCL17 (Fig. 3-7C), and CCL22 levels were equivalent in all groups (Fig. 3-7C). These results suggest that eosinophils may modulate the expression of CCL11,24/Eotaxin1,2, needed for recruitment of T cells into the lung during allergic airway inflammation.
Figure 3-5: Eosinophils are required for expression of CCL7, CCL11, and CCL24, and CD4+ T cell recruitment in the lungs during allergic airway inflammation in C57Bl/6 ΔdblGATA mice. (A) WT or ΔdblGATA mice were immunized, some ΔdblGATA mice given eosinophils, then challenged with OVA or PBS and lungs analyzed for mRNA for CCL7, CCL11, or CCL24 (n=4-5/group). *p<0.05 WT vs. ΔdblGATA + OVA. (B) Lungs from WT or ΔdblGATA mice treated as in (A) were analyzed for CD4+ T cells. *p<0.05 WT vs. ΔdblGATA + OVA (n=3/group). (C) BALF from mice treated similarly as in (A) was retrieved from the lungs of each group of mice and stained with antibodies against CD4+ T cells (n=3/group, repeated 3X). (D) Lung cytokine mRNA analysis from mice treated similarly to those in (A). *p<0.05 WT vs. ΔdblGATA + OVA (n=4-5/group). (E) BALF from mice treated as in (A) analyzed for the indicated cytokines by multiplex bead assay. *p<0.05 WT vs. ΔdblGATA + OVA (n=4).
Figure 3-6: IL-5 transgenic T cell transfer to ΔdblGATA mice and OVA challenge does not induce lung airway inflammation and T cell recruitment. (A) T cells were purified from IL-5 transgenic peripheral blood and washed 3X. On the first day of intranasal challenge, 1X10^6 cells were transferred i.v. into immunized ΔdblGATA mice as outlined in Materials and Methods. Twenty-four hours after the 4th intranasal challenge, mice were sacrificed, lungs fixed, stained with H&E and PAS, and analyzed for airway inflammation and mucous production (n=3/group). (B) Lungs from mice treated as in (A) were analyzed for the presence of CD4^+ T cells as outlined in Materials and Methods (n=3/group). (C) Lungs from WT or ΔdblGATA mice transferred with neutrophils as outlined in the Materials and Methods were analyzed for CD4^+ T cell infiltration (n=3/group).
Intranasal delivery of CCL11/Eotaxin-1 rescues CD4+ T cell recruitment to the lung and the development of AHR in C57Bl/6 ΔdblGATA mice

Our experiments described above revealed significantly reduced expression of the chemokine CCL11/Eotaxin-1 in the lungs of OVA-challenged C57Bl/6 ΔdblGATA mice. As we discovered that T cells migrating to lung during OVA provocation express CCR3, the receptor for Eotaxin-1, the reduction of this chemokine suggests a possible mechanism for the lower responses in these mice (Fig. 3-7D). To test if CCL11/Eotaxin-1 is able to rescue T cell recruitment and the development of AHR, we delivered this chemokine to the lungs of C57Bl/6 ΔdblGATA mice previously immunized with OVA over the 4 days of intranasal challenge along with OVA. We found that CCL11/Eotaxin-1 delivered with OVA and an isotype control antibody was sufficient to induce AHR in C57Bl/6 ΔdblGATA mice (Fig. 3-7A, 7F).

CCL11/Eotaxin-1 delivery with CCL11/Eotaxin-1 blocking antibody did not induce AHR in C57Bl/6 ΔdblGATA mice challenged with OVA, indicating that the rescue was specific to this chemokine (Fig. 3-7F). Analysis of the lungs of the C57Bl/6 ΔdblGATA mice given CCL11/Eotaxin-1 and OVA intranasally showed that this treatment also rescued T cell migration into the lungs, which did not occur in mice given intranasal CCL11/Eotaxin-1 in combination with CCL-11/Eotaxin-1 blocking antibody (Fig. 3-7B). As expected, OVA plus intranasal CCL11/Eotaxin-1 did not recruit eosinophils in the C57Bl/6 ΔdblGATA mice since these mice lack these cells (Fig. 3-7E). Our data suggest that, in the absence of eosinophils, exposing mice to an allergic airway challenge results in the lack of production of appropriate chemokines; particularly CCL11/Eotaxin-1, which allow recruitment of T cells into the lung and contribute to the pathology of the disease.
Figure 3-7: Chemokine CCL17/22 message levels in the lungs and anti-CCL11 add-back in C57Bl/6 ΔdblGATA mice. (A) Immunized ΔdblGATA mice were given CCL11/Eotaxin-1 during challenge with OVA. Alternatively, immunized WT C57Bl/6 or ΔdblGATA mice were just given CCL11/Eotaxin. This was followed by AHR analysis by mechanical ventilation (n=4). *p<0.05 for ΔdblGATA + Eot/OVA vs. WT or ΔdblGATA + Eot alone. (B) Lungs from immunized and IN OVA challenged WT and ΔdblGATA mice or ΔdblGATA mice delivered Eotaxin-1 with OVA, were analyzed for CD4+ T cells. Some mice challenged with Eotaxin/OVA also received anti-CCL11 blocking antibody IN (n=4/group) *p<0.05 WT vs. ΔdblGATA + OVA. (C) C57Bl/6 WT or ΔdblGATA mice were immunized, some ΔdblGATA mice given eosinophils, then challenged with OVA or PBS and lungs analyzed for chemokine RNA CCL17 and CCL22 as outlined in Materials and Methods (n=6/group). *= p<.05 for WT vs all ΔdblGATA groups. (D) Lungs from sensitized and OVA challenged WT mice were analyzed by flow cytometry for CD4 and CCR3. Histogram shows CCR3 expression on CD4+ T cells in the lung. (E) WT and ΔdblGATA mice were immunized and the ΔdblGATA group given CCL11/Eotaxin-1 during challenge with OVA. Lungs were analyzed for CD4+ T cells or eosinophils (n=3) *p<0.05 WT vs. ΔdblGATA + OVA/Eot. (F) OVA/alum immunized ΔdblGATA mice received 0.75 µg CCL11/Eotaxin-1 as well as .75 µg polyclonal anti-Goat IgG (Sigma) combined with the normal dose of 30 µl (2 mg/ml) OVA IN on days 12-15. Alternatively, ΔdblGATA mice received .75 µg CCL11/Eotaxin-1 blocking antibody (R&D Systems) in addition to .75 µg CCL11/Eotaxin-1 + OVA on days 12-15. Mice were analyzed for AHR 24 hours after the last intranasal challenge (n=4/group). *=p<.05 for ΔdblGATA + Eot/OVA/Ig vs. ΔdblGATA + OVA or ΔdblGATA + OVA/Eot/a-CCL11.
Discussion

In this investigation we have provided evidence that eosinophils are required for the development of allergic airway responses and recruitment of T cells into the lungs following allergen challenge in C57Bl/6 ΔdblGATA mice. These results add weight to other studies that have shown abrogation of the symptoms of allergic asthma in the absence or suppression of eosinophil function (47, 76, 77). Lee and colleagues have provided evidence in a transgenic eosinophil ablation approach in C57Bl/6 mice that eosinophils are required in order to develop AHR and airway inflammation, as well as mucus secretion (77). This is in contrast to data provided by Humbles, et al, in ΔdblGATA mutant Balb/c mice suggesting that eosinophils are required only for airway remodeling (76). Our data in the ΔdblGATA mutant mice on the Balb/c and C57Bl/6 backgrounds now indicate that the discrepancies observed between these two groups were most likely due to strain differences in C57Bl/6 and Balb/c mice. Indeed, our studies find that systemic Th2 responses are intact in the ΔdblGATA mutant mice on the C57Bl/6 background (Fig 3-4A-C), similar to that observed by Humbles, et al (76). The main difference we observe is the requirement for eosinophils in the acute model of allergic airway inflammation, and the recruitment of T cells into the lung. Indeed, we observed a reduction in the number of T cells recruited into the lungs of both C57Bl/6 and Balb/c ΔdblGATA mice (Fig 3-2C, 3D). However, although recruitment of T cells into the Balb/c ΔdblGATA mouse lungs was reduced compared to WT, the number of T cells in these lungs was statistically greater than controls, and the percentage of CD4+ T cells was similar to WT (Fig. 3-3C, 3D). Data from Voehringer, et al, support this, showing in the Th2 Nippostrongylus model that T cell recruitment to Balb/c ΔdblGATA lungs is intact (122). Also, reduced T cell numbers did not prevent Balb/c ΔdblGATA from producing Th2 cytokines IL-4 and IL-13 (Fig. 3-3E, 3F). While this is in agreement with Voehringer, et al, Fulkerson, et al, reported a reduction in IL-4 and IL-13
production in the lung in an *Aspergillus fumigatus* model of asthma (51). Whether these differences are due to the Balb/c strain having lower chemokine/cytokine requirements for recruitment of inflammatory cells in the airways and subsequent production of Th2 cytokines remains to be seen.

Our data in C57Bl/6 ΔdblGATA mice also address the influence that eosinophils have over CD4⁺ T cells in allergic asthma, suggesting that these cells are not just terminal effector cells, but are actively involved in the adaptive immune response by recruiting T cells to the lungs. This data supports results that propose eosinophils can modulate the function of T cells in the allergic lung. Eosinophils resident in the lung during allergic responses are able to present antigen and traffic to local lymph nodes where they co-localize with T cells (69, 71, 72). Other studies using the ΔdblGATA mice on the Balb/c background have suggested that eosinophils are not required for the accumulation of Th2 cells in the lungs during infection with *Nippostrongylus brasiliensis* (122). Eosinophils can also induce proliferation and cytokine secretion from T cells (2), as well as secrete T cell growth and chemotactic factors themselves, such as CCL11/Eotaxin-1 (2). In allergic mice deficient in Eotaxin-1 and IL-5, there is reduced production of cytokine IL-13, although these cells had normal cytokine production in general (39). These researchers did not investigate whether CD4⁺ T cells were actually recruited to the lungs in the Eotaxin-1/IL-5 double knockout mice, only that levels of Th2-type cytokines in the lung were reduced; innate immune cells can also produce such cytokines during allergic responses (39). We have shown here that ΔdblGATA mice have an absence of CD4⁺ T cells in the lung during allergen challenge, and that this rescued by delivery of CCL11/Eotaxin-1 in combination with allergen (Figs. 3-2C, 5B, 7B). These data lend support to the idea that CCL11/Eotaxin-1 is required for induced migration of T cells into the lung during allergic responses, and also underscores the need for eosinophils in the asthmatic lung. It is possible that eosinophils are required for low level of secretion of CCL11/Eotaxin-1/Th2 cytokines in the lung, which could induce T cell migration.
and secretion of effector cytokines by these cells that can further amplify the recruitment of eosinophils and T cells into the lung in a feed forward mechanism.

