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UNDERSTANDING HUMAN *IL33* EXPRESSION IN THE LUNGS DURING INFLAMMATION USING A REPORTER MODEL

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Abbreviations

ATAC-seq – Assay for transposase-accessible chromatin using sequencing

ARE – Adenylate-uridylate-rich elements

BAC – Bacterial artificial chromosome

CPE – Chromatin pellet extract

Cyto – cytoplasm

DC – Dendritic cell

GFP – Green Fluorescent Protein

GWAS – Genome wide association study

LPS – Lipopolysaccharide

HDM – House dust mite

HMVEC-L – Human lung microvascular endothelial cells

HUVEC – Human umbilical vein endothelial cell

IL – Interleukin

iLECs – Inducible lymphatic endothelial cells

i.t. – Intratracheal

Poly (I:C) – Polyinosinic:polycytidylic acid

qPCR – Quantitative real time polymerase chain reaction

Th1/Th2/Th17 – T-helper cell type 1/2/17

TLR – Toll-like receptor

TTP – trans-acting RNA binding proteins

SNP – Single nucleotide polymorphism

SNE – Soluble Nuclear Extract

Abstract

IL-33 is a tissue-derived cytokine that is abundantly expressed in the human lung, where it is involved in pathogen defense and barrier homeostasis. IL-33 alerts immune cells during allergen exposure and drives the downstream production of Th2 responses. Notably, while IL-33 has long been implicated in asthma, the sources of the cytokine have remained controversial. Here, we probed the cellular sources of human *IL33* using human donor lungs and show that basal cells and endothelial cells are the dominant producers. In stark contrast, murine *Il33* was found almost exclusively in lung epithelial type II pneumocytes. Further, the SNP-containing regulatory components in the human *IL33* locus are not conserved in the murine locus. Given these differences, we engineered a novel *IL33* reporter mouse containing the regulatory components from the human locus to reflect human *IL33* expression in a murine model. We show that the human *IL33* reporter is dramatically reduced by allergic inflammation and by direct administration of recombinant IL-33 *in vivo*, whereas *IL33* reporter expression was unaltered airway epithelium. Extracellular IL-33 negatively regulated intracellular *IL33* levels by inducing mRNA instability in endothelium, suggesting a mechanism for how IL-33 is able to dampen its inflammatory effects. Together, these data reveal a previously unrecognized cellular network comprised of crosstalk between the lung vasculature and the immune system that maintains tissue homeostasis and governs responses in allergic airway disease in humans.

Chapter 1 Introduction

The Immunology of Asthma

Asthma is a heterogenous, chronic respiratory disease that is impacted by a multitude of genetic and environmental factors (Kearley et al., 2015; Locksley, 2010; Network, 2014; Pavord et al., 2018; Umetsu et al., 2002; Wenzel, 2012). While healthy individuals do not mount immune responses to innocuous stimuli, asthmatic patients experience increased airway hypersensitivity, mucus production, and activation of cells that are resident and/or recruited to the lung (Umetsu et al., 2002; Wenzel, 2012). Over the years, studies of the asthmatic phenotype have uncovered the role of the immune system, a complex network of cells and tissues that protect the host organism from pathogens, in initiating and perpetuating disease.

The immune system is organized into two canonical arms: innate immunity and adaptive immunity (Chaplin, 2010). The innate arm has traditionally been defined as being comprised of cells that form physical barriers (such as epithelial and endothelial cell layers), soluble proteins of the complement system, and a repertoire of cells that include but are not limited to macrophages, monocytes, neutrophils, eosinophils, and dendritic cells. They represent the first line of host defense and carry germ-line encoded recognition molecules that are able to sense broad patterns found in invading pathogens. Conversely, the adaptive immune system is described as being highly antigen-specific (Chaplin, 2010). Cells of this arm carry somatically rearranged receptors that are highly specific for various antigens. These cell types, which include T and B lymphocytes, become activated after antigen recognition and amplify the immune response generated from the innate arm via the dendritic cell which serves as a bridge connecting the two arms (Hammad and Lambrecht, 2008). Depending on the nature of the tissue

environment, the status of the dendritic cell, and the cytokine milieu when the T cell is activated, T cells differentiate into distinct subsets. One of these subsets is called the T helper type 2 (Th2), characterized by the expression of the transcription factor GATA-3 and production of type 2 cytokines IL-4, IL-5, and IL-13 which promote the activity of other immune cells. Th2 responses are commonly found in helminth infections, such as intestinal nematode infection, where Th2 cytokines such as IL-4 and IL-13 facilitate expulsion of parasites by driving alternative macrophage activation (Chaplin, 2010; Halim et al., 2012; Woodruff et al., 2009). Outside of helminth infections, Th2 cells are also present in the lungs of asthmatics.

Asthma Endotypes

Traditionally, two phenotypes of asthma have been clinically observed: allergic asthmatics and nonallergic asthmatics (Thomas et al., 2010). However, the dogma of asthma as a binary condition has more recently been displaced by a broader umbrella diagnosis to include asthmatics' complex biological and environmental etiologies. While wheezing, shortness of breath, chest tightness, and airway obstruction all fall under asthma symptoms, disease severity varies widely across individuals. To account for the heterogeneity of asthma profiles, asthma is further segmented into endotypes, or distinct mechanistic pathways, that manifest into different phenotypes or clinical symptoms (Wenzel, 2012; Wenzel et al., 1999).

Th2 high asthma

Th2 high asthma is an endotype of severe asthma characterized by presence of eosinophils and antigen-specific IgE antibodies in serum and lung (Wenzel et al., 1999). Eosinophils, immune cells that are recruited to the lung by the release of IL-5 from Th2 cells, are found abundantly in the bronchial alveolar lavage (BAL) fluid as well as in tissue biopsies from these Th2 high patients (Robinson et al., 1992; Woodruff et al., 2009). These cells are released

from the bone marrow in response to Th2 cytokine stimulus and accumulate rapidly in lung tissue (Greenfeder et al., 2001). Once in circulation, they synthesize and release chemokines such as eotaxins, granule mediators, and cysteinyl leukotrienes that can cause edema, bronchoconstriction, and tissue damage (Greenfeder et al., 2001). Eosinophils also activate bronchial fibroblasts and have been associated with airway remodeling via the production of TGF-β (Broide, 2008). TGF-β induces TIMP-1 to stimulate fibroblasts to produce extracellular matrix (ECM) proteins and promotes myofibroblasts to produce collagen (Broide, 2008). Furthermore, TGF-β expression correlates with the degree of subepithelial fibrosis and is significantly increased in severe asthmatics with eosinophilia (Broide, 2008; Ohno et al., 1996).

Outside of eosinophil levels, serum IgE is used to identify atopic individuals. After allergen exposure, follicular helper T cells (Tfh) cells activate B cells via the production of IL-4, causing B cells to differentiate into plasma cells that generate the IgE antibody (Vijayanand et al., 2012). IgE binds high-affinity receptor FcεRI on mast cells and CD23 on dendritic cells to mediate degranulation and enhance antigen uptake, respectively. Further, data demonstrating the expression of FcεRI on airway smooth muscle indicate that the antibody may play a direct role in airway remodeling (Samitas et al., 2015).

Th2 high asthma can be further classified into three groups: individuals with early-onset allergic asthma, late-onset eosinophilic asthma, and aspirin-exacerbated respiratory disease (AERD) (**Table 1.1**) (Kuruvilla et al., 2018). Early onset asthma is found in children who exhibit wheezing, coughing, and shortness of breath, often before the first year of life. While there is some disagreement in the literature over the criteria of early onset, with some defining onset as asthma beginning before the 4$_{th}$ birthday and others before age 12 years, the consensus of these studies is some individuals with severe asthma experience asthma symptoms early on in life and

are diagnosed with positive allergy skin tests and increased serum-specific IgE (Miranda et al., 2004).

In contrast, individuals who develop steroid-resistant eosinophilic asthma later in life are often non-atopic (Peters et al., 2019; Tomassen et al., 2016). Instead, individuals often experience chronic rhinosinusitis with nasal polyps preceding asthma development and have high levels of IL-5 and IL-13 in their sinus mucosa (Tomassen et al., 2016). Among subjects with persistent eosinophilia, late onset asthmatics had a higher levels of tissue eosinophilia compared to individuals with early onset (Miranda et al., 2004). Lastly, a subset of Th2 high individuals with late-onset asthma falls under aspirin-exacerbated respiratory disease, characterized by chronic sinusitis with nasal polyps, COX-1 inhibitor-induced respiratory reactions, dysregulated arachidonic acid (AA) metabolism, and cysteinyl leukotriene overproduction (Buchheit et al., 2016; Kuruvilla et al., 2018; Liu et al., 2015). The result is severe persistent upper and lower airway disease with bronchoconstriction and nasal polyps.

Th2 low asthma

In contrast to Th2 high asthma, the endotype of Th2 low asthma is characterized by the presence of neutrophilic airway inflammation and a lack of response to corticosteroid therapy (Kuruvilla et al., 2018). BAL of severe asthmatics have higher frequencies of CD4+ T cells expressing IFN-γ and IL-17 in the airways compared with that in milder asthmatics (Chambers et al., 2015; Raundhal et al., 2015). The mechanisms that underpin Th2 low asthma remain unclear, but severe neutrophilic asthma has been associated with an imbalance of Th17/T regulatory cells as well as bacterial infections and inflammasome activation (Carr and Kraft, 2016; Simpson et al., 2014). Bacterial infections, such as with *Mycoplasma pneumoniae* and *Chlamydophila penumoniae*, can induce type 2 airway inflammation and manifest as asthma symptoms

(McCauley et al., 2019; Porter et al., 2014). Elevated expression of NLRP3, caspase-1, caspase-4, caspase-5 and IL-1β are observed in individuals with neutrophilic asthma, and protein levels of IL-1β are significantly higher in those with neutrophilic asthma compared to healthy controls (Simpson et al., 2014).

Individuals of the Th2 low endotype can be further subsetted by characteristics that include obesity, smoking, and age (Peters et al., 2019; Scott et al., 2011; Sutherland et al., 2008; Thomson et al., 2004) (**Table 1.1**). Obesity is a major risk factor and disease modifier of asthma, and individuals who fall into this subgroup exhibit severe symptoms despite preserved lung function and low eosinophilia (Beuther et al., 2006; Peters et al., 2019; Scott et al., 2011). Obese asthmatic patients do not respond as well to standard controller medications such as inhaled corticosteroids, likely due to increased production of inflammatory cytokines and altered glucocorticoid responses (Sutherland et al., 2008).

Morbidity and mortality from asthma are increased in smokers compared with nonsmokers. Asthmatic smokers have more severe asthma, and baseline FEV1 is directly related to the acute immediate response to inhaling cigarette smoke in these patients. While it has been hypothesized that smoking associated asthma develops as epigenetic modifications occur in neutrophils and macrophages due to oxidative stress, the mechanisms of corticosteroid resistance in asthmatic smokers are unexplained (Takahashi et al., 2018). Alterations in airway inflammatory cell phenotypes, overexpression of glucocorticoid receptor β, and increased activation of inflammatory transcription factor NF-κB have all been implicated in smoking associated-asthma.

Lastly, age has been associated with decreased lung function over time due to loss of elastic recoil and mechanical disadvantages. In elderly asthmatics, immunosenescence likely

has important consequences. When individuals age, irreversible loss of cellular replication and tissue repair occurs. Neutrophils and macrophages, which function in the initial clearance of pathogens through phagocytosis, lose both their chemotactic and phagocytic ability (Dunn et al., 2018). Interestingly, despite diminished neutrophil capabilities, Th17 cell frequency appears to increase with aging. Th17 cells were significantly increased in older individuals and correspondingly, CD4+ FoxP3+ Tregs were reduced in individuals >65 years (Schmitt et al., 2013).

Despite these endotype classifications, patients can exhibit features of both eosinophilic and neutrophilic pathways. A subset of adult onset Th2 high asthmatics have sputum neutrophilia in addition to eosinophilia, indicating that these endotypes are not mutually exclusive. A better understanding of the genetic and environmental factors at play during asthma onset will better explain Th2 asthma phenotypes. Outside of eosinophilic and neutrophilic asthma, asthma has been associated with exercise and environmental-related exposure to pollutants (Lambrecht and Hammad, 2015; McKinley et al., 2008; Shaw et al., 2007). The variability of asthmatic symptoms, lack of clear benchmarks of disease, and striking differences in environmental exposures among asthmatics complicate asthma disease etiology. To address the diversity in asthma endotypes, there has been a new push to using genetic and molecular markers in conjunction with observable symptoms to define disease.

Endotype	*Classifications*	*Clinical Characteristics*
Th2 high		
	Early onset	Atopic, steroid sensitive, high total IgE and allergen-specific IgE, blood eosinophilia (Miranda et al., 2004)
	Late onset	Chronic rhinosinusitis with nasal polyps, steroid refractory (Peters et al., 2019; Wenzel et al., 1999)
	AERD	Dysregulated arachidonic acid metabolism (Liu et al., 2015)
Th2 low		
	Smokers	Older adults, oxidative stress with elevated sputum neutrophil count (Takahashi et al., 2018)
	Obesity-related	Oxidative stress, common in females, increased neutrophils and serum IL-6 (Beuther et al., 2006; Scott et al., 2011)
	Elderly asthmatics	>50 years, immunosenescence, perturbation in Th1/Th17 pathway, steroid-resistant (Kuruvilla et al., 2018; Schmitt et al., 2013)

Table 1.1 The endotypes of asthma and clinical presentations.

Asthma GWAS

Genome Wide Association Studies (GWAS) have been instrumental in identifying the genetic causes of complex traits such as asthma (Laitinen et al., 2004; Ober and Hoffjan, 2006; Ramasamy et al., 2012). By examining the allele frequencies of single nucleotide polymorphisms (SNPs) across the genome and disease status of thousands of individuals, GWAS attempt to map genes and regulatory regions involved in susceptibility to asthma. The first genome-wide screen

for asthma susceptibility alleles was limited to a little over 200 SNPs and identified FCERB, a microsatellite in the β-chain of the high-affinity receptor for IgE, as being significantly associated with asthma (Daniels et al., 1996). Since then, more than a dozen of genome-wide surveys have been conducted to screen for more asthma-susceptibility loci among different ethnic groups. More recent attempts to stratify early onset versus late onset asthmatics have revealed distinct gene signatures (Pividori et al., 2019). A 2019 study by Pividori, and Schoettler *et al*. examining shared and distinct genetic risk loci for childhood-onset and adult-onset asthma revealed multiple independent asthma loci, 23 specific to childhood onset, one specific to adult onset, and 37 shared, with overall larger effect sizes for childhood-onset asthma at nearly all loci (Pividori et al., 2019). One of the SNPs shared between the two asthma cohorts fell at the *IL33* locus (Pividori et al., 2019). Moreover, a meta-analysis of North American asthma GWAS conducted in 2011 of individuals of European American, African American, or African Carribean, and Latino ancestry, termed the EVE Consortium, revealed SNPs near *IL1RL1* (also known as IL-33R and ST2), *TSLP*, and *IL33* as associated with disease in all three ethnic groups (**Table 1.2**) (Myers et al., 2012; Ramasamy et al., 2012; Torgerson et al., 2011). These results confirm the GABRIEL Study of 10,365 asthmatic European subjects whereby SNPs flanking the IL33 gene were top hits associated with disease (Moffatt et al., 2010a). Further, polymorphisms in the *IL33* are associated with Japanese allergic rhinitis and asthma-associated *IL33* SNPs were identified in a study of approximately 2,500 individuals (Sakashita et al., 2008).

Finally, a large-scale study designed to identify susceptibility alleles associated with elevated eosinophil counts identified *IL5*, *IL1RL1*, and *IL33* as important loci. A follow-up assessment of 8,000 asthmatics and more than 40,000 controls demonstrated variants in *IL33* were associated with disease (Gudbjartsson et al., 2009). Therefore, while various chromosomal

regions have been implicated in asthmatic disease, the reoccurrence of genetic polymorphisms on chromosome 9 short arm 9p24.1, or the *IL33* locus, reaching genome-wide levels of significance suggests a key role for the gene in asthmatic disease pathophysiology (Laitinen et al., 2004; Ober et al., 1998; Program et al., 2011). Despite the recurrence of *IL33* as a gene in asthma GWAS, most studies of genetic risk factors do not stratify patient cohorts into different endotypes. In fact, to this date, there have been only two asthma GWAS examining disease susceptibility in early onset versus adult onset cases (Moffatt et al., 2010a; Pividori et al., 2019). Delineating genetic risk factors affecting age of onset and environmental exposure at play could provide more insight into how IL-33 initiates and perpetuates the asthmatic response.

