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# The Role of IL-25 in Colitis-Associated Colon Cancer and Pulmonary Inflammation

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**Abstract**

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Epithelial cells located at mucosal barrier surfaces act as our front-line of defense against environmental toxins, pathogens, and helminth infections. Activation of pattern recognition receptors (PRRs) on epithelial cells stimulates them to produce IL-25, IL-33, and TSLP. These three cytokines, acting individually and/or in cooperation with one another, are capable of initiating and maintaining type-2 inflammation. There remain many unanswered questions in regard to the specific cell types IL-25 acts upon to produce pulmonary inflammation. In addition, whether the roles for IL-25, IL-33, and TSLP are interdependent and/or redundant has been largely unexplored. In this study, we selectively inhibited IL-25 signaling on CD4<sup>+</sup> cells, monocyte/macrophage populations, and CD11c<sup>+</sup> dendritic cells by deleting its receptor, IL-17RB. In each of these systems, we found IL-25 was able to generate type-2 inflammation despite the lack of direct signaling in any of these cells. Additionally, when IL-33 and TSLP

signaling (individually and in combination) were deleted, IL-25 was still capable of initiating an effective inflammatory response. Collectively, these results indicate a multifaceted role for IL-25 that is not dependent on its direct signaling through CD4<sup>+</sup> cells, monocytes/macrophages, or dendritic cells to create a response, nor is it dependent on IL-33 or TSLP. These studies are the first of their kind to look at IL-25 induced inflammation by deleting its receptor on specific cell types, and in the absence of other epithelial cytokines.

Chronic inflammation within the gastrointestinal tract results in an increased risk for developing colorectal cancer. Epithelial cell production of IL-25 within the colon is critical for protection from parasites, but can also be pathogenic in the context of inflammatory bowel diseases and allergy. Whether IL-25 is involved in the progression from inflammation to cancer is still largely unexplored. Using a well-established murine model for colitis-induced colon cancer; we aimed to determine the role of IL-25 in this process. We found that acute antibody mediated IL-25 blockade resulted in greater tumor burdens than did treatment with isotype control. Histologically,  $\alpha$ -IL-25 treated mice had increased colitis scores compared to mice receiving isotype control antibody, as well as decreased eosinophilia. This is the first study to explore the therapeutic potential of using an IL-25 blocking antibody during a chronic inflammatory setting. Taken together, these data suggest that IL-25 plays an inhibitory role in the growth and development of colonic tumors.

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## **DEDICATION**

For my mother, *Lana K. Thelen*

## Chapter 1. INTRODUCTION

### 1.1 PULMONARY IMMUNOLOGY

#### 1.1.1 *During Homeostasis*

Respiratory epithelial cells, along with innate immune cells within the lung, function cooperatively to maintain homeostasis. This is no trivial feat when considering the constant exposure to environmental particles, toxins, and microbial pathogens carried into the airway with every breath. The lungs have the tremendous responsibility of bringing in air to supply the entire body with enough oxygen to survive while maintaining a nearly sterile environment within the alveolar space. To accomplish this, epithelial cells utilize several mechanisms that are in constant operation.

One of the primary methods of maintaining homeostasis is through barrier function. Epithelial cells are able to form a 'wall' consisting of tight and adheren junctions facilitated by their intercellular junctional proteins: claudins, connexins, paranexins, adhesions, and zonula occludins<sup>1</sup>. Through homotypic and heterotypic binding of these proteins, tight connections are made that are linked to the actin cytoskeleton providing structural integrity to the respiratory epithelium<sup>1</sup>.

Secretory epithelial cells produce mucins and anti-microbial peptides to form a protective barrier from allergens and microbes within the airway. Epithelial cells secrete baseline levels of host defense molecules such as human  $\beta$ -defensin, lysozyme, lactoferrin, cathelicidin LL37, and surface proteins A & D; however, their secretion is also regulated by the pathogens, toxicants, and cytokines sensed within the environment<sup>2</sup>. This is also true for the many types of mucins produced within the lung.

Two main types of mucus exist, those that are ‘tethered’ to the epithelial cells (including MUC4, MUC13, MUC16, and MUC21) and those that are released into the airway (MUC5B, MUC5AC, and MUC2)<sup>1</sup>. Secreted mucins are able to disrupt bacterial aggregation and bind microbial pathogens for their eventual clearance up the airway through the beating of ciliated cells<sup>3</sup>. Ciliated cells are the predominant cell type lining the human trachea, bronchi, and bronchioles. Both ciliated and secretory cells are regenerated following injury to the epithelial layer by basal cells located directly beneath them. Ciliary clearance of environmental particles, toxins, and pathogens is a critical element in maintaining lung homeostasis.

The ability of epithelial cells to sense commensal and pathological microbes and respond appropriately by communicating with innate immune cells, is another essential component of minimizing inflammation within the lung. Lung epithelial cells have abundant surface expression of the pattern-recognition receptors (PRRs) toll-like receptors (TLRs) and nod-like receptors (NLRs) that sense pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively)<sup>4</sup>. The binding of ligands to the PRRs triggers signaling pathways via MAPK, IRFs, reactive oxygen species (ROS), and NF- $\kappa$ B. Depending on the nature of the initial signal, this results in the production of cytokines, chemokines, and interferons that influence the recruitment and activation of both innate and adaptive immune responses<sup>4,5</sup>. The lungs’ ability to continually respond to a wide variety of environmental insults and maintain host defense and homeostasis, while not amplifying the response enough to cause injury to the lung, requires further investigation in mucosal immunology.

### 1.1.2 *During Eosinophilic Asthma*

Asthma is a broad term used to describe a chronic inflammatory airway disorder that leads to airway hyper-responsiveness (AHR), airflow obstruction, overproduction of mucus, and remodeling of the airway wall. It is estimated that 39.5 million people (12.9%), including 10.5 million (14.0%) children, have been diagnosed with asthma in their lifetime<sup>6</sup>. Patients with asthma experience symptoms such as coughing, wheezing, breathlessness, and chest tightness. The estimated total cost of asthma to society, including medical expenses (\$50.1 billion per year), loss of productivity resulting from missed school or work days (\$3.8 billion per year), and premature death (\$2.1 billion per year), was ~\$56 billion in 2007<sup>7</sup>. Because of its complicated nature this disorder is now classified as 'Asthma Syndrome' and is subdivided first by its phenotypic characteristics and then further categorized into endotypes based on specific biological mechanisms<sup>8</sup>. For the purpose of this project, eosinophilic asthma was the main focus of these studies and will therefore be described further in this section.

Characteristics other than eosinophilia in eosinophilic asthma are those typically associated with type-2 inflammation, including increases in type-2 innate lymphoid cells (ILC2s) and Th2 cells, serum IgE production, mucus production, AHR, and type-2 cytokines (IL-4, IL-5, and IL-13). This process begins with PRR sensing of allergens and pollutants on airway epithelial cells and subsequent intracellular signaling events mentioned above. In response, epithelial cells produce the cytokines IL-25, IL-33, and TSLP that act upon innate immune cells to further augment the inflammatory response. ILC2s, which lack antigen-specific receptors but express IL-17RB and ST2 (IL-33R),

become activated in response to IL-25 and IL-33 to produce the initial sources of IL-5 and IL-13.

IL-5 mobilizes eosinophil precursors from the bone marrow, recruits them to the lung mucosa and interstitium via the chemokines eotaxin 1, 2, and 3, and promotes their activation and survival<sup>9</sup>. It is thought that the prolonged presence of eosinophilic toxins including neurotoxin, cationic proteins (eosinophil peroxidase) and major basic protein is responsible for damaging the structural cells in the lungs<sup>10,11</sup>. The many detrimental effects of IL-5 make it an attractive therapeutic target for asthmatic patients; accordingly, blockade of IL-5 in asthmatic patients resulted in reduced deposition of extracellular matrix proteins tenascin, lumican, and procollagen III, leading to less airway remodeling<sup>12</sup>.

IL-13, another key mediator in the inflammatory type-2 response, is both necessary and sufficient in triggering bronchial hyper-reactivity and for goblet cell metaplasia<sup>13,14</sup>. It is also able to prime the vessel wall for upregulation of the adhesion molecules VCAM-1 and ICAM-1, thus priming eosinophil exit<sup>9</sup>. TSLP and IL-13 act on dendritic cell (DC) populations to stimulate their migration into lymph nodes and prime them to polarize naïve T cells into Th2 cells.

T cells located within the lung are also stimulated in response to epithelial IL-25, inducing their expression of IL-4. Naïve T cells, in the presence of IL-4, are driven towards the Th2 lineage and subsequently increase production of IL-4, IL-5, and IL-13. The IL-4 driven Th2 activation induces class switching of immunoglobulins produced by B cells leading to elevated levels of antigen-specific IgE. The role of mast cells and basophils, which express the high-affinity IgE receptors, remains controversial. The

proven efficacy of asthma therapies directed against IgE suggest they do play an important role<sup>9</sup>.

This overview of the general mechanisms responsible for creating type-2 inflammation in asthma demonstrate the cooperation of structural cells within the lung and innate and adaptive immune cells. As a whole, asthma is a highly heterogeneous disease in which many of the individual components provide potential therapeutic targets. While genetic variability in patients will certainly influence the capacity of a particular therapy to resolve/prevent asthma attacks, knowing the individual contributions of the molecules involved will provide for more effective targeting strategies.

## 1.2 **GASTROINTESTINAL IMMUNOLOGY**

### 1.2.1 *Inflammatory Bowel Disease: Ulcerative Colitis*

As with lung mucosal epithelial cells, gut epithelial cells are now recognized for their active, rather than passive, role in maintaining homeostasis. With trillions of beneficial commensal bacteria residing in the gastrointestinal (GI) tract, the epithelial cells have developed a way to co-exist with these bacteria by becoming a tolerant immune barrier. The mechanisms described above for maintaining homeostasis in the lung (barrier function, secretion of mucus and antimicrobial peptides, microbial sensing, etc.), also exist for gut homeostasis. Tonic low-level sensing through the TLRs on gut mucosal epithelial cells provides a necessary signal for their survival, is required for homeostasis, and protects the gut epithelium from injury<sup>15</sup>. SIGIRR signaling, which negatively regulate the TLRs, is also required for maintaining homeostasis within the gut; SIGIRR<sup>-/-</sup> mice are highly susceptible to dextran sulfate sodium (DSS) induced

colitis<sup>16-18</sup>. The microflora-induced TLR signaling stimulates the production of IL-25 and TSLP from epithelial cells; this regulates the adaptive immune response by conditioning DCs to produce a non-inflammatory Th2 response<sup>19-21</sup>. However, if this delicate balance is lost and the epithelial immune barrier becomes disrupted, the commensal bacteria will cause inappropriate activation of the immune response, resulting in inflammatory bowel diseases, including ulcerative colitis (UC)<sup>22</sup>.

UC is a type of inflammatory bowel disease (IBD) that is chronic and relapsing, and affects the epithelial lining of the colon. UC patients have a severely depleted mucus layer and few mucin-producing goblet cells; without this protective barrier microbiota induce inflammation and disease pathology within the colon<sup>23-26</sup>. A well characterized mouse model of UC uses DSS to disrupt the epithelial barrier, causing colitis immune mechanisms, epithelial repair, and a cytokine profile similar to that seen in human IBD<sup>27</sup>. These mice also develop symptoms similar to that of IBD patients, including weight loss, diarrhea, and rectal bleeding<sup>28,29</sup>.

Th17 cells are known to play an important role in the pathogenesis of IBD. Because IL-23 controls the survival and expansion of Th17 cells, several studies have tried to determine the relative contribution of these cytokines in IBD. In one mouse model of UC, inhibition of IL-17 resulted in severe weakening of the intestinal epithelium, causing an exacerbation in colitis<sup>30</sup>. However, in this same model, inhibition of IL-23 enhanced regulatory T (Treg) cell accumulation and reduced colonic inflammation<sup>30</sup>. IL-25 was added to the IL-23/IL-17 axis when it was discovered that intestinal epithelial cell expression of IL-25 is dependent on commensal bacteria<sup>31</sup>. Germ free mice given IL-25 had reduced IL-23 expression and decreased frequency of

Th17 cells in the large intestine<sup>31</sup>. Increasing evidence supporting IL-17 as protective in intestinal inflammation was established by blocking IL-17R signaling, which resulted in enhanced inflammation<sup>32</sup>.  $\gamma\delta$ T cells were hypothesized to be the source of IL-17 which was independent of IL-23<sup>32</sup>.

Recently, when an IL-17A blocking antibody was used as a therapy to reduce inflammation in patients with IBD (specifically Crohn's disease), it instead exacerbated disease<sup>33</sup>. This further demonstrates the importance of studying the mechanisms of individual cytokines in various disease conditions rather than broadly classifying them as 'anti-inflammatory' or 'pro-inflammatory'. While the etiology of IBD is not completely understood, we are beginning to dissect some of the underlying mechanisms contributing to this complex disease process. Additional advances in this field could greatly enhance therapeutic options for UC patients, reducing their morbidity and mortality, as well as overall healthcare costs related to this disease.

### 1.2.2 *Inflammation-Associated Colon Cancer*

A chronic inflammatory environment localized within the colon can often lead to the development of colorectal cancer (CRC). In fact, UC patients have a 2.4-fold increased risk for developing CRC<sup>34</sup>. CRC is responsible for up to 15% of deaths in IBD patients<sup>35</sup>. 3.7% of patients with UC will eventually progress to CRC, with increasing cumulative probabilities of 2% by 10 years, 8% by 20 years, and 18% by 30 years from initial diagnosis<sup>36</sup>. Several risk factors are associated with the development of CRC in IBD patients, including long disease duration, extent of disease, young age at diagnosis, coexistence of primary sclerosing cholangitis, severity of inflammation, and family history of CRC<sup>35</sup>. While anti-inflammatories are believed to decrease the risk of CRC in

IBD patients, whether the degree of inflammation correlates to risk of developing CRC has not been directly studied<sup>37,38</sup>. With an early p53 mutation followed by dysplasia, the progression of IBD into CRC appears to be different than that of sporadic colon cancer<sup>35</sup>. The detection of dysplastic epithelium may allow for earlier diagnosis and less severe treatments with the proper surveillance.

Many of the discoveries being made in understanding the mechanisms involved in progression of IBD to CRC were accomplished with the use of mouse models. While several models exist, they typically involve the injection of a carcinogen [commonly azoxymethane (AOM)] in combination with DSS induced inflammation. Histologically, tumors in these mice resemble those of human CRC. Molecular changes including alterations in  $\beta$ -catenin, COX-2, and iNOS have been reported; however no changes were observed in p53 expression<sup>39</sup>.

TNF- $\alpha$  has been shown to promote inflammation and colitis-associated cancer through its ability to initiate DNA damage, stimulate angiogenesis, and induce expression of COX-2 (which also induces angiogenesis to enhance tumor growth)<sup>38</sup>. When mice deficient in TNF- $\alpha$  signaling were exposed to the AOM/DSS model, they had decreased inflammation and tumor development compared to wild-type (WT) mice<sup>40</sup>. Additional studies have confirmed neutralization of TNF- $\alpha$  is capable of reducing inflammation and colitis-associated cancer in mice; this is significant, considering anti-TNF- $\alpha$  treatments are already being used to control IBD<sup>41,42</sup>.

IL-6 has also been implicated as an important cytokine in promoting colitis-associated cancer. Mouse models of CRC as well as human patients have high levels of circulating IL-6<sup>43,44</sup>. Initiation of Stat3 signaling by IL-6 was shown to promote tumor

growth in CRC<sup>45</sup>. Colonic epithelial cells from patients with UC, dysplasia, or colon cancer had higher expression of IL-6 and p-STAT3 compared to controls<sup>46</sup>. While more studies are needed to determine the mechanism of IL-6 induced cancer, this appears to be an important pathway contributing to disease progression.

Indeed, there are many more factors influencing the evolution from chronic inflammation to cancer, particularly those from the adaptive T helper populations as well as the gastrointestinal microbiota. Various components of the immune system are being developed into drug targets for CRC (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, IL-21, and TNF- $\alpha$ ) and many more possible therapeutic targets exist. Because the molecular pathways of chronic inflammation vary widely from those of cancer, it is very difficult to identify targets that are beneficial in the chronic inflammatory stage of UC and that are also anti-tumorigenic. It will be important therefore to study these processes both together and separately to determine the best strategies for treatments, which will likely require combinatorial approaches.

### 1.3 INTERLEUKIN-25

#### 1.3.1 *Discovery*

IL-25 was initially discovered in 2001 by a group at Genentech Inc. looking for additional members of the growing IL-17 cytokine family<sup>47</sup>. It was therefore named IL-17E, a protein that is 177 amino acids in length and shares 16-20% homology with other IL-17 cytokine family members. They also confirmed its specific binding to IL-17BR, a receptor that had been previously described for IL-17B. While IL-25 and IL-17B are both capable of binding to IL-17BR (later renamed IL-17RB), IL-25 binding is more robust and capable of displacing IL-17B in a competing reaction<sup>47</sup>. These initial studies found

IL-25 was able to activate NF- $\kappa$ B and to induce production of the proinflammatory chemokine IL-8.

Shortly after its initial discovery, attempts to characterize the function of this new cytokine began. Two transgenic mouse models were developed: one overexpressing human IL-25, the other overexpressing mouse IL-25. In contrast to other members of the IL-17 cytokine family which typically induce neutrophilic inflammation, IL-25 generated a primarily eosinophilic reaction<sup>48,49</sup>. In addition to eosinophilia, these models reported increased blood serum levels of immunoglobulins IgE, IgG1, and IgM, as well as the type-2 inflammatory cytokines IL-4, IL-5, and IL-13. Elevated gene expression was detected for IL-4, IL-5, and IL-13 in many tissues, with noticeable inflammatory cell infiltrates, epithelial cell hyperplasia, and hypertrophy distinguished histologically<sup>48</sup>.

Other initial attempts to characterize the newest IL-17 family member were performed by the direct administration of high doses of IL-25, either intranasally or intraperitoneally. Both methods revealed similar results as those observed with the transgenic overexpression models, including increased gene expression of Th2 cytokines (IL-4, IL-5, and IL-13), increased serum IgE, IgG1, and IgA, eosinophilia and pathological changes primarily in the lung and gastrointestinal tract<sup>50,51</sup>. While both studies confirm the ability of RAG knock-out (KO) mice (lacking T and B lymphocytes) to produce an IL-4 independent type-2 inflammatory response, they have conflicting results on the dependencies of other down-stream cytokines. Specifically, Hurst et al. determine IL-13 to be required for the IL-25 mediated eosinophilia, while Fort et al. show it is IL-5 that is required for the eosinophilia and IL-13 required for the histological changes<sup>50,51</sup>. T and B lymphocytes, mast cells, and basophils were ruled out as the

cellular sources for IL-5 and IL-13; however the main cell types responding to IL-25 were still undetermined<sup>51</sup>.

### 1.3.2 *Signaling Pathway*

Upon stimulation with the protease allergens papain or DerP1, mouse lung epithelial cells were found to have increased expression of IL-25 both at the RNA and protein levels<sup>52</sup>. This activity was found to be through the Erk and p38 MAPK pathway and was dependent on the protease activities of these allergens. Protease dependent mechanisms, specifically protease activated receptor-2, were also found to be required when normal human bronchial epithelial cells were exposed to house dust mite<sup>53</sup>. IL-25 is constitutively produced and stored in the cytoplasm of these cells; upon allergen exposure, it is actively secreted into the extracellular space<sup>53</sup>.

As described above, IL-25 binds to and signals through the IL-17RB receptor. This receptor has been detected in a variety of tissues, including human lung, kidney, pancreas, liver, brain, and intestine<sup>54,55</sup>. However, IL-25 function also relies on the IL-17RA subunit<sup>56</sup>. While IL-25 does not bind to the IL-17RA subunit, both units of this heteromeric receptor complex are required for activation of signal transduction. Both the human and mouse IL-17RB subunit contain a TRAF6-binding motif that was found to be crucial for NF- $\kappa$ B activation in mouse embryonic fibroblasts<sup>55</sup>. Interestingly, the IL-17RB-mediated activation of ERK, JNK, and p38 in these cells was independent of TRAF6.

Another molecule critical in the IL-25 signaling pathway is the epithelial derived adaptor molecule Act1<sup>57</sup>. Recruited to the SEFIR domain on IL-17RB, Act1 is required for IL-25-induced eosinophilia and IL-4, IL-5, IL-13, and eotaxin-1 expression<sup>57</sup>. More recently, an Act1 independent mechanism was described in T<sub>H</sub>2 polarized cells through

STAT5 and JAK2 activation<sup>58</sup>. Upon ligand binding, phosphorylation of tyrosine residues 444 and 454 initiated STAT5 binding to the IL-17RB subunit, which led to downstream type-2 inflammatory responses<sup>58</sup>. STAT6 is another important component of the IL-17RB signaling pathway; STAT6 deficient mice lack eosinophilia in an IL-25 dependent lung inflammation model<sup>59</sup>.

It was recently discovered that T<sub>H</sub>2 responses were inhibited in *Traf4*<sup>-/-</sup> cells in response to IL-25<sup>60</sup>. In this study, the inhibitory molecule DAZAP2 was found to bind to tyrosine 355 within the SEFIR domain of IL-17RB, thereby directly inhibiting Act1's interaction. DAZAP2 was degraded by E3-ligase SMURF2 which was recruited by TRAF4.