Th2 cytokines have long been implicated in allergic airway responses, particularly cytokines IL-4, IL-5, and IL-13. Administration of IL-13 alone can induce asthmatic responses in mice, and is linked to mucus secretion (19). Transfer of T cells defective in IL-13 production into Eotaxin-1/IL-5 double knockout mice does not induce AHR, whereas transferring in vitro differentiated IL-13-producing T cells can overcome defects in Eotaxin-1/IL-5 double knockout mice and induce asthma suggesting that eosinophils may be linked to induction of IL-13 production in T cells during allergic airway responses (39). Our data support this, showing an absence of IL-13 message/protein and more importantly CD4+ T cells in ΔdblGATA lung challenged with OVA, and recovery of IL-13 message/protein and CD4+ T cell infiltration upon transfer of eosinophils into ΔdblGATA mice followed by OVA challenge (Fig. 3-2D, 2E, 5B, 5D, 5E).

ΔdblGATA mice also display defects in IL-4 and IL-5 production upon OVA challenge to the lung that can be rescued by eosinophil transfer (Fig. 3-5E). Blockade of IL-4 in humans can relieve some symptoms of asthma (14, 123), and administration of this cytokine to mice can induce asthmatic responses (14). Both T cells and eosinophils have been documented to produce this cytokine during asthmatic responses, but it is not clear if this cytokine production during this response is due solely to T cells, eosinophils, or a combination of these two cells. Notably, we have shown here that ΔdblGATA mice have normal Th2 humoral responses to OVA, suggesting that although T cells are not in the lung producing Th2 cytokines that contribute to AHR, they are still apparently able to produce sufficient amounts in the draining node or spleen to induce class switching of B cells (Fig 3-4C).

The role of IL-5 in asthmatic responses has been controversial. Deficiency in this gene reduces the numbers of eosinophils, but does not ablate them completely. Blockade of this
cytokine in vivo reduces the number of blood circulating eosinophils, but does not result in depletion of tissue resident eosinophils (22). Consequently, experiments investigating this cytokine have yielded mixed results; from no changes in AHR, minimal reduction of this symptom, or complete ablation of AHR (21, 22, 37, 124). It has also been suggested that the requirement for IL-5 is more likely due to its role in development and expansion of eosinophils from the bone marrow, rather than function of these cells. We observe a reduction in BALF protein for IL-5 in ΔdblGATA mice, suggesting that production of IL-5 is also under the influence of eosinophils either as a direct source or through stimulation of T cells to secrete this cytokine (Fig. 3-5E).

Simultaneous research that parallels that presented above was conducted in the Lee group, and also supports the above studies, in this case utilizing the PHIL eosinophil null mice (PHIL−/−) (125). These mice express diptheria toxin A (DTA) driven by the eosinophil peroxidase promoter so that eosinophils are specifically ablated while other lineages are still able to develop. In these mice, several characteristics such as reduced AHR, T cell recruitment, and Th2 cytokine production overlapped with findings presented here (125). However, eosinophil transfer alone followed by antigen provocation in the lung did not rescue the parameters outlined above. This result was attributed to a potential requirement for eosinophils to present antigen during the allergen sensitization phase, but this aspect of eosinophil involvement was left unexplored. However intravenous transfer of Th2 differentiated OT-II OVA specific T cells to PHIL−/− mice required eosinophils (instilled intratracheally) for migration into the lung (125). These two reports are therefore in agreement, suggesting that eosinophils are required to provide a signal or interact with T cells in order to promote Th2 response development and migration into the lung. The particular signal required for these responses remains to be seen. Both groups suggest a role for chemokine dependent migration in their analyses, however; the particular chemokine required differs between the two reports. This work determined that Eotaxins 1 and 2 are reduced in the
absence of eosinophils, and that Eotaxin-1 delivery to the lung in combination with OVA was sufficient to rescue AHR and T cell influx into the lung (Fig. 3-5A, 7A, 7B). Other chemokines analyzed; such as CCL17 and CCL22, did not appear to be directly affected by the presence or absence of eosinophils (Fig. 3-7C). In contrast, Jacobsen, et al, found the opposite results, that Eotaxins were not significantly reduced in the allergic lungs of PHIL−/− mice; however, CCL17 and CCL22 were reduced. Transfer of eosinophils intratracheally and OT-II cells intravenously recovered the levels of CCL17 and CCL22 in these mice (125).

The reason for the discrepancies in these two model systems is unclear, but could be due to several factors. While the ∆dblGATA mice lack mature granulated eosinophils that express CCR3, it has been suggested that these mice may harbor “granuleless” immature progenitors that could potentially be functional in some way, although this idea is as yet unexplored (51). Interestingly, bone marrow from these mice cultured ex vivo in the presence of IL-3, IL-5, and GM-CSF appear to develop into eosinophils, although there seems to be a cell intrinsic defect in vivo, as bone marrow transfer to wildtype hosts does not yield mature eosinophils (126). However, it is possible that these immature “granuleless” eosinophils may be able to present antigen or produce cytokines. Meanwhile, although the C57Bl/6 PHIL−/− mice are completely lacking eosinophils, there is some question about the consequences of expressing diptheria toxin in these mice. There is an increase in the lymphocyte compartment that is unexplained, and although T cells derived from these mice produce cytokines in response to nonspecific CD3/CD28 stimulation ex vivo, there has been no evidence of an intact antigen specific Th1 or Th2 response in vivo. The disparities amongst the different eosinophil deficient strains are presented in Table 3-1.

In summary, our data demonstrate that eosinophils are required for the recruitment of T cells to the lungs, probably through a mechanism involving eosinophil secretion of CCL11/Eotaxin-1. In the absence of eosinophils, this recruitment is not observed, but can be
rescued by the addition of CCL11/Eotaxin-1 to the lung environment. This reduction in T cell recruitment leads to questions regarding whether eosinophils are necessary only for T cell migration into the lung, or whether these granulocytes are important in activating and mobilizing T cells in the draining nodes as has been suggested. This migration defect precludes the ability of these T cells to secrete cytokines that amplify allergic responses in the lung environment, and subsequent airway inflammation and influx of inflammatory cells. Altogether, these data indicate that eosinophils are more than just terminal effector cells, and are important in modulating T cell function in response to allergic airway challenge. However, the particular signal that eosinophils elicit in order to drive migration of T cells to lung is still unknown. In this chapter, we presented data showing that Eotaxin-1 is able to induce recruitment of T cells to lungs in the absence of eosinophils, but production of this chemokine may be a secondary effect. It is known that IL-13 production induces epithelial cells to produce large amounts of Eotaxin-1 for the recruitment of T cells and other inflammatory cells. It is possible that eosinophils may produce this cytokine early in the allergic response in order to promote chemokine production and T cell recruitment. This possibility is explored in the next chapter.
Table 3-1: Strain-specific differences in the development of allergic asthma in eosinophil deficient mouse models.

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<th>ΔdblGATA Bl/6</th>
<th>ΔdblGATA Balb/c</th>
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<tr>
<td>Airway Inflammation</td>
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<td>Generation of AHR</td>
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<td>Chemokine Secretion</td>
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<td>T cell Recruitment to Lung</td>
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<tr>
<td>IgE</td>
<td>+</td>
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<td>ND</td>
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<td>Mucus Secretion</td>
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<tr>
<td>AHR rescue with Eosinophil Addback</td>
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<td>AHR rescue with Chemokine Adddack</td>
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<td>Long Term Remodeling</td>
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Chapter 4

Eosinophil production of IL-13 is integral to the development of allergic airway responses

Introduction

It is well established that IL-13 is a cytokine essential to the development of allergic responses (20). Knockout mouse models and antibody ablation studies of this cytokine have shown severe attenuation of the characteristic symptoms of allergic asthma, such as airway hyperresponsiveness (AHR), T cell recruitment to the lung, and eosinophilic infiltration of the lung parenchyma in the absence of IL-13 (20). Selective over-expression of IL-13 in the lungs results in allergic inflammation of the lungs as well as mucus hypersecretion (16). Moreover, administration of this cytokine alone to the airways of naïve mice is sufficient to induce AHR and goblet cell hyperplasia, and when administered along with antigen severe disease results (127).

Many different immune cells can contribute to the production of this cytokine. Of course, Th2-differentiated T cells are able to produce large amounts of IL-13 in addition to other cytokines, and are considered to be the main purveyors of disease in the pathogenesis of asthma (16, 127). However, some granulocytes are also able to produce IL-13, and contain preformed message that can be translated into the resulting cytokine rapidly (96). A role for mast cell-secreted IL-13 in the early phase of allergic asthma has been delineated, in which mast cells secrete this cytokine along with histamine to induce broncho-constriction and goblet cell hyperplasia, resulting in an acute attack of allergic asthma (5). Basophils and eosinophils also contain preformed message for this cytokine (96).
As IL-13 production is critical to the induction of the early phase of an allergic response, we wanted to determine whether it is also important in the induction of a late phase response. Knockout models have shown that without this cytokine, allergic diseases do not develop, but these data do not shed light on temporal needs for this cytokine or which cell may initially produce IL-13. Mast cells are not required for induction of a late phase allergic response, and as our previous work showed that eosinophils are integral to the recruitment of T cells to the lung in the late stage of this disease (128), we hypothesized that IL-13 production by eosinophils may facilitate initiation of the late phase allergic response.

Eosinophils, like other granulocytes, contain preformed message for IL-13, as well as having a supply stored in granules that can be instantly secreted (96). However, the only context in which IL-13 production by eosinophils has been studied has been as an end stage effector cell. Work presented in the previous chapter showed that eosinophils are able to act early in the late phase of allergic airway inflammation by facilitating the recruitment of T cells into the lung, although the method by which this occurs is not entirely clear. Evidence suggested that there was a failure in chemokine induction in the absence of eosinophils, and that addback of Eotaxin-1 could rescue T cell recruitment. There is much evidence in the literature to suggest that IL-13 can induce secretion of Eotaxins from airway epithelial cells, endothelial cells, and a variety of immune cells (90, 94, 129). Lastly, transgenic mice that over-express IL-13 in the lung have enhanced expression of Eotaxin-1 in the lung and infiltration of inflammatory cells (86).

In this chapter, we show that IL-13 production by eosinophils is integral to the development of allergic asthma. Reconstitution of ΔdblGATA mice with IL-13−/− eosinophils results in reduced T cell recruitment and Th2 cytokine production in the BALF. However, eosinophil production of IL-13 cannot sustain an allergic response without T cell recruitment and subsequent production of IL-13 to mobilize mass production and recruitment of inflammatory cells. Despite the dependence of eosinophils on T cells for maintaining their presence in the
lungs, even \textit{in vitro} differentiated T cells producing IL-13 are still dependent on eosinophils for recruitment to the lungs. Thus, IL-13 secretion by eosinophils early in the late phase of allergic asthma is able to facilitate the large-scale infiltration of Th2 inflammatory cells into the lung and perpetuation of the disease.