SNP	Chr.	Gene	Allele	European-American (P-value)	African-American/African-Caribbean (P-value)	Latino (P-value)	Meta (P-value)
rs4845783	1	*CRCT1*	G/A	0.0023	2.3×10^{-4}	0.048	5.6×10^{-7}
rs1102000	1	*PYHIN1*	T/C	NA	3.6×10^{-7}	0.12	3.6×10^{-6}
rs4653433	1	*SRP9*	A/G	0.0026	0.027	6.2×10^{-4}	5.4×10^{-7}
rs10173081	**2**	***IL1RL1***	**C/T**	**1.3×10^{-4}**	**0.07**	**4.5×10^{-5}**	**1.4×10^{-8}**
rs2017908	3	*RTP2*	A/C	0.24	0.67	4.4×10^{-9}	0.0049
rs11735820	4	*EPHA5*	G/T	1.5×10^{-4}	0.003	0.033	2.3×10^{-7}
rs1837253	**5**	***TSLP***	**C/T**	**1.1×10^{-5}**	**0.0023**	**0.0018**	**7.3×10^{-10}**
rs10064618	5	*GALNT10*	G/A	0.76	0.36	5.7×10^{-7}	4.4×10^{-4}
rs2453626	8	*FBXO43*	C/T	0.0044	2.1×10^{-4}	0.016	2.8×10^{-7}
rs2381416	**9**	***IL33***	**C/A**	**0.011**	**0.0022**	**6.6×10^{-4}**	**2.5×10^{-7}**
rs11214966	11	*C11orf71*	C/T	0.76	3.5×10^{-7}	0.59	0.0012
rs16929496	12	*RASSF8*	T/C	3.2×10^{-4}	0.025	0.0027	2.6×10^{-7}
rs9891949	17	*AURKB*	A/G	7.5×10^{-7}	0.68	0.73	4.5×10^{-4}
rs11078927	17	*GSDMB*	C/T	8.6×10^{-7}	0.0012	3.1×10^{-7}	1.2×10^{-14}
rs335016	19	*C19orf2*	A/C	0.16	0.68	2.6×10^{-7}	5.8×10^{-5}

Table 1.2 Frequencies of the most significant SNPs in each of the regions with at least one SNP associated with asthma at $p < 10^{-6}$ in at least one population or the combined populations from EVE Consortium (Torgerson et al., 2011).

IL-33, a pleiotropic tissue-derived cytokine

IL-33 signaling via ST2

IL-33, a member of the IL-1 family of cytokines, was originally identified in high-endothelial venules of human lymph nodes, though it is now well established that the cytokine is broadly expressed in a variety of tissue types including the gut, skin, brain, and the aortic vessels (Baekkevold et al., 2003; Chackerian et al., 2007; Moussion et al., 2008; Schmitz et al., 2005). It contains eight exons and spans more than 42kb, with many SNPs associated with asthma susceptibility located upstream of noncoding DNA, exon 1 or within the first intron (**Figure 1.1**) (Bønnelykke et al., 2014; Moffatt et al., 2010b; Torgerson et al., 2011). IL-33 binds with its receptor ST2 and this complex, along with the IL-1 receptor accessory protein (IL-1RAcP),

induces signaling via Toll-like domain (TIR)-containing receptors. TIR domains from ST2 and IL-1RAcP recruit adaptor molecules MyD88 and IRAK to mediate signaling through the NF-κB pathway (**Figure 1.2**) (Lingel et al., 2009; Liu et al., 2013; Palmer et al., 2008). This signaling is distinct from classical Th2 cytokines that signal via JAK-STAT pathways but is similar to the IL-1 family, where IL-33 is a member (Chackerian et al., 2007; Lingel et al., 2009).

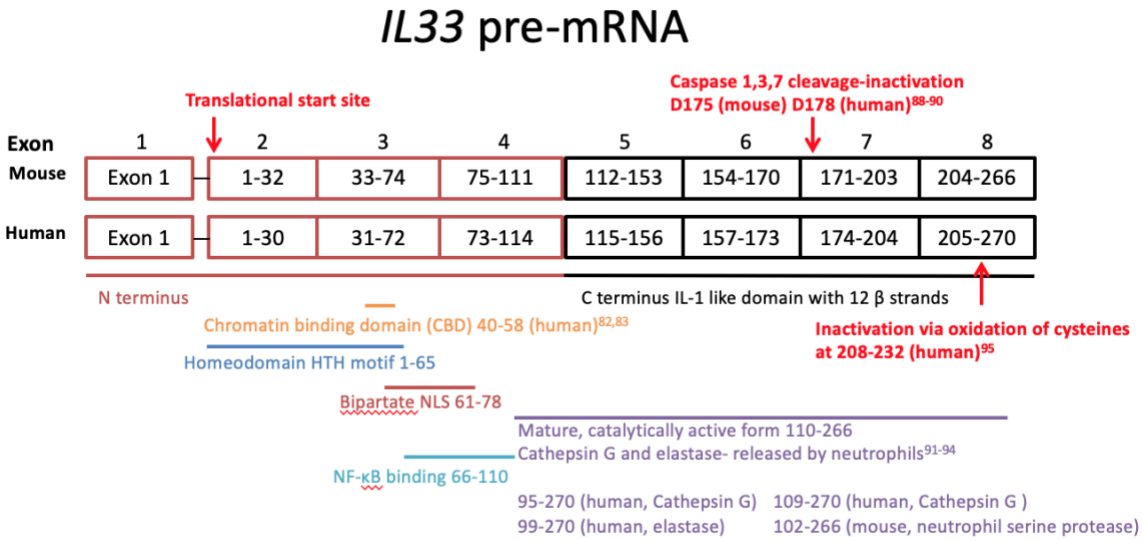

Figure 1.1 The structure of the murine and human *IL33* gene. The gene spans more than 42 kb, contains 8 exons, and is located on chromosome 9. Non-coding exon 1 is located 25.8 kb upstream of exon 2. An alternative exon 1b, located 4.6 kb upstream of exon 2 has also been described. Most single nucleotide polymorphisms (SNPs) associated with asthma are found in the promoter and intron 1 regions, as marked by the black dash preceding exon 2.

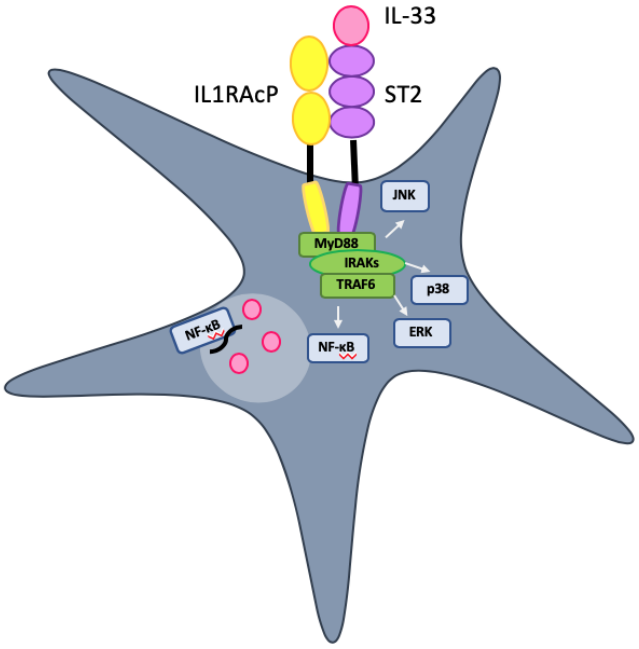

Figure 1.2 IL-33 binds to ST2 and the accessory protein IL-1RAcP, triggering a downstream signaling cascade that ultimately activates NF-κB and MAPK (ERK, JNK, p38).

ST2, located on chromosome 2q12, contains 13 exons with two different promoters, and has multiple isoforms (Hong et al., 2011; Tsuda et al., 2012; Tsuda et al., 2017). The two most common are a long, membrane bound (ST2L) isoform and a soluble (sST2) isoform (Bergers et al., 1994). Both sST2 and ST2L contain the same extracellular sequence, but sST2 lacks the transmembrane and cytoplasmic domains within the structure of ST2L and induces a unique 9 amino-acid C-terminal sequence. Soluble ST2 is released into the serum upon induction and acts as an inhibitor of IL-33, binding up excess IL-33 to prevent signaling and is thus termed a decoy receptor. In contrast, ST2L acts to transduce the IL-33 signal to the intracellular compartment (Lingel et al., 2009).

Binding of IL-33 to ST2L induces Th2-associated activity, such as eosinophil recruitment and mast cell degranulation (Coyle et al., 1999; Iikura et al., 2007; Silver et al., 2010). Type 2

innate lymphoid cells (ILC2s), basophils, mast cells, and eosinophils are major targets of IL-33 and express high levels of ST2 and respond to IL-33 by producing downstream cytokines such as IL-4, IL-5, and IL-13 (Barlow et al., 2012; Christianson et al., 2015; Kurowska-Stolarska et al., 2008; Kurowska-Stolarska et al., 2009; Louten et al., 2011; Neill et al., 2010; Rak et al., 2016; Stolarski et al., 2010; Xu et al., 2008). In addition to innate cells, a subset of T cells (Tregs) also express ST2 (Kurowska-Stolarska et al., 2008). In murine studies of allergen challenge, administration of antibodies against ST2 abrogated eosinophilia and IL-5 secretion (Liu et al., 2009). Further, mice deficient in either IL-33 or ST2 are protected from airway inflammation (Christianson et al., 2015; Oboki et al., 2010). Accordingly, intranasal administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs of mice even in the absence of adaptive immune system (Kondo et al., 2008; Kurowska-Stolarska et al., 2008).

Regulation and Processing of IL-33 Activity

Given that IL-33 signaling has profound effects on the immune system, it is unsurprising that there are multiple levels of regulation including nuclear sequestration, proteolytic processing, and oxidative inactivation (Cohen et al., 2015) (**Figure 1.1**). Full-length IL-33, proIL-33, is found in the nucleus of quiescent cells and associates with chromatin via H2A/H2B histone complex (Roussel et al., 2008). The N-terminus consists of a nuclear domain, and is followed by a central activation domain and C-terminus cytokine region. The nuclear domain consists of a homeodomain, which includes a helix turn helix DNA binding motif, that confers chromatin association and a nuclear localization signal (Carriere et al., 2007; Roussel et al., 2008). Together, they tether IL-33 to the nucleus where it may act as a transcriptional repressor (Choi et al., 2012; Lee et al., 2016; Roussel et al., 2008). In murine models where the nuclear domain of *IL33* is deleted, the cytokine is constitutively released and results in multi-organ

immune infiltration (Bessa et al., 2014). However, how IL-33 leaves the nucleus and is able to associate with its receptor remains unknown as IL-33 lacks a classical signal sequence and thus does not appear to be secreted via the ER-Golgi pathway.

It has been proposed that proIL-33 may be released during inflammatory cell death, either pyroptosis or necrosis, via membrane pores formed by the gasdermin family similar to other members of the IL-1 family such as IL-1β and IL-18 (Kayagaki et al., 2015; Schmitz et al., 2005). Interestingly, IL-33 is inactivated by caspases involved in apoptosis (Lüthi et al., 2009; Talabot-Ayer et al., 2009). Unlike other members of the IL-1 family, proIL-33 is not cleaved into a shorter, more bioactive isoform by caspase-1 cleavage (Lüthi et al., 2009). Instead, *in vitro* cultures of proIL-33 show that the cytokine is inactivated by the addition of recombinant caspase-1 (Cayrol and Girard, 2009; Lüthi et al., 2009). Therefore, processing of the full-length form is not required for the activation of IL-33. However, recent studies have demonstrated that full-length IL-33 can indeed be released extracellularly and cleaved into a shorter, mature form with 30-fold higher biological activity by protease-containing allergens, neutrophil elastases, as well as mast cell chymase (Cayrol et al., 2018; Hayakawa et al., 2009; Lefrançais et al., 2014; Lefrançais et al., 2012). The presence of high active, cleaved isoforms of IL-33 in the BAL of mice intranasally administered *Alternaria* extract corresponded to levels of neutrophil infiltration (Cohen et al., 2015).

Analysis of potential splice variants has revealed multiple isoforms (**Figure 1.3**). RT-PCR of human airway epithelial cells express a variant whereby exons 3, 4, and 5 are alternatively spliced out of the *IL33* transcript (Gordon et al., 2016). Splicing of exons 3 and 4 resulted in cytoplasmic localization of IL-33, though with intact cytokine signaling capacity that was capable of activating basophils and mast cells (Gordon et al., 2016). Epithelial brushings

from mild to moderate asthmatics demonstrated significant enrichment in the exon 3, 4 deletion variant that corresponded with epithelial activation (Gordon et al., 2016). Alternatively, splicing of exon 5 resulted in complete abrogation of cytokine activity and may be a mechanism to limit the effects of IL-33 (Gordon et al., 2016). Elucidating how these isoforms are regulated and whether or not they correspond to asthmatic disease endotype will be important in future studies, as these isoforms may serve as a method to classify disease subgroups. Patients with stable asthma have higher serum levels of IL-33 compared to healthy controls (Azazi et al., 2014; Tamagawa-Mineoka et al., 2014). One could thus hypothesize that airway epithelial cells are not inducing necrosis-related IL-33 induction and may be releasing an isoform lacking exons 3 and 4.

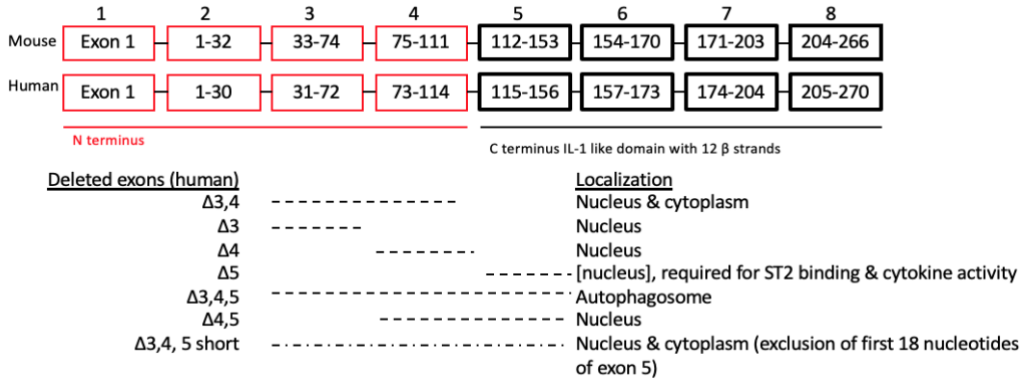

Figure 1.3 Regulation of IL-33 activity by mRNA splicing generates multiple isoforms. *IL33* pre-mRNA contains 8 exons and encodes for a full-length protein spanning exons 2-8. Depicted is a schematic of major IL-33 isoforms. Deletion of Exon 5 results in loss of IL-33 activity (Gordon et al., 2016).

In addition to necrosis, IL-33 can also be released from living cells upon exposure to allergen or ATP (Hudson et al., 2008; Kouzaki et al., 2011; Uchida et al., 2017). Stressed cells release ATP, which may serve as a signal to alert nearby cells of ongoing damage (Kouzaki et al., 2011). Exogenous ATP as well as the addition of *Alternaria* to *in vitro* cultures enhanced the secretion of IL-33 from human bronchial epithelial cells (hBE) (Byers et al., 2013; Doherty et

al., 2012; Uchida et al., 2017). Mechanistically, fungal allergen exposure facilitates IL-33 secretion by inducing extracellular ATP accumulation and increased Ca^{2+} via NADPH oxidate dual oxidase 1 (DUOX1) (Hristova et al., 2016). DUOX1 mediates the redox-dependent activation of SRC/EGFR/ERK signaling and thus oxidative stress seems to be an important pathway of IL-33 release by living cells (Hristova et al., 2016; Kakkar et al., 2012). Accordingly, treatment with Ca^{2+} chelators abrogated the extracellular release of IL-33 (Hristova et al., 2016). Despite its multiple pathways of release, however, IL-33 is quickly inactivated by oxidation of critical cysteine residues in the extracellular space (Cohen et al., 2015). IL-33 contains cysteine residues in its receptor-binding domain. While in the nucleus, IL-33 has cysteines that are kept in an unbound state with no disulfide formation (Cohen et al., 2015). However, as the cytokine is released into the extracellular environment, the reduced cysteines in IL-33 bind each other during disulfide bond formation. Cohen *et al.* showed that this oxidation results in a conformational change in IL-33, rendering the cytokine incapable of binding to ST2 (Cohen et al., 2015). Taken together, these data indicate the multifaceted regulatory components of IL-33 and underscore its importance as a cytokine capable of initiating an inflammatory immune response.

IL-33 in disease

Despite the resounding consensus that IL-33 and its receptor are heavily involved in allergic responses, the role of IL-33 extends beyond its involvement in the activation of the type 2 immune system and asthmatic disease. IL-33 has been implicated in response to viral infection, inflammatory bowel disease, and tissue repair (Beltrán et al., 2009; Burzyn et al., 2013; Kuswanto et al., 2016; Le Goffic et al., 2011; Monticelli et al., 2011; Tourdot et al., 2008). The first study implicating IL-33 in response to respiratory viral infection found that airway hyperresponsiveness was independent of the adaptive immune system (Tourdot et al., 2008).

Following infection with influenza and cytomegalovirus (CMV), IL-33 released from the lung mucosa enhances type 1 immunity by inducing IFNγ production from NKT cell, promotes antiviral responses via expansion of CD8+ T lymphocytes, and is required for restoration of epithelial barrier integrity (Bonilla et al., 2012).