Several cell types have been identified as being IL-17RB<sup>+</sup> and capable of responding to IL-25. Eosinophils isolated from human blood and incubated with IL-25 upregulate the chemokines and cytokines MCP-1, MIP-1 $\alpha$ , IL-8, and IL-6 by both gene transcript and protein levels<sup>54</sup>. This was attributed to regulation through JNK, p38 MAPK, and NF- $\kappa$ B pathways. Caruso et al. found the highest expression of IL-17RB in human blood samples was on CD14<sup>+</sup> monocytes. Furthermore, pre-incubation of these cells with IL-25 resulted in decreased gene expression and protein of IL-12, IL-1 $\beta$ , TNF- $\alpha$ , and IL-6<sup>61</sup>. This inhibitory effect was found to be through the p38 Map kinase pathway induction of Socs-3.

When IL-25 signaling was investigated in T<sub>H</sub> cells, it was discovered that co-stimulation with anti-CD3 and anti-CD28 was required for upregulation of IL-17RB prior to their induction of the cytokines IL-4, IL-5, IL-10, IL-6 and the chemokines CXCL10, CXCL9, and CCL5<sup>62</sup>. Furthermore, the upregulation of IL-17RB was mediated through

activation of JNK and NF- $\kappa$ B. Interestingly, it was found that only memory and not naïve T<sub>H</sub> cells were capable of cytokine and chemokine production upon co-stimulation and IL-25 activation<sup>62</sup>.

### 1.3.3 *Immunomodulatory Effects in the Lung*

During an allergic response, IL-25 is known to be induced and promote type-2 inflammation in the lungs of humans and mice. With the use IL-25 deficient mice, we now know IL-25 plays a crucial role in the generation of severe airway inflammation. The misdirected allergic response to otherwise innocuous antigens causes severe lung pathologies, including increased production of IL-4, IL-5, and IL-13, eosinophilia, B cell class switching to IgE, goblet cell hyperplasia, and increased mucus production. The role of IL-25 in creating this inflammatory state is multifaceted and involves an innate component during initial development, as well as an adaptive component for the prolonged maintenance and immunological memory response.

One of the first innate cell types activated in response to IL-25 is the ILC2s. In early attempts to characterize the role of IL-25 in initiating type-2 inflammation, it was discovered that mice lacking T and B cells were still capable of responding to IL-25 and potentiating an immune response<sup>51</sup>. Furthermore, it was the ILC2s within the lung that were the main producers of IL-5 and IL-13 in RAG<sup>-/-</sup> mice<sup>50,63</sup>. Recently, ILC2s were shown to be required for the differentiation of naïve T cells into T<sub>H</sub>2 cells in response to protease allergens in the lung, providing an important link between the innate and adaptive T<sub>H</sub>2 cell response<sup>64</sup>.

Upon stimulation with the allergens *Aspergillus oryzae* or ragweed, human and mouse epithelial cells upregulate IL-25 expression<sup>65</sup>. IL-17RB expression was detected

in both naïve T cells and at elevated levels in T<sub>H</sub>2 but not T<sub>H</sub>1 differentiated T cells<sup>65</sup>. Naïve T cells stimulated with IL-25 increase IL-4 production via transcription factors NFATc1 and JunB, leading to enhanced GATA-3 expression and T<sub>H</sub>2 differentiation, thereby further increasing IL-4, IL-5, and IL-13 production<sup>65</sup>. Angkasekwinai et al. further determined that this mechanism is dependent on STAT6 and the early production of IL-4 by T cells. Subsequent studies determined T cells incubated in vitro with IL-4 and TGF- $\beta$  have elevated expression of IL-17RB and produce IL-9 when treated with IL-25<sup>66</sup>. In an allergic airway model, mice deficient in IL-25 had greatly reduced IL-9 and attenuated allergic responses<sup>66</sup>. Unlike the T<sub>H</sub>2 cytokines, induction of IL-9 in this system was independent of IL-4<sup>66</sup>. These studies establish IL-25 as a regulator of T<sub>H</sub>9 cells and determine another mechanism by which IL-25 is capable of modulating the allergic response.

A subset of NKT cells positive for IL-17RB expression was found to be imperative in the development of AHR in an IL-25 induced lung asthma model<sup>67</sup>. These cells primarily produced IL-13, T<sub>H</sub>2 chemokines (CCL17, CCL22, and CCL6), and eosinophil chemotactic factor-L (ECF-L).

During asthma, exposure of macrophages to type-2 cytokines (IL-4 and IL-13), TGF- $\beta$ , or glucocorticoids causes them to undergo phenotypic changes whereby they are able to recruit T<sub>H</sub>2-type cells and produce IL-4 and IL-13, contributing to mucus production and turnover of local connective tissue<sup>68</sup>. When human peripheral blood monocyte-derived macrophages are stimulated with IL-4 and TGF- $\beta$ , they upregulate IL-17RB expression<sup>69</sup>. This was also true for IL-10 stimulated human myeloid regulatory DCs (DC<sub>reg</sub>-IL-10), suggesting antigen presenting cells (APCs) may also be contributing

to IL-25 induced type-2 inflammation<sup>69</sup>. More recently, Liu et al. showed that IL-25 could directly suppress the M1 phenotype of inflammatory macrophages, inhibiting their production of IL-6, IL-23, TNF- $\alpha$ , and IL-1 $\beta$ <sup>70</sup>.

Lungs from chronic asthmatic (as well as atopic dermatitis) patients were found to have substantially elevated levels of IL-25 and IL-17RB gene expression<sup>71</sup>. The main cellular sources of IL-25 in allergic donors were basophils and eosinophils<sup>71</sup>. This study found the highest expression of IL-17RB was in T<sub>H</sub>2 memory cells that had been previously stimulated with TSLP-activated DCs. In response to IL-25, the T<sub>H</sub>2 memory population underwent cellular expansion and produced T<sub>H</sub>2 cytokines. Wang et al. propose a possible mechanism in which innate cell production of IL-25 by eosinophils and basophils during an allergic response enhance the maintenance and functions of adaptive T<sub>H</sub>2 memory cells. These findings suggest IL-25 may be working together with other type-2 inducing cytokines, namely TSLP, to further enhance the allergic response.

When peripheral blood mononuclear cells (PBMCs) from patients with seasonal allergic rhinitis were analyzed, IL-17RB was found to be the most differentially expressed gene compared to healthy controls<sup>72</sup>. Further analysis of these cells determined basophils expressed IL-17RB and upregulated it upon exposure to IL-3. Basophils incubated with IL-25 had increased survival and enhanced IgE-mediated degranulation<sup>72</sup>.

There are now reports demonstrating a more direct link between IL-25 and lung resident dendritic cells. When researchers stimulated conventional CD11c<sup>+</sup> DCs with IL-25, they rapidly induced the chemokine CCL17 recruiting T<sub>H</sub> cells capable of producing IL-9 (T<sub>H</sub>9 cells)<sup>73</sup>. Using an in vivo mouse model, IL-25 signaling in DCs was found to be

required for the CCL17-dependent trafficking of T<sub>H</sub>9 cells into the lung<sup>73</sup>. Human studies found increased IL-17RB expression by airway myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) when mild atopic asthma patients were allergen challenged<sup>74</sup>. These patients also had an influx of mDCs and pDCs into sputum samples following allergen challenge. The effects of IL-25 were found to be primarily on pDCs through upregulation of TLR9 expression<sup>74</sup>. Because pDCs are major producers of type-1 interferons following TLR activation in response to viruses, this may provide a vital link between exacerbated allergic responses during or following viral infections.

Recently, stem cell factor (SCF) has been linked to IL-25 through an IL-4 producing myeloid cell. SCF is an important molecule in the development of severe lung pathology in airway inflammation. SCF is highly upregulated in the airways of asthmatics, mediates eosinophil activation, and has recently been shown to drive type-2 inflammation in the lung<sup>75-78</sup>. The IL-4<sup>+</sup> myeloid cells are induced during lung inflammation and are associated with increased IL-17RB expression and increased IL-25 production within the lung<sup>79</sup>. If SCF is either neutralized or blocked, the IL-4<sup>+</sup> myeloid cells, along with IL-25, are also reduced. This results in attenuation of the entire asthmatic response, including a decrease in type-2 cytokines, IgE, and mucus production. Additional studies indicate this myeloid population, now identified as type 2 myeloid (T2M) cells, appears to be resistant to steroid treatments and was present in the peripheral blood of asthmatic patients<sup>80</sup>.

To determine the therapeutic potential of targeting IL-25 during an allergic asthma reaction, an anti-IL-25 neutralizing antibody was developed. Treating mice with the neutralizing antibody during the sensitization phase of an allergic airway model

prevented AHR, reduced IL-5 and IL-13 production and decreased eosinophils, goblet cell hyperplasia, and serum IgE secretion<sup>81</sup>. Remarkably, AHR was also prevented when mice were given anti-IL-25 only during the challenge phase, indicating the possible therapeutic effects of targeting the IL-25 pathway after a type-2 inflammatory response has been established. However, these mice did still have increased serum IgE, eosinophils, IL-5, and IL-13, and mucus production. Interestingly, IL-25 induced AHR was found to be independent of typical type-2 cytokines; IL-4, IL-5, IL-9, and IL-13<sup>81</sup>. Significantly, IL-33 and TSLP production were ablated in allergen challenged mice treated with anti-IL-25, indicating an upstream role for the induction of these cytokines by IL-25<sup>82</sup>.

An anti-IL-25 blocking antibody was also used to look at the role of IL-25 in airway remodeling after chronic allergen exposure. As expected, these mice had reduced eosinophils and Th2 cytokines; in addition, the blockade of IL-25 resulted in abrogated peribronchial collagen deposition, airway smooth muscle hyperplasia, and AHR<sup>82</sup>. Furthermore, IL-25 impacted angiogenesis by recruiting endothelial progenitor cells and promoting neovascularization<sup>82</sup>. Endothelial cells expressing IL-17RB were significantly elevated in the bronchial mucosa of asthmatics. In culture, human vascular endothelial cells (HUVEC) upregulated their expression of IL-17RB upon exposure to TNF- $\alpha$ <sup>83</sup>. IL-25 was found to increase HUVEC expression of VEGF and VEGFR through PI3K/Akt and Erk/MAPK pathways<sup>83</sup>. HUVEC proliferation and the number, length, and area of microvessel structures were also increased in response to IL-25<sup>83</sup>. A variety of allergic airway studies using only IL-25 have supported its angiogenic properties,

illustrating elevated expression of amphiregulin, angiogenin, endothelin-1, bFGF, EGF, IGF-1, ERG, as well as VEGF<sup>84,85</sup>.

Fibroblasts and stromal cells residing in human lungs constitutively express IL-17RB, upregulating its expression upon exposure to TNF- $\alpha$ <sup>86</sup>. Culturing these lung fibroblasts with IL-25 stimulated them to produce CCL-11, CCL-5, GM-CSF, and CXCL-8, mediators known to favor tissue eosinophilia<sup>86</sup>. Fibroblasts have also been shown to directly respond to IL-25 to induce collagen secretion and promote airway remodeling<sup>82</sup>. Because eosinophils are known to produce TNF- $\alpha$  in asthma, IL-25 may be acting directly on fibroblasts to contribute to both the induction and maintenance of eosinophilic airway inflammation.

#### 1.3.4 *Role in Gastrointestinal Immune Function*

The type-2 inflammatory response is a critically important part of our immune system that exists to provide us protection from parasitic helminths. As infections with parasites have become increasingly rare in developing countries, the type-2 response is seen/experienced by most as an irritating allergic response they'd like to shut-down. However, as IL-25 is known to play an important role in initiating this response, it has also been found to be an important cytokine in the expulsion of helminths in the gastrointestinal tract. During infection with the parasitic helminth *Nippostrongylus brasiliensis*, type-2 inflammation was found to be induced independently of the adaptive immune system and a necessary initial source of IL-4 to generate an effective T<sub>H</sub>2 response<sup>87,88</sup>. Epithelial cells in the small intestine were found to be the major source of IL-25 during *N.brasiliensis* infection and this was dependent on IL-13 activation of STAT6<sup>89</sup>. With the generation of IL-25<sup>-/-</sup> mice, McKenzie et al. established an essential

role for ILC2s to efficiently expel an *N.brasiliensis* infection<sup>90</sup>. The absence of IL-25 resulted in a deficiency of ILC2s and therefore delayed production of type-2 cytokines necessary for fighting off the worms<sup>90</sup>. Importantly, they also found recombinant IL-25 treatments could induce ILC2s into the mesenteric lymph node (MLN) independently of T and B cells and type-2 cytokines<sup>90</sup>. Because ILC2s produce little or no IL-4, the initial source of IL-4 may be from another innate cell type. Notably, basophils are known to provide an essential source of this critical cytokine during *N.brasiliensis* infection<sup>91</sup>.

IL-25 also plays a protective role in response to infection with intestinal parasitic roundworms, *Trichinella spiralis* and *Trichinella muris*. IL-25 was able to promote T<sub>H</sub>2 cytokine production and goblet cell hyperplasia while limiting pro-inflammatory cytokines and chronic intestinal inflammation in response to a *T.muris* infection<sup>92</sup>. *T.spiralis* induced IL-25 was able to enhance worm expulsion by inducing an antigen-specific T<sub>H</sub>9 response<sup>93</sup>. IL-25 treatments in these mice correlated with alterations in mast cell numbers and the expression of the IL-9-regulated genes *mast cell protease-1*, paneth cell marker *Cryptdin* and *Ang4* in the intestine<sup>93</sup>. These results indicated IL-25 is able to modulate intestinal immunity through both T<sub>H</sub>2 and T<sub>H</sub>9 antigen-specific responses.

The effects of IL-25 in the GI tract are not restricted to helminth infections; it also plays an important role during inflammatory conditions such as UC and food allergies. If IL-25 is neutralized in an oxazolone-induced UC model, mice have reduced weight loss, colon ulceration, and ILC2 and NKT cells infiltrating the mucosa<sup>94</sup>. These improvements, associated with decreased IL-13 production, blood eosinophils, and IgE, demonstrate the pro-inflammatory role of IL-25 is able to exacerbate GI pathology.

Conversely, mice injected with IL-25 throughout the course of a DSS model exhibit less inflammation and increased survival, demonstrating a protective role for IL-25<sup>95</sup>.

IL-25 expression in the epithelial colon appears to be microbiota dependent, as gene expression in antibiotic treated mice is greatly reduced<sup>96</sup>. In a DSS model of colonic inflammation, IL-25 mRNA expression is significantly increased in colonic epithelial cells<sup>96</sup>. When colonic epithelial cells are stimulated with IL-25 in culture, they upregulate IL-6 expression in a dose dependent manner. IL-25<sup>-/-</sup> mice are protected from inflammatory destruction, tissue remodeling, and leukocyte infiltration in the DSS model<sup>96</sup>. However, when these mice were infected with *Citrobacter rodentium*, IL-25 was found to play a protective role<sup>96</sup>.

Recently, exciting work has surfaced from Locksley's group exploring the contribution of tuft cells, one lineage of epithelial cell, during parasitic helminth infection. These studies have revealed IL-25 expression is constitutively expressed in tuft cells to maintain ILC2 homeostasis within the resting lamina propria in mice<sup>97</sup>. Mice infected with *N. brasiliensis* have dramatic tuft cell hyperplasia, which was found to be mediated by IL-25 indirectly through IL-13 production in ILC2s<sup>97</sup>. These findings suggest type-2 inflammatory signals may be impacting epithelial cell fate decisions in the GI tract when activated by infection with parasitic worms.

### 1.3.5 *Clinical Significance*

Numerous studies have provided evidence for a causal link between respiratory viral exposure early in life to childhood asthma as well as exacerbating asthma attacks in adults, with rhinoviruses being the primary culprit<sup>98</sup>. In vitro experiments found increased IL-25 mRNA and protein levels when human bronchial epithelial cells were

infected with rhinovirus in asthmatics vs. healthy controls<sup>99</sup>. This was also confirmed with human in vivo rhinovirus infection; further experiments in mice established IL-25 was necessary for the rhinovirus-exacerbation of asthma<sup>99</sup>. Infection with respiratory syncytial virus (RSV) leads to increased IL-17RB and IL-25<sup>100</sup>. In RSV infected mice that were deficient in NK cells, suppressed IFN- $\gamma$  and development of an RSV-specific T<sub>H</sub>2 response led to subsequent allergic disease<sup>101</sup>. The T<sub>H</sub>2 response in these mice was found to be dependent on epithelial cell derived IL-25, which upregulated notch ligand Jagged1 on DCs<sup>101</sup>. IL-25 blockade (by neutralization or genetic deficiency) during RSV infection reduces pathology, AHR, and type-2 cytokine production<sup>100</sup>. This was also established in a model of RSV-driven asthma exacerbation<sup>100</sup>. Within the asthmatic population, the two main phenotypes classified as “T<sub>H</sub>2 high” and “T<sub>H</sub>2 low” have distinct responses to therapies. IL-25 expression is increased in a subset of asthmatics and correspondingly have greater AHR, increased eosinophils, higher serum IgE, and higher expression in T<sub>H</sub>2 genes<sup>102</sup>. When these patients are treated with inhaled corticosteroid, they have improved FEV<sub>1</sub> and hyper-responsiveness compared to the IL-25-low asthmatics, suggesting measurement of plasma IL-25 may be a predictive method of determining a patient’s potential to respond to therapy<sup>102</sup>.

Atopic dermatitis (AD) is another condition in which IL-25 appears to play a pro-inflammatory role. AD patients were found to have IL-25 producing cells within the dermis<sup>103</sup>. Loss of gene function in filaggrin, an epithelial barrier protein, is associated with an impaired skin barrier and is a predisposing factor for AD<sup>104</sup>. In vitro experiments found that keratinocytes stimulated with IL-25 have decreased filaggrin synthesis<sup>103</sup>. Hvid et al. suggest the IL-25 producing cells are DCs, and this is responsible for both

activation of type-2 inflammation and inhibition of filaggrin synthesis contributing to the AD phenotype. More recent studies attributed the inflammation in AD to the IL-25/IL-33 responsive ILC2s, which were enriched in lesional skin biopsies from atopic patients<sup>105</sup>. The specific cellular source(s) of IL-25 and cellular targets in atopic dermatitis are still undefined and require further investigation.

A patient with a profound combined immunodeficiency disorder was found to be suffering from recurrent Varicella infections, bronchitis, skin infections, upper respiratory tract infections, and otitis media. Molecular characterization of this novel disorder uncovered a hyperploid region on 14q11.2, associated with the IL-25 locus<sup>106</sup>. This increase in DNA copy number resulted in overexpression of IL-25 following T cell activation, producing a T<sub>H</sub>2 biased immune system<sup>106</sup>. Having an immune system with a T<sub>H</sub>2 bias rendered the patient susceptible to infections normally cleared by T<sub>H</sub>1 responses and pathologies associated with overactive type-2 inflammation. This case study further confirms the importance of a balanced immune system and the devastating effects if even one component of that system is dysfunctional. Interestingly, a genome-wide search for asthma susceptibility also found a link between the 14q11.2-13 and asthma in Caucasians<sup>107</sup>.

Patients with Churg-Strauss syndrome (CSS) experience systemic vasculitis, blood and tissue eosinophilia, and long-term history of asthma. When investigators studied the role of IL-25 in this rare disorder they found increased serum IL-25 in active CSS patients<sup>108</sup>. Further analysis revealed eosinophils to be the main source of IL-25 and IL-17RB<sup>+</sup> memory T cells to be primary effector cell responsible for creating the type-2 inflammation found within vasculitic lesions of CSS patients<sup>108</sup>. These findings

present the possibility of therapeutically targeting the eosinophil derived IL-25 in an attempt to moderate the T<sub>H</sub>2 response.

A small study measuring serum IL-25 levels in patients with relapsing-remitting multiple sclerosis (MS) found a significant reduction compared to healthy controls<sup>109</sup>. Molecular analysis of single nucleotide polymorphisms (SNP) in exon 2 of the IL-25 gene showed significant association to disease<sup>109</sup>. This finding correlates with mouse studies demonstrating IL-25<sup>-/-</sup> mice were highly susceptible to experimental autoimmune encephalomyelitis (EAE)<sup>110</sup>. In this study, mice with active EAE and treated with IL-25 had increased production of IL-13, which acts on DCs to suppress T<sub>H</sub>17 responses, resulting in suppression of disease<sup>110</sup>. This model suggests therapies aimed at increasing levels of IL-25 (perhaps by direct injection), could help to restore the T<sub>H</sub>2/ T<sub>H</sub>17 balance and reduce pathogenesis in this autoimmune condition.

While there have been many suggestions for the potential benefits of directly targeting IL-25 and/or its receptor, IL-17RB, none have been developed to date. There is, however, a drug awaiting FDA approval that is a monoclonal antibody against IL-17RA, called Brodalumab. This has shown significant benefits in clinical trials for patients with psoriasis; however no benefits were observed in severe asthmatics<sup>111,112</sup>. Because blockade of IL-17RA would also block the IL-25 signaling pathway, it will be critical to understand all the mechanisms affected when trying to reduce IL-17 inflammation in this way, as it may also result in blockade of the IL-25/ T<sub>H</sub>2 axis and inadvertently exacerbate a variety of diseases.