**Results**

\textbf{IL-13 addback to \textit{ΔdblGATA} mouse lungs rescues pathology associated with allergic asthma}

Previous work in our lab determined that eosinophils were required for the recruitment of T cells to the lungs in a model of OVA-induced allergic airway inflammation \cite{128}. We wanted to further identify the requirements for eosinophils in the pathogenesis of allergic asthma by determining how eosinophils facilitate migration of T cells to the lung, whether by secretion of a cytokine factor, chemokine, or direct interaction with the T cell. In pursuit of this goal, we sorted eosinophils from the peritoneum of IL-5 \textit{tg} mice, extracted RNA from the cells, and performed RT-PCR to determine if eosinophils contained any preformed cytokine or chemokine message. We found that eosinophils contained high levels of IL-4, and particularly IL-13 mRNA compared to \textit{in vitro} Th2-differentiated OT-II T cells (Fig. 4-1A), prompting us to hypothesize that production of IL-13 by eosinophils could help initiate recruitment of T cells into the lung during allergen challenge. Because IL-13 can affect different processes depending on where and when it is produced, we collaborated with Drs. Reka Albert and Juilee Thaker at Penn State University to develop a Boolean network computer model of allergic asthma wherein we could predict how production of IL-13 by various cell types in different mutant mouse models can affect the development of AHR and T cell recruitment into the lung, two hallmarks of allergic asthma. For
more information on network modeling in biological systems see reference (130). Simulations based on this model resulted in the development of late AHR in WT mice, while simulations of mice lacking eosinophils resulted in early AHR (due to mast cells and IgE), but not late AHR (Activity on the Y-axis is relative to development of AHR with higher values indicating more severe disease. The X-axis is labeled in arbitrary time units that may represent specific time periods, however, more specific knowledge about the temporal occurrence of events in allergic asthma is required in order to use defined time marks.) (Fig. 4-1B). The simulations also showed that mice lacking eosinophils are unable to recruit Th2 cells into the lung (Fig. 4-2A). These results matched our experimental observations on late AHR, validating the model. We next wanted to determine whether exposing the lungs of ΔdblGATA mice to IL-13 mice would bypass the requirement for eosinophil recruitment of T cells. In the simulation of this experiment, the model predicted that exposure of the lungs of eosinophil null mice to IL-13 leads to the development of AHR similar to WT (Fig. 4-1B). To experimentally test the model’s prediction, we sensitized ΔdblGATA or WT mice to OVA as previously described, followed by 4 consecutive days of intranasals of 40 µg of OVA combined with 2 µg of IL-13. Twenty-four hours after the last intranasal challenge, we performed mechanical ventilation on the different groups of mice and found that while there was little change in resistance in the lungs of ΔdblGATA mice challenged intranasally with PBS alone in response to increasing doses of methylcholine, both WT and ΔdblGATA mice challenged with OVA and IL-13 had significant increases in lung resistance (Fig. 4-1C), indicating that addition of IL-13 to the lungs of ΔdblGATA mice is able to rescue AHR in these mice. One lobe of the lungs from each mouse was fixed in paraformaldehyde, sectioned, and stained with H&E to determine inflammatory cell infiltrates, or PAS to determine levels of mucus secretion. ΔdblGATA lungs challenged with OVA and IL-13 displayed similar pathology in airway inflammation and cellular infiltration of
the lung tissue to WT lungs under the same conditions (Fig. 4-1D), while ΔdblGATA lungs challenged with PBS achieved significantly less inflammation and fewer inflammatory cell infiltrates. PAS stained sections correlated with these data, showing much less mucus production in ΔdblGATA lungs challenged with PBS compared to WT and ΔdblGATA lungs challenged with both IL-13 and OVA (Fig. 4-1D). These data further validate the model and confirm the model’s prediction.

As previous data in our lab showed that ΔdblGATA mice are defective in the recruitment of T cells to the lung in the absence of eosinophils, we also used our network model to predict whether IL-13 add-back to the lungs of these mice could rescue this defect (Fig. 4-2A). The simulations showed that T cell recruitment equal to WT levels occurs when IL-13 was added back to the lungs of eosinophils null mice, while eosinophils null mice receiving only PBS had much lower T cell recruitment (Fig. 4-2A). We tested these predictions by subjecting ΔdblGATA and WT mice to the protocol described above and removing lungs 24 hours after the last intranasal of OVA and IL-13. We then determined the number of T cells in the lungs of these mice by flow cytometry. We saw that administering IL-13 was able to rescue T cell infiltration into the lungs, as ΔdblGATA mice treated with IL-13 and OVA had numbers of T cells in the lung equivalent to those of WT lungs receiving the same treatment, while ΔdblGATA mice that received PBS treatment had a significantly lower number of T cells in the lungs (Fig. 4-2C). It also followed that ΔdblGATA mice and WT mice treated with IL-13 and OVA had similar lung mRNA levels for chemokines CCL11 (Fig. 4-2D) and CCL7 (Fig. 4-2E), as well as cytokines IL-4 and IL-13 (Fig. 4-2B), indicating that administration of IL-13 can compensate for the absence of eosinophils and induce an allergic Th2 response in the lung.
Figure 4-1: AHR in ΔdblGATA mice is rescued by IL-13 add-back to the lungs. (A) Purified eosinophils were lysed, and RNA extracted. T cells from the lymph nodes of OT-II mice were differentiated in vitro in the presence of IL-4 and anti-IFN-gamma, cells lysed, and RNA extracted. Real-time PCR for Th2 and Th1 cytokines was performed on both cell types. (B) Simulations for the development of AHR in WT mice (filled circles) and ΔdblGATA mice with (filled triangles) or without (filled squares) IL-13 add-back in a computational model of allergic asthma. (C) WT or ΔdblGATA mice were administered 2µg of IL-13 intranasally for 5 days and then mechanically ventilated to determine AHR. Some ΔdblGATA mice received PBS intranasally as a control. (D) Lungs from OVA-sensitized WT or ΔdblGATA mice challenged intranasally with OVA/IL-13 or PBS were fixed, sectioned, and stained with H&E to determine airway inflammation or PAS to determine mucus secretion (bar=50µm). *= p<.005. n=5 per group.
Figure 4-2: T cell recruitment, cytokine production, and chemokine production are rescued by IL-13 add-back to the lungs in ΔdblGATA mice. (A) Computational model simulations for the recruitment of CD4\(^+\) T cells to the lungs in WT mice (filled circles) and ΔdblGATA mice with (filled triangles) or without (filled squares) IL-13 add-back. (B) Lungs from OVA-sensitized WT or ΔdblGATA mice challenged intranasally with OVA/IL-13 or PBS were homogenized, RNA extracted, and real-time PCR conducted to determine mRNA levels of cytokines IL-4, IL-13, and IFN-gamma (n=5 per group). (C) Lungs from groups of mice in (B) were analyzed for CD4\(^+\) T cells by flow cytometry (n=4 per group). (D) Lungs from groups of mice treated as in (B) were analyzed for mRNA levels of CCL11 (n=5 per group). (E) Lungs from groups of mice treated as in (B) were analyzed for mRNA levels of CCL7 (n=5 per group). * = p<.005.
IL-13 production by eosinophils is requisite in order to rescue Th2 responses in the lungs of ΔdblGATA mice

As we saw that eosinophils contained preformed mRNA for IL-13 and administration of this cytokine to ΔdblGATA lungs was able to rescue T cell infiltration and cytokine production in the lungs of these mice, we wanted to more specifically determine whether there was a role for eosinophil production of IL-13 in the generation of allergic asthma. We modeled IL-13 deficient eosinophils into our computational model of allergic asthma, to simulate the effect of the function of eosinophils lacking the capacity to secrete IL-13, while T cells are still able to do so. The model indicated that ΔdblGATA mice would be able to initiate an early phase AHR response as was previously predicted (Figure 4-1B), but when IL-13 deficient eosinophils were provided in an attempt to rescue this response, these mice would be unable to generate the late phase AHR seen in WT mice (Fig. 4-3A). To test this prediction, we crossed C57Bl/6 IL-13⁻/⁻ mice to C57Bl/6 IL-5 tg mice to obtain mice that overproduce IL-13⁻/⁻ eosinophils. We then purified WT or IL-13⁻/⁻ eosinophils, transferred them into OVA-sensitized ΔdblGATA mice and challenged these intranasally with OVA as previously described and analyzed mice for AHR. We found that ΔdblGATA mice that were recipients of IL-13⁻/⁻ eosinophils and were challenged intranasally with OVA developed low AHR, similar to ΔdblGATA mice challenged with OVA alone. By contrast, both WT and ΔdblGATA mice that had received WT eosinophils, and were challenged with OVA had significantly increased AHR (Fig. 4-3B). These data suggest that IL-13 production by eosinophils contributes to the initiation of allergic responses in the lung. Lung sections stained with H&E from these mice revealed that ΔdblGATA mice that had received IL-13⁻/⁻ eosinophils had lower levels of airway inflammation and numbers of inflammatory cell infiltrates than WT mice or ΔdblGATA mice that had received WT eosinophils. However airway inflammation did seem to be increased in ΔdblGATA mice that had received IL-13⁻/⁻ eosinophils.
compared to sections from ΔdblGATA mice + OVA alone (Fig. 4-3C). ΔdblGATA mice + IL-13−/− eosinophils lung sections stained with PAS also exhibited higher levels of mucus production than ΔdblGATA mice + OVA, but had significantly lower levels compared to lung sections from WT mice or ΔdblGATA mice + WT eosinophils (Fig. 4-3D). Increased airway inflammation and mucus production in the lungs of ΔdblGATA mice that had received IL-13−/− eosinophils may be as a result of eosinophils still being able to produce IL-4, which can also activate goblet cells to secrete mucus as well as cause airway inflammation. Regardless, our data support the computational model simulations that predict that the generation of AHR is dependent on eosinophil-derived IL-13.

We also simulated the outcome of the effect of IL-13−/− eosinophils on the ability of T cells to be recruited to the lungs of ΔdblGATA mice (Fig. 4-4A). The results indicated that IL-13 deficient eosinophils would not be able to recruit T cells to the lungs at levels similar to WT (Fig. 4-4A). As we saw before, T cell recruitment occurs in WT mice, but not in the presence of eosinophils that are unable to make IL-13 (Fig. 4-4A). We tested this prediction experimentally by examining the lungs from WT, ΔdblGATA mice, ΔdblGATA mice + WT eosinophils, or ΔdblGATA mice + IL-13−/− eosinophils for the presence of CD4+ T cells. We found significantly lower numbers of CD4+ T cells in the lungs of ΔdblGATA mice challenged with OVA alone or those ΔdblGATA mice that had received IL-13−/− eosinophils and then challenged with OVA, compared to the lungs of WT or ΔdblGATA mice that had received WT eosinophils (Fig. 4-4B). This indicates that without production of IL-13 by eosinophils, these mice were deficient in the ability to recruit T cells to the lungs. Interestingly, these mice also had lower numbers of eosinophils in lungs, prompting the question of whether IL-13−/− eosinophils were able to home to the lung (Fig. 4-4B). However, when we transferred CFSE-labeled WT or IL-13−/− eosinophils into ΔdblGATA mice, we found that these eosinophils had equal ability to migrate to the lung
(Fig. 4-5). The data suggest that without the support of T cells migrating to the lung and producing survival factors for eosinophils, such as IL-5, recruitment or survival of eosinophils in the lungs is not maintained.