Yet experimental models of inflammatory bowel disease (IBD) show IL-33 as a negative regulator of wound healing and colon permeability (Beltrán et al., 2009; Kobori et al., 2010; Pastorelli et al., 2010; Pushparaj et al., 2013; Sedhom et al., 2013; Seidelin et al., 2010). Indeed, IL-33-deficient mice have reduced levels of intestinal inflammation compared to controls after dextran sodium sulphate (DSS) treatment (Groβ et al., 2012). More recently, IL-33 has been implicated in the restoration of tissue integrity following damage (Duan et al., 2012). After skeletal muscle damage, IL-33 recruits highly suppressive regulatory T cells to the site of injury that produce amphiregulin and other factors critical to the resolution of inflammation (Burzyn et al., 2013; Kuswanto et al., 2016). Thus IL-33 appears to be an important immune mediator with pleiotropic effects. However, while the potent effects of IL-33 have been extensively studied in murine models of disease, much less is known about the expression of human IL-33.

IL-33 expression and cellular sources in the lung

Originally, IL-33 was identified in human high endothelial venules, specialized blood vessels that mediate entry of lymphocytes into lymph nodes (Schmitz et al., 2005). Abundant *IL33* transcripts are found in multiple barrier sites such as endothelial cells, smooth muscle, keratinocytes, adipocytes, cardiomyocytes and epithelial cells (Baekkevold et al., 2003; Chen et al., 2015; Hammad and Lambrecht, 2015; Lambert et al., 1993; Pastorelli et al., 2010; Pichery et al., 2012a; Préfontaine et al., 2009; Préfontaine et al., 2010). Extensive analyses of the cellular sources of human IL-33 demonstrate little IL-33 expression in CD45+ hematopoietic cells, either

during steady state or inflammation (Hardman et al., 2013; Lloyd, 2010; Pichery et al., 2012b). Given that most of IL-33 expression resides in anatomical barriers, it is unsurprising that areas such as the skin, gut, and lung appear to be prominent IL-33-expressing tissues. Constitutive nuclear expression of IL-33 has also been observed in fibroblastic cells of lymphoid tissue and epithelial cells in tonsillar crypts and salivary glands. In the lung, the sources of IL-33 expression remain contentious. Analysis of epithelial cells of the bronchus and small airways by RT-PCR confirm *IL33* transcripts, though it is unclear how much IL-33 protein these cells express. IL-33 protein can be readily detected in airway smooth muscle cells, with augmented levels in the smooth muscles of asthmatics compared to healthy subjects (Lambert et al., 1993; Préfontaine et al., 2009). Current models of IL-33, based largely on murine models of airway inflammation, show airway epithelium as the primary source of IL-33, though it is now clear that murine hematopoietic cells such as bone marrow derived macrophages and dendritic cells are capable of producing of IL-33 and mediating airway disease (**Figure 1.4**) (Hardman et al., 2013; Pichery et al., 2012b). The degree to which species-specific differences exist in the cellular sources and in the regulatory regions of murine and human IL-33 remains unknown.

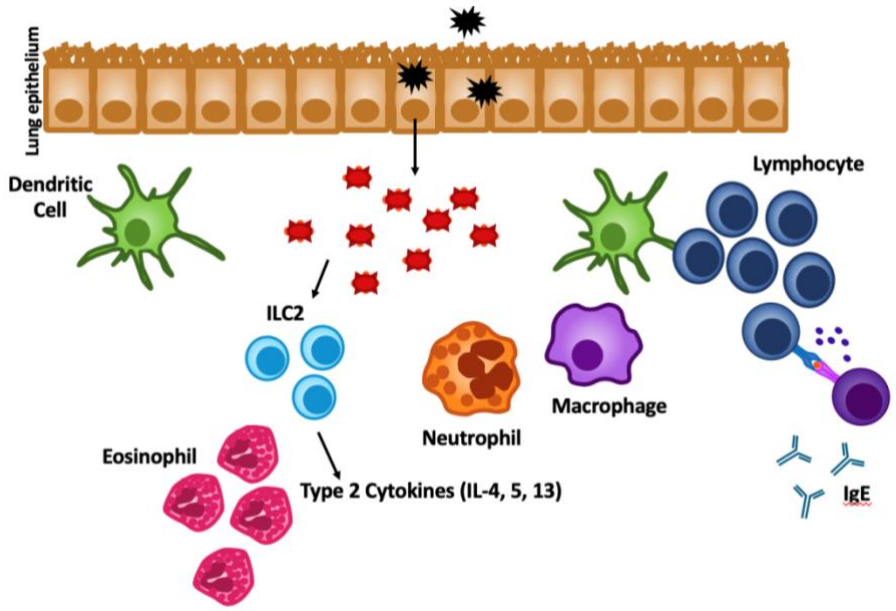

Figure 1.4 Current paradigm suggests epithelium as lung reservoir of IL-33 during allergen exposure. IL-33 (red circles) is released from epithelial cells at barrier surfaces in response to cell damage, allergen, infection or other cellular stresses. IL-33 then shapes innate and adaptive immune responses by acting on several cell types that express ST2 and mediate CD4+ T cell maturation. ST2-expressing ILC2s are responsive to IL-33 and produce Th2 cytokines, such as IL-4, IL-5 and IL-13. IL-33 can also act directly on ST2-expressing Treg that restore barrier integrity by producing amphiregulin (not pictured).

IL-33 therapeutics

Given the important role of IL-33 in driving Th2 immune responses, multiple immunotherapies have been generated with the goal of blocking the IL-33 pathway in asthma (NCT03207243). Inhibition of the IL-33/ST2 signaling pathway either with neutralizing antibodies or recombinant sST2 showed alleviation of murine models of asthma and ulcerative colitis. Further, in a murine model of peanut food allergy, neutralizing antibodies against IL-33 and not against other barrier cytokines such as TSLP or IL-25, protected mice from allergic immunity (Chu et al., 2013). Mechanistically, IL-33 is able to induce high levels of OX40L expression in dendritic cells *in vivo* and expand innate lymphoid cells that perpetuate downstream type 2 responses.

Currently, there are multiple monoclonal antibodies in early stage clinical trials (**Table 1.3**). Genentech's ST2-targeting antibody (AMG 282, RG6149) is entering Phase 2 clinical trials for asthma, while AnaptysBio's anti-IL-33 neutralizing antibody (ANB020) is concluding its Phase 2a trials for the treatment of moderate-to-severe atopic dermatitis, eosinophilic asthma, and adult chronic rhinosinusitis with nasal polyps. Janssen, in partnership with GSK, also developed an anti-ST2 monoclonal antibody (CNTO 7160) entering Phase 2a trials for severe asthmatic populations. Outside of using monoclonal antibodies against IL-33 and ST2, soluble receptor drugs may represent a platform to target Th2 responses. The existence of sST2 as a naturally occurring isoform *in vivo* demonstrates a level of IL-33 regulation that could be replicated using recombinant sST2. This approach has been used with Etanercept, a recombinant TNF receptor IgG1 fusion protein that works by competitively binding to TNF and preventing activation of the inflammatory cascade. If sST2 could be administered similarly, it may serve to outcompete ST2L binding and abrogate Th2 signaling.

The main drawback with therapies targeting IL-33 and ST2 is how these drugs will impact the patient systemically. Safety and efficacy are primary concerns, and given the pleiotropic effects of IL-33 and widespread distribution of ST2, it is unclear what long-term impact immunotherapy may have on the individual. Care should be taken when designing antibodies that target ST2, as inadvertently neutralizing sST2 may exacerbate the effects of IL-33. Furthermore, sST2 has been implicated in other immunomodulatory pathways outside of its interaction with IL-33. Production of the inflammatory cytokine IL-6 was dampened by sST2 in LPS-stimulated THP1, a human monocytic cell line (Nagata et al., 2012). ST2 suppressed the process of IκB degradation, such that NF-κB is rendered unable to translocate to the nucleus and to subsequently bind to the IL-6 promoter. Therefore, though sST2 has key implications in

asthma disease pathophysiology, it is a component of a complex web of immunomodulatory networks. It is crucial to evaluate what effect removing or enhancing this gene would have on the overall immune landscape and determine the optimal threshold for effective treatment.

Finally, given its dual function in driving Th2 inflammation as well as tissue repair, abrogating IL-33 function may have lasting consequences. The tissue environment, disease state, and genetic profile of subjects need to be considered during drug development. Despite being an initiator of Th2 inflammation, IL-33 has a protective function in maintaining adipose tissue homeostasis (Miller et al., 2010). IL-33-deficient mice develop obesity even on a normal diet, as IL-33 expands a subset of innate lymphoid cells that are important in metabolic homeostasis (Brestoff et al., 2015; Miller et al., 2010). Furthermore, IL-33-deficient mice also have decreased ILC2 numbers in white adipose tissue (WAT) accompanied by a reduction in downstream type 2 cytokines. During allogeneic hematopoietic cell transplantation, IL-33 levels from nonhematopoietic cells are augmented and promote type 1 alloimmunity and lethal acute graft-versus-host disease (GVHD) (Matta et al., 2016). However, IL-33 also exhibits immunoregulatory properties by increasing the frequency of Tregs which prevent the accumulation of effector T cells in GVHD-target tissue (Matta et al., 2016). Therefore, IL-33 may offer therapeutic potential to prevent GVHD after alloHCT in alleviating tissue damage or in promoting tolerance in organ transplantation. Collectively, these data underscore the multifaceted nature of an IL-33 response and thus extreme caution should be exercised when exploiting the IL-33/ST2 pathway for therapeutic potential. It is of utmost importance to identify where and when human IL-33 is released and to understand the splice variant of IL-33 that is induced, as IL-33 the most upstream mediator of inflammation acts on a broad range of cell types and cytokines.

Project	Company	Disease Indication	Trial	Phase Completion as of 2019
REGN3500 (SAR440340)	Sanofi/Regeneron	Asthma	NCT03387852	
		COPD	NCT03546907	Dec 2019
		Atopic Dermatitis	NCT03736967, NCT03738423	Both Mar 2020
Etokimab	AnaptysBio	Atopic Dermatitis	Atlas, NCT03533751	Jun 2019
		Nasal Polyps	NCT03614923	Dec 2019
		Asthma	Phase 2b planned 2019	
GSK3772847	Glaxosmithkline/J&J	Asthma	NCT03207243, NCT03393806	Feb 2019, Oct 2019
Phase I				
MEDI3506	Astrazeneca	COPD	NCT03096795	Sep 2019

Table 1.3 The IL-33 pipeline. *Several monoclonal antibodies to target IL-33/ST2 signaling in atopic diseases and asthma are currently in clinical trials.*

Summary

Given the dichotomy between murine and human IL-33, studying murine IL-33 may not accurately inform us of human disease. In this thesis, I address how human *IL33* is regulated and expressed in the lungs during homeostasis and inflammation. To explore the genetic regulation and expression of human *IL33*, I use a novel transgenic mouse strain generated from Bacterial Artificial Chromosome (BAC) technology containing the human *IL33* locus with a fluorescent reporter, termed BAC (+). This model closely mirrors human *IL33* expression in lung tissue and allowed me to 1) determine the constitutive and inducible cellular sources of IL-33 and 2) the regulatory regions that govern responses during airway inflammation.

Here, I demonstrate negative regulation of human *IL33* reporter in lung endothelium during house dust mite (HDM)-induced allergic, airway inflammation. This response is governed

by a novel regulatory region in the human *IL33* locus previously demonstrated in our earlier studies (Swanson and Decker et al., manuscript in preparation). IL-33 is able to autoregulate itself via a negative feedback loop in primary pulmonary vessels, as IL-33 administration downregulated the expression of human *IL33* in lung endothelial cells. ATAC-Seq analysis of endothelial cells treated with IL-33 identified regions of differential nucleosome positioning at the endogenous locus containing CTCF sites and transcription factors associated with long-range chromatin organization. Together, these data emphasize a novel role of pulmonary endothelium during allergic airway disease and pinpoint a region in the human *IL33* locus that underlies this distinct response.

Chapter 2 Materials and methods

Mice

6-12 week old C57Bl/6 (B6) mice were purchased from Harlan Industries (Indianapolis, IN). B6.ST2$^{-/-}$ mice were provided by Dr. A. McKenzie (Medical Research Laboratory, University of Cambridge, UK). IL-33$^{-/-}$ mice were generously provided to Dr. P.J. Bryce (Northwestern University, Chicago, IL) by Dirk Smith (Amgen, Seattle, WA) and to Dr. H.A. Turnquist (University of Pittsburgh, Pittsburgh, PA) by Dr. S. Nakae (The Institute of Medical Science, Tokyo, Japan). For the BAC (+) mice, a BAC clone (RP11-725F15) was purchased from the NCBI Clone Registry and modified to include an E2-Crimson (Clontech) reporter gene at ATG start codon in Exon 2 using standard recombineering techniques. A stop codon was inserted after the fluorescent reporter. The DEL-BAC (+) is identical to the full-length construct but with the removal of the 5kb CTCF region at position Chr9: 6194500-6199500. The full-length construct in the BAC was able to recapitulate *IL33* expression in 5 separate founder lines by qPCR. All mice were bred and housed in specific pathogen-free facilities maintained by the University of Chicago Animal Resource Center. The studies described conform to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals in biomedical research.

Cell culture, cells, and reagents

Human umbilical vein-derived endothelial cells (HUVECs) were purchased from Lonza (Cat #: C2519A) and cultured in EBM™ Basal Medium (Cat #: CC-3156) and EGM™-2 SingleQuots™ Supplements (Cat #: CC-4176). Conditionally immortalized dermal LECs (iLECs; Immortomice) were gifted from Dr. Melody Swartz at the University of Chicago's Institute for Molecular Engineering (Chicago, IL). Human Lung Microvascular Endothelial Cells

(HMVEC-L) were purchased from Lonza (Cat #: CC-2527) and cultured in EBM™ Basal Medium (Cat #: CC-3156) and EGM™-2 MV Microvascular Endothelial Cell Growth Medium SingleQuots™ supplements (Cat #: CC-4147). All cells were maintained at 37°C in 95% humidity/5% CO_2 atmosphere and were split at a ratio of 1:3 before reaching confluence. The cultures were used at passage level one to five. All experiments were performed on confluent cultures.

Stimulation of HUVECs, iLECs, and HMVEC-L

Cells were plated at $2x10_4$/mL in a 24 well plate and treated with varying concentration of recombinant IL-33 (Biolegend). Cells were pre-treated with 10µg/mL of cycloheximide for 30 min before the addition of 100ng of recombinant IL-33 to culture for 6 hours.

RNAscope

RNA in situ hybridization technology (RNAscope®) was used to hybridize IL-33 mRNA probes (Cat #: 400118-C2) and ST2 probes (Cat #: 603498) customized from ACD using the RNAscope® 2.5 LS Duplex Reagent Kit (Cat #: 322440) onto at least 7 sections from lung samples from 10 donors by the Human Tissue Research Center (HTRC) of the University of Chicago. Non-transplantable control lungs were obtained from the Gift of Hope/Regional Organ and Tissue Donor Network (GOH) organ and tissue donor network, an organ procurement organization that provides services regionally to 12 million people within the national donation system. Subjects from whom control specimens (GOH) were obtained had no known history of lung disease or immunologic disorder. Lung tissue was taken from left upper lobe and formalin-fixed, paraffin-embedded for use. H&E staining to confirm asthma phenotype was performed on the formalin-fixed, paraffin-embedded specimens.

Tissue Fixation

Mouse lungs were removed, and a lobe from the left side was saved for microscopy studies. Lobes were fixed in a 2% methanol-free formaldehyde solution (Cat#28906, Thermo Scientific, Rockford, IL) for 60 to 90 minutes at room temperature with agitation. After a brief wash in PBS, lobes were placed in a 30% sucrose solution. The solution was changed two to three times over a period of 24 hours, after which lobes were allowed to sit in the sucrose until they sank in the tube. At this point, lobes were embedded in Optimal Cutting Temperature compound (OCT 4583) and stored at -80$_{o}$C until sectioning.

Tissue sectioning and staining

Frozen tissues were sliced into sections 7μm thick and dried onto Superfrost Plus slides (Cat#12-550-15, Fisher Scientific, Pittsburg, PA) overnight at room temperature. Slides were washed in PBS, permeabilized with 0.5% IGEPAL CA-630 (Cat#I7771, Sigma-Aldrich, St. Louis, MO) for 5 minutes, washed in PBS, and quenched/ blocked for 50 minutes in 50mM NH_4Cl with 10% normal goat serum (NGS, Cat#G9023, Sigma-Aldrich, St. Louis, MO). Tissues were immunostained with primary antibodies for 2 hours at room temperature. After washing with 1% NGS in PBS, sections were stained with secondary antibodies and the nucleic acid stain Hoechst 33342 (Life Technologies, Thermo Fisher, Waltham, MA) for 1 hour at room temperature in the dark. Slides were washed in PBS, dipped in H_2O, and No.1.5 thickness coverslips were set with ProLong Diamond Antifade Mountant (Life Technologies, ThermoFisher, Waltham, MA). Coverslips were allowed to cure at room temperature overnight before being sealed. Antibodies included Living Colors DsRed Polyclonal anti-E2 Crimson (Clontech [now Takara Bio], Mountain View, CA); anti-mouse CD45 (clone 30-F11, Biolegend, San Diego, CA); anti-mouse Lyve1 (eBioscience, clone ALY7, Thermo Fisher Scientific,

Rockford, IL); polyclonal anti-GFP:FITC (Novus Biologicals, Centennial, CO); goat anti-rabbit IgG:Alexa Fluor 633 and goat anti-rat IgG:Alexa Fluor 680 (Invitrogen, Thermo Fisher, Rockford, IL).