## 1.4 OUTSTANDING QUESTIONS

IL-25, primarily derived from epithelial cells, is an essential inducer of type-2 inflammation at mucosal barrier surfaces. It is capable of stimulating both innate and adaptive immune responses that are critical in fighting off helminth infections and yet become pathogenic when directed against innocuous antigens. The IL-25 receptor, IL-17RB, has been detected on a variety of innate and adaptive cell types. However, which of these cells respond to IL-25 and augment type-2 inflammation in vivo is not well understood. To more conclusively establish specific cell types that are IL-25 responsive, several mouse systems were investigated. First, a model of IL-25 induced type-2 inflammation was established. Then, to delete IL-25 signaling on specific cell types, the Cre-lox recombination system was used. In this system, our mice had the IL-17RB gene flanked with LoxP sites and were crossed to mice containing the Cre recombinase driven by CD4, Lysm, or CD11c promoters. This allows for the specific deletion of IL-25 signaling on CD4<sup>+</sup> T cells, monocytes/macrophages/neutrophil populations, or DCs, respectively. Applying our IL-25 induced model of pulmonary inflammation to these genetically modified mice allowed us to determine the relative contributions of each cell type individually.

IL-25, IL-33, and TSLP are generally grouped together as epithelial cytokines important in the generation of type-2 inflammation. To establish any possible interdependence and/or cross-talk between these three cytokines, we treated mice deficient in IL-33 receptor (IL-33R<sup>-/-</sup>), TSLP receptor (TSLPR<sup>-/-</sup>), or both receptor knock-out mice with our model of IL-25 induced pulmonary inflammation. Using this approach,

we were able to identify the relative contributions of each of these cytokines and to define their interdependence.

In addition, IL-25 has been implicated in modulating the immune response within the tumor microenvironment. There are very few studies investigating the specific role of IL-25 in this unique environment. However, there is an abundance of evidence for the importance of IL-25 expression within the GI tract. Therefore, we sought to determine whether IL-25 is significantly impacting the growth and development of tumors within the colon. To accomplish this, we treated IL-25<sup>-/-</sup>, IL-25<sup>+/-</sup>, and WT mice with the previously established AOM/DSS model of colitis-induced colon cancer. In another approach, WT mice undergoing AOM/DSS treatment were also injected with either anti-IL-25 blocking antibody or an isotype control for the duration of the experiment. Using both the acute blockade of IL-25 and its genetic disruption, we identify a potential role for IL-25 in an inflammation-induced cancer model.

## Chapter 2. MATERIALS AND METHODS

### 2.1 PULMONARY INFLAMMATION METHODS

#### 2.1.1 *Animals*

BALB/cJ mice were originally purchased from Charles River Laboratory. CD4-Cre and Lysm-Cre mice were purchased from The Jackson Laboratory. CD11c-Cre mice were from Dr. Boris Reizis, ST2 KO mice were from Dr. Andrew McKenzie, and TSLPR KO mice were from Dr. Jim Ihle. All acquired strains were on the BALB/cJ background. IL-17RB<sup>flx/flx</sup> mice were made in our lab from C57BL/6N-A/A ES clones purchased from The European Conditional Mouse Mutagenesis Program (EUCOMM) and backcrossed onto BALB/cJ. All mice were bred and maintained at the Benaroya Research Institute Animal Facility under specific pathogen-free conditions. Experimental mice were 7-10 weeks of age with littermates used as controls. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Benaroya Research Institute, and were performed in accordance with the approved guidelines for animal experimentation at the Benaroya Research Institute.

#### 2.1.2 *IL-25 Induced Pulmonary Inflammatory*

Mice were anesthetized with isoflurane and given intranasal (i.n) injections every other day for 2 weeks (8 total). 24 hours after the final treatment, mice were euthanized via intraperitoneal (i.p.) injection of Avertin (2.5%) in PBS and airway inflammation was assessed. Control mice were treated with either PBS or 25 µg OVA (Sigma) in PBS and experimental mice were given 100 ng IL-25 (R&D Systems, generous gift of Amgen) in PBS, or a combination of IL-25 + OVA in a total volume of 40 µl PBS. Mice depleted of

CD4 T cells were given i.p. injections of 200 µg anti-CD4 antibody (GK1.5) or control IgG in a total volume of 200 µl PBS on days -7, -1, and 7.

### 2.1.3 *Single Cell Isolation and Flow Cytometry*

Bronchoalveolar lavage (BAL) fluid was collected by lavaging the lung with 1 mL PBS 4 times. The fluid was pooled and centrifuged to obtain a cell pellet; this was re-suspended in 1 mL staining buffer (PBS plus 2% bovine serum albumin (Sigma)) to perform cell counts and differential staining by flow cytometry. Differential cell counts were obtained in the lung by removing the left lobe, cutting it into fine pieces, and digesting with 50 µg/mL Liberase TM (Roche) and 10 U/mL DNase I (Sigma) in RPMI-1640 at 37°C for 40 min. Lung suspensions were then passed through a 100 µm cell filter and washed with complete RPMI (RPMI plus 2.05 mM L-glutamine, 10% fetal bovine serum, 50 units/l penicillin, 50 mg/mL streptomycin, 50 mg/mL gentamycin, 1 mM sodium pyruvate, 1 mM HEPES, 50 µM β-mercaptoethanol). Red blood cells were lysed using ACK lysis buffer, washed with complete RPMI and resuspended in 1 mL staining buffer.

For surface staining, cells were first incubated with anti-CD16/32 (clone: 2.4G2; BD Biosciences) for 20 minutes at 4°C to prevent non-specific binding, followed by a 30 minute incubation at 4°C with antibodies to surface proteins. The cells were washed 2x with staining buffer and fixed with 4% formaldehyde (Sigma).

Intracellular cytokine staining following restimulation was performed by incubating cells in complete RPMI containing phorbol 12-myristate 13-acetate (Sigma), ionomycin (Sigma), and GolgiStop (BD Biosciences) for 5 hours at 37°C, 5% CO<sub>2</sub> prior to staining. Surface markers were stained as above; cells were washed and

permeabilized for 20 minutes in Fix/Perm buffer (eBioscience) at 4°C. Cells were incubated for 30 minutes at 4°C with antibodies against intracellular markers, washed and resuspended in staining buffer. Data were acquired on an LSR II (BD Biosciences) flow cytometer and analyzed using FlowJo software (Treestar).

The following antibodies were purchased from eBiosciences: anti-CD11c PE-Cy7 (N418), anti-CD3 eFluor450 (17A2), anti-MHCII AlexaFluor700 (MS/114.15.2), anti-CD11b APC-e780 (M1/70), anti-IFN- $\gamma$  PE-Cy7 (XMG1.2), anti-CD45R PE-Cy5 (RA3-6B2), anti-IL-13 FITC (eBlo13A), anti-CD44 AlexaFluor700 (IM7). Antibodies purchased from BioLegend; anti-Ly6G FITC (1A8), anti-CD45.2 Pacific Blue and APC (104), anti-CD4 BV605 (RM4-5), anti-Ly6C PerCpCy5.5 (HK1.4), anti-IL-5 PE (TRFK5), anti-TCR- $\beta$  PerCpCy5.5 (H57-597), and anti-CD11c PE-Cy7 (N418). The following antibodies were purchased from BD Biosciences: anti-SiglecF PE (E50-2440), anti-IL-17A BV650 (TC11-18H10.1).

#### 2.1.4 *Histology*

The inferior lung lobe was removed and fixed in 10% neutral buffered formalin. Tissue was embedded in paraffin, sectioned, and stained with hematoxylin & eosin (H&E) or periodic acid-Schiff's reagent (PAS).

#### 2.1.5 *Quantification of mRNA Levels by Real-time PCR*

The right upper lung lobe was placed in 0.5 mL RA1 lysis buffer (TaKaRa Bio) and frozen until further processing. Total RNA was isolated from frozen lung tissue using NucleoSpin RNA kit (TaKaRa Bio) following the manufacturer's instructions. cDNA was synthesized using PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio) with oligo-

dT primers following the manufacturer's instructions. cDNA was amplified using SYBR® Premix Ex Taq™, ROX Plus (Takara Bio). Real-time PCR analysis was performed on the Applied Biosystems Prism 7900HT Fast RT-PCR System. Samples were performed in triplicate and relative expression of each gene to *Gapdh* gene expression was determined by SDS 2.2.3 (Applied Biosystems) and calculated using  $\Delta\Delta CT$  method. The following primer pairs were used to determine gene expression in mouse lung tissue; *Muc5ac*, 5'-AAAGACACCAGTAGTCACTCAGCAA-3' and 5'-CTGGGAAGTCAGTGTCAAACCA-3'; *Ifn- $\gamma$* , 5'-CCTGCGGCCTAGCTCTGAG-3' and 5'-GCCATGAGGAAGAGCTGCA-3'; *il-4*, 5'-TCATCGGCATTTTGAACGAG-3' and 5'-TTTGGCACATCCATCTCCG-3'; *il-5*, 5'-TGCCTGGAGCAGCTGGAT-3' and 5'-TGGCTGGCTCTCATTCACT-3'; *il-13*, 5'-ATTCCCTGACCAACATCTCCA A-3' and 5'-CGGTTACAGAGGCCATGCAA-3'; *il-17a*, 5'-ATCAGGACGCGCAAACATGAGT-3' and 5'-ACGCTGAGCTTTGAGGGATGAT-3'.

#### 2.1.6 *Quantification of Protein Levels by ELISA*

Blood was collected by cardiac puncture and the serum was frozen for ELISA.

Sandwich ELISA for IgE was performed following the manufacture's protocol using IgE capture antibody (clone R35-72) and IgE detection antibody (clone R35-118), both from BD Biosciences.

#### 2.1.7 *Statistics*

All statistical analyses were performed using GraphPad Prism software, version 6.0.

Results are expressed as mean values  $\pm$  SEM. Statistical significance was determined by one-way ANOVA with Tukey post-test, two-tailed unpaired t-test, or two-way ANOVA

with Tukey post-test. P values of < 0.05 were considered statistically significant, with \* = p<0.05; \*\* = p<0.01; \*\*\* = p<0.001; \*\*\*\* = p<0.0001.

## 2.2 COLITIS INDUCED COLON CANCER METHODS

### 2.2.1 *Ethics Approval*

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Benaroya Research Institute, and were performed in accordance with the approved guidelines for animal experimentation at the Benaroya Research Institute.

### 2.2.2 *Animals*

BALB/cJ mice were originally purchased from Charles River Laboratory. IL-25 deficient mice were a generous gift from Dr. Andrew N.J. McKenzie<sup>90</sup>. These mice had been backcrossed for 10 generations onto the Balb/cJ background prior to receiving them into our facility. All mice were bred and maintained in the Benaroya Research Institute Animal Facility under specific pathogen-free conditions.

### 2.2.3 *Colitis-Induced Colon Cancer*

Mice received one intraperitoneal (i.p.) injection of azoxymethane (AOM) (10mg/kg) on day 0; mice were given two rounds of a 2% DSS solution in their drinking water for 7 days starting on day 7 and day 28. Mice were monitored/weighed throughout the experiment and sacrificed at day 70. Colons were removed, cut lengthwise and rinsed with cold PBS. Digital photographs were taken for tumor counting, after which, sections were collected for further analysis. Dextran Sodium Sulfate (36,000-50,000) MP grade

was purchased from MP Biomedicals (Santa Ana, CA). AOM was purchased from Sigma-Aldrich (St Louis, MO).

#### 2.2.4 *Antibody Treatment*

BALB/c mice were given 500 µg α-IL-25 (Amgen) via i.p. injection on days 7, 10, 14, 17, 21, 28, 35, 42, 49, 56, and 63 of the protocol for colitis induced colon cancer. Littermate control animals were given an equivalent dose of isotype control antibody (Sigma-Aldrich). Anti-IL-25 was a gift from Amgen (Thousand Oaks, CA).

#### 2.2.5 *Histology*

Using the Swiss-roll technique, colon tissue was placed in 10% neutral buffered formalin (Fisher BioTech), fixed at room temperature overnight, and embedded in paraffin<sup>113</sup>.

Paraffin blocks were sectioned and stained with H&E.

#### 2.2.6 *Cell Isolation and Flow Cytometry*

Colon tissue was cut into small pieces and digested with liberase (50 µg/mL) (Roche) and DNase (2 units/mL) (Sigma-Aldrich) in RPMI 1640 (Sigma-Aldrich) for 30 min at 37°C followed by filtration through a 20 µm filter. Single cell suspensions were stained with fluorescently labeled antibodies fixed in 2% paraformaldehyde (Sigma) and analyzed using the BD LSRII. The following antibodies were purchased from BD Biosciences and eBioscience: anti-CD45.2, anti-CD4, anti-CD16/32, anti-SiglecF, anti-MHCII, anti-CD11c, anti-CD11b, anti-CD8, anti-Ly6C, anti-Ly6G, and anti-F4/80.

### 2.2.7 Quantification of mRNA Levels by Real-time PCR

Total RNA was isolated from frozen colon tissue using NucleoSpin RNA kit (TaKaRa Bio) following the manufacturer's instructions. cDNA was synthesized using PrimeScript First Strand cDNA Synthesis Kit (TaKaRa Bio) following the manufacturer's instructions. Genes amplified using SYBR® Premix Ex Taq™, ROX Plus (Takara Bio). Real-time PCR analysis was performed on the Applied Biosystems Prism 7900HT Fast RT-PCR System. Samples were performed in triplicate and relative expression of each gene to *Gapdh* gene expression was determined by SDS 2.2.3 (Applied Biosystems) and calculated using  $\Delta$ CT method. The following primer pairs were used to determine gene expression in mouse colon tissue with SYBR green; *Mmp2*, 5'-CACCACCGAGGACTATGACC-3' and 5'-TCCTTGGTCAGGACAGAAGC-3'; *Mmp9*, 5'-TTCGACACTGACAAGAAGTGG-3' and 5'-CCACGACCATACAGATACTGG-3'; *Areg*, 5'-GACTCACAGCGAGGATGACA-3' and 5'-GGCTTGGCAATGATTCAACT-3'; *Vegfa*, 5'-TTACTGCTGTACCTCCACC-3' and 5'-ACAGGACGGCTTGAAGATG-3'; *Hif1a*, 5'-CGACACCATCATCTCTCTGG-3' and 5'-TGATTCAGTGCAGGATCAGC-3'; and *Ptgs2* (*Cox2*), 5'-CAGTCAGGACTCTGCTCACG-3' and 5'-TTGACATGGATTGGAACAGC-3'; *Tnf- $\alpha$* , 5'-CCCCAAGGGATGAGAAGTTC-3' and 5'-TGTGAGGGTCTGGGCCATAG-3'; *Ifn- $\gamma$* , 5'-CCTGCGGCCTAGCTCTGAG-3' and 5'-GCCATGAGGAAGAGCTGCA-3'; *il-10*, 5'-CCTCAGGATGCGGCTGAG-3' and 5'-GCTCCACTGCCTTGCTCTTATT-3'; *il-4*, 5'-TCATCGGCATTTTGAACGAG-3' and 5'-TTTGGCACATCCATCTCCG-3'; *il-5*, 5'-TGCCTGGAGCAGCTGGAT-3' and 5'-TGGCTGGCTCTCATTCACT-3'; *il-13*, 5'-ATTCCCTGACCAACATCTCCA A-3' and 5'-CGGTTACAGAGGCCATGCAA-3'; *il-17a*, 5'-ATCAGGACGCGCAAACATGAGT-3' and 5'-ACGCTGAGCTTTGAGGGATGAT-3'; *il-*

23p19, 5'-ATCACCCCCGGGAGACCCAA-3' and 5'-TGCTGCTCCGTGGGCAAAGAC-3'; and *tslp*, 5'-AGGCTACCCTGAAACTGAGA-3' and 3'-GGAGATTGCATGAAGGAATAC-5'. Primer pairs and probes using TaqMan Gene Expression Assays (Life Technologies) for mouse; *Gapdh* (Mm99999915\_g1), *il-9* (Mm01235642\_g1), *Bcl2* (Mm00477631\_m1), *Reg3γ* (Mm01181783\_g1), and *S100A8* (Mm00496696\_g1).

### 2.2.8 *Statistics*

All statistical analyses were performed using GraphPad Prism software version 6.0. Results are expressed as means  $\pm$  SEM. The statistical test used to analyze data sets between two groups was the Student t test and a one-way ANOVA to compare difference between three or more groups. P values of  $< 0.05$  were considered statistically significant.

## Chapter 3. THE ROLE OF IL-25 IN PULMONARY INFLAMMATION

### 3.1 INTRODUCTION

Maintenance of immune homeostasis requires the ability to discern harmful pathogens from innocuous environmental stimuli through the coordinated interaction of several cell types. Epithelial cells located at mucosal sites play an important role in this process by producing inflammatory cytokines IL-25, IL-33, and TSLP<sup>114</sup>. These cytokines are important immunomodulators of both innate and adaptive immunity that are known to be associated with T<sub>H</sub>2 mediated inflammation at mucosal sites. IL-25 provides protection during enteric helminth infections but can also cause pathology in inflammatory responses, including allergy and asthma<sup>50,89</sup>. While IL-25 promotes T<sub>H</sub>2 responses, it also inhibits pro-inflammatory T<sub>H</sub>1 and T<sub>H</sub>17 cytokine responses<sup>92</sup>. However, the specific role for IL-25 in the initiation and progression of type-2 inflammation remains poorly understood. IL-25, a member of the IL-17 cytokine family (IL-17E), is known to induce T<sub>H</sub>2 cytokines (IL-4, IL-5, and IL-13), thereby enhancing the T<sub>H</sub>2 response. It also induces IgE production and eosinophilia, demonstrating an important role in host defense to helminth infections; however this response becomes pathogenic in allergic disorders. IL-25 signaling occurs through its high affinity binding to the IL-17RB subunit<sup>47</sup>, though both IL-17RA and IL-17RB subunits mediate its downstream effects<sup>56</sup>. IL-25 receptor expression has been detected on a variety of cells including T cells, macrophages, type-2 myeloid cells, epithelial cells, DCs, and ILC2s<sup>115</sup>.

The focus of this study is to more specifically define which cells are important in driving IL-25 induced type-2 inflammation. We accomplish this in a novel way by specifically deleting IL-25 signaling on CD4 cells, monocytes/macrophages, or DCs

using the Cre-Lox system. As mentioned previously, each of these cell types has increased IL-17RB expression in various asthma/allergy settings. We therefore hypothesize that the selective deletion of IL-17RB using CD4-, Lysm-, or CD11c-Cre mice will result in attenuated type-2 inflammation in response to IL-25.

Additionally, we wanted to determine the role of IL-33 and/or TSLP in response to IL-25 induced pulmonary inflammation. While the functions of IL-25, IL-33, and TSLP have been explored individually and as a group, whether they are interdependent has not been explored. We assessed this using IL-33R<sup>-/-</sup>, TSLPR<sup>-/-</sup>, and IL-33R<sup>-/-</sup>/TSLPR<sup>-/-</sup> double KO mice. We hypothesized IL-25 would be capable of inducing type-2 inflammation independently of IL-33 and TSLP; however we expected to see slight variations when lacking contributions from either of these two cytokines.

## 3.2 RESULTS

### 3.2.1 *IL-25 Induces Type-2 Pulmonary Inflammation*

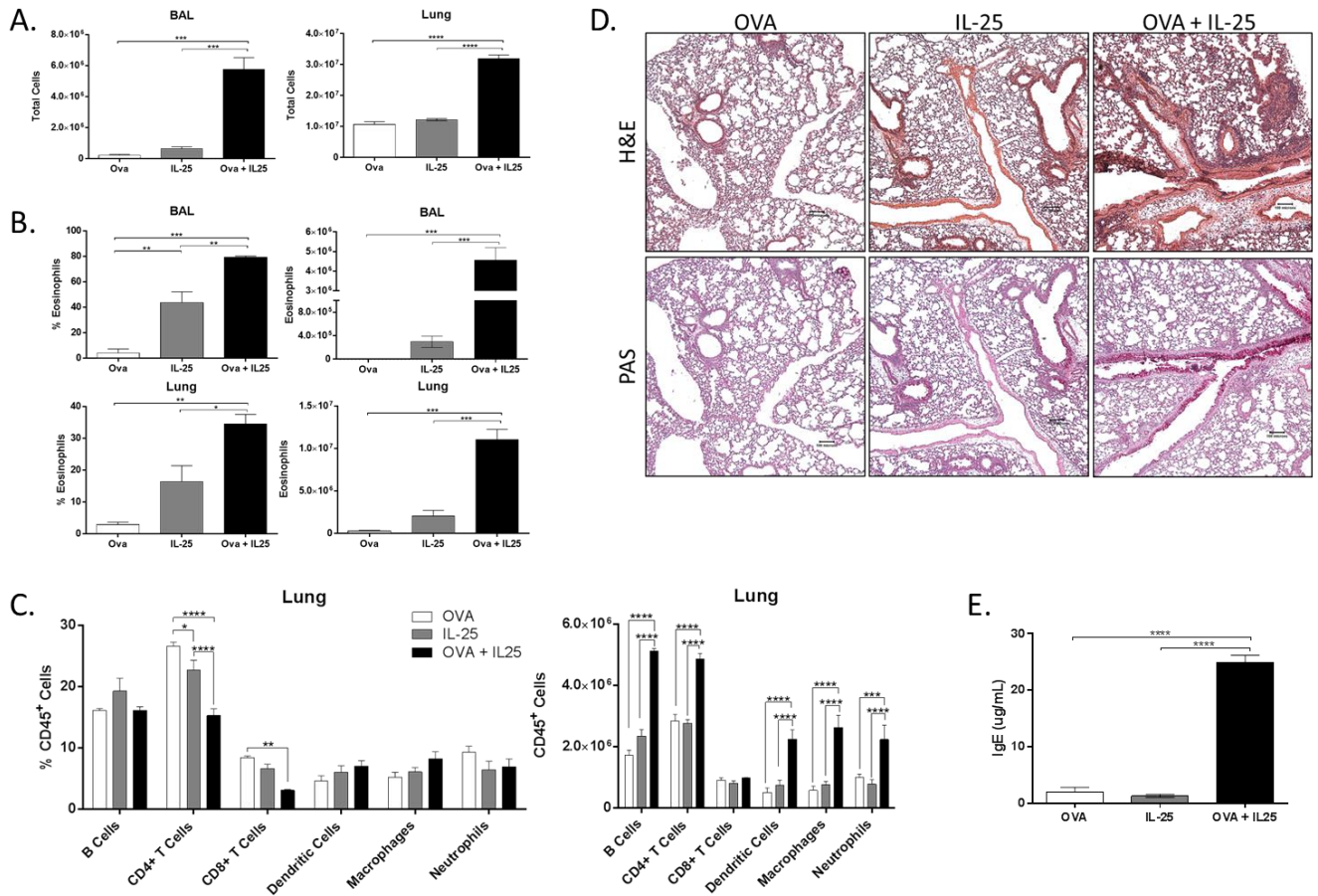
To establish a model of IL-25 induced pulmonary inflammation with and without antigen, WT mice were administered either OVA, IL-25, or OVA+IL-25 intranasally every other day for 2 weeks for a total of 8 treatments. Assessment of lung inflammation was performed 24 hours after the final treatment. Total cellularity in both the BAL fluid and lung revealed significant increases when mice were treated with OVA+IL-25 compared to OVA or IL-25 treatments alone (Fig.3.1a). Because eosinophils are the hallmark of a typical asthmatic reaction, these as well as other white blood cell populations infiltrating the lung, were analyzed using flow cytometry. In the BAL fluid, eosinophil percentages rose significantly from an average of 3.8% in OVA treated mice to 43.5% in mice given IL-25 alone, and 79.1% in mice given both OVA and IL-25. Increases in eosinophils were also observed in whole lung tissue and remained significant by percentage and number when IL-25 was administered with OVA (Fig.3.1b).