Given that there was a decrease in the number of T cells being recruited to the lung, we expected that there would also be a decrease in Th2 cytokine production in the lungs of ΔdblGATA that had received IL-13−/− eosinophils and challenged with OVA. Indeed, when we recovered BALF from these mice as well as ΔdblGATA mice challenged with OVA alone, we found that there was a drastic decrease in the amount of IL-4, IL-5, and IL-13 produced in the lungs of these mice compared with WT or ΔdblGATA mice that had received WT eosinophils (Fig. 4-4C), although there was no significant difference in the production of IFN-γ (Fig. 4-4D). This confirmed our expectation that low numbers of T cells in the lungs of these mice would lead to reduced Th2 cytokine production.
Figure 4-3: IL-13 deficient eosinophils are not able to facilitate induc- 
tion of late AHR. (A) Computer simulations for the development of AHR upon allergen provocation in WT mice (filled 
circles) and mice that lack eosinophil-derived IL-13 (filled squares). (B) OVA-sensitized WT 
mice and ΔdblGATA mice + WT eosinophils or IL-13+/− eosinophils were administered 40µg of 
OVA intranasally for 4 days and then mechanically ventilated to determine AHR. Control 
ΔdblGATA mice challenged with OVA did not receive eosinophils (n=15 per group). (C) Lungs 
from OVA-sensitized WT mice and ΔdblGATA mice +/- WT eosinophils or IL-13−/− eosinophils 
challenged intranasally with OVA were fixed, sectioned, and stained with H&E to determine 
airway inflammation or (D) PAS to determine mucus secretion (bar=50µm) (n=6 per group).
Figure 4-4: IL-13 deficient eosinophils are not able to induce T cell recruitment, cytokine production, and chemokine production in the lung. (A) Computer simulations of the recruitment of CD4\(^+\) T cells to the lungs in WT mice (filled circles) or mice lacking eosinophil-derived IL-13 (filled squares). (B) Lungs from OVA-sensitized WT or ΔdblGATA mice +/- WT eosinophils or IL-13\(^{-/-}\) eosinophils and challenged intranasally with OVA were analyzed for CD4\(^+\) or CCR3\(^+\) cells by flow cytometry to determine the numbers of T cells and eosinophils respectively (C&D) BALF was collected and concentrated from each group of mice mentioned in (A) and protein levels of cytokines IL-4, IL-13, and IFN-gamma were determined by multiplex bead assay. *=p<.05. n=5 per group.
Eosinophils are unable to induce an allergic response in the lungs if they are the sole source of IL-13 in an in vivo model of allergic asthma

The finding that eosinophil production of IL-13 was required during the generation of an allergic response in the lungs was significant in that, prior to this discovery, it was assumed that T cells were the only required source of IL-13. This prompted us to examine simulation in the computational model, where eosinophils are the sole source of IL-13 during an allergic response. The model predicted that eosinophil production of IL-13 would be insufficient to generate AHR, if T cells are unable to secrete this cytokine (Fig.4-6A). To experimentally test this prediction, we transferred $2 \times 10^6$ WT eosinophils into IL-13$^{-/-}$ C57BL/6 mice that had been sensitized to
OVA, and then challenged these mice intranasally with OVA. Note that in these mice, all cells lack the ability to secrete IL13, and providing them with WT eosinophils would provide a source of IL-13 from these cells. We found that transferring WT eosinophils into these mice and challenging them with OVA led to reproducible increases in AHR over IL-13−/− mice challenged with OVA alone; however, these responses were not significantly higher than that seen in IL-13−/− mice alone, and were statistically lower than AHR responses in WT mice challenged with OVA (Fig. 4-6C). We also found increased airway inflammation and infiltration of inflammatory cells into the lung tissue of IL-13−/− mice transferred with WT eosinophils and challenged with OVA intranasally when we examined lung sections stained with H&E (Fig. 4-6B). However, this inflammation was lower than inflammation found in WT lungs, and when sections were stained with PAS to detect mucus production, there was very little mucus found in lung sections from both IL-13−/− mice without eosinophil transfer, and IL-13−/− mice injected with WT eosinophils when compared with WT mice that had been challenged with OVA (Fig. 4-6B). These data indicate that IL-13 production by eosinophils is not sufficient to induce symptoms of an allergic asthma response.

Next we simulated the effect of eosinophils as the sole source of IL-13 on T cell recruitment to the lungs in IL-13−/− mice provided with WT eosinophils. The model predicted that while WT conditions would yield the expected high levels of T cell recruitment and the IL-13 deficient system would have low levels of T cell recruitment, adding back eosinophil-derived IL-13 to IL-13−/− mice would lead to an increased but unstable pattern of T cell recruitment to the lungs. This prediction supports the view that stable T cell recruitment to the lung itself is dependent on eosinophil derived IL-13 (Fig. 4-6A). To test this prediction, we determined whether T cells intrinsically incapable of producing IL-13 could still be recruited to the lung of IL-13−/− mice that had received WT eosinophils and challenged intranasally with OVA, compared to those mice that had not received eosinophils. We found that IL-13−/− mice
challenged with OVA had significantly lower numbers of T cells recruited to the lung compared to IL-13−/− mice transferred with WT eosinophils and challenged with OVA (Fig. 4-6D). This correlated with our data from ΔdblGATA mice transferred with IL-13−/− eosinophils that suggests that T cell recruitment to the lung is facilitated by eosinophil-derived IL-13 (Fig. 4-4B). IL-13−/− mice reconstituted with IL-13 competent eosinophils were able to recruit T cells to the lung, although these T cells were unable to produce cytokines to sustain the allergic response, and the quantity produced by eosinophils is not able to drive the allergic response on its own. In fact, when levels of IL-13 mRNA from IL-13−/− mice challenged with OVA and IL-13−/− mice transferred with WT eosinophils and delivered OVA were examined, there was no significant difference in the amount being produced, however, there were significantly higher levels of IL-4 message (Fig. 4-6E). It is possible that T cells, or possibly eosinophils, recruited to the lung were able to produce IL-4 and induce some level of allergic airway inflammation as well as AHR in these mice, accounting for the higher, although not significant, increases we observed in these IL-13−/− recipients of WT eosinophils.
Figure 4-6: Eosinophil IL-13 production alone is not sufficient to generate AHR in IL-13−/− mice. (A) Computational simulations for the development of allergic asthma and T cell recruitment to the lung upon allergen provocation in WT mice (filled circles) IL-13−/− mice (filled squares), and IL-13−/− mice reconstituted with WT eosinophils (filled triangles). (B) Lungs from OVA-sensitized WT mice and IL-13−/− mice +/- WT eosinophils and challenged intranasally with OVA were fixed, sectioned, and stained with H&E to determine airway inflammation or PAS to determine mucus secretion (bar=50µm). (C) Mice from groups treated as in (B) were mechanically ventilated to determine AHR (n=4 per group). (D) Lungs from groups of mice in (B) challenged with OVA were analyzed for the number of T cells by flow cytometry. (E) Lungs from groups of mice treated as in (B) were analyzed by real-time PCR to determine mRNA levels of cytokines IL-4, IL-13, and IFN-gamma. *=p<.05. (n=5 per group).

Eosinophil production of IL-13 is required for recruitment of in vitro differentiated Th2 T cells to the lungs of ΔdblGATA mice

Since we found that T cell production of IL-13 appeared to be integral to the development of an allergic response in the lung, regardless of whether eosinophils were secreting
IL-13, we wanted to determine whether elevated numbers of Th2-differentiated T cells already capable of producing IL-13 could bypass the requirement for eosinophil production of this cytokine in the lung. To test this we differentiated T cells from OT-II mice, a transgenic mouse in which the majority of T cells are OVA-restricted and CD4+, to the Th2 phenotype. We then transferred 5 X 10^6 of these cells intravenously into naïve WT or ΔdblGATA mice. Some ΔdblGATA mice also received 5 X 10^6 WT or IL-13−/− eosinophils intravenously in a different vein, 6 hours prior to T cell transfer. We challenged each group of mice intranasally with OVA and then analyzed AHR development by mechanical ventilation. We found that, even with the presence of elevated numbers of differentiated OVA-specific Th2 T cells, ΔdblGATA mice required eosinophils to generate AHR, as ΔdblGATA mice provided OT-II Th2 cells alone had significantly reduced AHR compared to WT mice transferred with OT-II Th2 cells (Fig. 4-7B). Furthermore, although in vitro differentiated Th2 cells were capable of making their own IL-13, IL-13 production by eosinophils was still required, as ΔdblGATA mice transferred with IL-13−/− eosinophils along with OT-II Th2 cells had significantly reduced AHR compared with ΔdblGATA mice transferred with WT eosinophils along with OT-II Th2 cells or WT mice transferred with OT-II Th2 cells (Fig. 4-7B).

ΔdblGATA mice transferred with OT-II Th2 cells and IL-13−/− eosinophils also had significantly reduced airway inflammation in the lung compared with ΔdblGATA mice transferred with OT-II Th2 cells and WT eosinophils, or WT mice injected with OT-II Th2 cells, as denoted by H&E staining of lung sections from these mice (Fig. 4-7A). ΔdblGATA mice transferred with OT-II Th2 cells alone and challenged intranasally with OVA also had drastically reduced airway inflammation, similar to ΔdblGATA mice transferred with OT-II Th2 cells and IL-13−/− eosinophils (Fig. 4-7A). We also stained sections from these lungs with PAS to detect mucus production and found that ΔdblGATA mice transferred with OT-II Th2 cells and IL-13−/−
eosinophils had little to no mucus in the lungs compared to WT mice transferred with OT-II Th2 cells or ΔdblGATA mice transferred with WT eosinophils and OT-II Th2 cells (Fig. 4-7A), indicating that IL-13 production by eosinophils is required to induce airway inflammation and mucus production in the lung, even when elevated numbers of antigen specific Th2-differentiated T cells are circulating in the system.

To determine if the reason AHR and airway inflammation were not being induced was lack of T cell recruitment to the lung in the absence of eosinophils, we determined the numbers of eosinophils and CD4$^+$ T cells in the lungs of these mice. We found significantly lower numbers of eosinophils and CD4$^+$ T cells in the lungs of ΔdblGATA mice transferred with OT-II Th2 cells and IL-13$^{-/-}$ eosinophils compared with ΔdblGATA mice that received OT-II Th2 cells and WT eosinophils or WT mice that received OT-II Th2 cells (Fig 4-6C,D). ΔdblGATA mice transferred with OT-II T cells alone and challenged with OVA also had significantly reduced infiltration of CD4$^+$ T cells to the lung (Fig. 4-6C), suggesting that even when the numbers of Th2 cells are elevated, these cells still have a requirement for the production of IL-13 by eosinophils in order to migrate to the lung.