Imaging

Imaging was performed at the University of Chicago Integrated Light Microscopy Facility. Images were captured with a Leica SP8 laser scanning confocal microscope (Leica Microsystems, Inc., Buffalo Grove, IL) using a 20x/0.7 multi-immersion objective and LAS_X Leica acquisition software. Further processing of images was completed using ImageJ software (National Institutes of Health, Bethesda, MD).

Air-Liquid Interface Cultures

Human donor lungs that were not suitable for transplantation were obtained from deceased individuals through Gift of Hope Organ & Tissue Donor Network (Itasca, IL). Primary Airway Epithelial Cell (AEC) cultures were established from these lungs. To obtain either basal cell or differentiated cell phenotypes, AEC were grown in submersion or ALI culture (21 days) respectively. Passage 1 cells used in ALI culture were seeded at 1.0 x 10_5 cells onto Corning™ Transwell™ Multiple Well Plate with Permeable Polyester Membrane Inserts collagen IV-coated, 12 mm diameter (pore size 0.4 μm) (Corning, Inc. Cat #: 3462). Cells were grown to 80% confluence, the apical medium was removed and cells were fed with PneumaCult™-ALI Medium (Stem Cell STEMCELL Technologies Inc. Cat #: 05001) via the basolateral side every other day to day 21. Passage 1 cells used in submersion culture were seeded similarly onto collagen IV-coated 6-well plate, and grown to 100% confluence using Airway Epithelial Cell Growth Medium with supplement mix (PromoCell GmbH Cat #: C-21060). Confluent cells were treated with IL-5 (BioLegend Cat#: 560701) and harvested at different time points.

Sensitization and challenge models

House dust mite (Greer Laboratories) in phosphate-buffered saline (PBS) was administered to mice i.t. on day 0 with 50 μg of HDM and again on day 7, 8, 9 and 10 with 25 μg HDM, then killed 24 hours after the last challenge. For LPS and Poly (I:C), administration, mice were weighed one day prior to i.t. to determine appropriate unit/kg dose. At killing, lungs were dissociated by mechanical mincing followed by digestion with 150 U ml^{-1} Collagenase IV (Sigma-Aldrich) and 1mg/mL of DNase I (Worthington Biochemical Corporation, Lakewood, NJ) in 10 ml for 60 min. Samples were then washed and red blood cells were lysed using ACK lysis buffer, resuspended in a single-cell suspension, and counted. For all cell populations, 0.5×10^6 to 1×10^6 cells were used for staining, with anti-CD16/32 (clone 2.4G2) used for blocking. Cells were washed with PBS containing 0.1% sodium azide and 0.2% bovine serum albumin, fixed overnight in 1% paraformaldehyde. All data were collected using a BD Biosciences LSRFortessa and analyzed using FlowJo software (Tree Star, Inc.). All instruments are maintained by the University of Chicago Flow Cytometry and Antibody Technology Core Facility.

Flow cytometric analysis

For FACs analysis, 5×10^5 cells were resuspended in 100 μL of FACs buffer (PBS containing 0.1% sodium azide and 1% BSA). They were then blocked with 20 μL of 2.4G2 (anti-CD16/32) and stained with fluorescently conjugated antibodies (**Table 2.1**).

Non-hematopoietic/Structural Cells (CD45-)	Hematopoietic Cells (CD45+)
CD45 BV421 Clone: 30-F11 Biolegend	CD45 PE Clone: 30-F11 Biolegend
gp38 (podoplanin) PE Clone: 8.1.1 Biolegend	CD11c PE-Cy7 Clone: N418 Biolegend
EpCAM BV605 Clone: G8.8 Biolegend	Siglec-f BV421 Clone: E50-2440 BD
CD31 PE-Cy7 Clone: MEC13.3 Biolegend	Ly6G BV711 Clone: 1A8 Biolegend
Sca-1 (Ly-6A/E) BV711 Clone: D7 Biolegend	CD11b FITC Clone: M1/70 eBioscience
MHC-II PerCy/Cy5.5 Clone: M5/114.15.2 Biolegend	MHC-II PerCy/Cy5.5 Clone: M5/114.15.2 Biolegend
Zombie Aqua™ Fixable Viability Kit BV510 Biolegend	F4/80 BV605 Clone: BM8 Biolegend
ST2 PE DIH9 Clone: Biolegend	Zombie Aqua™ Fixable Viability Kit BV510 Biolegend

Table 2.1 Antibodies used for flow cytometry.

Gating Strategies

Endothelial cells in the lung were identified as live, $CD45^{-}$ $EpCAM^{-}$ $CD31^{+}$. Endothelial cells were further subsetted into lymphatic endothelial cells or vascular endothelial cells based on expression of gp38 (**Figure 2.1**). Lymphatic endothelial cells were phenotypically $CD45^{-}$ $EpCAM^{-}CD31^{+}gp38^{+}$ while vascular endothelial cells were $CD45^{-}EpCAM^{-}CD31^{+}gp38^{-}$. For the detection of epithelial cells, cells were first gated on positive expression of EpCAM. Type 2 penumocytes were subsetted as $EpCAM^{+}$ $MHC\text{-}II^{+}$. For the detection of fibroblasts, the cells were first gated on $CD45^{-}$ $EpCAM^{-}$ $CD31^{-}$. Each cell population was then examined for Crimson expression compared to negative littermate controls.

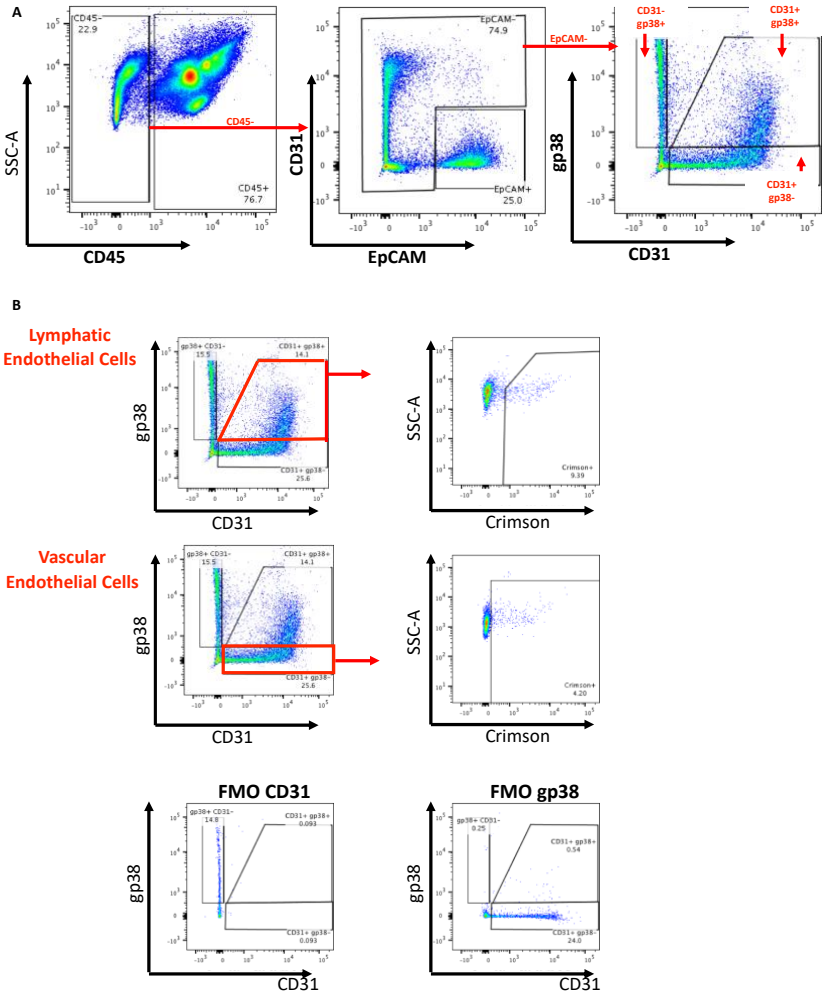

Figure 2.1 Gating strategy for subsetting stromal cell populations in the lung. (A) Gating strategy for flow cytometric analysis of lung cell subsets. Live CD45- lung cells were gated for their surface expression of EpCAM, CD31, gp38, and (B) intracellular expression of Crimson. (C) Subsets of Crimson expressing stromal cells as defined by their surface marker expression. Data are representative of 3 or more experiments. BAC (-) littermate mice used as negative control in analysis of all Crimson expression.

Histologic analysis

The left lobe was removed from the mice after perfusion and fixed by immersion into 10% formalin. Lobes were embedded in paraffin, sectioned sagitally, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E staining) for analysis of cellular infiltrates. Images were taken and analyzed with Pannoramic Viewer (3DHISTECH).

Quantitative PCR analysis

Total cellular RNA extraction was carried out using the quick-RNA MicroPrep (Zymo Research) and RNA was reverse-transcribed with Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Cat #: 4368814). For quantitative PCR, a total volume of 25 μl containing 1 μl cDNA template, 0.5 μM of each primer (**Table 2.2**), and SYBR Green PCR Master Mix (Applied Biosystems) was analyzed in quadruplicate. Gene expression was analyzed with an ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.9.1 (Applied Biosystems). Results were normalized by division of the value for the unknown gene by that obtained for *GAPDH* or *B2m*.

Mouse Primers

Gene	Sequence
ST2L F	5’ GCATGATAAGGCACACCATAA 3’
ST2L R	5’ ATCGTAGAGCTTGCCATCGT 3’
sST2 F	5’ GCATGATAAGGCACACCATAA 3’
sST2 R	5’ ACACAGAGAGGGGAAGGATA 3’
Crimson F	5’ GCC AAG CTG CAA GTG ACC AA 3’
Crimson R	5’ GCC TTG GAG CCG TAG AAG AA 3’
β2M F	5′ GGG TGG AAC TGT GTT ACG TAG 3’
β2M R	5’ TGG TCT TTC TGG TGC TTG TC 3’

Table 2.2 qPCR primer design for murine and human genes.

Human Primers

Gene	Sequence
IL33 F	5’ TGA GTC TCA ACA CCC CTC AAA TG 3’
IL33 R	5’ GGC ATG CAA CCA GAA GTC TTT T 3’
ST2L F	5’ GAAGGCACACCGTAAGACTA 3’
ST2L R	5’ TTGTAGTTCCGTGGGTAGAC 3’
GAPDH F	5’ GAA GGG TGA AGG TCG GAG TC 3’
GAPDH R	5’ GAA GAT GGT GAT GGG ATT TC 3’

Table 2.2, continued.

Assay of transposase-accessible chromatin using sequencing

ATAC-Seq was done as described. HUVECs ($2x10_4$) treated with 30ng/mL of recombinant murine IL-33 (BioLegend) were used for each ATAC-Seq assay. To prepare nuclei, we centrifuged cells at 500*g* for 5 min, washed them with ice-cold PBS and centrifuged them again at 500*g* for 5 min. Cells were lysed with cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% IGEPAL CA-630). Immediately after lysis, nuclei were spun at 500*g* for 10 min at 4 °C. Supernatant was carefully pipetted away from the pellet after centrifugation. Immediately after the nuclei prep, the pellet was resuspended in the transposase reaction mix (25 µl 2× Tagment buffer, 2.5 µl Tagment DNA enzyme (Illumina, FC-121-1030) and 22.5 µl nuclease-free water). The transposition reaction was carried out at 37 °C for 30 min. After transposition, the sample was purified with a Qiagen MinElute kit. After purification, we amplified library fragments using Nextera PCR Primers (IlluminaNextera Index kit) and NEBnext PCR master mix (New England BioLabs, 0541) for a total of 10–12 cycles followed by purification with a Qiagen PCR cleanup kit.
The amplified, adaptor-ligated libraries were size-selected with Life Technologies' E-Gel SizeSelect gel system in the range of 150–650 bp. We quantified the size-selected libraries

with an Agilent Bioanalyzer and via qPCR in triplicate using the KAPA Library Quantification Kit on the Life Technologies Step One System. Libraries were sequenced on the Illumina HiSeq2000 system to generate $7.5\times 10_7$ to $10 \times 10_7$ 50-bp paired-end reads.

QC and DNA alignment

All raw sequence data was quality trimmed to a minimum phred score of 20 using trimmomatic. Alignment to reference genome hg38 was done with BWA; for ATAC-Seq data, read pairs where one pair passed quality trimming but the other did not were aligned separately and merged with the paired-end alignments. PCR duplicates were removed using Picard Mark Duplicates and alignments with an edit distance greater than two to the reference, or that were mapped multiple times to the reference, were removed.

ATAC-Seq analysis

Read alignments were first adjusted to account for TAC transposon binding: +4 bp for +strand alignments, -5bp for -strand alignments. The open chromatin enrichment track was generated by first creating a bedGraph from the raw reads using bedtools genomcov, then converted to bigWig using UCSC tool bedGraphToBigWig; tracks were normalized by the sum of alignment lengths over 1 billion. The start position track was generated by taking either the first base of the alignment for +strand alignments or the last base of the alignment for - strand alignments, then creating bedGraph and bigWig tracks as for the open chromatin; tracks were normalized to the alignment count over 1 million. Open chromatin peaks were called using Macs2 with --nomodel set and no background provided; peaks with a score >5 were retained. Furthermore, we used the peak calling results as a guide to identify regulatory elements, then quantified enrichment in these regulatory elements and ran differential analysis to compare

samples. Reproducibility was factored into the differential statistics that we calculated using estimates of dispersion.

For nucleosome positioning, properly paired alignments were filtered by their fragment size. Fragment sizes <100 bp were considered nucleosome free, and replaced with a single BED region, and used as a background. Sizes between 180 and 247 bp were considered mononucleosome and replaced with a single BED region; sizes between 315 and 473 bp were considered dinucleosomes and replaced with two BED regions, each spanning half the overall fragment length; and sizes between 558 and 615 bp were considered trinucleosomes and replaced with three BED regions, each spanning one third of the overall fragment length; the mononucleosome, dinucleosome, and trinucleosome regions were concatenated and used as the nucleosome signal. The resulting BED regions were analyzed using DANPOS with the parameters –p 1 –a 1 –d 20 —clonalcut 0 to identify regions enriched or depleted for nucleosomes.

Differential Expression

Differential expression statistics (fold-change and p-value) were computed using edgeR on raw expression counts obtained from quantification. Importantly, edgeR allows multi-group and multi-factor analyses to prioritize which genes show the biggest effects overall, as well as pair-wise tests between sample conditions to determine the context of the changes as a post-hoc evaluation. In all cases, p-values were adjusted for multiple testing using the false discovery rate (FDR) correction of Benjamini and Hochberg. Significant genes were determined based on an FDR threshold of 5% (0.05) in the multi-group comparison.

Cell Fractionation for RNA Stability

Fractionation of human nuclei was performed following Bhatt et al. (2012). Briefly, $2x10^4$ pooled Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza and treated 9 hours *in vitro* with recombinant IL-33. Cell pellets were rinsed in 1× PBS/1 mM EDTA and then the plasma membranes were lysed by resuspension in ice-cold NP-40 lysis buffer (10 mM Tris-HCl [pH 7.5], 0.05% [LA-N-5]–0.15% [HeLa] NP40 [Sigma], 150 mM NaCl), for 5 min. The lysate was then layered on top of 2.5 volumes of a chilled sucrose cushion (24% sucrose in lysis buffer) and centrifuged for 10 min, 4°C, 14,000 rpm in an Eppendorf 5415C microfuge. The supernatant (cytoplasmic fraction) was collected and treated with proteinase K for 1 h at 37°C, then phenol/chloroform extracted and ethanol precipitated. The nuclei pellet was gently rinsed with ice-cold 1× PBS/1 mM EDTA, then resuspended in a prechilled glycerol buffer (20 mM Tris-HCl [pH 7.9], 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol) by gentle flicking of the tube. An equal volume of cold nuclei lysis buffer (10 mM HEPES [pH 7.6], 1 mM DTT, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 0.3 M NaCl, 1 M UREA, 1% NP-40) was added, the tube was gently vortexed for 2 × 2 sec, incubated for 2 min on ice, and then centrifuged for 2 min, 4°C, 14,000 rpm as above. The supernatant (soluble nuclear fraction) was treated with proteinase K for 1 h and 37°C, then phenol/chloroform extracted and ethanol precipitated. The chromatin pellet was gently rinsed with cold 1× PBS/1 mM EDTA and then dissolved in TRIzol (Invitrogen). Chromatin-associated RNA was purified according to the TRIzol protocol, but an additional phenol/chloroform extraction step was performed prior to precipitation. All RNA fractions were resuspended in TE (pH 7) and quantified using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies) and tested for DNA contamination by RT-PCR lacking reverse transcriptase.