Single cell suspensions obtained from whole lung tissue were stained using surface markers specific for lymphocyte subsets (including B cells, CD4<sup>+</sup> and CD8<sup>+</sup> T cells), DCs, macrophages, and neutrophils. Significant decreases in the percentage of CD4<sup>+</sup> T cells were measured in mice treated with either IL-25 alone or OVA+IL-25 compared to Ova treated mice. Decreases in CD8<sup>+</sup> T cells were also observed in OVA+IL-25 treated mice. However, when examining total cell numbers, there were significant increases in B cells, CD4<sup>+</sup> T cells, DCs, macrophages, and neutrophils in mice treated with OVA+IL-25 vs. OVA or IL-25 alone (Fig.3.1c).

To establish whether pathological changes were also occurring, whole lung sections were preserved in formalin, embedded in paraffin, sectioned, and stained for histological analysis. H&E staining revealed increased cellular infiltrates surrounding the airways, with the highest infiltration in mice treated with OVA+IL-25. Increases in mucus production were observed by PAS staining in mice treated with IL-25 and OVA+IL-25 compared to mice receiving OVA only (Fig.3.1d; H&E top, PAS bottom).

Elevated serum IgE concentrations are typically observed in various allergic situations; it was therefore important to also measure IgE levels in this experimental model. Blood was taken by cardiac puncture at the time of sacrifice and serum collected for IgE measurement by ELISA. Serum IgE concentrations increased significantly from an average of 2.01  $\mu\text{g/mL}$  in OVA treated mice to 24.9  $\mu\text{g/mL}$  in mice treated with OVA+IL-25; no measurable differences were detected when mice were treated with IL-25 alone (Fig.3.1e).

Taken together, these data support the conclusion that repeated exposure of IL-25 alone is capable of creating an inflammatory environment within the lung. Furthermore, this pulmonary inflammation is exacerbated when a foreign antigen is also present. This established model of pulmonary inflammation will allow a more detailed examination of the mechanisms contributing to IL-25 induced inflammation.



**Figure 3.1. IL-25 Induces Type-2 Pulmonary Inflammation.** (A) Total cell counts in BAL (left) and Lung (right) from WT mice treated with intranasal OVA, IL-25, or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and Lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes (including B cells, CD4<sup>+</sup>- and CD8<sup>+</sup>-T cells), DCs, macrophages, and neutrophils by percentage (left) and absolute number (right), measured by flow cytometry. (D) Histology lung sections stained for H&E (top) and PAS (bottom) taken from mice treated with intranasal OVA, IL-25, or OVA+IL-25. (E) Serum IgE measured by ELISA. Results are presented as mean  $\pm$  SEM and are representative of three independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.005, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

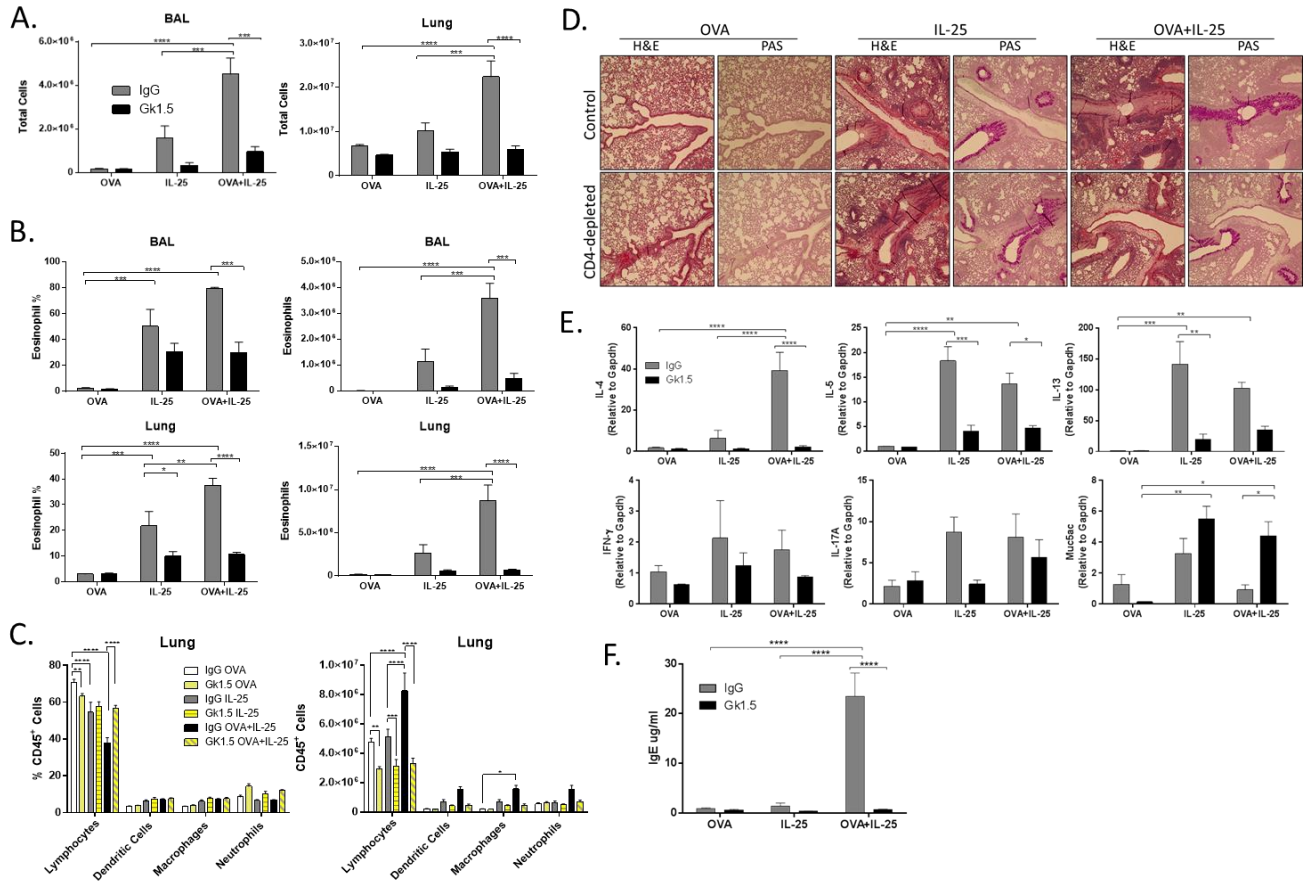
### 3.2.2 *IL-25 Induced Pulmonary Inflammation is Partially Dependent on CD4<sup>+</sup> T Cells*

T<sub>H</sub>2 cells are important contributors to type-2 inflammation in many chronic lung inflammatory settings. To determine if they are required in our IL-25 induced inflammatory model, mice were given i.p. injections of an anti-CD4 antibody (GK1.5) to deplete CD4 T cells and treated with OVA, IL-25, or Ova+IL-25 as described above. Control mice were given i.p. injections of IgG in concentrations corresponding to that of the GK1.5 antibody. As seen previously, total cell counts were increased in the BAL and lung of control mice treated with IL-25 alone, and exacerbated further in Ova+IL-25 treated mice. However, mice depleted of CD4 T cells showed significantly reduced cellular infiltrates when treated with OVA+IL-25 (Fig.3.2a). Eosinophils were profoundly impacted by the lack of CD4 T cells, exhibiting decreases in both the BAL fluid and lung by percentage and absolute numbers from mice treated with either IL-25 alone or OVA+IL-25 compared to OVA treated mice (Fig.3.2b). Assessment of other cellular infiltrates revealed decreased lymphocyte populations in all treatment conditions, as expected. Interestingly, mice depleted of CD4 T cells that were treated with OVA+IL-25 also had decreases in DCs, macrophage, and neutrophil populations (Fig.3.2c). The cellular infiltrates measured by flow cytometry correlate with what is observed histologically by H&E; however mucus production assessed by PAS staining did not appear to be significantly changed in mice treated with the anti-CD4 antibody (Fig.3.2d).

To determine the effects of CD4 depletion on inflammatory cytokine expression levels, RNA was isolated from lung sections and real-time PCR was performed. Levels of IL-5 and IL-13 expression were significantly increased in IgG control mice treated with IL-25 and OVA+IL-25 over mice treated with OVA alone and this increased

expression was diminished when mice were depleted of CD4 T cells (Fig.3.2e). While IL-4 expression levels were only increased when control mice were treated with OVA+IL-25, this was also significantly reduced when mice were treated with the anti-CD4 antibody. Neither IFN- $\gamma$  nor IL-17A expression levels were affected by treatment with IL-25 or OVA+IL-25; as expected, depleting CD4 cells also had no effect on these cytokines. Mucus production, as assessed by gene expression of Muc5ac, was upregulated in mice treated with IL-25 $\pm$ Ova and this was unaffected by CD4 depletion, correlating with what was observed histologically (Fig.3.2e). These results suggest the CD4 T cells are necessary for the increased expression of type-2 inflammatory cytokines: IL-4, IL-5, and IL-13. They also reveal a dispensable role for CD4 T cells in the promotion of goblet cell hyperplasia resulting in increased mucus production.

Serum IgE concentrations were elevated in control mice treated with OVA+IL-25. As shown in Fig.3.1, this effect was completely abrogated when the mice were also treated with the anti-CD4 depleting antibody (Fig.3.2f). This result indicates that CD4 T cells are required for IgE production.



**Figure 3.2. IL-25 Induced Pulmonary Inflammation is partially dependent on CD4 T Cells.** (A) Total cell counts in BAL (left) and Lung (right) from WT mice ± CD4 depleting antibody (GK1.5) treated with i.n. OVA, IL-25, or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and Lung (bottom) by percentage (left) and absolute numbers (right) measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right), measured by flow cytometry. (D) Histology lung sections stained for H&E and PAS taken from IgG controls (top) and CD4 depleted (bottom) mice treated with i.n. OVA, IL-25, or OVA+IL-25. (E) Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN-γ, IL-17A, and Muc5ac (bottom) as measured by RT-PCR. (F) Serum IgE measured by ELISA. Results are presented as mean ± SEM and are combined from two independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.005, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

### 3.2.3 *IL-25 Induced Pulmonary Inflammation is not due to Direct Effects on CD4 T Cells*

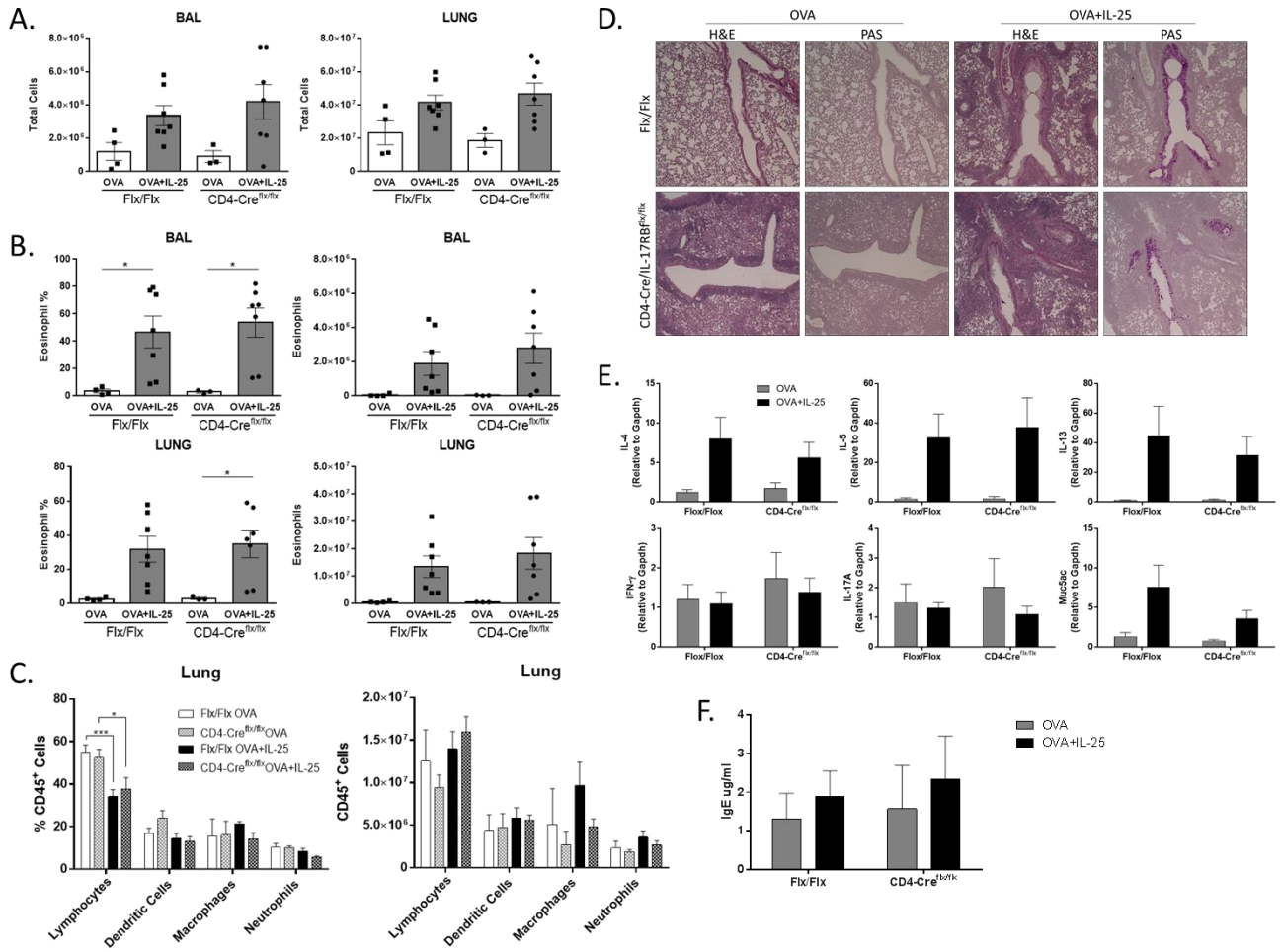
To determine if the effects observed in the CD4 depleting experiments were due to a direct response of T cells to IL-25, CD4-Cre mice were crossed to mice with the gene for the IL-25 receptor (IL-17RB) flanked with LoxP sites. T cells from these mice, referred to as CD4-Cre<sup>flx/flx</sup> mice, are unable to respond directly to IL-25. CD4-Cre<sup>flx/flx</sup> mice and littermate controls (mice homozygous for IL-17RB LoxP sites, referred to as Flx/Flx) were treated with either OVA or OVA+IL-25 as described above. In contrast to our findings in WT mice, only slight increases in total cell numbers were observed in both the BAL fluid and lung from both groups of mice (Fig.3.3a). There were significant increases in eosinophil percentages in both BAL fluid and lung in mice treated with OVA+IL-25; however, there was no difference between CD4-Cre<sup>flx/flx</sup> and Flx/Flx mice (Fig.3.3b). Consistent with previous findings, the percentages of lymphocytes infiltrating the lung was decreased; however changes in absolute numbers of lymphocytes, DCs, macrophages, and neutrophils were unaffected (Fig.3.3c).

Histologically, both groups of mice treated with OVA+IL-25 had greater inflammatory infiltrates and increased mucus production compared to mice treated with OVA only (Fig.3.3d). The latter finding correlates with gene expression for mucus production, in which there was also a slight increase when mice were treated with OVA+IL-25 (Fig.3.3e). No noticeable differences were observed between CD4-Cre<sup>flx/flx</sup> and Flx/Flx mice.

Genetic expression levels of type-2 cytokines (IL-4, IL-5, and IL-13) showed measurable increases in all mice treated with OVA+IL-25. However, the fold increase in gene expression was not as consistent or as elevated as seen previously, nor was there

a significant difference between CD4-Cre<sup>flx/flx</sup> and Flx/Flx mice (Fig.3.3e). As expected, there were no changes in the levels of IFN- $\gamma$  or IL-17A expression.

Interestingly, serum concentrations for IgE were unchanged in either mouse group when treated with OVA+IL-25, compared to OVA only controls (Fig.3.3f). The diminished production of IgE, combined with reduced eosinophils and type-2 cytokine expression levels reveal possible differences in this mouse strain compared to WT mice. Overall, the inflammatory effects that were observed do not appear to be due to direct effects on CD4 cells as CD4-Cre<sup>flx/flx</sup> mice had similar responses to those seen in littermate control Flx/Flx mice. This was also consistent with experiments in which only IL-25 was administered (data not shown).



**Figure 3.3. IL-25 induced pulmonary inflammation is not due to direct effects on CD4 T cells.** (A) Total cell counts in BAL (left) and lung (right) from Flx/Flx and CD4-Cre<sup>flx/flx</sup> mice treated with i.n. OVA or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and Lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right) measured by flow cytometry. (D) Histology lung sections stained for H&E and PAS taken from Flx/Flx controls (top) and CD4-Cre<sup>flx/flx</sup> (bottom) mice treated with i.n. OVA or OVA+IL-25. (E) Fold change in mRNA gene expression for the cytokines IL-4, IL-5, and IL-13 (top) and IFN-γ, IL-17A, and Muc5ac (bottom), as measured by rt-PCR. (F) Serum IgE measured by ELISA. Results are presented as mean ± SEM and are combined from two independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*\*, P < 0.001.