We also analyzed the amount of Th2 cytokine mRNA in the lungs of the different groups of mice by qRT-PCR for IL-4, IL-13, and IFN-γ. We found that ΔdblGATA mice transferred with OT-II Th2 cells alone or OT-II Th2 cells and IL-13$^{-/-}$ eosinophils had statistically lower levels of IL-4 and IL-13 in the lungs, when compared with WT mice injected with OT-II Th2 cells or ΔdblGATA transferred with OT-II Th2 cells and WT eosinophils (Fig. 4-7E). These data indicate that lack of T cell migration into the lungs of ΔdblGATA mice transferred with OT-II Th2 cells and IL-13$^{-/-}$ eosinophils also results in reduced Th2 cytokine production in these mice. Overall, these data suggest that regardless of the number of T cells that are able to produce IL-13, they still maintain a requirement for eosinophil-derived IL-13 in order to migrate to the lung.
Figure 4-7: Elevating the number of Th2-differentiated T cells is not able to compensate for the requirement of eosinophil-derived IL-13 in the generation of allergic asthma. (A) Lungs from OVA-challenged naive WT mice transferred with in vitro differentiated OT-II T cells or AdblGATA mice transferred with in vitro differentiated OT-II T cells and +/- WT or IL-13^-^ eosinophils were fixed, sectioned, and stained with H&E to determine airway inflammation or PAS to determine mucus secretion (bar=50 µm). (B) Mice from groups mentioned in (A) were administered 40 µg of OVA intranasally for 4 days and then mechanically ventilated to determine AHR. (C&D) Lungs from groups in (A) were analyzed for numbers of T cells or eosinophils by flow cytometry. (E) RNA was extracted from lungs of mouse groups mentioned in (A), and levels of IL-4, IL-13, and IFN-gamma were analyzed by real-time PCR. *= p<.05. n=4 mice per group.
Discussion

In this investigation we have provided evidence that IL-13 secretion by eosinophils is an early event integral to the recruitment of T cells to the lung during the pathogenesis of allergic asthma. This work underpins our previous research, as well as that of others, that suggested eosinophils were required for the recruitment of T cells to the lungs (125, 128). In these previous studies, we showed in ΔdblGATA-1 mutant eosinophil deficient mice on the C57Bl/6 background that, upon allergen challenge, T cell recruitment to the lung was reduced in the absence of eosinophils, as was Th2 cytokine production and chemokine production of Eotaxin-1/Eotaxin-2. These defects could be rescued by transferring eosinophils back into ΔdblGATA mice, or by administering the chemokine Eotaxin-1 directly to the lung in combination with OVA allergen. While add-back of Eotaxin-1 was able to rescue recruitment of T cells to the lungs, as well as some Th2 cytokine production, it is possible that this event would normally occur secondarily to IL-13 cytokine production. It is known that IL-13 is a strong inducer of Eotaxin-1 production in \textit{in vivo} allergic models and when IL-13 is administered to endothelial cells, it promotes production of Eotaxin-1 (17, 45, 52). IL-13\textsuperscript{−/−} mice produce very little Eotaxin-1 and have severely attenuated symptoms of allergic airway inflammation, while transgenic mice that over-express IL-13 in the lungs exhibit large increases of this chemokine in the BALF and lung (86).

To help determine how different sources of IL-13 can affect disease development, we developed a network model of allergic asthma and ran simulations probing the requirements for different components of the disease, by creating various deficiencies. We first ran simulations testing our model to see if adding IL-13 into the system could bypass the need for eosinophils in eosinophil deficient systems. We administered this cytokine in combination with allergen to the intranasally to ΔdblGATA mice and determined that IL-13 could rescue AHR, chemokine and cytokine production, and T cell recruitment to the lung. This correlated with data derived from
other mouse models that were defective in Th2 responses. Similar results have been found in IL-5 and Eotaxin-1 double deficient mice, although of course Eotaxin-1 expression is not rescued in these mice (39). It is possible in this case, that Eotaxin-2 is induced in the absence of Eotaxin-1 and is able to facilitate influx of inflammatory cells.

Given that both human and mouse eosinophils have preformed message for IL-13 as well as intracellular granule stores of this cytokine, it should not have been unexpected that IL-13 production by these cells can significantly contribute to the generation of allergic asthma. Our computational simulations and data suggest that eosinophils deficient in IL-13 are unable to generate AHR and airway inflammation due to an inability to recruit T cells to the lung, and are defective in subsequent Th2 cytokine production. This is likely due in part to an inability to induce production of chemokines such as Eotaxin-1 and Eotaxin-2 that play a major role in recruiting immune cells to the lung as well as Th2 cytokine production during allergen challenge. Eotaxin/CCR3 signals are able to induce eosinophils to release Th2 cytokines from secondary granules that can affect immediate activation of cells such as mast cells, which can secrete inflammatory mediators that contribute to lung pathology (5, 47). Mice lacking both Eotaxin-1 and Eotaxin-2 have reduced inflammatory cell recruitment to the lung as well as defects in Th2 cytokine production in the lung (51). Similarly, mice double deficient in IL-5 and Eotaxin-1 are also unable to recruit inflammatory cells to the lungs due to lack of eosinophils and reduced chemokines, however transferring eosinophils back into the lungs of these mice restores the ability of inflammatory cells to migrate to the lung and secrete Th2 cytokines (39).

It was also interesting that both naïve and OVA-sensitized ΔdblGATA mice provided with IL-13−/− eosinophils had reduced numbers of these cells in the lungs compared with ΔdblGATA mice that were given WT eosinophils. Both types of eosinophils were equally able to migrate to the lungs in ΔdblGATA mice when examined 16 hours after intravenous transfer and intranasal OVA challenge. However when lungs from OVA-sensitized ΔdblGATA mice
challenged with OVA were analyzed, we found reduced numbers of IL-13\(^+\) eosinophils. Thus T cells require eosinophils to migrate into the lungs, and eosinophils may require T cell production of cytokines, such as IL-5, in order to survive for extended periods in the lungs, or to maintain or recruit these cells into the lung. This hypothesis is supported by the fact that even when ΔdblGATA mice received elevated numbers of Th2-differentiated T cells along with IL-13\(^-\) eosinophils, they were still unable to recruit T cells to the lungs (Fig. 4-7C). Conversely, when WT eosinophils were transferred into IL-13\(^-\) mice, they were able to induce migration of T cells to lung, but as these recruited cells were unable to produce IL-13, a large-scale allergic response could not occur. Unfortunately, we have not yet examined how many eosinophils are able to infiltrate the lungs in these mice, or whether are able to maintain a presence in the lungs in the absence of T cell-derived IL-13. More studies are needed to determine this, as well as whether the computational model’s prediction that T cells continually cycle in and out of the lungs in the presence of eosinophils the sole source of IL-13 is correct. It would also be interesting to more closely model the dynamic interactions between eosinophils and T cells in the allergic lung.

There is much indirect evidence to support this interdependent relationship between eosinophils and T cells in the generation of an allergic response. IL-5 and Eotaxin-1 double deficient mice cannot produce IL-13 in the lungs when challenged with allergen (39). PHIL\(^-\) mice have defective T cell migration to the lung and Th2 cytokine production in the absence of eosinophils, as do ΔdblGATA mice (76, 77). Mice lacking the common βε receptor that is required for the development of eosinophils, mast cells, and basophils also have defects in T cell migration to the lung as well, as Th2 T cell differentiation (28). At the same time, antibody blockade of CD4\(^+\) T cells in the lung reduces secretion of chemokines and Th2 cytokines as well as attenuating eosinophilic inflammation in the lung (3, 5, 6). Rag\(^-\) mice lacking T and B cells also fail to induce significant eosinophilic inflammation in the lungs, as do CD-4 deficient mice (131).
Collectively, these data suggest that there is a unique interdependence between eosinophils and T cell during the course of an allergic airway response. T cells require eosinophil secretion of IL-13 in order to migrate to the lung in response to allergen challenge, and eosinophils most likely require T cells to secrete cytokines for sustained eosinophil survival in the lung environment, as well as to induce mass mobilization of eosinophils from the bone marrow. We found our computational model to be a useful tool in helping to decipher this complicated dynamic between eosinophils and T cells, and recognize its ability to aid in further exploration of the contributions of eosinophils and T cells to allergic asthma, as well as identifying potential therapies to counteract their effects. This interconnected relationship presents an opportunity for targeting the main effector cells in an allergic asthma response. If one were able to simultaneously ablate these two cells, it may result in abrogation of allergic asthma symptoms that afflict millions of people. In the next chapter, I discuss a potential mechanism for disrupting the function of both eosinophils and T cells in allergic disease.
Eosinophilia activation and migration to the lung have been correlated with allergic asthma disease development for many decades. These cells are able to secrete a wide range of cytokines, as well as chemokines and lipid mediators, that significantly contribute to exacerbation of allergic asthma. Despite this knowledge, very few relevant therapeutics have been developed to treat eosinophil associated pathology in asthma. Mepolizumab is a drug that targets eosinophils by depriving them of a cytokine, IL-5, that is critical to their survival and differentiation. Clinical trials with this drug revealed little efficacy in alleviating the symptoms of allergic asthma, although it seemed to decrease long term lung remodeling (22, 124). It was found that while numbers of circulating eosinophils were decreased by 90%, tissue resident eosinophils were only decreased by 50%, allowing some cells to remain and become activated locally in the lung (22). It may be that residual signaling through the common gamma chain that IL-5 shares with IL-3 and GM-CSF is enough to allow a small number of eosinophils to survive, or that the antibody delivered systemically has difficulty penetrating the lung environment. Thus, while anti-cytokine therapy against IL-5 seems to work well in a systemic fashion, it cannot counteract the local effects that are caused during an allergic response in the lung, perhaps due to inability to penetrate well into the lung environment.

Small molecule inhibitors have been used successfully to treat a variety of diseases in both in systemic and local fashion. Their small size enables them to infiltrate areas that larger molecules cannot, and also to be taken up by cells fairly easily. This approach may be an attractive alternative that could be used to target eosinophils in allergic asthma if an appropriate
target molecule could be identified, such as an eosinophil intracellular signaling component. Unfortunately, many of the signaling pathways that eosinophils are known to use are common to many different immune cells, as well as nonimmune cells. CCR3, one of the main chemokine receptors expressed constitutively on the eosinophil surface, is a G-protein coupled receptor that signals through $\mathrm{G}\alpha$ and $\mathrm{G}\beta\gamma$ subunits. The $\mathrm{G}\alpha$ subunit is able to directly activate Src-family kinases (132), which leads to activation of MAPKs and protein kinase B (PKB) that can activate transcription of genes that result in eosinophil degranulation and secretion of cytokines and lipid mediators (133). The $\mathrm{G}\beta\gamma$ subunit activates several pathways, including $\mathrm{Ca}^{++}$ mobilization downstream of PLC-$\gamma$, and PKB and MAPKs through phosphotidylinositol 3 kinase-$\gamma$ (PI3K-$\gamma$). These pathways are not unique to eosinophils, but are expressed in many different cell types, thus represent problematic therapeutic targets.