Statistics

All statistical analyses were performed with GraphPad Prism software, and *P* values less than 0.05 were considered significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$). For comparisons of 2 groups, an unpaired Student's 2-tailed *t* test (parametric) or Mann-Whitney test (nonparametric) was performed. To determine whether data were parametric or nonparametric, variances were compared using an *F* test. For comparisons of 3 or more groups, a 1-way ANOVA with Sidak's multiple-comparisons post hoc test was conducted.

Study approval

All animal procedures were approved by the University of Chicago Institutional Animal Care and Use Committee.

Chapter 3 Species specific analyses reveal endothelium and basal cells, not epithelium, as main sources of *IL33* in human lung

Introduction

Murine models of disease have been the cornerstone of most *in vivo* immunological experimentation. Mice mirror complex human systems remarkably well, with only 300 or so non-shared genes between the two species (Consortium, 2002). Despite these similarities, however, important species-specific differences exist in the regulatory network and cellular expression profiles in mice and humans. To this, several regulatory features of the human *IL33* locus are nonhomologous to the murine *Il33* locus in the ENCODE portal. We have previously shown in our in silico epigenetic analysis of the human *IL33* landscape that a 23kB region containing asthma GWAS SNPs, two CTCF sites, H3K4me1 and H3K27Ac chromatin marks, and multiple DNase hypersensitivity sites are absent from the murine *Il33* locus. Chromatin immunoprecipitation with antibodies specific to the transcription factor, Oct1, followed by sequencing of the precipitated DNA (ChIP-seq), showed co-binding of RAD21 and SMC-3 at the CTCF site (Swanson and Decker et al., manuscript in preparation). The presence of large, multi-transcriptional complexes in the human *IL33* locus is area, absent in the murine *Il33* locus, suggests that there may be functional differences in the expression profiles of murine and human IL-33.

Species-specific differences in the expression and regulation of epidermal IL-33 have been reported (Sundnes et al., 2015). IL-33 in the murine epidermis has been shown to be constitutively expressed in keratinocyte nuclei and rapidly lost during inflammation (Sundnes et al., 2015). In contrast, human and porcine IL-33 are weakly expressed or absent in keratinocytes

during homeostasis and induced during inflammation (Sundnes et al., 2015). Moreover, using a novel murine *Il-33–LacZ* Gt reporter strain, Pichery *et al*. demonstrated that murine *Il33* promoter was constitutively expressed in epithelial cells from barrier tissues such as the vagina, skin, lung, stomach and salivary glands, but absent from high endothelial venules of mice. This is in stark contrast to human IL-33 expression, because IL-33 was originally discovered in HEVs based on its abundant expression (Pichery et al., 2012a).

Given that *Il33* expression in murine models does not replicate data from human IL-33 studies, it is essential to parse out comparative data to bridge the gap between murine experimental models and IL-33 function in human clinical settings. This is extremely difficult due to the lack of a working model of human IL-33 expression and the use of non-specific human IL-33 antibodies, the multiple IL-33 isoforms in the lung, ST2 usage bias, and the absence of negative controls in human IL-33 studies. Use of various ELISA kits to detect levels of IL-33 in the serum of well-characterized patients with asthma proved futile, as the level of IL-33 was below the lower limit of detection in serum of asthma patients (Ketelaar et al., 2016). Another problem with the many IL-33-specific antibodies on the market is that post-translational modifications, such as cleavage by proteases or extracellular oxidation, may preclude the cytokine from detection. In addition, depending on the time frame in which IL-33 is captured, binding to sST2 may prevent an accurate readout of IL-33 levels. As new information on the processing and regulation of human IL-33 develop, the hope is that assays with higher sensitivity and specificity are also developed to capture human IL-33 expression. Clearly, with so many paradigms that do translate well between the two species, murine models are an effective tool at studying components of the immune system.

Our goal for this chapter is not to invalidate the use of murine models, but to dissect the key differences between human and murine IL-33 and to better understand the phenotypic profile of human IL-33 expression using a model that addresses some of the regulatory limitations of murine models. Here, we generate a novel murine model of human *IL33* expression using BAC transgenic technology that enables us to directly compare murine and human *IL33* expression patterns during homeostasis and inflammation. We find that unlike murine *Il33* reporter which is expressed largely by type II pneumocytes, human *IL3*3 reporter is constitutively expressed by lung endothelium. In addition, we found in human donor lungs a population of basal stem cells that express *IL33* and populate the lower airway. Moreover, the nonhomologous asthma-associated regulatory region in the human *IL33* locus governs expression in lung microvasculature, as removal of this region abrogated all expression in smaller vessels. Here, we develop a novel reporter mouse model, containing the human *IL33* locus and its regulatory components, that faithfully recapitulates the cellular expression of *IL33* as found in human lungs.

Results

Analysis of human lung tissue reveals endothelium and basal cells as the predominant sources of IL-33.

We sought to identify the cell types that produce IL-33 and maintain lung tissue homeostasis in humans. To explore the relevant sources of human IL-33, we turned toward the Lung Gene Expression Analysis (LGEA) web portal to bioinformatically query single-cell gene expression and quantitative RNA expression of *IL33* in each lung cell type from both human and mouse lungs. Human *IL33* was expressed almost exclusively by lung endothelial cells from birth to adulthood (**Figure 3.1A**). In contrast, RNA expression of murine *Il33* was found most abundantly in lung epithelial cells and to a lesser extent, in lung endothelium and fibroblasts

(**Figure 3.1B**). While epithelium upregulated murine *Il33* transcripts from embryogenesis to post-natal day 28, endothelium and fibroblasts expression of *Il33* remained low.

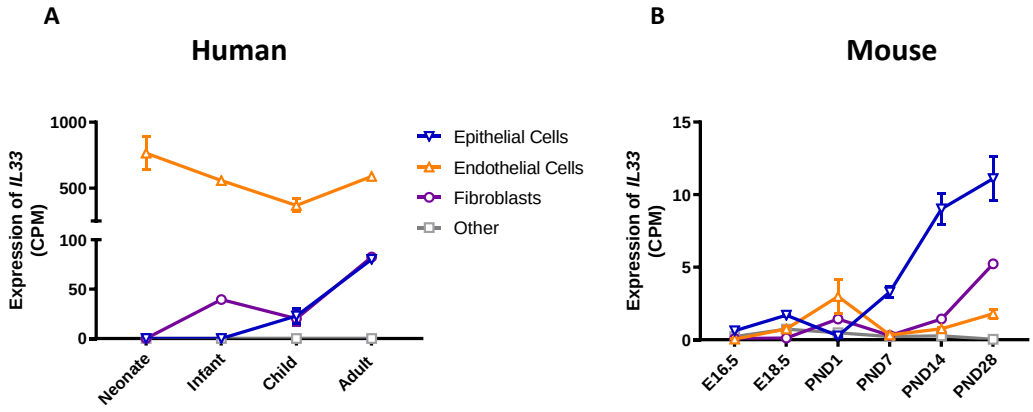

Figure 3.1 Human *IL33* is highly expressed by the lung vasculature but is not detectable in EpCAM+ lung epithelium. Expression of human *IL33* (A) and murine *Il33* (B) from single-cell RNA-seq data courtesy of the LungGens database.

Given the literature reporting human *IL33 transcript* expression in epithelial cells, we turned toward examining human donor lungs obtained through the Gift of Hope Organ Tissue & Donor Network (Otto, 2002; Préfontaine et al., 2010; Soumelis et al., 2002). Non-transplantable control lungs were obtained through an organ procurement organization that provides services regionally to 12 million people within the national donation system. These subjects had no known history of lung disease or immunologic disorder. *In situ* hybridization of *IL33* RNA probes demonstrated that *IL33* was expressed in large vessels of lung endothelium and not lung epithelium of control donors (**Figure 3.2**). Interestingly, in these donor lungs, *IL33* was found along the basement membrane of large airways where pluripotent stem cells, called basal cells, typically localize (**Figure 3.2**).

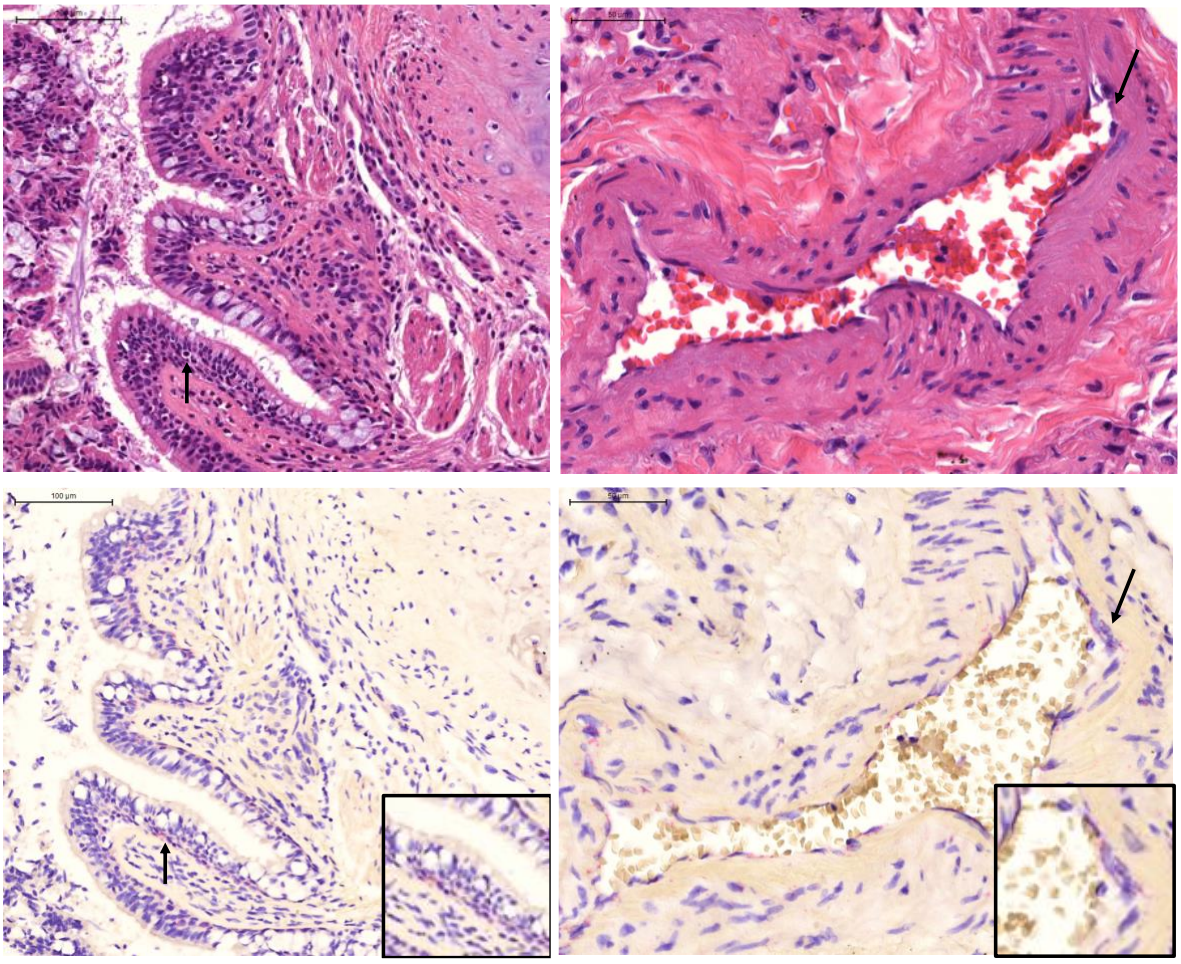

Figure 3.2 *IL33* mRNA evaluation in human donor lungs obtained through the Gift of Hope Organ Tissue & Donor Network. *IL33* mRNA probes were used to label expression in airway epithelium (left) and vessels (right). Positive cells (red) are indicated by arrows. Insets show *IL33* positive cells.

Consistent with this, *IL33* mRNA has been found in abundance from *ex vivo* basal cell cultures of whole lung explants where expression was associated with an ITGA6$_{hi}$NGFR$_{lo}$ subpopulation of basal cells, characteristic of epithelial progenitor stem cells, in isolated tissue from COPD and non-COPD subjects (Byers et al., 2013). To this end, we cultured basal cells from three human donor lungs. Primary basal cell cultures were submerged in air-liquid interface (ALI) cultures to allow for cellular differentiation into ciliated, secretory, and basal epithelial cells. While basal epithelial cultures expressed moderate amounts of *IL33* in two of the donors, expression was

significantly downregulated upon differentiation into terminal epithelial cells (**Figure 3.3**). Moreover, *IL33* mRNA levels varied greatly between donors, suggesting that *IL33* levels may be dependent upon genetic variation and environmental exposure. Taken together, these results suggest that the cellular sources of human IL-33 are basal cells and the lung vasculature.

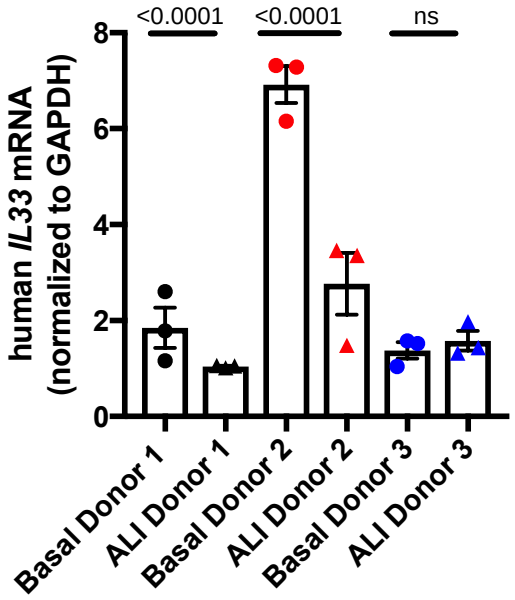

Figure 3.3 Basal lung cells downregulate *IL33* mRNA as they differentiate in ALI epithelial cultures *in vitro*. Human donor lungs that were not suitable for transplantation were obtained from deceased individuals through Gift of Hope Organ & Tissue Donor Network, Itasca, IL. Primary AEC cultures were established from these lungs. AEC were grown in submersion or ALI culture (21 days) respectively.

Development of a novel human IL33 reporter mouse model recapitulates the endothelial-derived IL33 expression profile found in humans.

The lack of conservation between the human and murine *Il33* loci and between protein expression necessitated the development of a model to include the regulatory features of human *IL33*. To this end, we used a human BAC (clone RP11-725F15), approximately 166 kb in length, spanning the entire coding region of *IL33*, and recombineering technology to generate a murine model that reports transcription from the human *IL33* locus as Crimson (**Figure 3.4**). We recombined a cassette containing *Crimson* in frame with the *IL33* translational start site in Exon

2. A stop codon was inserted after the Crimson sequence such that only the Crimson transcript and not human *IL33* was made into protein. Therefore, Crimson expression in this system, termed BAC (+) mice, serves as a surrogate for human *IL33* expression. In addition to the human *IL33* reporter, we dissected the cellular sources of murine *Il33* using a GFP reporter where GFP is targeted to exon 2 of the murine *Il33* locus. In these mice, GFP marks transcription from the murine *Il33* locus but no functional murine IL-33 is made.

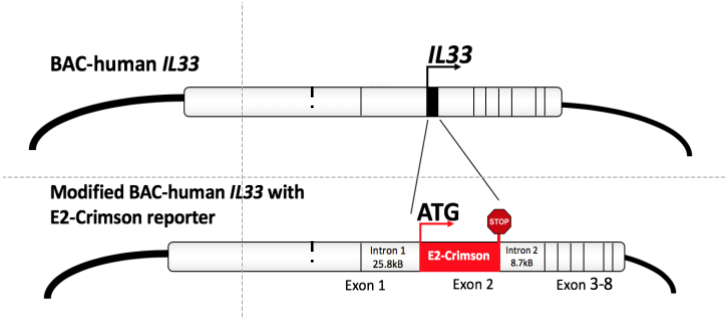

Figure 3.4 Schematic of BAC (+) transgenic strain. Bolded black bar represents upstream regulatory region containing asthma associated SNPs. Thin black bars represent the exons of *IL33*. The ATG site is located at the beginning of the 2nd exon. The human *IL33* locus was modified with a fluorescent Crimson reporter knocked into the ATG translational start site of *IL33*.

IL-33 was first identified as a nuclear factor in human lymph-node associated high endothelial venules (Schmitz et al., 2005). Thus, we examined human *IL33* reporter expression in the lymph nodes of BAC (+) mice. Indeed, immunohistochemistry confirmed constitutive Crimson expression in in CD31+ (PECAM-1) high endothelial venules of BAC (+) mice (**Figure 3.5A**). Analysis of BAC (+) lung tissue using immunohistochemistry from naïve mice showed that CD45+ hematopoietic cells did not colocalize with Crimson expression (**Figure 3.5B**) and flow cytometry analysis demonstrated that lung structural cells accounted for <30% of Crimson expression (**Figure 3.5C**). Crimson reporter colocalized largely with LYVE-1, a marker for lymphatic endothelial cells (**Figure 3.5D**).