### 3.2.4 *IL-25 Induced Pulmonary Inflammation is not due to Direct Effects on Monocyte, Macrophage, or Granulocyte Populations*

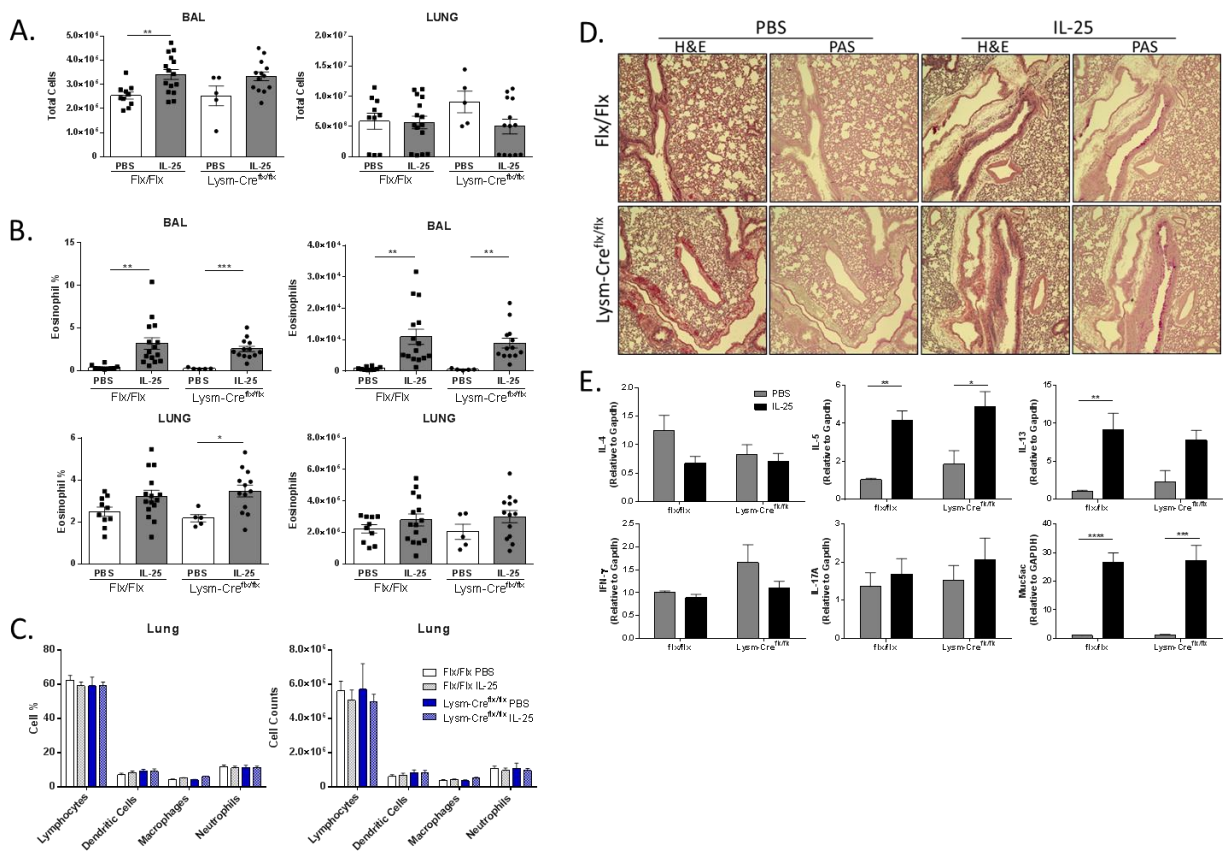
The effects of IL-25 signaling through T cell-independent mechanisms are known to play an essential role in the induction of a T<sub>H</sub>2 response<sup>50,94</sup>. Along with the ILC2s, a newly described population termed type 2 myeloid (T2M) cells were shown to be capable of producing IL-4 and IL-13 upon stimulation with IL-25<sup>116,117</sup>. T2M cells, which were also found in the peripheral blood of asthmatic patients, appear to be both pathogenic and steroid resistant. To determine whether IL-25 mediated pulmonary inflammation is due to a direct effect on monocyte, macrophage, or granulocyte populations, we crossed the IL-17RB<sup>flx/flx</sup> mouse to a Lysm-Cre mouse and treated with IL-25 as described above. When given IL-25 alone, increases in total cell numbers were detected in BAL fluid of both Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice compared to PBS treated controls (Fig.3.4a). Increases in eosinophils by percentage and absolute number account for the increased cell numbers in BAL of IL-25 treated mice; no other cell type had noticeable differences by percentage or number in the lungs of these mice (Fig.3.4b&c). Increases in total cells were further exacerbated in both BAL fluid and lung when the mice were given OVA+IL-25; however there was no difference between Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice (Fig.3.5a). In OVA+IL-25 treated mice, significant increases in eosinophils were found in both BAL fluid and lung by percentage and number. These mice also had increased total numbers of lymphocytes, DCs, macrophages and, to a lesser extent, neutrophils (Fig.3. 5b&c).

Histologically, Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice maintain normal lung architecture with little to no mucus production visible by PAS staining in PBS and OVA treated controls. Increasing concentrations of inflammatory infiltrates are visible by H&E

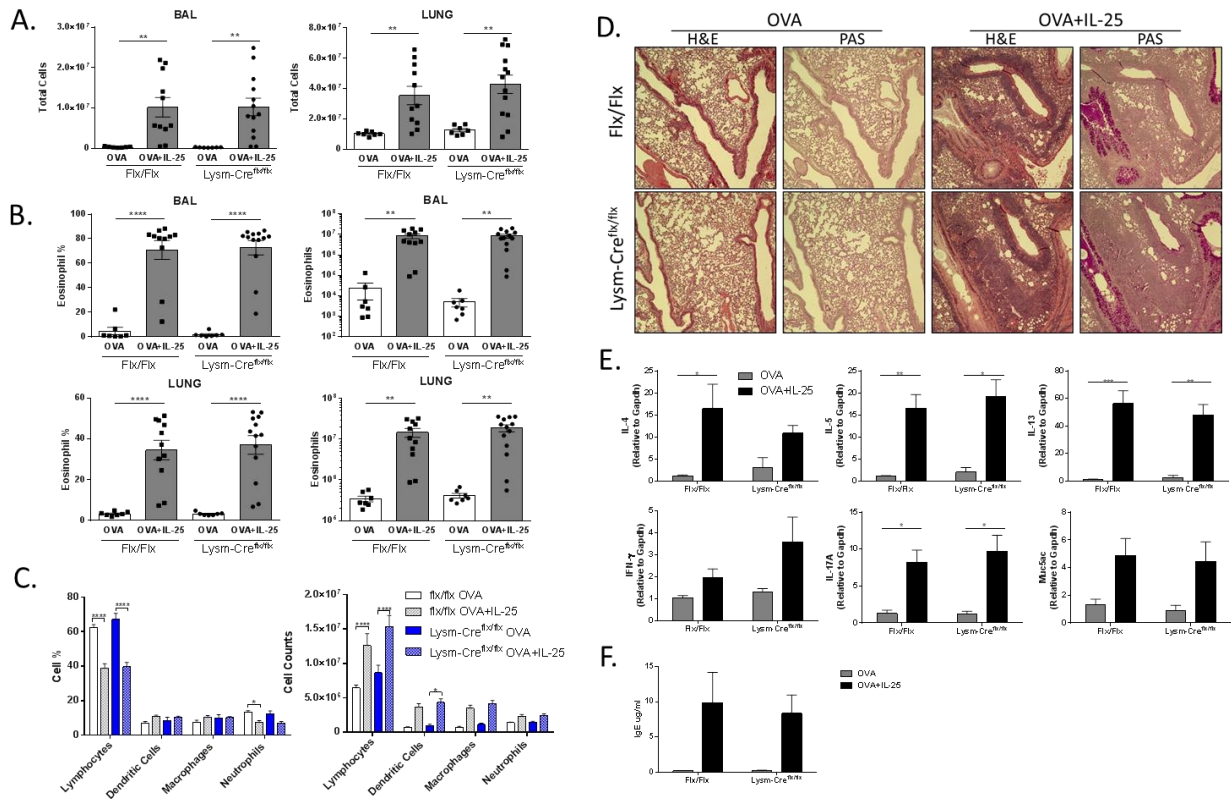
staining when mice are treated with IL-25 and OVA+IL-25, as well as increasing mucus production (Fig.3.4d & 3.5d). No discernable changes were observed between Flx/Flx and Lysm-Cre<sup>flx/flx</sup>.

Both Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice treated with IL-25 had increased levels of IL-5, IL-13, and Muc5ac gene expression, with no significant variation between the two groups; little to no changes were detected in IL-4, IFN- $\gamma$ , or IL-17A expression (Fig.3.4e). However, in mice treated with both OVA and IL-25, significant increases were found in IL-4, IL-5, IL-13, Muc5ac, and, interestingly, IL-17A (Fig.3.5e). The fold increase for each gene was similar between the Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice. OVA+IL-25 treated mice also had increased serum IgE concentrations, with similar levels detected in Flx/Flx and Lysm-Cre<sup>flx/flx</sup> mice (Fig.3.5f).

Collectively, these data confirm previous findings, including increases in eosinophils into the BAL and lung, mucus production, and type-2 cytokine gene expression when mice are given IL-25 i.n.. These characteristic changes are further exacerbated when IL-25 is administered with foreign antigen, adding to this the increased IgE production. However, none of our findings indicates that these changes are due to the direct action of IL-25 signaling on a monocyte, macrophage, or granulocyte cell population.



**Figure 3.4. IL-25 induced pulmonary inflammation is not due to direct effects on monocyte, macrophage, or granulocyte populations.** (A) Total cell counts in BAL (left) and lung (right) from Lysm-Cre/IL-17RB<sup>flx/flx</sup> or Flx/Flx control mice treated with i.n. PBS ± IL-25. (B) Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right) measured by flow cytometry. (D) Histology lung sections stained for H&E and PAS taken from Flx/Flx (top) and Lysm-Cre/IL-17RB<sup>flx/flx</sup> (bottom) mice treated with i.n. PBS ± IL-25. (E) Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN-γ, IL-17A, and Muc5ac (bottom) as measured by rt-PCR. Results are presented as mean ± SEM and are combined from three independent experiments with 2-5 mice per group. \*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001.



**Figure 3.5. Ova+IL-25 induced pulmonary inflammation is not due to direct effects on monocyte, macrophage, or granulocyte populations. (A)** Total cell counts in BAL (left) and lung (right) from Lysm-Cre/IL-17RB<sup>flx/flx</sup> or Flx/Flx control mice treated with i.n. OVA  $\pm$  IL-25. **(B)** Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. **(C)** Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right) measured by flow cytometry. **(D)** Histology lung sections stained for H&E and PAS taken from Flx/Flx (top) and Lysm-Cre/IL-17RB<sup>flx/flx</sup> (bottom) mice treated with i.n. OVA  $\pm$  IL-25. **(E)** Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN- $\gamma$ , IL-17A, and Muc5ac (bottom) as measured by rt-PCR. **(F)** Serum IgE measured by ELISA. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments with 2-5 mice per group. \*, P < 0.05, \*\*, P < 0.005, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

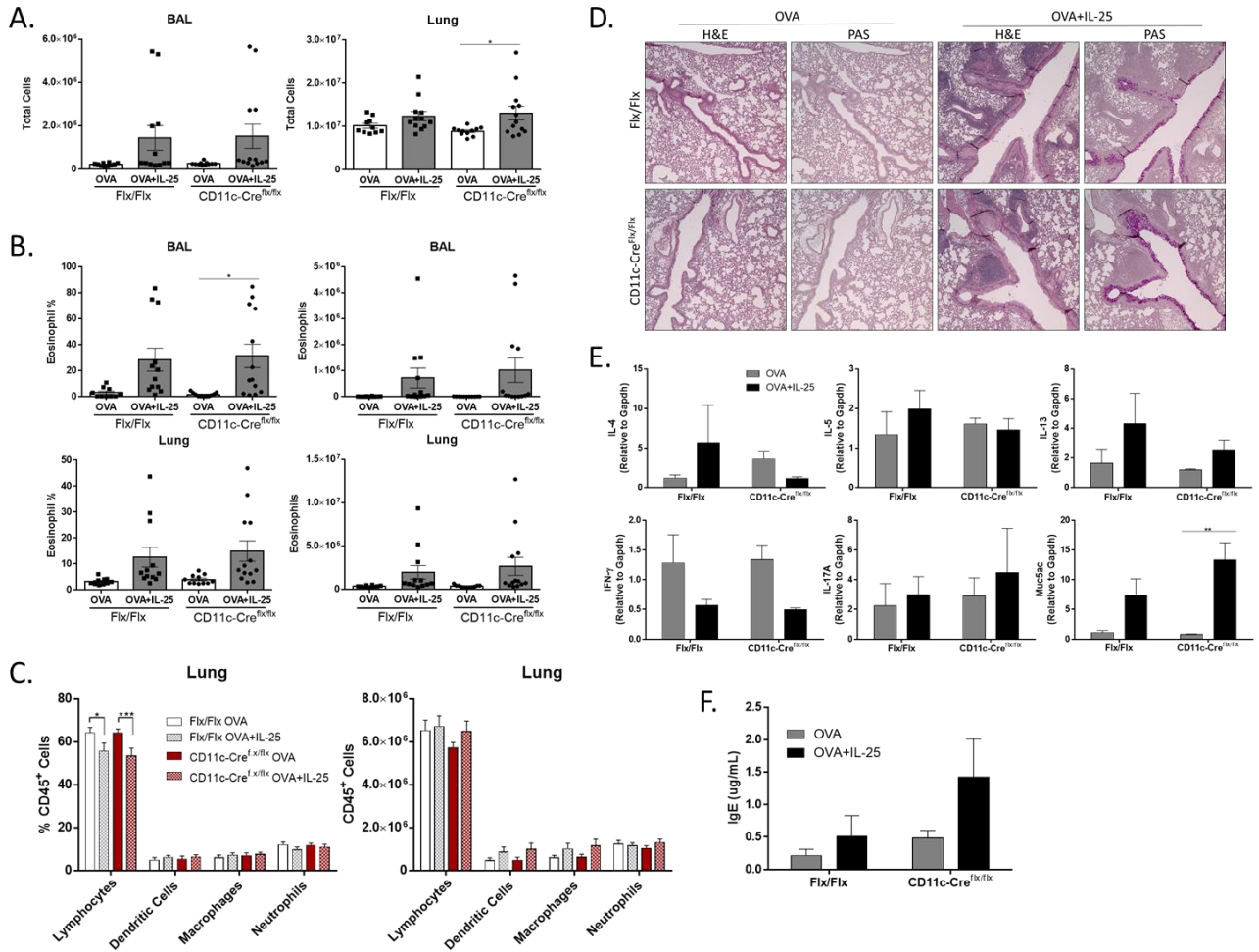
### 3.2.5 *IL-25 Induced Pulmonary Inflammation is not due to Direct Effects on CD11c Dendritic Cells*

IL-17RB is also known to be expressed on CD11c<sup>+</sup> DC populations<sup>73,74</sup>. Studies in our lab have revealed that i.n. administration of TSLP, another epithelial cytokine, is sufficient to induce an inflammatory airway disease. This response is dependent on CD11c<sup>+</sup> DCs, as mice with specific deletion of the TSLP receptor in CD11c<sup>+</sup> cells have a significant reduction in pulmonary inflammation (unpublished data). Given the overlapping roles for these epithelial cytokines, we sought to determine if IL-25 was also capable of acting directly through CD11c DCs to mediate the type-2 inflammatory response. To accomplish this, the IL-17RB<sup>flx/flx</sup> mice were crossed to CD11c-Cre mice to obtain a strain in which DCs would be unable to respond directly to stimulation by IL-25.

We anticipated any differences seen in these mice would be apparent only when treated with antigen +IL-25; therefore, experiments were only performed comparing OVA to OVA+IL-25. Both Flx/Flx and CD11c-Cre<sup>flx/flx</sup> mice had slight increases in total cells infiltrating the BAL fluid and lung when treated with OVA+IL-25 compared to OVA only controls (Fig.3.6a). This correlated with increases in eosinophil percent and absolute counts from BAL fluid and lung, with Flx/Flx and CD11c-Cre<sup>flx/flx</sup> mice having similar increases (Fig.3.6b). Results shown are combined from three experiments, where there was a noticeable amount of variation between experiments and an apparent overall diminished inflammatory response in both groups of mice when compared to previous experiments. While percentages of lymphocytes in the lung were significantly lower in mice treated with OVA+IL-25, no differences in absolute numbers were detected in lymphocytes, DCs, macrophages, or neutrophils (Fig.3.6c).

Despite the overall reduced response to OVA+IL-25 treatments, histological examination revealed areas of inflammatory infiltrates and increased mucus production visualized by H&E and PAS staining (Fig.3.6d). The increased mucus production correlates with increases in Muc5ac gene expression in both Flx/Flx and CD11c-Cre<sup>flx/flx</sup> mice treated with OVA+IL-25 compared to Ova treated controls (Fig.3.6e lower right panel). No significant differences in type-2, T<sub>H</sub>1, or T<sub>H</sub>17 cytokine gene expression were observed for either Flx/Flx or CD11c-Cre<sup>flx/flx</sup> mice treated with OVA+IL-25 (Fig. 3.6e). There was also no measureable differences in serum IgE concentrations for either group of mice when treated with OVA+IL-25 as was detected in previous experiments (Fig.3.6f).

Although the response in both the CD11c-Cre<sup>flx/flx</sup> mice and their Flx/Flx littermate controls was somewhat variable, the lack of significant differences between the two strains suggests that direct signaling triggered by IL-25 in DCs is not a driving factor in the development of pulmonary inflammation.



**Figure 3.6. IL-25 induced pulmonary inflammation is not due to direct effects on CD11c+ DCs.** (A) Total cell counts in BAL (left) and lung (right) from Flx/Flx and CD11c-Cre<sup>flx/flx</sup> mice treated with i.n. OVA or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right), measured by flow cytometry. (D) Histology lung sections stained for H&E and PAS taken from Flx/Flx controls (top) and CD11c-Cre<sup>flx/flx</sup> mice treated with i.n. OVA or OVA+IL-25. (E) Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN- $\gamma$ , IL-17A, and Muc5ac (bottom) as measured by rt-PCR. (F) Serum IgE measured by ELISA. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments with 2-5 mice per group. \*, P < 0.05, \*\*\*, P < 0.001.

### 3.2.6 *IL-25 Mediated Pulmonary Inflammation is Independent of TSLP*

In a recent study, IL-25 was shown to be responsible for the induction of the epithelial-derived cytokines TSLP and IL-33 in an allergic response to house dust mite<sup>82</sup>.

Previously, the effects of IL-25 on memory T<sub>H</sub>2 cells were shown to be dependent upon TSLP activated DCs<sup>71</sup>. An area that requires further study is the ability of IL-25 to mediate type-2 inflammation *independently* of TSLP and IL-33. To answer this question, we applied our established model of IL-25 induced pulmonary inflammation to mice deficient in TSLP and/or IL-33 signaling.

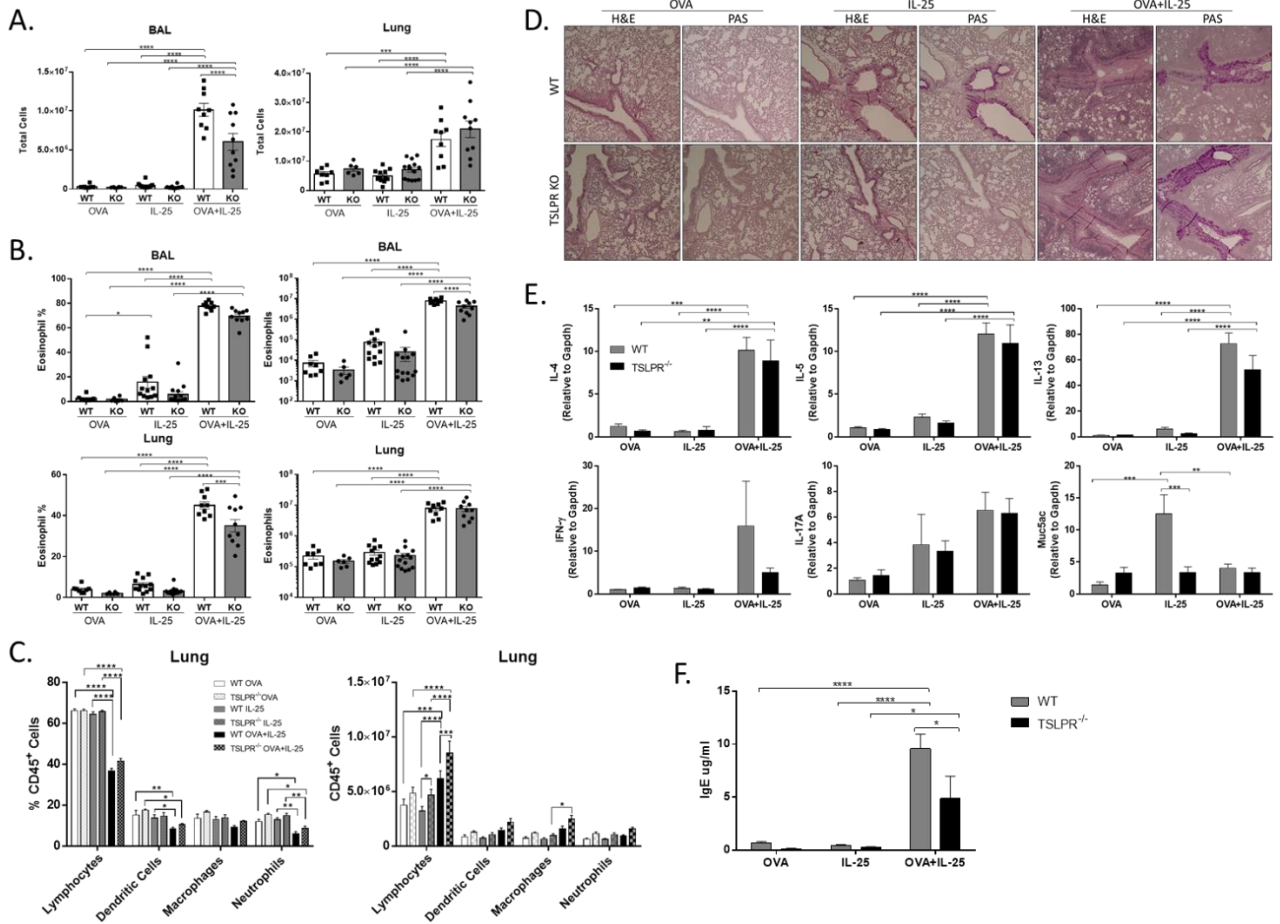
In TSLP receptor deficient mice (TSLPR<sup>-/-</sup>), total cell counts in the BAL fluid of OVA+IL-25 treated mice were significantly reduced compared to WT mice. This decrease was specific to the BAL, as the lungs had no change in total cellularity compared to WT mice (Fig.3.7a). Looking specifically at eosinophils, slight decreases were apparent in both IL-25 and OVA+IL-25 treated mice in the lung and BAL fluid by percentage and absolute numbers (Fig.3.7b). By percentage, significant decreases were observed in both WT and TSLPR<sup>-/-</sup> mice treated with OVA+IL-25 in lymphocytes, DCs, and neutrophils. Interestingly, by absolute numbers, the lymphocytes were significantly increased in TSLPR<sup>-/-</sup> mice treated with IL-25±OVA (Fig.3.7c). A trend toward increased dendritic cells, macrophages and neutrophils was also noted in TSLPR<sup>-/-</sup> mice; however, not to statistically significant levels.