ITK is a molecule that is expressed solely in immune cells upstream of PLC-$\gamma$ and MAPKs. It has been shown in T cells that absence of ITK results in impaired $\mathrm{Ca}^{++}$ signaling and reduced MAPK activation as well as a lower number of CD4$^+$ T cells (99). Responses to Th1 diseases in mice lacking ITK are intact, while Th2 disease responses are severely attenuated, particularly allergic asthma, making this molecule a viable drug target (109). If eosinophils were to express ITK, it may also be an effective target to counteract the actions of eosinophils in allergic asthma. In T cells, ITK has been shown to phosphorylate PLC-$\gamma$ to induce $\mathrm{Ca}^{++}$ mobilization, and this process is also a PLC-$\gamma$–dependent event in eosinophils (99). ITK may also have a role in PKB activation, a process linked to survival and cytokine production in eosinophils. Eosinophils express ITK and it could potentially be integral to eosinophil functions in allergic asthma in vivo. Here, we have generated IL-5 transgenic ITK$^{+/}$ mice that overproduce eosinophils lacking ITK and determined that these eosinophils have attenuated function in allergic asthma, potentially due to a defect in ERK1/2 signaling that could possibly lead to a defect in effector functions.
Results

**IL-5 transgenic ITK<sup>−/−</sup> mice have reduced CD4<sup>+</sup> T cell numbers**

Because previous strains of mice that were created lacking ITK have shown defects in the development of CD4<sup>+</sup> T cells, we characterized the T cell compartment to determine if IL-5 transgenic ITK<sup>−/−</sup> mice also have this defect or whether the inherent overproduction of IL-5 in these mice can restore the CD4<sup>+</sup> T cell population (103). Using age-matched 10-week-old mice, we enumerated the number of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the thymus, lymph nodes, and spleen of C57Bl/6 WT, IL-5 transgenic (IL-5 tg) and IL-5 transgenic ITK<sup>−/−</sup> mice (IL-5 tg ITK<sup>−/−</sup>). We found that there was no defect in the overall number of CD8<sup>+</sup> T cells in any of these tissues; however, the number of CD4<sup>+</sup> T cells was significantly reduced in the lymph nodes of IL-5 tg ITK<sup>−/−</sup> mice (Fig 5-1A). When we further characterized the different subsets of T cells by staining for activation markers CD44 and CD62L, we found a reduction in the number of naïve CD4<sup>+</sup> T cells expressing low levels of CD44 and high levels of CD62L in the lymph nodes (Fig. 5-1A), similar to numbers found in ITK<sup>−/−</sup> spleens (Fig. 5-1C). However, there was not a significant reduction in this population in the thymus. Perhaps there is increased apoptosis of these cells in the periphery due to ITK<sup>−/−</sup> T cells’ reduced ability to make IL-2, a survival cytokine for T cells. However, this defect cannot be fully explained by this reason as the number of CD4<sup>+</sup> T cells expressing a memory phenotype, CD44<sup>hi</sup>/CD62L<sup>lo</sup>, were at numbers similar to WT (Fig. 5-1A). The number of naïve CD8<sup>+</sup> T cells in the lymph nodes of IL-5 ITK<sup>−/−</sup> mice was also significantly reduced compared with IL-5 tg mice (Fig. 5-1B), again displaying characteristics much like ITK<sup>−/−</sup> mice (data not shown). The memory phenotype population in IL-5 tg ITK<sup>−/−</sup> mice was larger than that of IL-5 tg mice, though not statistically so, as is seen in ITK<sup>−/−</sup> mice (Fig. 5-1F).
Figure 5-1: IL-5 tg ITK−/− mice have reduced numbers of CD4+ T cells in the peripheral lymph nodes. (A) Lymph nodes were harvested from WT, IL-5 tg, and IL-5 tg ITK−/− mice, homogenized to a single cell suspension through a screen, enumerated on a hemocytometer, and stained with antibodies against CD4, CD44, and CD62L, then analyzed by flow cytometry. (B) Lymph nodes from WT, IL-5 tg, and IL-5 tg ITK−/− mice processed as in (A) were stained with antibodies against CD8, CD44, and CD62L, and then analyzed by flow cytometry. (C) Spleens from WT or ITK−/− mice were treated as in (A) and were stained with antibodies against CD4, CD44, and CD62L, and then analyzed by flow cytometry. (D) Thymi from WT, IL-5 tg, and IL-5 tg ITK−/− mice were treated as in (A) and stained with antibodies against cell surface markers as in (A), then analyzed by flow cytometry. (E) Thymi from WT, IL-5 tg, and IL-5 tg ITK−/− mice were treated as in (A) and stained with antibodies against cell surface markers as in (B), then analyzed by flow cytometry. (F) Spleens from WT or ITK−/− mice were treated as in (A) and were stained with antibodies against CD8 and CD44, and then analyzed by flow cytometry. * = p < 0.05.
**IL-5 tg ITK^- mice display high levels of serum IgE**

It is known that ITK^- mice have large quantities of serum IgE due to gamma delta T cells inducing a high level of class switch in B cells to IgE and overproducing this isotype of antibody (Fig. 5-2B) (unpublished results). To determine if IL-5 tg ITK^- mice also have high levels of serum IgE, we took blood from 10-12 week-old C57Bl/6 WT, IL-5 tg, and IL-5 tg ITK^- mice, spun it down to separate out serum, and quantified the amount of serum IgE via ELISA. We found that IL-5 tg ITK^- mice had a 5 fold increase in this antibody, indicating that expression of the IL-5 transgene did not alter the expected ITK^- phenotype.

![Graphs showing increased serum IgE levels](image)

**Figure 5-2:** IL-5 tg ITK^- have increased levels of serum IgE. (A) Blood from IL-5 tg, or IL-5 tg ITK^- mice was harvested via cardiac puncture, centrifuged, and serum collected. The amount of serum IgE was analyzed via an IgE ELISA kit. (B) Blood from WT or ITK^- mice treated as in (A) was analyzed for serum IgE via an IgE ELISA kit. * = p = <.005.
IL-5 tg ITK<sup>−/−</sup> mice have comparable numbers of resting and activated eosinophils to IL-5 tg mice

It is possible that ITK is an important component of eosinophil signaling during development in the bone marrow as it is for T cells in the thymus. If this is the case, we would have expected to see lower numbers of these cells in the peripheral immune system. Hence, we determined the number of eosinophils in the thymus, lymph nodes, and spleen of 10-12 week-old IL-5 tg and IL-5 tg ITK<sup>−/−</sup> mice by staining cells from these tissues with CCR3. We found similar overall numbers of eosinophils in the thymus, lymph nodes and spleens of these mice compared to IL-5 tg mice (Fig. 5-3A). Both types of mice had larger numbers of eosinophils in the thymus and spleen compared to the lymph nodes, which is a trend that occurs normally in WT mice, demonstrating that although the numbers of eosinophils are drastically increased in IL-5 tg mice, they are still distributed at a similar frequency throughout the body compared to WT (Fig. 5-3A).

We next stained cells from thymus, lymph nodes, and spleens with CD69 or CD62L and CCR3 to determine the number of activated eosinophils in IL-5 tg ITK<sup>−/−</sup> mice compared to IL-5 tg mice. IL-5 tg ITK<sup>−/−</sup> mice displayed similar numbers of CCR3<sup>+</sup>CD69<sup>+</sup> eosinophils in thymus, lymph node, and spleen to IL-5 tg mice, indicating that these mice have similar numbers of activated eosinophils (Fig. 5-3B). When we stained cells with CCR3 and CD62L, we found increased numbers of CD62L<sup>LO</sup> eosinophils in the lymph nodes of IL-5 tg ITK<sup>−/−</sup> mice compared to IL-5 tg mice, contradicting the data from above and indicating that there are more eosinophils in IL-5 tg ITK<sup>−/−</sup> lymph nodes that have an activated phenotype (Fig. 5-3C). The reason for the discrepancy between CD69 and CD62L<sup>lo</sup> expression is not clear, but more repetitions of this experiment may reveal whether these trends repeat. The number of eosinophils expressing the CD62L<sup>LO</sup> phenotype in the thymus and spleen were not significantly different between the two groups.
Figure 5-3: ITK is not required for eosinophil development. (A) Thymus, lymph nodes, and spleens from IL-5 tg mice or IL-5 tg ITK−/− mice were homogenized to a single cell suspension and stained with CCR3 antibody along with forward and side scatter analysis to identify eosinophils. (B) Thymus, lymph nodes and spleens from IL-5 tg and IL-5 tg ITK−/− mice processed as in (A) were stained with antibodies against CCR3 and activation marker CD69 to determine activation status of eosinophils. (C) Thymus, lymph nodes and spleens from IL-5 tg and IL-5 tg ITK−/− mice processed as in (A) were stained with antibodies against CCR3 and activation marker CD62L to determine activation status of eosinophils. *= p<.05. n=4 mice per group.
**ITK**<sup>−/−</sup> eosinophils have reduced function in an *in vivo* model of OVA-induced allergic asthma

Although eosinophils are able to develop normally in IL-5 tg ITK<sup>−/−</sup> mice, they may still have functional defects in response to disease. Using the model previously described in Chapters 3 and 4, we determined the ability of ITK<sup>−/−</sup> eosinophils to induce AHR and facilitate recruitment of CD4<sup>+</sup> T cells during a model of OVA-induced airway inflammation. We transferred WT or ITK<sup>−/−</sup> eosinophils into OVA sensitized ΔdblGATA mutant mice and then gave mice 40 µl of 2 mg/ml OVA intranasally on four consecutive days. Twenty-four hours after the last intranasal challenge, we mechanically ventilated mouse and analyzed the resistance of their lungs in response to a methylcholine dose curve. As observed previously, ΔdblGATA mice challenged with OVA alone had significantly lower increases in resistance compared to WT mice (Fig 5-4B). When ΔdblGATA mice were transferred with WT eosinophils and challenged with OVA, AHR was rescued in these mice to near WT levels (Fig. 5-4B). However, when ΔdblGATA mice were transferred with ITK<sup>−/−</sup> eosinophils and challenged with OVA, they displayed statistically lower resistance in the lungs compared to either WT or ΔdblGATA mice transferred with WT eosinophils (Fig 5-4B), indicating that there is indeed a functional defect in the ability of ITK<sup>−/−</sup> eosinophils to respond during the course of an allergic asthma response.

After mechanical ventilation, mice were sacrificed, lungs excised and some fixed in paraformaldehyde and embedded in paraffin for sectioning and staining. Lungs from ΔdblGATA mice transferred with ITK<sup>−/−</sup> eosinophils and challenged with OVA that were stained with H&E showed fewer inflammatory infiltrates and reduced airway inflammation, similar to ΔdblGATA +
OVA alone, than lungs from ΔdblGATA mice transferred with WT eosinophils and administered with OVA (Fig. 5-4A). In contrast, ΔdblGATA mice transferred with WT eosinophils showed levels of airway inflammation and inflammatory cell infiltrates that were comparable to WT + OVA lungs (Fig. 5-4A). Lung sections stained with PAS to detect mucus also showed a decrease in mucus production in the lungs of ΔdblGATA mice transferred with ITK−/− eosinophils and challenged with OVA compared with either WT or ΔdblGATA mice transferred with WT eosinophils (Fig. 5-4A). The lower airway inflammation, reduced inflammatory infiltrates, and decreased mucus production in the lungs of ΔdblGATA mice transferred with ITK−/− eosinophils correlate with the reduced AHR found in these mice, overall indicating that there is a functional disability in ITK−/− eosinophil responses.