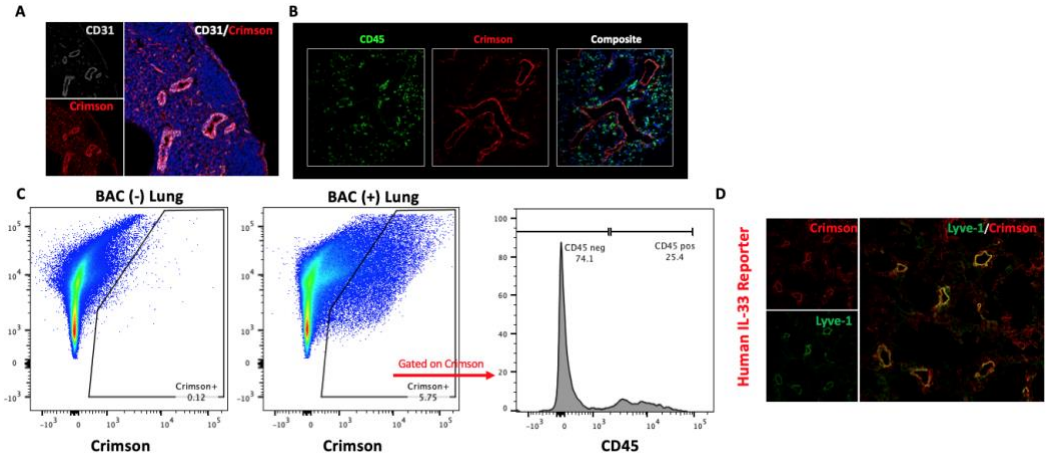

Figure 3.5 Crimson is constitutively expressed by lung structural cells. (A) Confocal microscopy of lymph node (A) and lung (B) from 6-8 week old naïve BAC (+) mouse. Red indicates Crimson expression. White indicates CD31 endothelial cells. Green indicates CD45. Blue indicates Hoescht staining. (C) Flow cytometric analysis of lung digest from 6-8 week old naïve BAC (-) mouse (left) and BAC (+) mouse (right). Data are representative of 3 or more experiments. BAC (-) littermate mice used as negative control in analysis of all Crimson expression. (D) Confocal microscopy of lungs from BAC (+) mice. Red indicates Crimson expression. Green indicates LYVE-1. Blue indicates Hoescht staining.

Flow cytometric analysis demonstrated that vascular and lymphatic endothelial cell compartments were nearly a quarter of the source of Crimson at baseline (**Figure 3.6**). Strikingly, lung epithelial cells, which have long been thought to be the dominant cell type expressing and storing IL-33 during homeostasis, were only a small source of Crimson in non-hematopoietic lung cells of BAC (+) mice (**Figure 3.6**). These data confirmed that Crimson, like human IL-33, was abundantly expressed in high endothelial venules from lymphoid organs and that lung endothelium, not epithelium, was the primary source of human *IL33* expression at baseline.

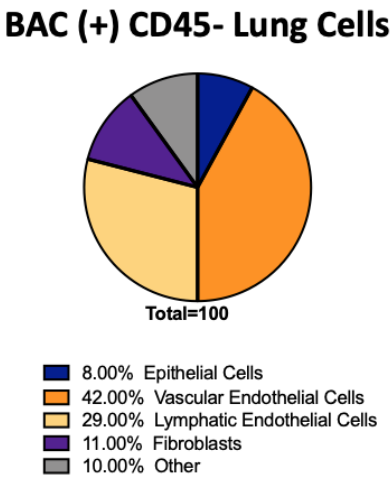

Figure 3.6 Flow cytometric analysis of Crimson expression in structural cells of BAC (+) lung. Subsets of Crimson expressing stromal cells as defined by their surface marker expression. Data are representative of 3 or more experiments. BAC (-) littermate mice used as negative control in analysis of all Crimson expression.

Given minimal human *IL33* reporter expression by lung epithelium, we aimed to address whether studies demonstrating *lL33* derived from epithelium were a unique feature of murine models. Indeed, immunohistochemistry of lung sections at steady state demonstrated that GFP was present almost exclusively in type II pneumocytes (**Figure 3.7**). Unlike the human *IL33* reporter, GFP failed to colocalize with LYVE-1 (**Figure 3.7**). Flow analysis of murine reporter lungs demonstrated that epithelial cells, not lung endothelial cells, are predominantly the source of lung *Il33* (data not shown).

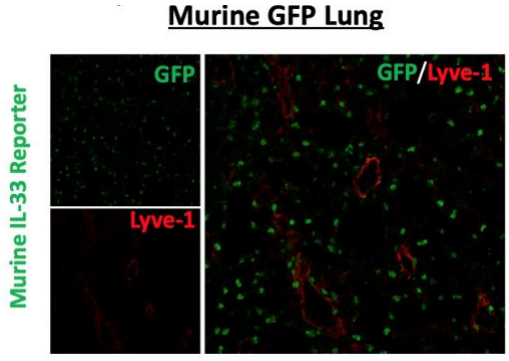

Figure 3.7 Confocal microscopy of lungs from GFP$^{+/+}$*Il33*$^{-/-}$ reporter mice. Green indicates GFP expression. Red indicates LYVE-1. Blue indicates Hoescht staining.

When we examined the CD45+ hematopoietic compartment for Crimson expression, we found that alveolar macrophages were by far the largest source of Crimson, accounting for nearly 75% of total expression (**Figure 3.8**). Neutrophils, lymphocytes, and dendritic cells also expressed Crimson, though to a much lesser degree. To better understand the Crimson expression by the hematopoietic compartment, we turned to analyzing the bone marrow of BAC (+) mice.

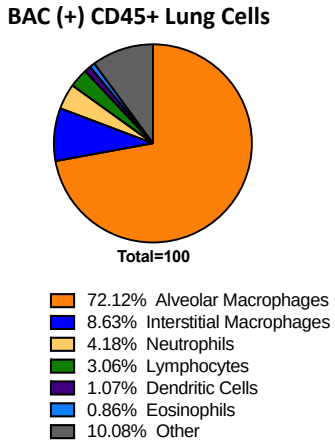

Figure 3.8 Flow cytometric analysis of Crimson expression in hematopoietic cells of BAC (+) lung. Subsets of Crimson expressing hematopoietic lung cells as defined by their surface marker expression. Data are representative of 3 or more experiments. BAC (-) littermate mice used as negative control in analysis of all Crimson expression.

However, flow cytometric analysis of bone marrow demonstrated very little Crimson expression (**Figure 3.9, top**), and virtually all B cells in the bone marrow were negative for Crimson expression regardless of developmental stage (**Figure 3.9, bottom)**.

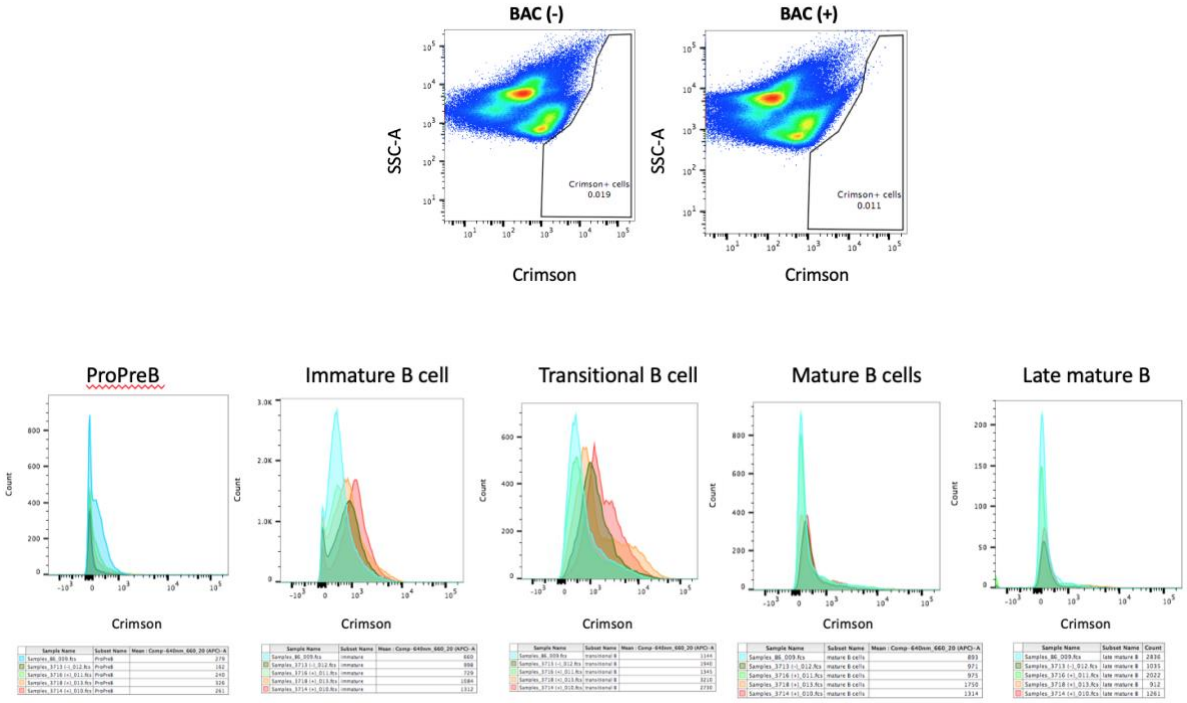

Figure 3.9 B cells from BAC (+) bone marrow do not express human *IL33* reporter. (Top) Flow cytometric analysis of bone marrow from 6-8 week old naïve BAC (-) (left) and BAC (+) (right) mice. Data are representative of 3 or more experiments. BAC (-) littermate mice used as negative control in analysis of all Crimson expression. (Bottom) MFI of Crimson expression on B cell subsets in the bone marrow.

In contrast to Crimson, GFP, the murine reporter for *Il33*, was highly expressed by bone marrow B cells (**Figure 3.10**). Almost all cells that expressed GFP in the bone marrow were pre/proB and immature B cells. GFP was lost in B cells as the population matured in the bone marrow. Taken together, these results demonstrate that 1) our novel BAC (+) human *IL33* reporter mice faithfully replicate patterns observed in primary human tissue and gives us a novel platform to assess human *IL33* etiology and expression and 2) studies on murine IL-33 expression may not accurately translate to studies of human disease.

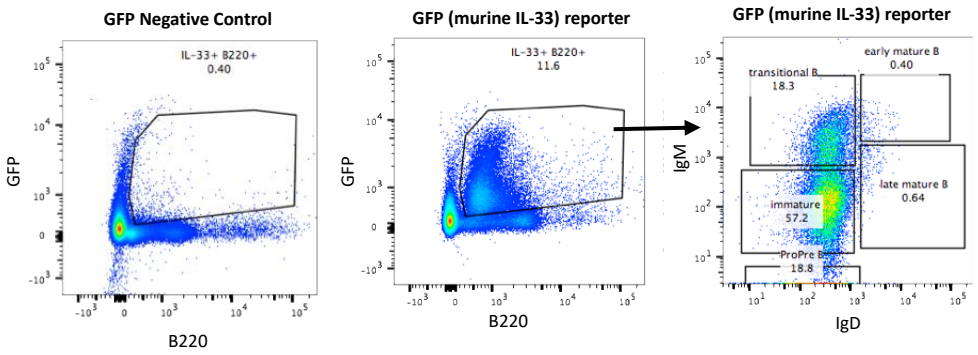

Figure 3.10 Pre/proB and immature B cells in the bone marrow of GFP$^{-/-}$ mice express high levels of murine *Il33* reporter. Flow cytometric analysis of bone marrow from 6-8 week old naïve GFP negative control mice (left) and GFP$^{-/-}$ reporter mice. Data are representative of 3 or more experiments.

5kB regulatory site, containing asthma-associated SNPs, from the upstream region of IL33 confers tissue specific expression of the human IL33 reporter.

Given that *IL33*-expressing cells in the lung differ between mice and humans, we next probed the human *IL33* locus to understand human *IL33* regulation in more detail. Our previous *in silico* interrogation of the human *IL33* locus identified an aforementioned non-conserved 5kB region upstream of the human *IL33* translational start site (Swanson and Decker et al., manuscript in preparation). We investigated to what extent this 5kB region upstream of the *IL33* locus played an important role in further regulation of the cytokine. To understand the role of the 5kB regulatory region identified in ENCODE, another BAC transgenic mouse strain was developed in which the 5kB regulatory region was removed from the human *IL33* locus as previously described (Swanson and Decker et al., manuscript in preparation). This mouse, termed the DEL-BAC (+), is genetically identical to the BAC (+) except with the excision of the 5kB site (**Figure 3.11**).

Figure 3.11 ENCODE analysis identifies an upstream regulatory region within the human *IL33* locus, not conserved in mice. Schematic of DEL-BAC (+).

A 5kB regulatory region upstream of the ATG translational start site was deleted.

We previously demonstrated that removal of the 5' region completely abrogated baseline Crimson expression from lymph node high endothelial venules of DEL-BAC (+) mice, confirming that this region governs tissue specific expression of human *IL33* (Swanson and Decker et al., manuscript in preparation). However, while Crimson expression was significantly diminished in the lymph nodes of DEL-BAC (+) mice, Crimson expression appeared to be retained in the larger vessels of the lungs of DEL-BAC (+) (**Figure 3.12**). Upon further interrogation of the cellular components of DEL-BAC (+) mice, we observed Crimson costained with LYVE-1+ lymphatic endothelial cells, but there was a loss of Crimson expression unique to pulmonary microvasculature (**Figure 3.12**). Larger endothelial architecture remained Crimson positive in the lungs, but smaller capillaries and veins lost their Crimson expression in the DEL-BAC (+) (**Figure 3.13**).

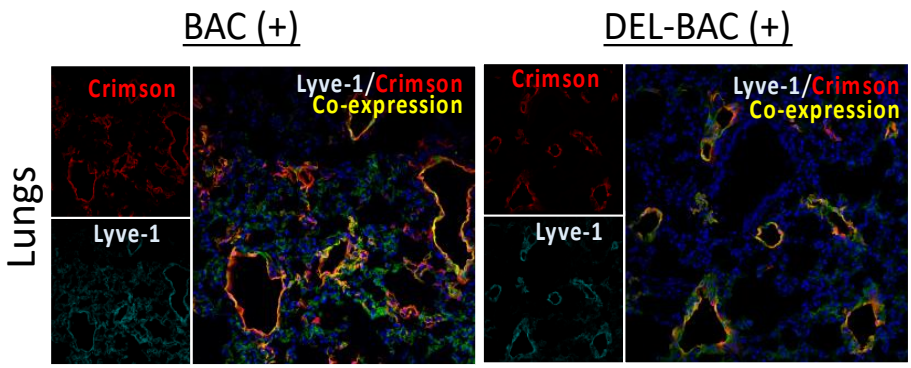

Figure 3.12 Crimson expression is retained in larger airways of DEL-BAC (+) lungs. Confocal microscopy of lungs from BAC (+) mice and DEL-BAC (+) mice. Cyan indicates LYVE-1+ cells. Red indicates Crimson expression. Blue indicates Hoescht staining.

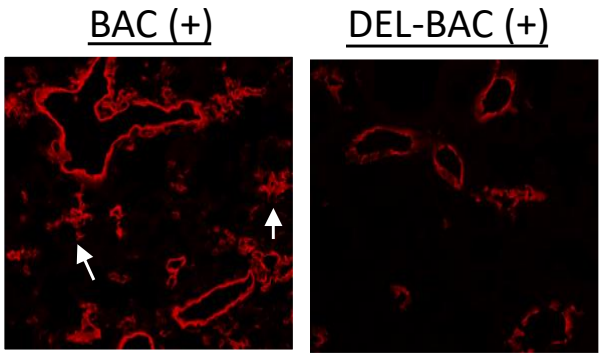

Figure 3.13 Crimson expression is lost in smaller vessels of DEL-BAC (+) lungs. Confocal microscopy of lungs from BAC (+) mice and DEL-BAC (+) mice. Red indicates Crimson expression. White arrows indicate microvasculature.

To understand the nature of the cells governed by the 5kB region, we turned toward flow cytometric analysis of Crimson expressing cells from the lungs of DEL-BAC (+) and BAC (+) mice. Unsupervised clustering analysis generated 30 distinct clusters of Crimson expressing cells (**Figure 3.14, left**). Of the 30 distinct clusters, two were significantly reduced in the lungs of DEL-BAC (+) mice. Populations 14 and 27 in the DEL-BAC (+) mice accounted for less than half of total Crimson expression compared to their expression in BAC (+) mice (**Figure 3.14, right**).