Histologically, TSLPR<sup>-/-</sup> mice did appear to have slightly less inflammatory infiltrates after IL-25 and OVA+IL-25 treatments compared to WT controls by H&E staining. Remarkably, when TSLPR<sup>-/-</sup> mice were treated with IL-25 alone, a loss of mucus was observed by PAS staining (Fig.3.7d). This correlates with a significant

decrease in Muc5ac gene expression compared to WT mice treated with IL-25 alone (Fig.3.7e lower right panel).

Gene expression for the type-2 cytokines IL-4, IL-5, and IL-13 was significantly increased in both WT and TSLPR<sup>-/-</sup> mice when treated with OVA+IL-25, however there were no significant differences between the two strains (Fig.3.7e). While slight variations were seen in IFN- $\gamma$  and IL-17A, these changes were not significant. As expected, serum concentrations of IgE were elevated when WT mice were treated with OVA+IL-25; however, surprisingly these levels were reduced in TSLPR<sup>-/-</sup> mice (Fig.3.7f).

Based on these data, it is clear that IL-25 (particularly in the presence of antigen) is capable of creating a type-2 inflammatory environment without TSLP signaling. However, there are contributions from TSLP signaling that appear to be important in creating a robust response including the production of mucus and elevation of IgE secretion.



**Figure 3.7. IL-25 mediated pulmonary inflammation is independent of TSLP.** (A) Total cell counts in BAL (left) and lung (right) from WT (white bars) or TSLPR<sup>-/-</sup> (grey bars) mice treated with i.n. OVA, IL-25, or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right), measured by flow cytometry. (D) Histology lung sections stained for H&E and PAS taken from WT control (top) and TSLPR<sup>-/-</sup> (bottom) mice treated with i.n. OVA, IL-25, or OVA+IL-25. (E) Fold change in mRNA gene expression for the cytokines IL-4, IL-5, and IL-13 (top) and IFN- $\gamma$ , IL-17A, and Muc5ac (bottom), as measured by rt-PCR. (F) Serum IgE measured by ELISA. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

### 3.2.7 *IL-25 Mediated Pulmonary Inflammation is Independent of IL-33*

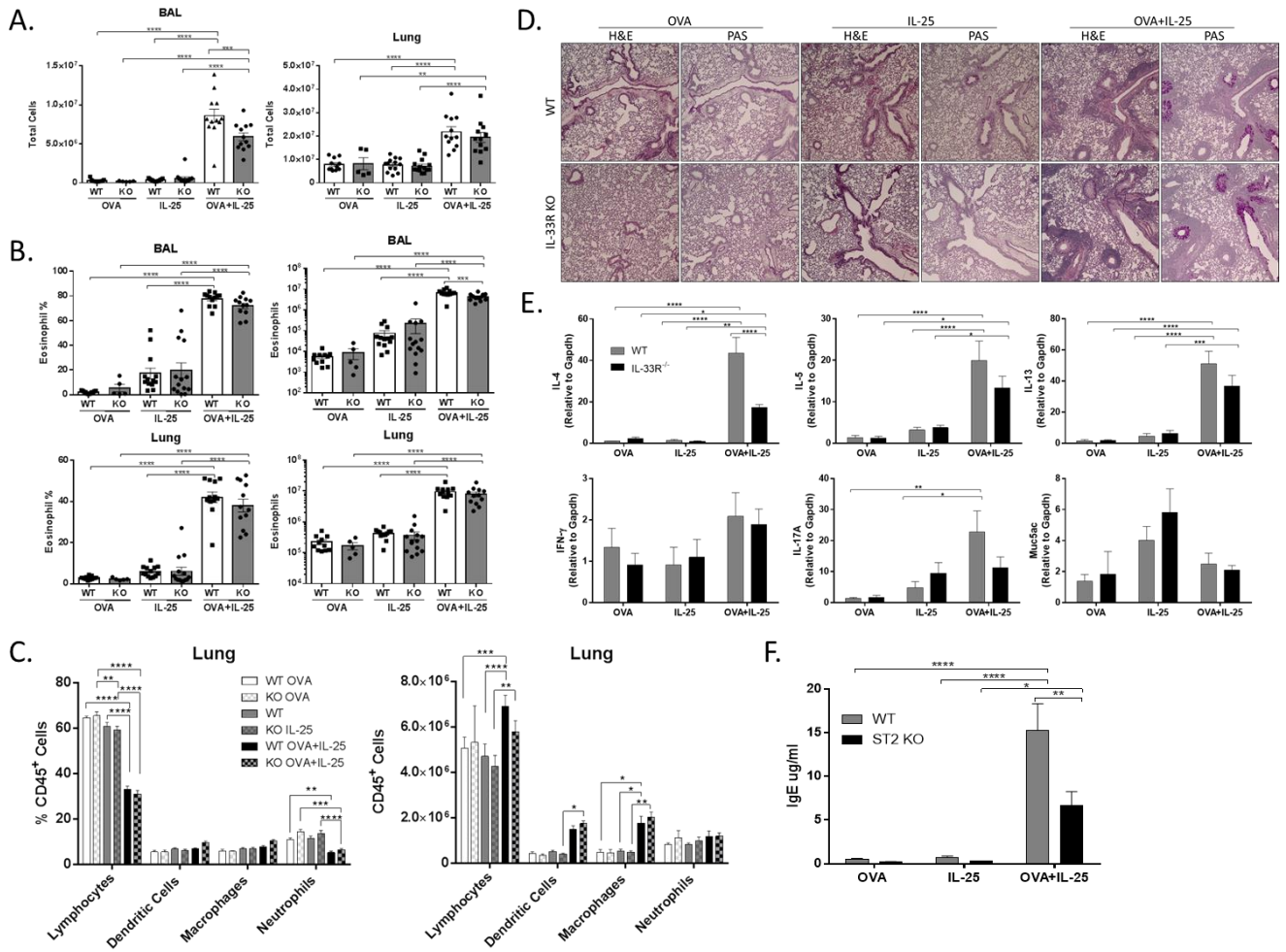
To test if IL-33 is necessary for IL-25 induced inflammation, we used ST2 deficient mice (IL-33R<sup>-/-</sup>) because IL-33 signals through the ST2 receptor. Initial examination of total cellular infiltrates into the BAL fluid and lung revealed increases only in mice treated with OVA+IL-25 and a significant reduction in the BAL of IL-33R<sup>-/-</sup> mice compared to WT controls (Fig.3.8a). Assessment of individual inflammatory cell types revealed significant increases in eosinophils in Ova+IL-25 in BAL fluid and lung by percentage and absolute number. These effects were similar for WT and IL-33R<sup>-/-</sup> mice (Fig3.8b). Decreases in the percent of lymphocytes and neutrophils were detected in both strains of mice when treated with OVA+IL-25. However, by absolute number, there was an increase in lymphocytes, DCs, and macrophages. These changes were consistent in both WT and IL-33R<sup>-/-</sup> mice (Fig.3.8c).

Histologically, by H&E and PAS staining, both strains of mice appear to be unchanged comparing IL-25 treated mice to OVA controls with limited inflammatory cells and little to no mucus staining. Increases in both inflammation and mucus production are evident in Ova+IL-25 treated mice, with comparable changes in WT and IL-33R<sup>-/-</sup> strains (Fig.3.8d). The lack of mucus staining corresponds with gene expression for Muc5ac when comparing IL-25 to OVA controls; however, there is also limited expression in mice treated with OVA+IL-25. Expression levels are similar over all treatments in both groups of mice (Fig.3.8e lower right panel).

IL-33 signaling may be capable of enhancing type-2 cytokine expression, as IL-4, IL-5 and IL-13 all had decreased expression in IL-33R<sup>-/-</sup> compared to WT controls in mice treated with OVA+IL-25 (Fig.3.8e). IFN- $\gamma$  expression remained unchanged,

however, IL-17A was increased significantly in OVA+IL-25 treated mice, with a notable decrease in IL-33R<sup>-/-</sup> mice compared to WT mice.

As was seen with TSLPR<sup>-/-</sup> mice, IL-33R<sup>-/-</sup> mice also had significantly decreased serum IgE concentrations compared to WT mice when they were treated with OVA+IL-25 (Fig.3.8f). The decreases in eosinophils coming into the lung space, decreased type-2 cytokine expression, and decreased IgE production found in IL-33R<sup>-/-</sup> mice suggest the IL-33 signaling pathway is capable of enhancing IL-25 mediated pulmonary inflammation. While IL-33 does not appear to be required to induce the inflammatory response, it may be acting down-stream of IL-25 to augment the response.



**Figure 3.8. IL-25 mediated pulmonary inflammation is independent of IL-33.** (A) Total cell counts in BAL (left) and lung (right) from WT (white bars) and IL-33R<sup>-/-</sup> (grey bars) mice treated with i.n. OVA, IL-25, or OVA+IL-25. (B) Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. (C) Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right), measured by flow cytometry. (D) Histology of lung sections stained for H&E and PAS taken from WT controls (top) and IL-33R<sup>-/-</sup> (bottom) mice treated with i.n. OVA, IL-25, or OVA+IL-25. (E) Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN- $\gamma$ , IL-17A, and Muc5ac (bottom), as measured by rt-PCR. (F) Serum IgE measured by ELISA. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

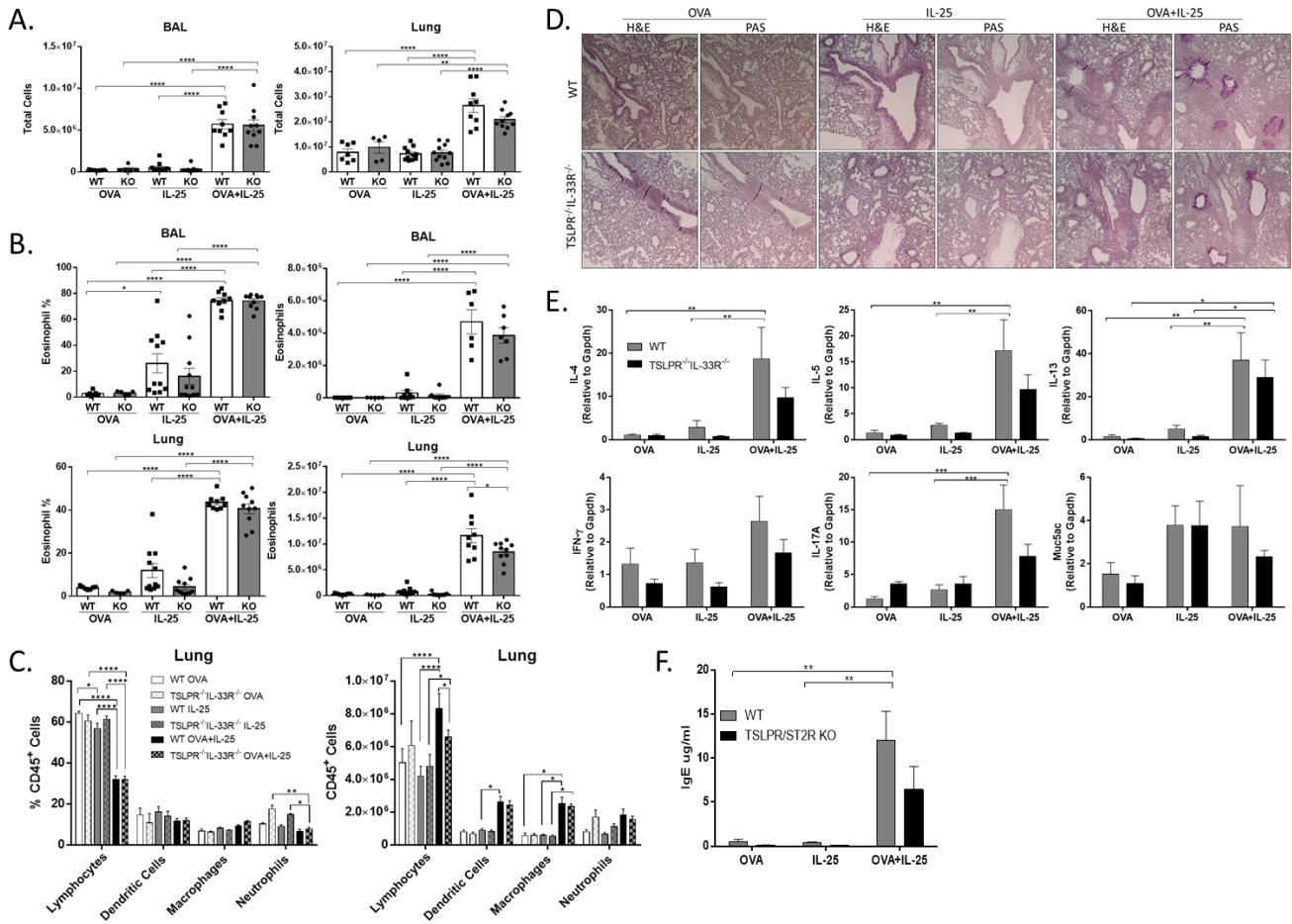
### 3.2.8 *IL-25 Mediated Pulmonary Inflammation is Independent of the Combined TSLP/IL-33 Signaling Pathways*

Our data suggest TSLP and IL-33 both make contributions downstream of IL-25 to enhance the type-2 inflammatory response. To determine whether a lack in one of these cytokines is compensated for by another, we crossed the TSLPR<sup>-/-</sup> and IL-33R<sup>-/-</sup> mice to obtain a double KO (TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup>). When these mice were treated using the protocol described above, total cellular infiltrates were increased only in mice treated with OVA+IL-25, with no significant differences between TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> and WT mice (Fig.3.9a). TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> had slight decreases in eosinophils in both BAL fluid and lungs of mice treated with IL-25±OVA (Fig.3.9b). Consistent with previous data, decreases in percentages of lymphocytes with an increase in absolute number were found in both TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> and WT mice when treated with OVA+IL-25. Both strains also had increases in absolute numbers of DCs and macrophages with OVA+IL-25 treatments (Fig.3.9c).

As with IL-33R<sup>-/-</sup> mice, the TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> mice had little observable changes to lung architecture or mucus production in IL-25 treated mice compared to OVA controls. Inflammatory infiltrates as well as mucus staining appeared similar in OVA+IL-25 treated mice in TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> versus WT controls (Fig.3.9d). Gene expression for Muc5ac was unchanged in all conditions with both strains of mice (Fig.3.9e lower right panel).

There were significant increases in IL-4, IL-5, and IL-13 in WT mice treated with OVA+IL-25 compared to OVA controls, however only IL-13 had significant levels of increased expression in the TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> mice. Although not significant, there was a trend towards decreased expression of IL-4, IL-5, IL-17A, and serum IgE concentrations

in TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> mice compared to WT controls treated with OVA+IL-25 (Fig. 3.9e). Collectively, these results indicate that a loss in one of these epithelial cytokines is enough to reduce IL-25-induced pulmonary inflammation. However there does not appear to be a synergistic effect of losing both TSLP and IL-33 signaling. Furthermore, IL-25 is still capable of inducing a type-2 inflammatory response independently of these two cytokines.



**Figure 3.9. IL-25 mediated pulmonary inflammation is independent of the combined TSLP/IL-33 signaling pathways. (A)** Total cell counts in BAL (left) and lung (right) from WT and TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> mice treated with i.n. OVA, IL-25, or OVA+IL-25. **(B)** Eosinophil infiltrates into BAL (top) and lung (bottom) by percentage (left) and absolute numbers (right), measured by flow cytometry. **(C)** Lung differential cell analysis for lymphocytes, DCs, macrophages, and neutrophils by percentage (left) and absolute number (right) measured by flow cytometry. **(D)** Histology of lung sections stained for H&E and PAS taken from WT controls (top) and TSLPR<sup>-/-</sup>IL-33R<sup>-/-</sup> (bottom) mice treated with intranasal OVA, IL-25, or OVA+IL-25. **(E)** Fold change in mRNA gene expression for cytokines IL-4, IL-5, and IL-13 (top) and IFN-γ, IL-17A, and Muc5ac (bottom), measured by rt-PCR. **(F)** Serum IgE measured by ELISA. Results are presented as mean ± SEM and are combined from three independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.01, \*\*\*, P < 0.001, \*\*\*\*, P < 0.0001.

### 3.3 DISCUSSION

It is increasingly appreciated that IL-25 induction of type-2 inflammation is mediated through both innate and adaptive cell types. While various populations of innate and adaptive cells are known to express the IL-25R, whether these cells are directly responding to IL-25 in vivo remains poorly understood. Previous studies have explored this matter *indirectly* by using KO mice, blocking antibodies, or in vitro analyses. If IL-25 induced inflammation is reliant on direct signaling through CD4 T cells, myeloid cells, or DCs, we would expect to see a diminished response when IL-25 signaling is deleted only in these cells. We therefore designed our study to test this hypothesis using the Cre-Lox system to exclusively delete IL-17RB expression in CD4<sup>+</sup> cells, myeloid cells, and DCs using CD4<sup>-</sup>, Lysm<sup>-</sup>, and CD11c-Cre mice, respectively.

We first established a model of pulmonary inflammation that was dependent on IL-25. Consistent with what is known regarding the ability of IL-25 to initiate type-2 inflammation, we found increased cellular infiltrates within the lungs (particularly eosinophils), increased mucus production, and increased serum IgE (when given in combination with antigen). The fact that we were able to induce type-2 inflammation with IL-25 alone is a feature that is unique to this epithelial cytokine as previous studies with TSLP have shown a requirement for the presence of antigen<sup>118</sup>. Additionally, we were able to establish that when antigen was given along with IL-25, this further enhanced the inflammatory response, indicating innate and adaptive cells are capable of responding to IL-25. To initially establish a role for CD4 T cells in this model, we used a CD4 depleting antibody. While we anticipated the lack of CD4 cells would reduce type-2 inflammation in the mice treated with OVA+IL-25 to the level observed with IL-25

alone, we were surprised that it also had an influence on the mice treated with IL-25 alone. Not only were the eosinophils decreased in these mice, but so were IL-5 and IL-13 expression levels. However, there did appear to be enough IL-5 and IL-13 that eosinophils were not at baseline and there was still increased mucus production compared to control mice. While others have shown RAG<sup>-/-</sup> mice are capable of creating type-2 inflammation in response to IL-25, these intriguing results from this experiment indicate that CD4 T cells augment the response even without foreign antigen.

However, when we examined the ability of CD4 cells to respond directly to IL-25 using CD4-Cre/IL-17RB<sup>flx/flx</sup> mice, we were surprised to find they had an equivalent inflammatory response to that of control mice. This was true for mice treated with IL-25 alone or in addition to OVA. In vitro experiments<sup>62</sup> have indicated that T cells are capable of responding directly to IL-25; our model suggests that type-2 inflammation can progress even without IL-25 signaling in CD4 cells. This emphasizes the importance of delineating what cells are capable of in vitro versus how they actually respond in vivo. We speculate innate cells (presumably ILC2s) are able to respond to IL-25 in this environment, generating initial sources of IL-5 and IL-13 which can then further enhance the inflammatory cascade independent of any T cells responding directly to IL-25.

IL-17RB expression has been detected on both myeloid/macrophage populations as well as CD11c DC populations<sup>69,70,72,116,117</sup>. To determine whether IL-25 signaling in these cells is required to mediate type-2 inflammation in our model, we crossed Lysm- and CD11c-Cre mice to the IL-17RB<sup>flx/flx</sup> mice. Both the Lysm-Cre/IL-17RB<sup>flx/flx</sup> and CD11c-Cre/IL-17RB<sup>flx/flx</sup> mice were capable of producing an inflammatory response that

was equivalent to that of IL-17RB<sup>flx/flx</sup> control mice. While we don't argue against the role of these cell types in contributing to type-2 inflammation, our data suggest this is not due to their direct response to IL-25. It is likely that while IL-25 can signal through myeloid and DC populations (as shown by others), it is not required to induce inflammation; it may also be that in a situation where IL-17RB is absent on these cells, other cell types are able to compensate, resulting in an equally robust response.

Detection of environmental toxins, allergens, and helminths at mucosal barrier surfaces by epithelial cells stimulates their production of IL-25, IL-33, and TSLP. For that reason, these three cytokines tend to be grouped together as type-2 inducing epithelial cytokines. We used IL-33R<sup>-/-</sup> and TSLPR<sup>-/-</sup> mice to determine whether IL-25 is capable of initiating pulmonary inflammation independently of IL-33 and TSLP. Our studies revealed IL-25 can drive type-2 inflammation independently of these two cytokines separately or in combination using a double IL-33R/TSLPR KO. Decreased serum IgE in the IL-33R<sup>-/-</sup>, TSLPR<sup>-/-</sup>, and the double KO demonstrates a possible downstream role for these cytokines in the ability of T<sub>H</sub> cells to induce class switching in B cells. Interestingly, IL-4, IL-5, and IL-13 expression was reduced in IL-33R<sup>-/-</sup> mice suggesting this cytokine is enhancing type-2 inflammation downstream of IL-25. However, the mechanism of this needs to be explored further. Collectively, these studies indicate IL-25 is sufficient to initiate type-2 inflammation within the lung independently of IL-33 and/or TSLP. However, we do not claim that IL-25 is required for this inflammatory process to develop. Additional studies would need to explore the role of TSLP- and IL-33-driven inflammation in the absence of IL-25R to make such a statement. It is our hunch that if any one of these cytokines is present, type-2

inflammation can progress. While there may be additional contributing factors and an augmented response when IL-25, IL-33, and TSLP are all present, we don't believe any one of them is 'required' for its initiation.