Previously our lab showed that ΔdblGATA mice are unable to recruit T cells to the lung in response to OVA challenge, but upon WT eosinophil transfer, this defect is rescued. It is possible that eosinophils lacking ITK are unable to provide a necessary signal to recruit T cells to the lung. To test this, we reconstituted OVA-sensitized ΔdblGATA mice with either WT or ITK−/− eosinophils and challenged them intranasally with OVA for four consecutive days. Twenty-four hours after the last intranasal challenge, we excised the lungs, digested them with collagenase, and stained them for CD4+ T cells. We found no significant difference between the numbers of T cells infiltrating the lungs of ΔdblGATA mice transferred with WT or ITK−/− eosinophils, indicating that ITK−/− eosinophils are just as capable as WT eosinophils in recruiting T cells to the lungs (Fig. 5-4C). One caveat with this experiment is that the proper WT and ΔdblGATA controls were not present and that it was only performed once with 3 mice per group. Subsequent repetitions may or may not agree with the result found here.

Even though eosinophils appear to be able to recruit T cells to the lung, it is still possible that they are required to provide a signal in order to induce T cells to secrete cytokines once there.
To determine this, we took lungs and draining lymph nodes from ΔdblGATA mice transferred with either WT or ITK<sup>−/−</sup> eosinophils and challenged with OVA. We homogenized the lungs and extracted RNA for real time PCR (RT PCR). We conducted RT PCR for Th2 cytokines IL-4 and IL-13 as well as Th1 cytokine IFN-γ, and found that all three of these cytokines were reduced in the lungs of ΔdblGATA mice transferred with ITK<sup>−/−</sup> eosinophils compared with WT eosinophil transfers or WT mice (Fig. 5-4D), suggesting that while ITK<sup>−/−</sup> eosinophils are able to induce T cell migration to the lung during an allergic response, these T cells are lacking an appropriate signal, presumably derived from eosinophils, to promote production of Th2 cytokines. These data also correlated with findings derived from <em>ex vivo</em> OVA restimulation of draining lymph node T cells from WT, ΔdblGATA mice, ΔdblGATA mice + WT eosinophils, and ΔdblGATA mice + ITK<sup>−/−</sup> eosinophils. We found that while T cells from ΔdblGATA mice transferred with WT eosinophils secreted IL-4 and IL-13 cytokine levels significantly higher than ΔdblGATA mice, although lower than WT mice, T cells from ΔdblGATA mice transferred with ITK<sup>−/−</sup> eosinophils secreted statistically lower amounts of IL-4 and IL-13 compared with T cells from WT or ΔdblGATA + WT eosinophils (Fig. 5-4E). This correlates with the above RNA data from the lungs, indicating that ITK<sup>−/−</sup> eosinophils may not be able to provide additional signals required for Th2 production of cytokines from T cells either activated in the lungs or draining lymph nodes.
Figure 5-4: ITK<sup>-/-</sup> eosinophils cannot facilitate induction of an allergic asthma response in ΔdblGATA mice. (A) Lungs from OVA-sensitized WT or ΔdblGATA mice transferred with WT or ITK<sup>-/-</sup> eosinophils were fixed, sectioned, and stained with H&E to determine airway inflammation or PAS to determine mucus secretion (bar=50 µm). (B) Mice from groups in (A) were administered 40 µg of OVA intranasally for 4 days and then mechanically ventilated to determine AHR. (C) Lungs from groups in (A) were analyzed by flow cytometry to determine the numbers of T cells. (D) RNA was extracted from lungs of mouse groups mentioned in (A), and levels of cytokines were analyzed by real-time PCR. (E) Lymph nodes from groups in (A) were collected and restimulated ex vivo for three days with 100 µg/ml OVA. Supernatants were collected and cytokine levels analyzed by multiplex cytokine bead assay. *= p<.05. (n=3 per group)
**ITK<sup>-/-</sup> eosinophils have reduced Erk1/2 signaling in response to Eotaxin-1 stimulation in vitro**

To determine whether the reduced *in vivo* responses to OVA in our allergic asthma model were due to a defect in eosinophil signaling downstream of chemokine stimulus, we performed *in vitro* stimulation of eosinophils with Eotaxin-1. This chemokine induces signal transduction through CCR3, the main chemokine receptor by which eosinophils are stimulated to produce cytokines during an allergic response. If phosphorylation of proteins downstream of CCR3 were dysfunctional due to the absence of ITK, this might cause impairment of eosinophil production of cytokines or chemokines that stimulate Th2 T cells to produce cytokines, such as IL-4 and IL-13 that are involved in allergic responses.

To test whether signaling downstream of CCR3 was intact, we purified eosinophils from the peritoneum of IL-5 tg or IL-5 tg ITK<sup>-/-</sup> mice and stimulated them with 10 ng/ml Eotaxin-1 in RPMI 1640 media in a time course from 0 to 30 minutes at 37 degrees Celsius. After stimulation, cells were washed, lysed, and 20 µg loaded on an SDS-PAGE gel. After transferring the proteins to PVDF membrane, we probed with anti-phospho-Erk1/2. We found that, compared to WT eosinophils, there is delayed and reduced Erk1/2 signaling downstream of CCR3 in ITK<sup>-/-</sup> eosinophils (Fig. 5-5A). Total levels of Erk1/2 appeared to be equal in all samples, indicating that the observed differences in phosphorylation were not due to unequal protein loading (Fig. 5-5A). We quantified this further using a modified ELISA MSD protein assay, in which total Erk and phosphorylated Erk are measured in each well, allowing for normalization of protein levels between wells. We quantified the amount of phosphorylated protein using this assay at the time points we observed the highest difference in phosphorylated Erk1/2, 1 minute and 5 minutes. We
determined that there was about a 50 percent reduction in Erk1/2 phosphorylation in ITK<sup>−/−</sup> eosinophils at these time points (Fig 5-5B), correlating with the data from the Western blot, and perhaps providing a partial explanation as to why T cells recruited to the lungs of ΔdblGATA mice by ITK<sup>−/−</sup> eosinophils are not able to produce Th2 cytokines.
Figure 5-5: ITK<sup>−/−</sup> eosinophils have reduced ERK 1/2 activation downstream of CCR3. (A) WT or ITK<sup>−/−</sup> eosinophils were purified and 5 X 10⁶ cells per time point were treated with 10 ng/ml of Eotaxin-1 for the indicated amount of time, and then washed, cells pelleted and lysed, and run on an SDS PAGE gel. After transfer to PVDF membrane, blots were blocked, probed with mouse monoclonal phospho-ERK 1/2 or total ERK 1/2, and then anti-mouse HRP secondary antibody (n=3). (B) Eosinophils were treated as in (A) and cell lysates applied to an MSD plate coated with antibodies against phospho-ERK1/2/total ERK, and a modified ELISA technique for quantification of phospho-ERK1/2 was followed as per manufacturer protocol (n=1).


**Discussion**

In this investigation we have provided evidence for ITK being integral to the function of eosinophils during an allergic asthma response. This data lends support to findings from other ITK deficient systems that are defective in Th2 responses. Although ITK\(^{-/-}\) eosinophils appear to be functionally deficient in this model, IL-5 tg ITK\(^{-/-}\) mice have similar numbers of eosinophils compared to IL-5 tg mice (Fig. 5-3). However, they exhibit some defects in T cell development similar to ITK\(^{-/-}\) mice; i.e. they have reduced numbers of CD4\(^+\) T cells in the peripheral tissues, although this defect does not apply to the thymus as it does in ITK\(^{-/-}\) mice (103). The reason for this discrepancy is not clear. IL-5 tg ITK\(^{-/-}\) mice also exhibit high levels of IgE, much like other ITK deficient strains (Fig. 5-2) (109). It may be that gamma delta T cells in these mice have similar functional characteristics to ITK\(^{-/-}\) mice, resulting in increased B cell class switch to the IgE isotype.

Although IL-5 ITK\(^{-/-}\) mice produce equal numbers of eosinophils from the bone marrow compared to IL-5 tg mice, there is some difference in the activation status of these cells. In the lymph nodes but not thymus or spleens of IL-5 ITK\(^{-/-}\) mice, there are a significantly higher number of CD62L\(^{LO}\) eosinophils, but not CD69\(^+\) cells (Fig. 5-3B, 3C). It is possible that CD62L is a more reliable marker for eosinophil activation. A literature search revealed that CD69 is not a marker commonly used to denote eosinophil activation, whereas CD62L is utilized frequently (134). It is also possible that these results are an anomaly and the study should be repeated.

Further study of eosinophil maturation from the bone marrow in IL-5 tg ITK\(^{-/-}\) mice is warranted as well. Recently, research from Voehringer, *et al*, showed that eosinophils develop in distinct stages that are characterized by varying expression of CCR3, Siglec-F, FIRE, and CD62L (134). To determine whether this process occurs normally, characterization of these markers is required in IL-5 tg ITK\(^{-/-}\) mice. However, ITK does not play a significant role in the development of mast...
cells from the bone marrow, so it is possible that ITK expression does not affect eosinophil maturation either (114).

ITK’s role in Th2-dependent diseases has been well characterized in mice where all cells are deficient in ITK. Of course, these mice are unable to mount an effective Th2 response in the course of an allergic asthma response, exhibiting attenuated AHR, fewer cellular infiltrates to the lung, including T cells, as well as decreased mucus and Th2 cytokine production in the lung (109). Similarly, these mice have reduced capacity to respond to parasitic infections, again due ultimately to their inability to produce Th2 cytokines (99, 103). However, most of these defects have been attributed to the T cell’s inability to produce IL-4 and IL-13. It is only now that the particular contribution of each type of ITK-expressing cell to the development of disease is being ascertained. The research presented above delineates a clear role for eosinophils in the course of allergic asthma, and potentially other Th2 diseases. ITK−/− mice have impaired Th2 responses in vivo, exhibiting reduced AHR, just as we observe in ΔdblGATA mice reconstituted with ITK−/− eosinophils (Fig. 5-4B) (109). Also similar to ITK−/− mice, we discovered reduced airway inflammation and mucus production in histological analysis of H&E and PAS-stained lung sections, respectively, of ΔdblGATA mice transferred with ITK−/− eosinophils (Fig 5-4A). It may be possible that attenuated T cell activation in response to allergen challenge may be partially due to ITK−/− eosinophils being unable to induce Th2 T cell activation in vivo.

The inability of ITK−/− T cells to differentiate into Th2 T cells precludes the development of allergic airway inflammation in ITK−/− mice (103), however, we now present data that suggest that even if these cells were Th2 cytokine-sufficient, interactions or lack thereof with ITK−/− eosinophils would not result in Th2 T cell activation during airway allergen challenge. ΔdblGATA mice transferred with ITK−/− eosinophils had reduced cytokine mRNA levels in the lung compared with IL-5 tg mice (Fig. 5-4D), indicating that ITK deficient eosinophils are incapable of delivering a necessary signal, perhaps IL-13 production, that induces large scale
production of Th2 cytokines, presumably by T cells. Further evidence that there is a defect in Th2 T cell cytokine secretion in ΔdblGATA mice transferred with ITK<sup>−/−</sup> eosinophils is derived from OVA restimulations of draining lymph node cells from these mice (Fig. 5-4E). ΔdblGATA mice transferred with ITK<sup>−/−</sup> eosinophils produced less IL-4 and IL-13 in lymph node restimulations, again suggesting that ITK<sup>−/−</sup> eosinophils were not able to provide a necessary signal for T cell secretion of Th2 cytokines, and perhaps are not able to produce Th2 cytokines themselves. This supposition would make sense considering that ITK<sup>−/−</sup> mice are defective in parasite clearance as well. For instance, in a *Schistosoma mansoni* model of infection, ITK<sup>−/−</sup> mice have defective Th2 responses, exhibiting poor T cell migration to the lung and reduced production of Th2 cytokines IL-4, IL-13, and IL-5 (135). Another example of compromised extracellular parasite Th2 responses in ITK<sup>−/−</sup> mice is infection with the nematode *N. brasiliensis*, in which ITK<sup>−/−</sup> are unable to clear the worms due to a suboptimal Th2 response (103). It would be interesting to see whether transfer of WT eosinophils into ITK<sup>−/−</sup> mice would be able to rescue the immune response to these parasites, or whether Th2 T cells would still be required.