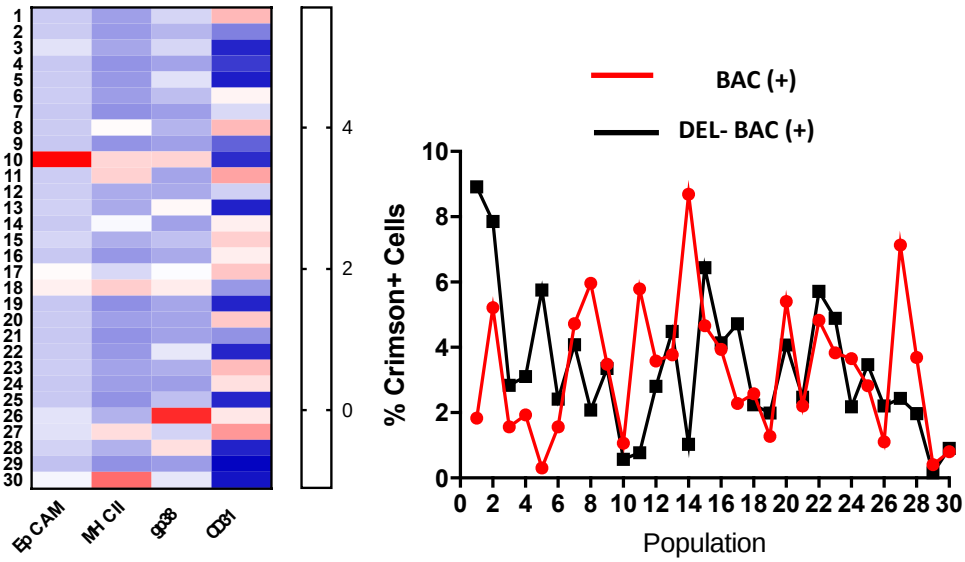

Figure 3.14 Upstream regulatory region within the human *IL33* locus, not conserved in mice, confers expression in CD31+ MHC-II+ microvasculature. (Left) Heatmap view of metaclusters generated from Phenograph from whole lung digest of 6-8 week old naïve BAC (+) and DEL-BAC (+) mice. (Right) Quantification of the percentage of Crimson expression within each metacluster generated from Phenograph.

Analysis of surface markers on these two populations revealed expression of both CD31 and MHC-II, indicating these populations were a subtype of lung endothelial compartment. These results demonstrate that the 5kB regulatory region we identified in the human *IL33* locus is important for constitutive Crimson expression in both the lymph nodes and pulmonary microvasculature, and may serve to confer tissue-specific expression to human *IL33*.

Lung epithelial-derived IL33 is a feature of murine model systems.

To better characterize human and murine IL-33 cellular sources in the lung, we generated dual reporter mice (F1) by crossing our human *IL33* Crimson BAC (+) mice to our murine *Il33* GFP murine reporter. The F1 mice recapitulated human *IL33* reporter expression as observed in BAC (+) mice and murine *Il33* reporter expression from the GFP-mice. Lung tissue immunostaining revealed very little coexpression between human and murine *Il33* in these

unchallenged mice. Crimson was again detected in LYVE-1+ lung lymphatic endothelial cells, while GFP was present distinctly in type 2 pneumocytes within the alveoli (**Figure 3.15, left**). Flow cytometric analysis of Crimson and GFP-expressing cells demonstrated that almost all GFP expressing cells were negative for the endothelial marker CD31, while Crimson-expressing cells were all positive for CD31 (**Figure 3.15, right**). 75% of murine-derived *IL33* came from epithelial cells, while endothelial cells (combined from CD31+ gp38+ lymphatic and CD31+ gp38- vascular endothelial) made up <10% of murine reporter expression.

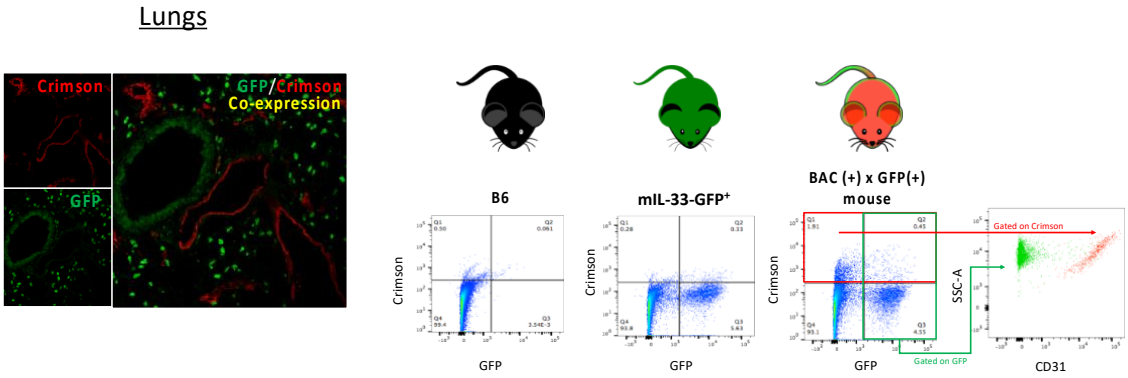

Figure 3.15 *In vivo* analysis of human *IL33* and murine *Il33* expression using a novel BAC (+) GFP double reporter strain. (Left) Confocal microscopy of lung from 6-8 week old naïve F1 mouse. Red indicates Crimson expression. Green indicates GFP. Yellow indicates co-expression of both Crimson and GFP. (Right) Flow cytometric analysis of lung digest from 6-8 week old naïve B6 mouse (left), naïve GFP mouse (center) and naïve F1 reporter mice (right).

To gain an unbiased insight into the sources of Crimson and GFP, we used unsupervised clustering analysis to visualize non-hematopoietic human *IL33* expressing subsets in the lung (**Figure 3.16, left**). We identified 32 clusters, where GFP+ clusters expressed high amounts of EpCAM and MHC-II, genes associated with type II pneumocytes, while Crimson+ clusters were enriched in moderate amounts of CD31 and gp38, consistent with LYVE-1 and Crimson imaging data (**Figure 3.16, right**). tSNE analysis of whole lung digest from BAC (+) mice revealed distinct cellular populations where islands were marked by well-defined surface expression of

CD31 and EpCAM and non-overlapping expression of Crimson and GFP (**Figure 3.17**).

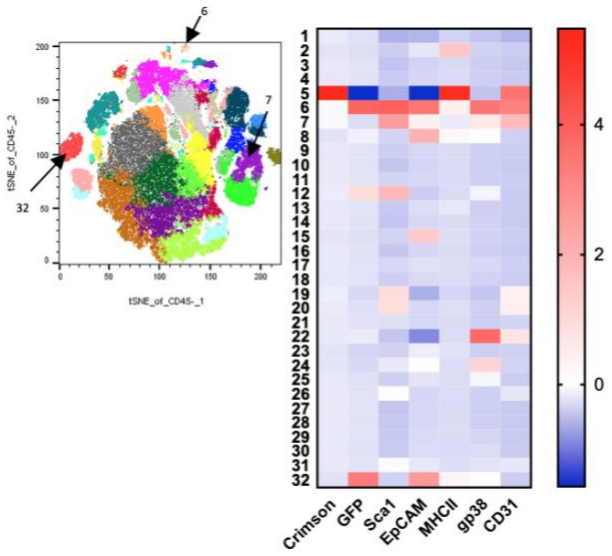

Figure 3.16 Unsupervised clustering analysis of lungs from BAC (+) GFP double reporter strain. (Left) Unsupervised clustering analysis of CD45- cells from naïve, F1 mouse lung visualized with Phenograph. Each dot indicates an individual cell. Arrows correspond to population on the right. Total cell number: 282,583. (Right) Heatmap view of metaclusters generated from Phenograph of lung digest from 6-8 week old naïve F1 mice.

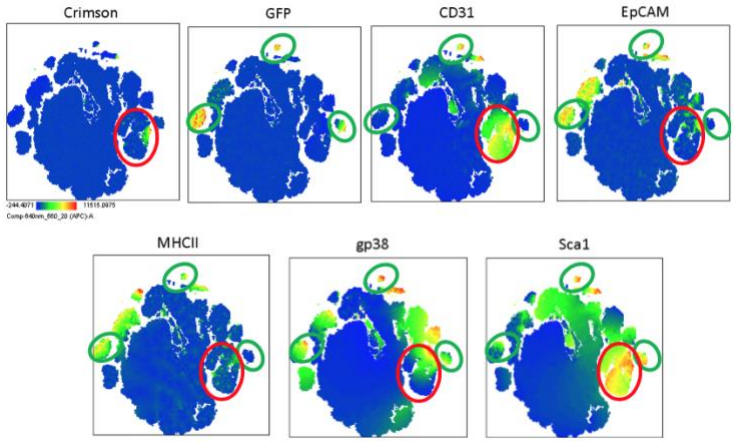

Figure 3.17 Unsupervised clustering analysis of CD45- cells from naïve, F1 mouse lung visualized with tSNE. Each dot indicates an individual cell. Total cell number: 282,583.

We previously hypothesized GFP-expressing lung epithelium represented a homogenous population. However, EpCAM-positive cells clustered to two separate islands that were positive for MHC-II, only one of these islands expressed GFP and suggests heterogeneity among type II

pneumocytes. Consistent with our imaging data, Crimson expression was enriched on the island marked by CD31+ cells, with the majority of Crimson+ cells clustering with the CD31+gp38+ lymphatic endothelial sub-cluster (**Figure 3.18**). In contrast to murine *Il33* reporter, human *IL33* reporter was observed almost exclusively in lung endothelial cells, with a negligible percentage of EpCAM+ epithelial cells comprising Crimson reporter expression (**Figure 3.18**).

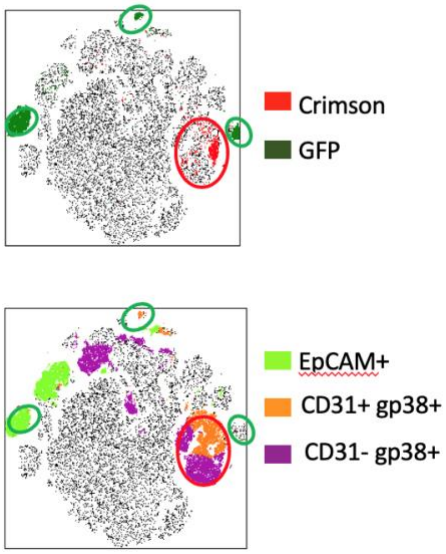

Figure 3.18 Unsupervised clustering analysis of CD45- cells from naïve, F1 mouse lung visualized with t-SNE and overlaid with parent gates from FlowJo. Each dot indicates an individual cell. Total cell number: 282,583.

Interrogation of the Lung Gene Expression Analysis (LGEA) web portal for *IL33* expression patterns in mice and humans demonstrates remarkable similarity to the patterns of expression observed in both our Crimson reporter mice and GFP reporter mice. RNA expression of *IL33* at the single cell resolution from the lungs of postnatal day 28 old mice closely mirrors the expression profile of GFP observed in our F1 reporter mice. Murine *Il33* was found most abundantly in lung epithelial cells and to a lesser extent in lung endothelium and fibroblasts (**Figure 3.19)**. In contrast, human *IL33* was expressed almost exclusively by lung endothelium and comparable to the Crimson expression profile observed in the lungs of F1 reporter mice

(**Figure 3.19**). Taken together, these results suggest that the cellular sources for human and murine IL-33 in the lung are distinctly unrelated.

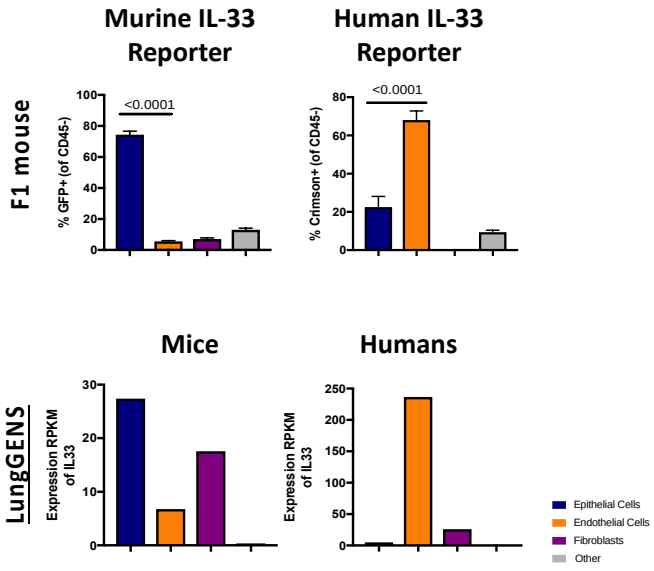

Figure 3.19 Expression of Crimson and GFP expression by CD45- lung cells of F1 mice (top) and human *IL33* (20 mos. old) and murine *Il33* (P28) expression from LungGens database (bottom).

Discussion

In this section, we show that the reservoirs for *IL33* expression in the unperturbed human lung are endothelial cells and multipotent basal cells. *IL33* was abundantly expressed by the vasculature in explanted human donor lungs and by multipotent stem cells. Using an improved murine model that contains the human *IL33* locus and its regulatory components to report the expression of human *IL33* crossed to a murine *Il33* reporter mouse, we demonstrate that murine *Il33* reporter and human *IL33* reporter are expressed by distinct cellular compartments. The human *IL33* reporter was highly expressed by lung lymphatic and blood vessels while murine *Il33* reporter was expressed by type II pneumocytes. Strikingly, murine *Il33* reporter was absent from the endothelial network in mice, revealing the existence of important species-specific differences between humans and mice. In addition to differences in *IL33* expression, we find that a 5kB regulatory region in the human *IL33* locus, absent in mice, not only governs 3D chromatin architecture of *IL33* but also confers *IL33* reporter expression to MHC-II+ microvasculature in the lungs. Together, our data strongly suggest that human *IL33* encodes an endothelial, not epithelial, cytokine whose expression profile is not captured by preexisting murine models.

Given that the lungs of DEL-BAC (+) mice still retain human *IL33* reporter expression in larger vessels, but not microvascular vessels, these data indicate that lung endothelium is not comprised of a homogenous population. True to their heterogeneity, endothelial cells vary vastly in cell morphology, function, gene expression and antigen composition even between sites of the same tissue (Aird, 2007, 2012). Endothelial cells that line arteries, for example, are distinctly oriented from endothelial cells that line veins (Aird, 2012). Moreover, vascular permeability varies across the endothelial network, with movement of material during homeostasis primarily occurring at the level of smaller capillaries (Aird, 2012). During inflammation, permeability can

occur at larger veins and arterioles to permit leukocyte trafficking (Aurrand-Lions et al., 2002; Butcher, 1991; Springer, 1994). In addition to blood endothelium, lymphatic endothelial cells have been shown to alter their homogeneity. Our data also reveal *IL33* reporter expression in high endothelial venules (HEV), specialized endothelium involved in leukocyte transmigration between blood and underlying tissue (Aird, 2012). Importantly, expression is also abrogated in these venules upon removal of the upstream regulatory region. Given that we see a loss of *IL33* reporter expression selectively in CD31+ MHC-II+ lung microvasculature and in HEVs through which cells traffic, it is entirely possible that this network of smaller endothelial cells surveys the immune landscape via antigen sampling to maintain homeostasis and release IL-33 upon damage or cellular stress. In support of this, the endothelial network is capable of capturing exogenous and endogenous antigens through phagocytosis and can transfer antigens to dendritic cells in close proximity to enhance T cell anergy (Rouhani et al., 2015; Rouhani et al., 2014). Therefore, regulating IL-33 levels may be a possible mechanism by which endothelial cells alert the adaptive immune system of cellular stress.

Our human lung tissue analysis revealed that lung basal cells are an important source of *IL33*. Further, basal cell cultures from human donor lungs demonstrate highest *IL33* expression in their multipotent state, with a significant reduction in *IL33* mRNA levels upon terminal differentiation into ciliated and secretory epithelial cells. These data suggest that IL-33 could be important in tissue repair, as basal cells have also been implicated in the restoration of barrier tissues following injury. TRP63+ KRT5+ basal cells isolated from normal human airways proliferate and give rise to ciliated and secretory cells in *ex vivo* air-liquid interface cultures (Dvorak et al., 2011; Hackett et al., 2008; Ross et al., 2007). Furthermore, FACs sorted ITGA6+ NGFR+ basal cells from human lung tissue are able to develop into bronchospheres with a

visible lumen and ciliated cells when seeded in 3-dimensional sphere-forming assays (Rock et al., 2009). Lineage tracing experiments demonstrate the regenerative capacity of basal cells following SO_2 inhalation (Borthwick et al., 2001; Rock et al., 2009; Stripp et al., 1995). Given that repetitive rounds of damage to lung barrier tissue occurs in asthmatics, it would be important to examine the basal cell niche over time compared to non-asthmatics. Loss of homeostatic IL-33 expression by basal cells and subsequent inability of lung tissue to regenerate may explain asthmatic airway remodeling.

Given their multipotent nature and importance in maintaining lung integrity, we were surprised to find an absence of *IL33*-expressing basal cells in the murine lung. This, however, is due to important differences in the anatomical architecture of basal cells between mice and humans. In humans, the basal cell-containing compartment extends distally to the terminal bronchioles (Hong et al., 2004; Rock et al., 2009). In contrast, basal cells do not extend beyond the trachea in mouse lungs (Hong et al., 2004; Mitzner et al., 2000; Rock et al., 2009). Thus, an important consideration from our analysis of human lung tissue is the anatomical dichotomies that exist in IL-33 compartments. However, to our knowledge, our mouse model is the first of its kind to not only incorporate *IL33* and its regulatory regions but also to demonstrate human *IL33* reporter expression by lung vasculature. Therefore, this model is a useful platform to investigate how *IL33* is expressed in the cells that orchestrate differentiation, maintenance and repair not mirrored in previous murine models.