In summary, our studies have examined the capabilities of IL-25 using an in vivo pulmonary inflammation model. First, using the Cre-Lox system to delete IL-25 signaling in specific cell types and secondly, in mice deficient for epithelial cytokine signaling. The combined results of these experiments reveal a multifaceted role for IL-25 that is not dependent on any one cell type or on the other epithelial cytokines, IL-33 and TSLP.

## Chapter 4. ACUTE BLOCKADE OF IL-25 IN A COLITIS-ASSOCIATED COLON CANCER MODEL LEADS TO INCREASED TUMOR BURDEN

### 4.1 INTRODUCTION

UC is a type of IBD in which immune dysregulation promotes development of ulcerations in the lining of the colon. UC has an incidence of 1.2-20.3 cases per 100,000 people per year and a prevalence of 7.6-246.0 cases per 100,000 people per year<sup>119</sup>. Chronic inflammation observed in patients with UC is associated with an increased risk for colorectal cancer (CRC) by as much as 2.4-fold<sup>34</sup>. Despite the clear correlation between UC and CRC, the immunological factors contributing to the progression from inflammation to cancer are largely unknown.

Mucosal inflammation in UC is characterized as an atypical type-2 response. IL-25, a member of the IL-17 cytokine family (IL-17E), initiates production of type-2 cytokines (IL-4, IL-5, and IL-13), thereby enhancing a T<sub>H</sub>2 response<sup>48,49,51,120</sup>. Through its ability to induce IgE production and eosinophilia, IL-25 plays an essential role in host defense to helminth infections. However, its production can also result in a pathogenic role in allergic disorders. IL-25 is expressed in both the colon and specific macrophage and epithelial cells located in the gut<sup>61,120</sup>, is detected in the colons of mice under steady-state conditions, and is significantly elevated upon acute exposure to a DSS-induced colitis model<sup>96</sup>.

While little is known about how IL-25 modulates tumor pathogenesis, recent studies have revealed it could prove to be an important therapeutic target. IL-25 was shown to induce cell death in breast cancer cells, whereas non-malignant cells were left unaffected<sup>121</sup>. This effect was hypothesized to be due to the increased levels of IL-25

receptor (IL-17RB) expressed in malignant tumor cells, which correlates with previous findings associating low levels of IL-17RB with aggressive breast cancers and decreased overall survival<sup>122,123</sup>. Along with breast cancers, IL-25 was shown to exert antitumor effects in melanoma, lung, colon, and pancreatic cancers with a dependence on both B cells and increased levels of IL-5 inducing eosinophilia<sup>124</sup>. IL-25 influences both innate and adaptive immunity to induce type-2 inflammation. Because a Th2 phenotype is generally associated with tolerance in the setting of tumors, it will be critical to define the functional significance of this important cytokine in the context of tumor immunity.

There are conflicting reports on the ability of IL-25 to be either protective or detrimental in models of UC. In the United States, 1-1.3 million people suffer from IBD<sup>125</sup>; given the development of new therapeutic strategies for inhibiting chronic inflammation in the GI tract, we sought to establish the role of IL-25 in the context of colitis-driven colon cancer. We hypothesized the increased IL-25 in colitis, resulting in type-2 inflammation, would create an environment more favorable for tumor growth and development. To test this hypothesis, WT mice were treated with an  $\alpha$ -IL-25 blocking antibody in a model of colitis-induced colon cancer. Contrary to our hypothesis, antibody suppression of IL-25 resulted in increased tumor burden compared to controls. Mice with increased tumor burden also exhibited an increased overall colitis score and decreased eosinophil infiltrates in colonic tissue. Interestingly, genetic ablation of IL-25 had no effect on tumor growth. These data suggest that while IL-25 may be promoting the type-2 inflammation associated with UC, it is also inhibiting the pro-tumorigenic potential associated with long term inflammation.

## 4.2 RESULTS

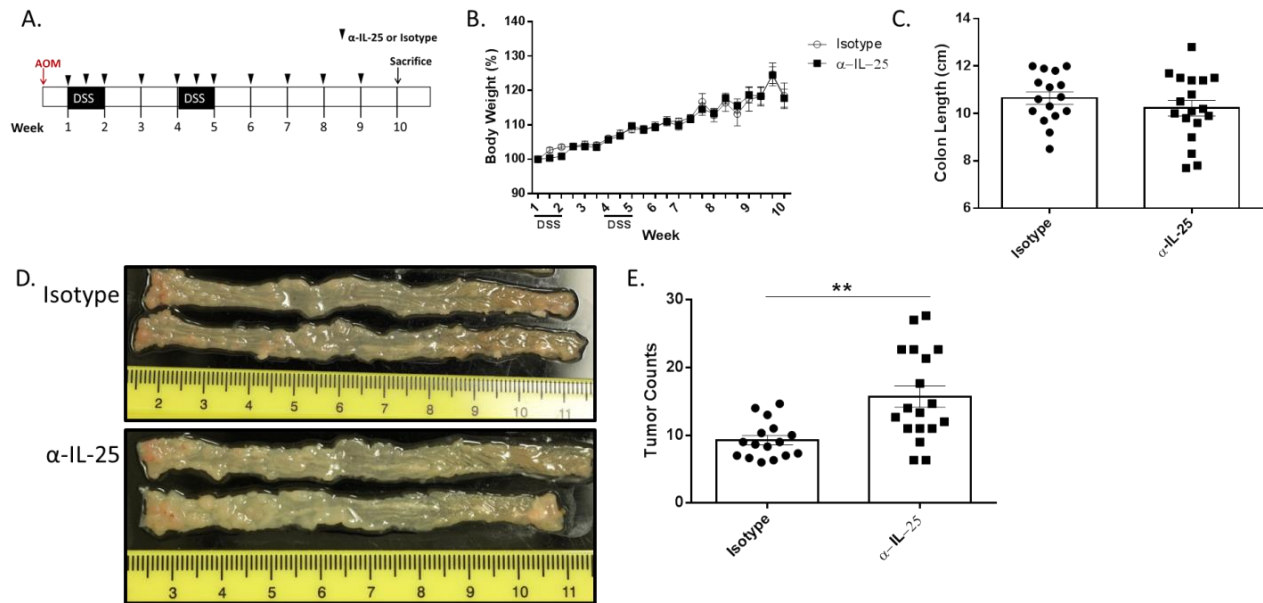
### 4.2.1 *IL-25 Neutralizing Antibody Increases Tumor Burden in a Murine Colitis-Associated Cancer (CAC) Model*

To investigate the role of IL-25 in colitis-induced colon cancer, a previously established model<sup>126,127</sup> was utilized in which mice were treated with the carcinogen azoxymethane (AOM), followed by two rounds of 2% DSS in their drinking water. Previous studies have shown that BALB/cJ mice develop 4-12 colonic neoplasms per mouse after 10 weeks. To establish what role IL-25 plays in the progression of colitis into cancer, the mice were given i.p. injections of either  $\alpha$ -IL-25 or an isotype control, starting with the initial dose of DSS, 3 days later, and weekly thereafter (Fig. 4.1a).

Mice were monitored and weighed 2-3 times per week for the duration of the experiment, with similar weight patterns observed between  $\alpha$ -IL-25 treated and control groups (Fig. 4.1b). At week 10, the mice were euthanized and the colon was removed for further analysis. DSS-induced colitis is associated with a shortening of the colon; however, the lengths of the colons from the two groups are comparable ( $10.66 \pm 0.34$  N=12 in control group versus  $10.27 \pm 0.43$  N=14 in  $\alpha$ -IL-25) (Fig. 4.1c).

Upon removal, the colons were cut lengthwise, and tumors were visualized using digital photography. Macroscopically, colonic tumors were observed in all mice with a higher concentration toward the distal rectum, which was expected as this is the area known to endure the greatest injury with DSS treatments. A moderate number of tumors were observed in the mid-region, though none were detected in the proximal colon of either group (Fig. 4.1d). Interestingly, there was greater overall tumor burden in the colons of mice treated with the  $\alpha$ -IL-25 antibody ( $16.79 \pm 1.95$  N=14 versus  $9.61 \pm 0.87$

N=12 in control group, p=0.004) (Fig. 4.1d,e), suggesting that IL-25 is limiting the growth and development of colonic tumors. To understand how IL-25 might be regulating this process, further evaluation of the colonic tissue was executed.

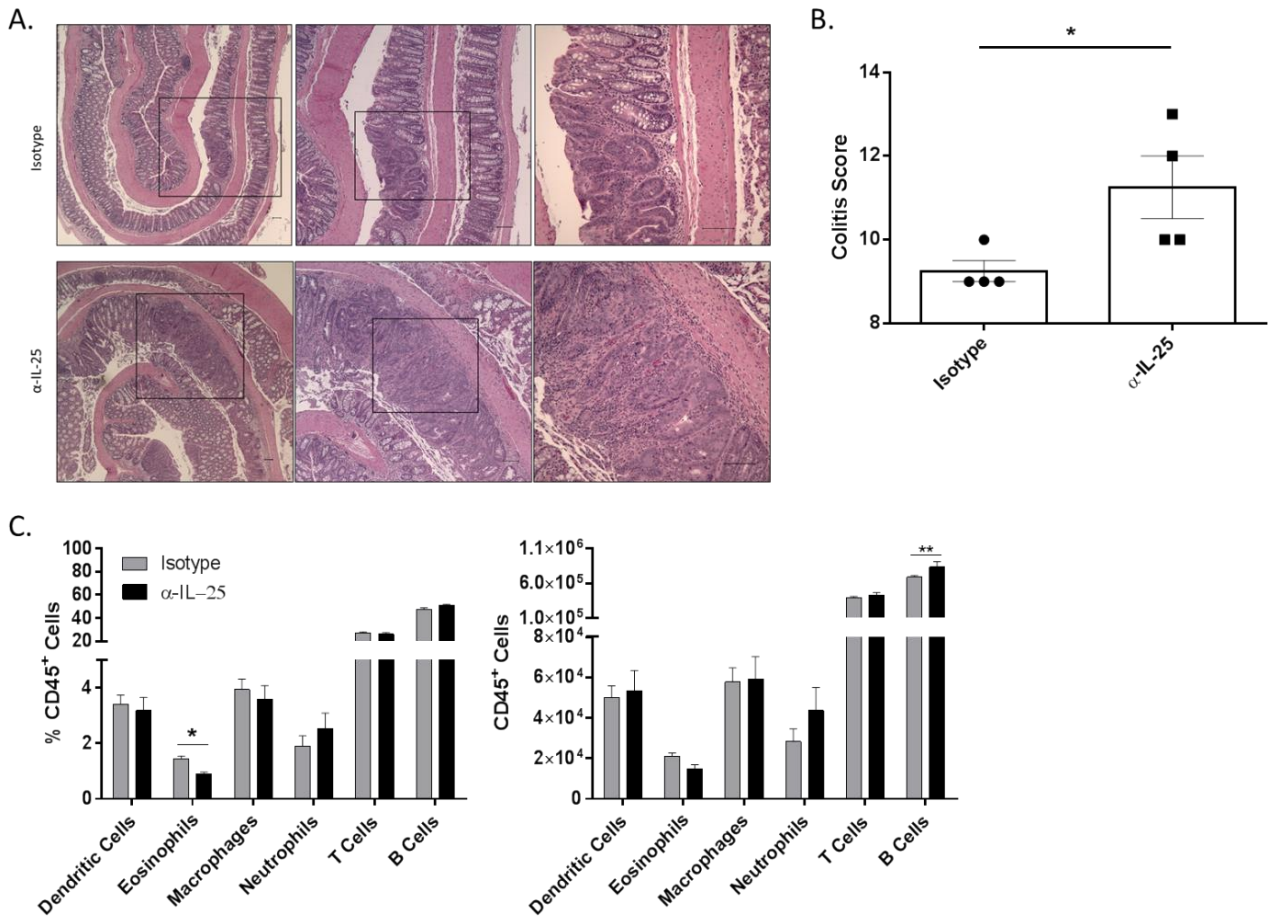


**Figure 4.1. IL-25 neutralizing antibody increases tumor burden.** Balb/cJ mice were given an i.p. injection of AOM on day 0, 2% DSS in drinking water for 7 days starting on days 7 and 28, and were euthanized on day 70. **(A)** WT Balb/c mice were given i.p. injections of either  $\alpha$ -IL-25 or isotype control antibody on days 7, 10, 14, 21, 28, 31, 35, 42, 49, 56, and 64. **(B)** Body weight was monitored from the initial dose of DSS onward and expressed as a percentage of initial weight. **(C)** Length of colon at euthanasia. **(D)** Colon was removed, cut lengthwise, washed with PBS, and digitally photographed. **(E)** Tumor counts based on gross analysis. Results are presented as mean  $\pm$  SEM and are combined from four independent experiments with 3-5 mice per group. \*\*, P < 0.005.

#### 4.2.2 *Increased Colitis and Decreased Eosinophils in Colons from $\alpha$ -IL-25 Treated Mice*

To examine the extent of disease in the colon, sections were obtained from the colons of tumor-bearing mice and stained with H&E (Fig. 4.2a). Colon sections underwent histological assessment for intestinal tumors as well as overall inflammation and assigned a combined colitis score<sup>128</sup>. Mice receiving  $\alpha$ -IL-25 throughout the course of the CAC model had a significantly increased colitis score ( $11.25 \pm 0.75$ , N=4) compared to mice receiving isotype control antibody ( $9.25 \pm 0.25$ , N=4) ( $p=0.0447$ ) (Fig. 4.2b).

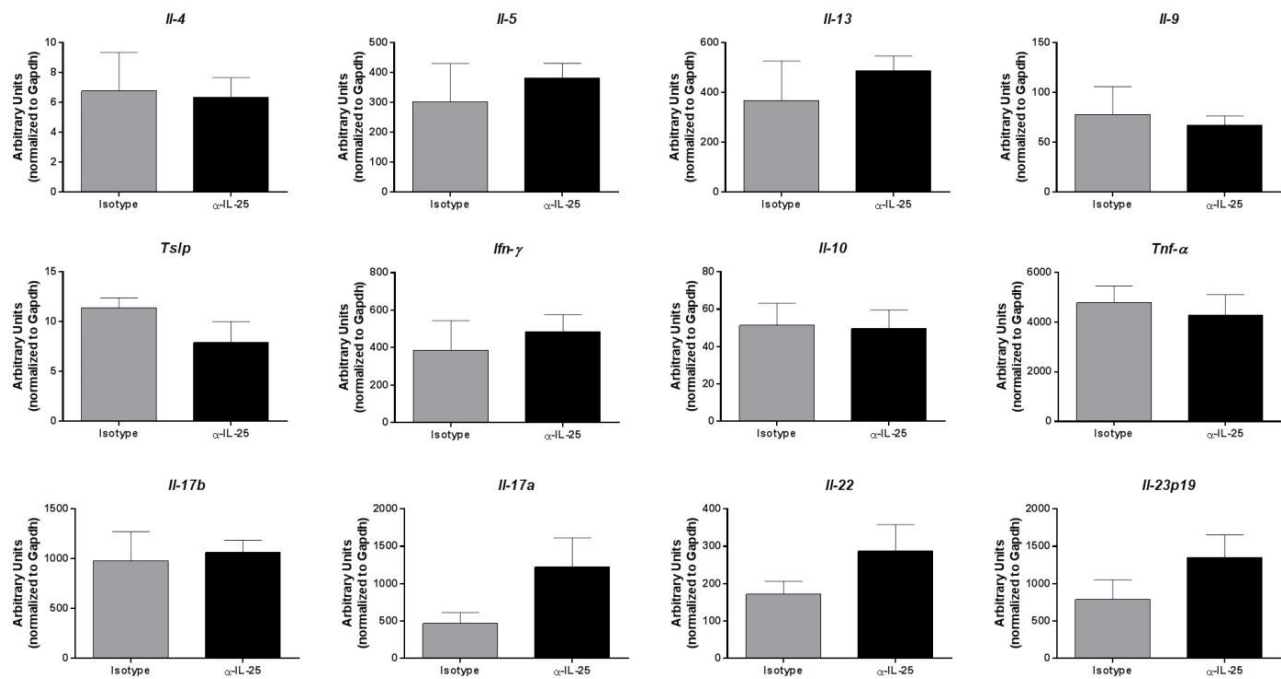
To determine which cell types contribute to increased tumor incidence in mice treated with  $\alpha$ -IL-25, single cell suspensions from colonic tissue sections were stained and analyzed by flow cytometry. CD45<sup>+</sup> white blood cells were gated based on surface markers to determine frequencies of DCs, eosinophils, macrophages, neutrophils, T cells, and B cells. While the majority of these cell types were nearly equivalent between the two groups, there was a significant decrease in the percentage of eosinophils found in mice treated with  $\alpha$ -IL-25 compared to control mice (0.9% and 1.45% respectively,  $p=0.00098$ ) (Fig. 4.2c). These results suggest blockade of IL-25-mediated migration of eosinophils into tissues may be partially responsible for the increased tumor burden in these mice. To determine whether IL-25 blockade was also able to exert its effects at the genetic level, mRNA levels were quantified for various inflammatory cytokines as well as tumor-associated genes.



**Figure 4.2. Increased colitis and decreased eosinophils in CAC mice treated with IL-25 neutralizing antibody. (A)** H&E stained histology sections of colons from isotype (top) and  $\alpha$ -IL-25 treated mice (bottom). Black boxes represent successive higher magnification from left to right with the black bar representing 100 $\mu$ m. **(B)** Histological colon sections analyzed for colitis severity. **(C)** Single cell suspensions were made from colon tissue and labeled with fluorescently labeled antibodies to distinguish dendritic cells, eosinophils, macrophages, neutrophils, T cells and B cells and were analyzed by flow cytometry (percentage on left, absolute number on right). Results are presented as mean  $\pm$  SEM and are representative of three independent experiments with 3-5 mice per group. \*, P < 0.05, \*\*, P < 0.005.

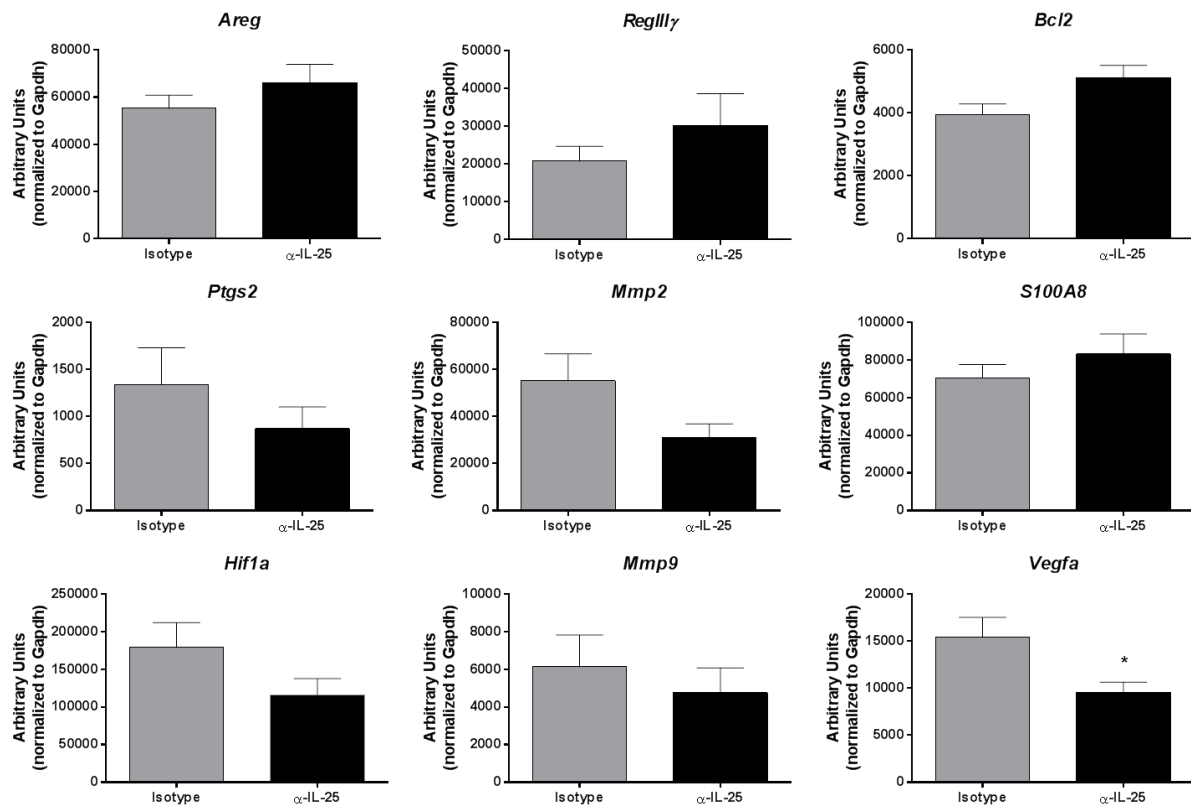
#### 4.2.3 Gene Expression in CAC Mice Treated with $\alpha$ -IL-25 Antibody

IL-25 is able to modulate the inflammatory environment by downregulating IL-17A production via IL-23<sup>31</sup>, as well as to promote Th2 responses and reduce IFN- $\gamma$  production during helminth infections<sup>129,130</sup>. Mice deficient in IL-25 are highly susceptible to EAE with increases in numbers of IL-17, IFN- $\gamma$ , and TNF- $\alpha$  producing cells that invade the central nervous system<sup>110</sup>. Studies have found reduced T<sub>H</sub>17 cells and increased T<sub>H</sub>9 cells in response to IL-25<sup>31,66</sup>. To determine whether cytokine expression was affected and contributed to tumor outcome in mice treated with the IL-25 blocking antibody, a section of the distal colon was processed for rt-PCR to measure levels of gene expression for various inflammatory cytokines. Although not statistically significant, there was a trend toward increased expression levels in genes encoding cytokines of the Th17 pathway, including *il-17a*, *il-22*, and *il-23p19*, in anti-IL-25 treated mice compared to controls (Fig. 4.3). This correlates with the previously established ability of IL-25 to down-regulate Th17 cytokines as mentioned above, and is consistent with the recently observed role for the IL-23 pathway in colon cancer<sup>131,132</sup>.