Determining how ITK affects immune cell function has been a strong focus of research, and many direct and indirect results of ITK deficiency have been found. For example, ITK<sup>−/−</sup> mice have decreased mast cell function *in vivo* due to saturation of Fcε receptors on their cell surface by high circulating levels of IgE in these mice (114). However, when ITK<sup>−/−</sup> mast cell signaling is examined *in vitro*, these cells have similar phosphorylation levels of p38 and Erk1/2 downstream of the Fcε receptor, unlike Erk1/2 phosphorylation downstream of the TCR in CD4<sup>+</sup> T cells (114). Our results in eosinophil signaling downstream of CCR3 show reduced activation of Erk1/2 and may account for the reduced function of ITK<sup>−/−</sup> eosinophils *in vivo* (Fig. 5-5). Interestingly, ITK<sup>−/−</sup> mast cells have heightened translocation of NFATc1/NFATc2 to the nucleus, and are able to secrete higher amounts of cytokine than WT mast cells when stimulated *in vitro*, indicating that ITK deficiency results in a gain of function in these cells, unlike the deficit that is...
created in eosinophils and T cells upon loss of this signaling molecule (114). The true implications of inhibiting ITK in immune cells remains to be seen, but several small molecule inhibitors have been created against non-receptor tyrosine kinases, such as ITK, as well as MAPKs p38 and Erk1/2 that are currently undergoing research trials.

Itk-selective inhibitors BMS-488516 and BMC-509744 when given to mice are able to suppress the production of IL-2 induced by cross-linking the TCR (136). In addition, when BMC-509744 is administered to mice during a model of allergic asthma, it is able to dose-dependently decrease allergen-induced immune cell infiltration into the lung. The effects of these inhibitors on other disease models remain to be seen (136). Some inhibitors, U0126 for example, a potent and selective MEK1/2 inhibitor through ablation of ERK1/2 activation, is able to significantly block antigen-induced inflammatory cell infiltration of the airway, levels of IL-4, IL-5, IL-13 and eotaxin in BALF, serum antigen-specific IgE levels, mucus production, VCAM-1 expression, and airway hyperresponsiveness in a dose-dependent manner in a mouse asthma model (137).

Inhibitors against p38 MAPK, such as oral SB239063, significantly inhibit antigen- and leukotriene D4-induced eosinophil infiltration in both mouse and guinea pig models of asthma as well as promoting eosinophil apoptosis in guinea pig BALF (138). However, using inhibitors designed to depress MAPK signaling run the risk of inducing more general immune suppression as these molecules operate downstream of many pathways associated with disease. ITK may be a better target in this regard as its primary function appears to be in the development and function of Th2 T cells. Targeting ITK in allergic asthma holds a lot of promise, and brightens the future of fighting allergic diseases in a broader context, as ITK appears to be integral to the function of several immune cells that are involved in a variety of allergic diseases.
Chapter 6

Conclusions and Future Directions

Eosinophilia has been observed in conjunction with the development of allergic asthma for over a century, almost dating back to Paul Erlich’s discovery of this granulocyte in 1879. Since then, a vast number of studies have revealed just how diverse the functions of this cell are: secretion of numerous cytokines, chemokines, granules, lipid mediators, antigen presentation, and T cell polarization. Despite this knowledge, or perhaps because of it, a clear role for the function of eosinophils in allergic asthma has never been delineated, although researchers assumed that eosinophils were a terminal effector cell recruited to the airways by T cells later in the development of allergic asthma. In this thesis, I presented evidence that suggests that not only are eosinophils integral to the development of allergic asthma, but that IL-13 secretion by these cells is required to initiate Th2 T cell-dependent responses.

With the advent of eosinophil deficient strains of mice, it seemed only a matter of time before the role of eosinophils in the development of allergic asthma would be divulged. Lee, et al., created PHIL/− mice via an eosinophil ablation approach on a Bl/6 background and found that when subjected to model antigen, OVA, these mice did not develop allergic asthma (77). However, Humbles, et al., used a mouse model, ΔdblGATA mutant mice on a Balb/c background, under similar OVA challenge conditions, and determined that AHR was intact in these mice, but that eosinophils were required for induction of lung remodeling that occurs in chronic cases of asthma (76). Hence, these studies added more controversy to an already convoluted field of study.

Because strain specific variation in allergic responses has been reported in other knockout and transgenic mouse models, such as IL-4 and IL-13 deficient mice (117), we obtained
ΔdblGATA mutant mice on the C57Bl/6 background to determine whether the same trend occurs in the ΔdblGATA mutants. We found that this was indeed the case, and that ΔdblGATA mutant mice on the C57Bl/6 background were defective in the generation of allergic asthma (Fig. 3-1). Surprisingly, we also found that, in the absence of eosinophils, recruitment of T cells to the lung was drastically reduced, as was Th2 cytokine production in the lung, and when we added eosinophils back into the system, these defects were rescued (Fig. 3-2). This indicated that eosinophils were recruited or present in the lung before T cells and that they were responsible for driving T cell recruitment and perhaps responses. In retrospect, there had been prior clues that this was true. In 2006, Fulkerson, et al., reported reduced Th2 cytokine production in a parasitic model of A. fumigatus, as well as reduced mucus hypersecretion in the lungs of Balb/c ΔdblGATA mice (51). Lower T cell responses were also found in the βc-receptor knockout mice, mice unable to produce eosinophils, basophils, or mast cells, although these data did not definitely prove that eosinophils were responsible for this phenotype (28). Also, IL-4Rα+/IL-5−/ mice have impaired IL-13 production in a model of allergic asthma, whereas IL-4Rα−/ mice alone have only a slight defect in this ability (97). Despite these clues, no one further explored the possibility that eosinophils were influencing the function of T cells in allergic disease until we made our observations in the C57Bl/6 ΔdblGATA mice.

The data presented here have greater implications than the discovery that eosinophils are required for the development of allergic asthma because they provide a model system in which eosinophil function in disease can be examined in detail. We have already manipulated this model to determine that eosinophil-derived IL-13 is essential to T cell recruitment to the lungs during an allergic response, but eosinophils can secrete a myriad of other factors, including cytokines and chemokines, as well as lipid mediators that could be playing a role in allergic asthma development. For example, eosinophils have large amounts of preformed message for IL-
4 and have been shown to be able to secrete this cytokine in Th2 parasitic models of disease, other cells can also produce IL-4, and the specific contribution of eosinophil-derived IL-4 has never been determined in allergic asthma. Eosinophils are also able to present antigen to T cells, although the importance of this function to development of allergic asthma has not yet been explored. Given that several eosinophil deficient mouse models have T cells defective in Th2 cytokine production, antigen presentation by eosinophils could be a major factor in activation and differentiation of Th2 cells. This hypothesis could easily be tested in our model by reconstituting ΔdblGATA mice with eosinophils deficient in MHC Class II expression and subjecting them to a Th2 polarizing immunization protocol.

Our computational model of asthma is another useful tool developed in the course of these studies. This system allows us to simulate the removal of different components of an allergic asthma response and can predict the outcome of that deficiency in terms of an end result such as generating AHR, recruitment of inflammatory cells, or mucus production. We used computational simulation in order to determine different effects of eosinophil and T cell-derived IL-13 in the generation of allergic asthma, which led to the discovery of a unique interdependent relationship between eosinophils and T cells in allergic asthma. These cells have a unique interdependence during the generation of allergic asthma, although further study into the temporal and spatial requirements of this relationship is needed to more closely define this correlation. For instance, we know that eosinophils are required for the migration of T cells to the lungs, but we don’t know when exactly eosinophils are recruited there, or whether it is mostly tissue resident eosinophils that are activated and secrete IL-13 to induce chemokine secretion and subsequent T cell infiltration. This system could also be used to remove sources of other cytokines from the simulated allergic response to provide a starting point for developing in vivo experiments. Computational networks could also be useful in modeling the complex effects of eosinophils on lung remodeling in chronic asthma. These systems may prove to be a valuable component of
allergic asthma research and present interesting possibilities within allergic systems that may have otherwise been unexplored.

We also used our eosinophil deficient mouse model to determine whether ITK is integral to the function of eosinophils in allergic asthma. We showed that ITK, a non-receptor tyrosine kinase essential to the development of Th2 T cell responses, appears to be required for eosinophil signaling and function as well. This deficiency may be due in part to reduced MAPK signaling in eosinophils, as we found reduced Erk1/2 activation in these cells upon stimulation with Eotaxin-1, however much more detailed analysis of this system is required before we know the exact mechanism by which ITK affects eosinophil activation and function. For instance, it is known that T cells lacking ITK have reduced migratory capacity in response to chemokines both in vitro and in vivo. These cells also have reduced Ca^{++} signaling when stimulated through the T cell receptor. It is possible that eosinophils may have similar sorts of deficiencies. Degranulation of these cells may be decreased, as well as efficient generation of preformed cytokines stores. These processes are not affected in ITK^{−/−} mast cells, however Erk1/2 signaling is also normal in these cells.

It is also important to determine how ITK functions in signaling networks within the eosinophil. ITK may potentially interact directly with the intracellular domain of chemokine receptors to phosphorylate tyrosine residues to induce downstream MAPK signaling, or it may interact with other proteins downstream of the receptor. In T cells, ITK is known to complex with LAT downstream of the TCR and is responsible for phosphorylation of PLC_{γ−1}, as well as being thought to interact with several other proteins in the T cell signaling cascade.

Taken together, these data have provided further insight into the role of eosinophils in allergic asthma, by showing that they are integral to the recruitment of T cells to the lung during an allergic asthma response. We have also suggested a mechanism involving IL-13 secretion by which eosinophils induce T cell recruitment in this disease, and presented a potential strategy for
attenuating the function of these cells in allergic responses by targeting ITK. There is still much
to be discovered about this cell and its functions, as well as the development of allergic diseases
in general. However, these data, as well as other current eosinophil studies, offer new perspective
on how eosinophils may be affecting allergic asthma development and represent a paradigm shift
in that these cells will now be viewed as instigators of disease rather than only a terminal effector
cell. This knowledge opens new possibilities for the development of effective therapeutic agents,
and brightens the prospect for future treatment of allergic asthma.
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