**Figure 4.3. Gene expression in CAC mice treated with IL-25 neutralizing antibody.** RT-PCR analysis for various inflammatory cytokines in colon sections from AOM/DSS treated mice, expressed as arbitrary units relative to Gapdh housekeeping gene. Results are presented as mean  $\pm$  SEM and are representative of three independent experiments with 3-5 mice per group.

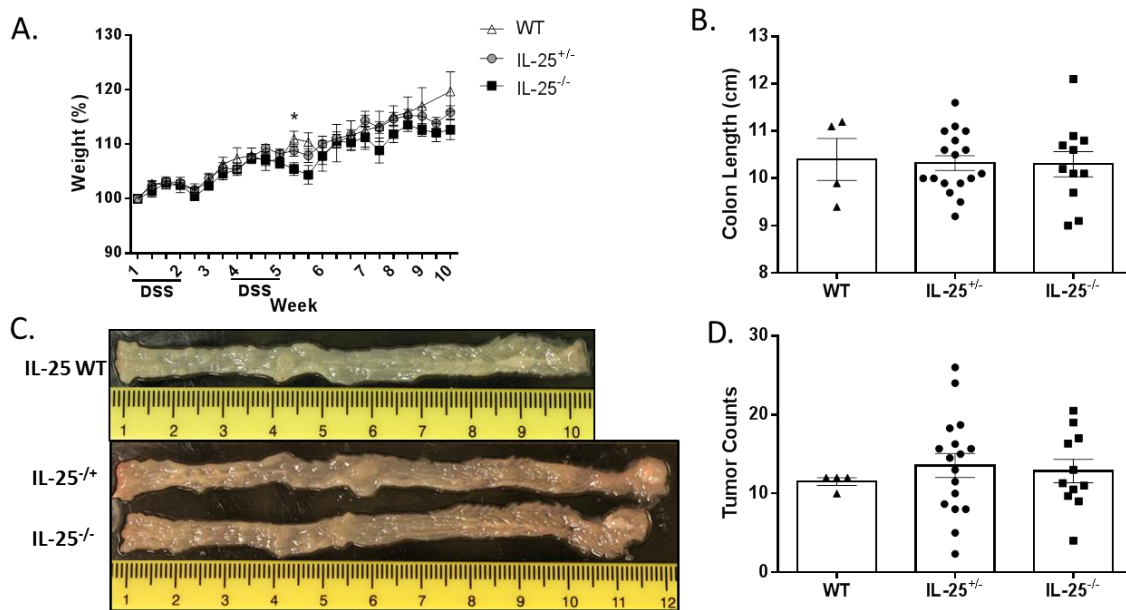
It is unknown what effect IL-25 has on tumor-associated gene expression levels during colonic tumor development. Therefore, mRNA levels of tumor-associated genes known to be important in the AOM/DSS model were assessed to establish whether IL-25 is capable of regulating genes involved in tumor development. Genes involved in colonic epithelial neoplasia include amphiregulin (*Areg*), (epidermal growth factor (EGF) receptor ligand and mediator of proliferation), hypoxia-inducible factor 1-alpha (*Hif1a*), a regulator of cellular and developmental response to hypoxia, prostaglandin-endoperoxide synthase 2 (*Ptges2*), a modulator of apoptosis, angiogenesis, and tumor invasiveness, vascular endothelial growth factor A (*Vegfa*), which increases vascular permeability, angiogenesis, cell migration and inhibits apoptosis, matrix metalloproteinase-2 (*Mmp2*) and matrix metalloproteinase-9 (*Mmp9*), both involved in invasion and angiogenesis, B-cell lymphoma 2 (*Bcl2*), with anti-apoptotic activity, and regenerating islet-derived protein 3 gamma (*RegIIIγ*) and calgranulin A (*S100A8*), both antimicrobial peptides. Mice treated with the  $\alpha$ -IL-25 antibody had similar expression levels of all these genes, with a marginally significant decrease in *Vegfa* compared to isotype treated mice (Fig. 4.4). These findings suggest IL-25 is unable to modulate tumor development at the genetic level.



**Figure 4.4. Gene expression in CAC mice treated with IL-25 neutralizing antibody.** RT-PCR analysis for tumor related genes in colon sections from AOM/DSS treated mice, expressed as arbitrary units relative to Gapdh housekeeping gene. Results are presented as mean  $\pm$  SEM, with asterisks representing statistical significance relative to isotype treated mice. Data are representative of three independent experiments with 3-5 mice per group.

#### 4.2.4 *IL-25 Deficient Mice Show No Difference in Tumor Burden*

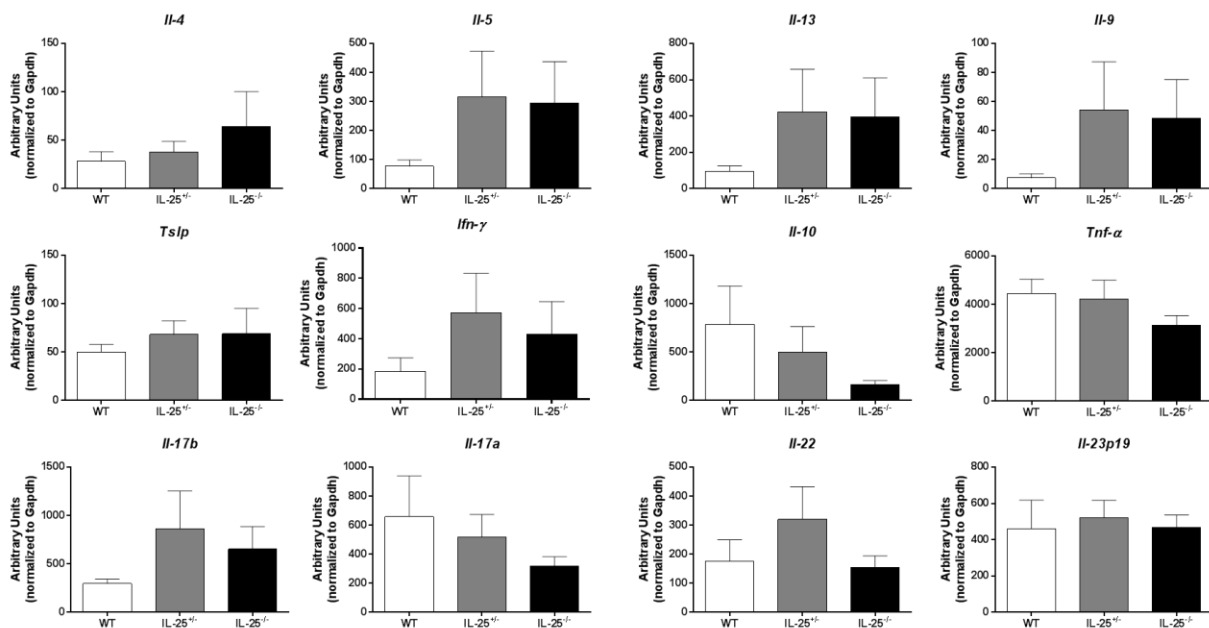
The unexpected effect of IL-25 blockade resulting in an increase in grossly evident colonic tumors led to the hypothesis that a genetic deletion of IL-25 would also reveal an increase in tumor development. Because of the vital role GI microbiota play in this model, it was critical to compare littermate mice of the same parents and genetic background. To test the hypothesis, IL-25 heterozygous mice were bred in-house to obtain littermates that were WT, heterozygous, and full KOs. The mice were given one i.p. injection of AOM followed by two rounds of 2% DSS and monitored/weighed throughout the experiment. Interestingly, genetic deletion of IL-25 had no effect on tumor burden or other aspects of disease development (Fig. 4.5), suggesting that these mice are able to compensate for genetic, but not acute, IL-25 deficiency.



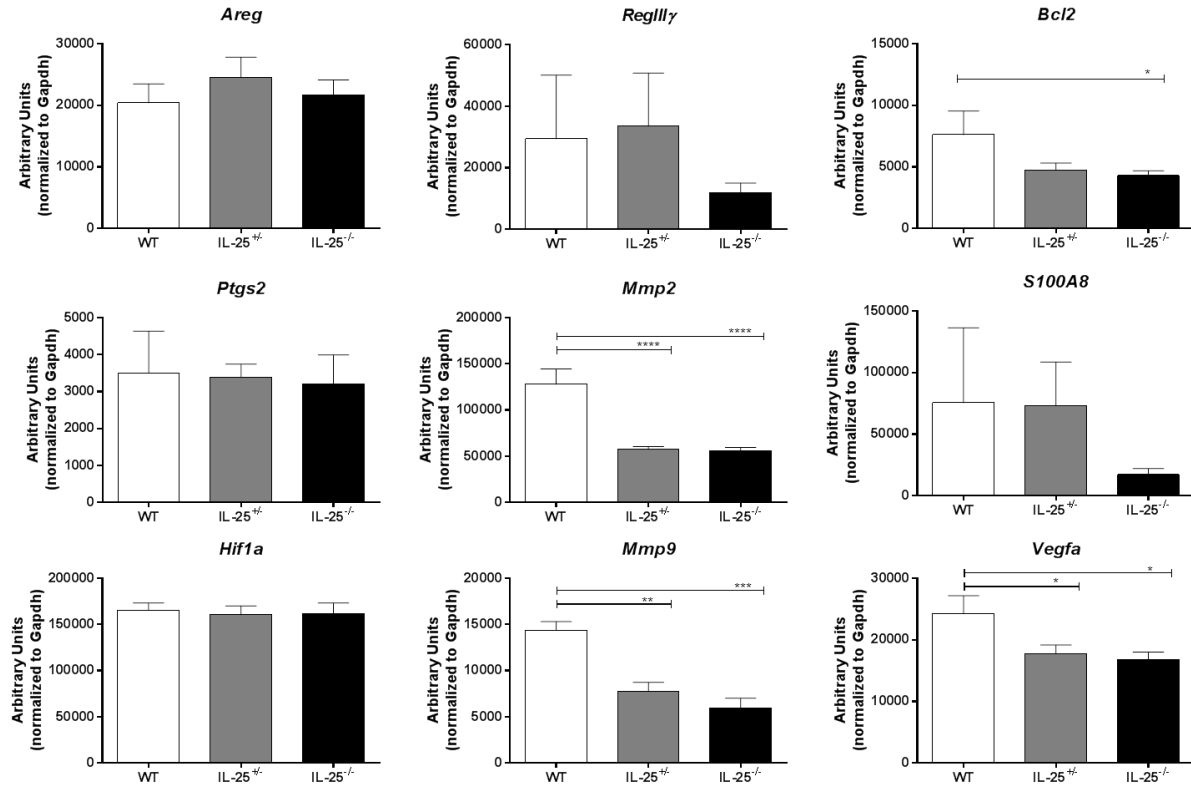
**Figure 4.5. Genetic deletion of IL-25 has no effect on tumor burden.** IL-25<sup>-/-</sup> or littermate control mice were given an i.p. injection of AOM on day 0, 2% DSS in drinking water for 7 days starting on days 7 and 28, and euthanized on day 70. **(A)** Body weight was monitored from the initial dose of DSS onward and expressed as a percentage of the initial weight. **(B)** Length of colon at euthanasia. **(C)** Colon was removed, cut lengthwise, washed with PBS, and digitally photographed. **(D)** Tumor counts based on gross analysis. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments.

#### 4.2.5 Gene Expression in AOM/DSS Treated IL-25<sup>-/-</sup> Mice

As with the Balb/c mice treated with anti-IL-25 (or isotype), gene expression levels were assessed for various inflammatory cytokines using colon samples from IL-25<sup>-/-</sup> mice in the AOM/DSS model. Interestingly, genetic ablation of IL-25 had no significant impact on the expression of any of the cytokine genes we measured (Fig. 4.6). However, there were several tumor-associated gene expression levels that were significantly reduced in IL-25<sup>-/-</sup> (and some IL-25<sup>+/-</sup>) mice (Fig. 4.7). These data suggest that, although genetic ablation does not affect tumor burden, there are changes in the gut microenvironment that appear to be IL-25 specific.



**Figure 4.6. Expression of cytokine genes in AOM/DSS treated IL-25<sup>-/-</sup> mice.** RT-PCR analysis for various inflammatory cytokines in colon sections from AOM/DSS treated IL-25<sup>-/-</sup> or littermate control mice, expressed as arbitrary units relative to Gapdh housekeeping gene. Results are presented as mean  $\pm$  SEM and are combined from three independent experiments.



**Figure 4.7. Gene expression in AOM/DSS treated IL-25<sup>-/-</sup> mice.** RT-PCR analysis for tumor related genes in colon sections from AOM/DSS treated IL-25<sup>-/-</sup> or littermate control mice, expressed as arbitrary units relative to Gapdh housekeeping gene. Results are presented as mean  $\pm$  SEM, with asterisks representing statistical significance relative to WT mice. Data are combined from three independent experiments.

### 4.3 DISCUSSION

We have examined the role of IL-25 in an inflammation-induced colon cancer model using both antibody blockade of the cytokine and genetic deletion of signaling via IL-25 knock-out mice.

IL-25 blockade was shown to be protective in a mouse model of colitis resulting in decreased type-2 cytokines, blood eosinophils, and IgE<sup>94</sup>. In various models of allergic asthma, antibody-blockade of IL-25 inhibited its established ability to increase eosinophil infiltration, concentrations of IL-5 and IL-13, AHR, and to induce goblet cell hyperplasia and serum IgE secretion<sup>82,94,133</sup>. Given the anti-inflammatory benefits discovered in both lung and gut inflammation, the use of an IL-25 blocking antibody has been suggested as a possible therapeutic treatment for human patients suffering from these types of inflammation. However, when we applied this same approach to an established murine model of colitis induced colon cancer, the tumor development surprisingly increased when mice were treated with an IL-25 blocking antibody. While colitis studies generally focus on shorter time-points post DSS treatment for evaluation, mouse colons assessed after long-term blockade of IL-25 revealed an increased colitis score compared to control mice. Our studies suggest that using an  $\alpha$ -IL-25 antibody as a therapeutic approach could have adverse effects in colitis patients, whereby it actually promotes tumorigenesis.

IL-25 injections in human tumor xenograft models including melanoma, breast, lung, colon, and pancreatic cancers were shown to have antitumor efficacy with increased eosinophils in the peripheral blood of these mice<sup>124</sup>. This is in alignment with the current study where we found decreased eosinophil infiltration into the colons of

mice treated with an IL-25 depleting antibody. While the role of eosinophils in various tumors remains controversial, they have been found to be associated with a good prognostic indicator in gastrointestinal cancers<sup>134-136</sup>.

Our evaluation of mRNA expression in the colons of mice treated with  $\alpha$ -IL-25 showed a trend toward increased expression of genes encoding inflammatory cytokines in the Th17 pathway (*il-17a*, *il-22*, and *il-23p19*). Previous studies in animal models of colorectal carcinoma have established Th17 cells, IL-22, and IL-23 as important drivers of inflammation leading to colonic tumor formation. Furthermore, blockade of this pathway could decrease tumorigenesis<sup>131,137,138</sup>. In humans, colorectal tumor samples showed increased IL-17 producing cells compared to non-tumor regions<sup>132</sup>. While our study showed a slight decrease in *Vegfa* expression in  $\alpha$ -IL-25 treated mice, Liu et al. identified IL-17 induction of VEGF production by tumor cells as the main pro-angiogenic factor responsible for the development and progression of colorectal carcinoma. The high expression levels of *Vegfa* in both isotype and  $\alpha$ -IL-25 treated mice indicate it likely plays a role in this model, however the mechanism of whether IL-25 is able to directly or indirectly affect its expression requires further investigation. Overall, it is possible that IL-25 blockade is allowing for increased Th17 inflammation which is promoting tumor development in these mice.

Within the GI tract, IL-25 is an important regulator of type-2 immunity. Owyang et al. used IL-25<sup>-/-</sup> mice to establish IL-25 as a critical factor in protection during *Trichuris* infections, not only by inducing a type-2 response but also by dampening IFN- $\gamma$  and IL-17 expression<sup>130</sup>, demonstrating an additional ability for IL-25 to limit pathologic inflammation within the gastrointestinal tract. Consistent with this, when IL-25<sup>-/-</sup> mice

were used in another model of helminth infection, *Nippostrongylus brasiliensis*, the protective role of IL-25 was dependent on its ability to induce type-2 cytokine production<sup>90</sup>. Models of colitis have shown IL-25 to either reduce<sup>139</sup> or promote<sup>140</sup> inflammation in the GI tract. However, when IL-25<sup>-/-</sup> mice were used in the colitis-associated colon cancer model, we found no difference in the ultimate outcome. We speculate that while the acute blockade of IL-25 is able to impact the inflammatory environment and promote tumorigenesis, in the complete ablation of this pathway, other mechanisms are able to compensate and overcome the lack of IL-25 signaling. The mechanism by which this may be occurring is currently being investigated.

In summary, due to the known role for IL-25 to augment disease pathogenesis in UC and because UC often progresses into colon cancer, an AOM/DSS-induced murine model of colon carcinogenesis was used to establish the role for IL-25 in this environment. This is the first study of its kind to specifically examine the influence of IL-25 in a long-term inflammation-induced cancer model. We show herein that mice treated with an IL-25 blocking antibody developed greater tumor burdens and increased pathology than isotype treated control mice. These results demonstrate that the use of a blocking antibody against IL-25, while potentially therapeutic in the context of UC, should be approached with caution as it may prove to be detrimental if used long term and could actually promote tumor development.

## Chapter 5. CONCLUDING REMARKS

Epithelial cells located at mucosal surfaces, including the skin, lungs, and GI tract act as our front-line of defense against environmental toxins, viruses, microbial pathogens, and helminth infections. These cells, once thought to provide merely a physical barrier, are now recognized as playing an active role in modulating the immune response. Epithelial cells are able to secrete antimicrobial peptides and mucins, form tight structural junctions, as well as sense and respond to various environmental insults. Activation of PRRs on epithelial cells stimulates their release of cytokines IL-25, IL-33, and TSLP. These three cytokines, acting individually and/or in cooperation with one another, are capable of initiating and maintaining type-2 inflammation. They do this by activating ILC2s, CD4<sup>+</sup> T cells, mast cells, basophils, and IgE-producing B cells. The focus of this study was to determine the specific role of IL-25 in two very different mucosal environments: first, in a model of chronic pulmonary inflammation and second, in a model of colitis-induced colon cancer.

Human asthmatic patients have increased expression of IL-25 within the lung and systemically. In mice, IL-25 is able to mediate pulmonary type-2 immunity in response to allergens and viruses. While many advances have been made in the field of IL-25 biology, there are still many unanswered questions regarding the specific cell types IL-25 acts upon to produce an asthmatic inflammatory response. In addition, whether the roles for IL-25, IL-33, and TSLP are interdependent and/or redundant has been largely unexplored. In this study, we established a model of pulmonary inflammation and applied this model to several genetically-modified mouse strains to resolve some of these unanswered questions. Using the Cre-Lox system, we selectively

deleted IL-17RB signaling in CD4<sup>+</sup> cells, monocyte/macrophage populations, and CD11c<sup>+</sup> DCs. In each of these systems, we found IL-25 was able to generate type-2 inflammation despite the lack of direct signaling in any of these cells. Additionally, when IL-33 and TSLP signaling (individually and in combination) were deleted, IL-25 was still capable of initiating an effective inflammatory response. Collectively, these results indicate a multifaceted role for IL-25 that is not dependent on its direct signaling through CD4<sup>+</sup> cells, monocytes/macrophages, or DCs to generate a response. We also established that IL-25 is not reliant on IL-33 or TSLP to mediate type-2 inflammation. These studies are the first of their kind to look at IL-25 induced inflammation by deleting its receptor on specific cell types. Our findings suggest the role of IL-25 is multifaceted and likely relies on a combination of cells and inflammatory mediators that contribute to an inflammatory response. The ability of IL-25 to create a response in lieu of TSLP and IL-33 indicates that therapeutic targeting of one of these epithelial cytokines within the asthmatic pathway will likely not be efficacious. It may prove to be more successful therapeutically to target all three of these cytokines or to aim for something further upstream, perhaps on/in the epithelial cells themselves.

Chronic inflammation within the GI tract results in an increased risk for developing colorectal cancer. Epithelial cytokines, including IL-25, are produced in the colon and are critical for protection from parasites, but can also be pathogenic in the context of IBDs and allergy. Whether IL-25 is involved in the progression from inflammation to cancer is still largely unexplored. Using a well-established murine model for colitis-induced colon cancer, we aimed to determine the role of IL-25 in this process. We found that acute IL-25 blockade resulted in greater tumor burdens compared to

isotype control treated mice. Histologically,  $\alpha$ -IL-25 treated mice had increased colitis scores compared to mice receiving isotype control antibody, as well as decreased eosinophilia. This is the first study to explore the therapeutic potential of using an IL-25 blocking antibody during a chronic inflammatory setting. Taken together, these data suggest that IL-25 plays an inhibitory role in the growth and development of colonic tumors.

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