Chapter 4 Extracellular IL-33 negatively regulates endothelial *IL33* mRNA by modulating RNA stability

Introduction

Allergic sensitization to inhaled allergens such as house dust mites (HDMs) is a prevalent feature of atopic asthma. Pattern recognition receptors (PRRs), which recognize conserved features among pathogens, are expressed by multiple barrier tissue cells as well as innate immune cells from the myeloid lineage. In the traditional paradigm of allergic airway inflammation, epithelial cells are the initiators of the inflammatory cascade given that they sit at the interface between the host and the outside environment (Hammad and Lambrecht, 2015). Microbial activation of epithelial TLRs, which recognize microbial patterns, results in the production of cytokines and chemokines that recruit additional arms of the immune system. In a series of elegant experiments using bone marrow chimeras, Hammad *et al*. showed that TLR4 expression on airway epithelial cells was required for the recruitment, activation, and intraepithelial migration of dendritic cells in response to intratracheal instillations of LPS and HDM (Hammad et al., 2009a). Other studies have shown that the amount of LPS administered with antigen is the critical determinant of CD4+ T cell differentiation during the sensitization phase of allergen exposure (Hammad et al., 2009a; Tan et al., 2010). Eisenbarth *et al.* showed that low doses of inhaled LPS are necessary for the induction of Th2 responses to inhaled antigens during allergic sensitization, while higher doses of LPS induced a Th1 response (Eisenbarth et al., 2002; Tan et al., 2010). The absence of TLR4 on structural cells, but not on hematopoietic cells, abrogated Th2 allergic airway inflammation (Hammad et al., 2009b; Tan et al., 2010). While these results emphasize the importance of TLR4 and airway epithelium in murine models of airway inflammation, they do not sufficiently address other types of stimuli

and cell types that may affect allergen induced airway inflammation in humans.

Many allergens are often classified by the primary antigen implicated in the allergic response; for example, HDM (*Dermatophagoides pteronyssinus*) responses are often measured by Der p1 or Der p2 antibody responses. However, both Der p 2 and Der p 7 are capable of activating TLR4 signaling, and there are 30 proteins in HDM known to elicit IgE (Hales et al., 2006; Thomas et al., 2010). Furthermore, inhaled allergens can simultaneously trigger other PRRs. In fact, respiratory syncytial virus (RSV), a common cause of lower respiratory tract infections which signal via TLR3, exhibits a Th2 biased immune response in the lungs. Eosinophilia, mucus hyperproduction, and airway remodeling have been observed in human infants with severe RSV (Saravia et al., 2015). Moreover, numerous studies show high correlation between genetic variation in the *IL33* and *ST2* genes and risk for asthma and severe RSV disease (Akhabir and Sandford, 2010; Bønnelykke et al., 2013; Faber et al., 2012; Tulah et al., 2013). Airway epithelial cells of neonatal mice infected with RSV rapidly secrete IL-33 in response to live RSV infection and subsequently expand their lung ILC2 population (Saravia et al., 2015). Consistent with viral-induced IL-33, Le Goffic and colleagues found that infection of murine type II pneumocyte MLE-15 and human bronchial epithelial A549 lines with influenza resulted in the release of IL-33 (Le Goffic et al., 2011), and rhinovirus replication was significantly greater in human airway epithelial cells *in vitro*-cultured from asthmatic patients versus controls (Werder et al., 2018). While these studies suggest a synergistic interplay between viral and allergen exposure, they are limited by their use of murine models and *in vitro* cultures of human epithelium. Moreover, there are no studies demonstrating increased lung IL-33 levels in asthmatics following RSV infection, although there are studies that show RSV can trigger an asthma exacerbation.

Because epithelial cells are the biggest source of IL-33 in the murine lung, most studies have focused exclusively on examining their role in airway inflammation. This is problematic, however, as they fail to consider other sources of lung IL-33 that may be at play. Our data demonstrate that the lung vasculature abundantly expresses human *IL33*. Indeed, previous studies examining IL-33 expression using human endothelial cell cultures demonstrate that IL-33 is abundantly expressed in the nuclei of endothelial cells (Choi et al., 2009; Küchler et al., 2008; Moussion et al., 2008; Sundlisæter et al., 2012). In these experiments, nuclear IL-33 was down-regulated at the earliest onset of angiogenesis during wound healing or when endothelial cell cultures were treated with either tumor necrosis factor-α or vascular endothelial growth factor (Gautier et al., 2016). Thus, downregulation of IL-33 appears to be a feature of activated endothelial cells, with nuclear IL-33 expression as a marker of vascular quiescence and tissue homeostasis.

Given that the lung vascular network is the prime source of human *IL33*, it is essential to examine the vascular response in asthma when angiogenesis may be occurring. The lung vasculature also expresses PRRs (Asosingh et al., 2018). In mice, HDM protease allergens have been shown to penetrate the airway mucosa and activate PAR-2 on endothelial cells, and lung endothelial cells respond by upregulating levels of GM-CSF and IL-1α (Asosingh et al., 2018). In bronchial biopsies from asthmatics, airway vascularity correlates with disease severity (Li and Wilson, 1997; Salvato, 2001; Vrugt et al., 2000). Patients with severe asthma had significantly more vessels than those with moderate disease, and asthmatic capillaries and venules had oedematous walls and thickening of the subendothelial basement membrane (Salvato, 2001). Asthmatic vessels showed eosinophil recruitment, activation, and intravascular lysis. Higher eosinophil count was associated with more marked vascular structural changes, suggesting there

is an interaction between inflammation and vascular remodeling. Based on our finding of endothelial-derived IL-33 in humans and the importance of the innate response in asthma, we hypothesized that through its ability to produce IL-33, pulmonary endothelium may initiate and perpetuate the response to airway inflammation.

Here, we examine the IL-33 response of the endothelial cells to asthma triggers using human BAC (+) reporter mice. We find that signaling through TLR3 and TLR4 alone, via intratrachael instillations of Poly (I:C) and LPS to the lungs of BAC (+) mice, fails to alter the expression of human *IL33* reporter in lung endothelial cells. Surprisingly, HDM-induced allergic airway inflammation was accompanied by a significant reduction in human *IL33* reporter expression only in lung endothelium. Importantly, no observable change in human *IL33* reporter was detected in airway epithelial cells in response to sensitization and challenge with HDM. We hypothesized that IL-33 signaling, downstream of HDM administration, may regulate human *IL33* reporter expression in the lung vasculature. Indeed, administration of exogenous IL-33 led to a significant reduction in human *IL33* reporter expression. Analysis of *in vitro* primary human endothelial cell cultures treated with recombinant IL-33 demonstrated that exogenous IL-33 directly reduced the stability of nuclear mRNA and promoted changes to nucleosome landscape at the *IL33* locus. Together, these results suggest that IL-33 mediated responses to complex allergens involves human lung endothelium, not epithelium.

Results

House dust mite-stimulated allergic airway inflammation downregulates human IL33 reporter expression in lung endothelium while murine Il33 reporter expression remains unaffected.

Because anatomical barriers are constantly exposed to natural environmental allergens and are the first lines of defense against microbial particles, we examined the Crimson expression in lung structural cells following intratracheal instillation of LPS and Poly (I:C) to

mimic bacterial and viral responses, respectively (Jackson et al., 2014; Le Goffic et al., 2011; Michel, 2003; Tourdot et al., 2008). Airway instillation of LPS was given and twenty-four hours later, mice were sacrificed and cellular infiltration and Crimson expression was assessed using flow cytometry. LPS did not significantly affect Crimson expression in either vascular endothelium, lymphatic endothelium, fibroblasts, or airway epithelium (**Figure 4.1, top**), though exposure to LPS markedly increased the percentage of neutrophils in the BAC (+) lungs. Given that respiratory virus infection is a major cause of human asthma exacerbations, we examined the role of viral signaling on human *IL33* reporter expression using Poly (I:C), a synthetic analog of viral dsRNA and a TLR3 ligand. Repeated intratracheal instillations of Poly (I:C) to the lungs of BAC (+) mice failed to upregulate Crimson expression in endothelial, epithelial, or lung fibroblasts (**Figure 4.1, bottom**). These data suggest that neither TLR4 nor TLR3 signaling alone are sufficient to alter the expression of human *IL33* reporter in lung structural cells.

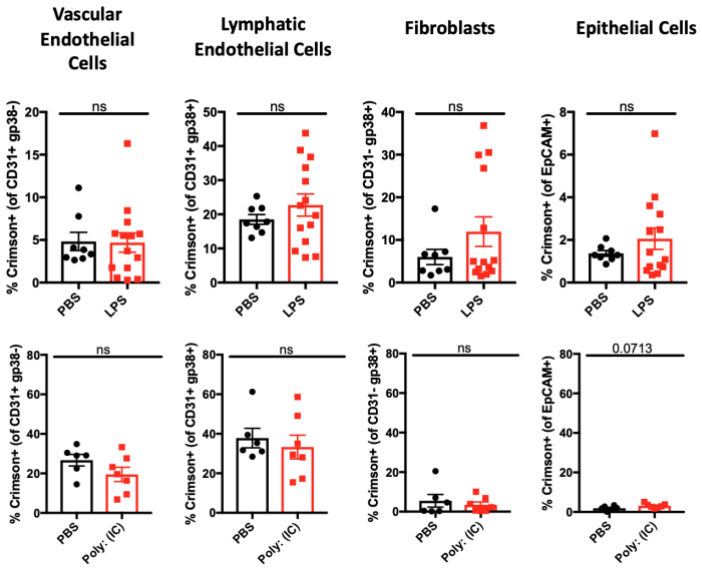

Figure 4.1 Expression of Crimson in BAC (+) lung following intratrachael administration of LPS and Poly (I:C). (Top) Flow cytometric analysis of whole lung digest from 6-8 week old BAC (+) mice intratracheally challenged with 0.63mg/kg of LPS or 50μL of PBS 24 hours before sacrifice (Bottom) Flow cytometric analysis of whole lung digest from 6-8 week old BAC (+) mice intratracheally challenged three times sequentially with 1mg/kg of Poly (I:C) or 50μL of PBS 9 hours before sacrifice. Data are representative of 3 or more experiments.

House dust mite is one of the most common allergens in the environment, and extracts from this allergen have been shown to elicit potent IL-33 and subsequent Th2 allergenicity in mice (Gregory and Lloyd, 2011; Hammad et al., 2009a; Thomas et al., 2010). Thus, to examine the role of HDM-induced allergic airway inflammation in the expression of human *IL33*, we intratrachaeally sensitized BAC (+) mice with 50μg of HDM and challenged four sequential times daily, a week later at 25μg. HDM administration led to airway inflammation (**Figure 4.2**) and a marked increase in hematopoietic cells that did not express Crimson (**Figure 4.3**). Our previous studies demonstrate that HDM drives Th2 inflammation via murine IL-33 production in mice. However, HDM-induced allergic airway inflammation was accompanied by a significant reduction in Crimson expression in total lung tissue of BAC (+) mice. When we quantified the amount of Crimson expression observed by microscopy in the entire left lobe of BAC (+) mice sensitized with HDM, we found a significant reduction in Crimson per total lung surface area (**Figure 4.4 top)** Flow cytometric analysis of lung stromal cells confirmed this finding, where the percentage of CD45- Crimson expressing cells was far less following HDM compared to PBS controls **(Figure 4.4 bottom**) (Tjota et al., 2014). While our previous studies demonstrate that HDM drives Th2 inflammation via murine IL-33 production, HDM induced allergic airway inflammation was accompanied by a significant reduction in the human *IL33* reporter. When we interrogated the subsets that downregulated human *IL33* reporter expression in response to HDM via flow cytometry, we observed significant reductions in the vascular endothelial and lymphatic endothelial cell populations both in percentage and total cell number (**Figure 4.5A and 4.5B**). Moreover, this effect seemed to be limited to lung endothelial cells as epithelium and fibroblasts were unaffected. Endothelial downregulation of Crimson was not due to increased cell death, as HDM treated mice had comparable levels of live lung cells nor to an increase in total endothelial

cell numbers **(Figure 4.5C)**. Together, these results suggest that exposure to complex allergens, though not TLR signaling alone, drives down expression of human *IL33* reporter in lung endothelium.

We previously observed distinct cellular sources between murine and human *IL33* reporter in naïve mice, thus we hypothesized that the cellular sources governing HDM-induced allergic airway inflammation would be distinct between human IL-33 and murine IL-33. Endothelial cells again constituted a negligible proportion of GFP-expressing cells in the lung while lung epithelium was the predominant source of GFP. Further, while lung endothelium responded to allergen challenge by downregulating human *IL33* reporter, no observable change in GFP was found in lung endothelial compartments after allergen sensitization (**Figure 4.6**). While HDM administration significantly decreased Crimson levels in lung stromal cells, epithelial cells did not downregulate GFP expression to allergen treatment despite being the predominant source of murine *Il33* at homeostasis. Thus, while murine epithelium and human endothelium may represent reciprocal sources of IL-33 in each species, only lung endothelium directly responds to allergen exposure.

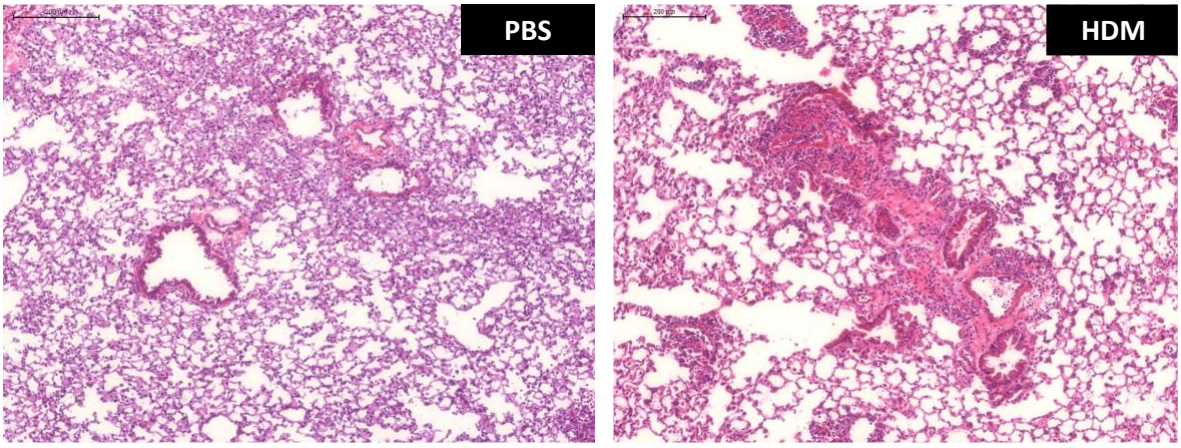

Figure 4.2 H&E staining of left lobe of 6-8 week old BAC (+) mice intratracheally sensitized and challenged with PBS or HDM.

BAC (+) mice were sensitized with 50µL of PBS or 50µg/mouse of HDM and challenged four sequential times a week later with PBS or 25µg/mouse of HDM. Data are representative of 3 or more experiments.

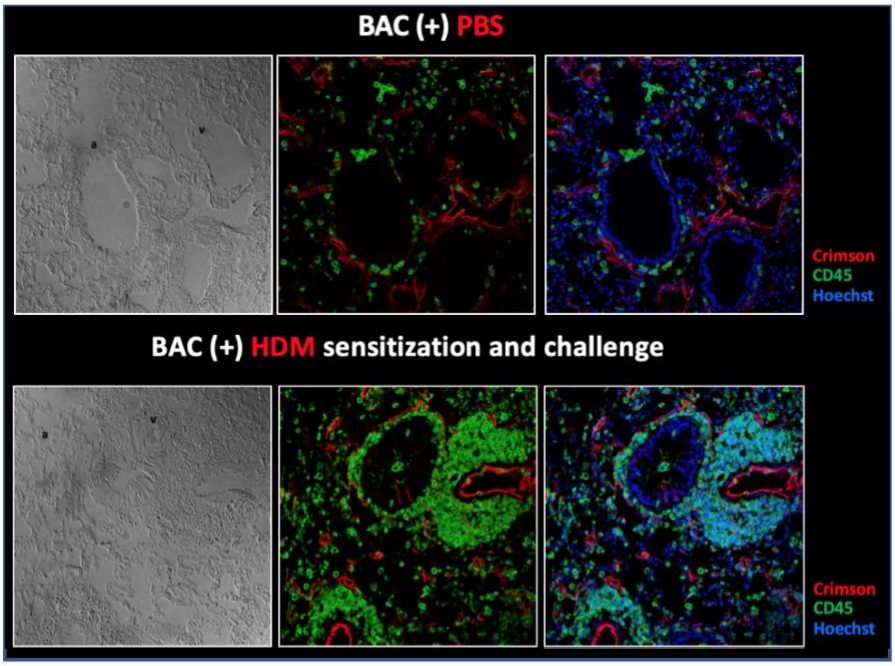

Figure 4.3 Confocal microscopy of lungs from BAC (+) mice sensitized and challenged with PBS or HDM. BAC (+) mice were sensitized with 50µL of PBS or 50µg/mouse of HDM. Green indicates CD45 (top) or LYVE1 (bottom) cells. Red indicates Crimson expression. Blue indicates Hoescht staining.

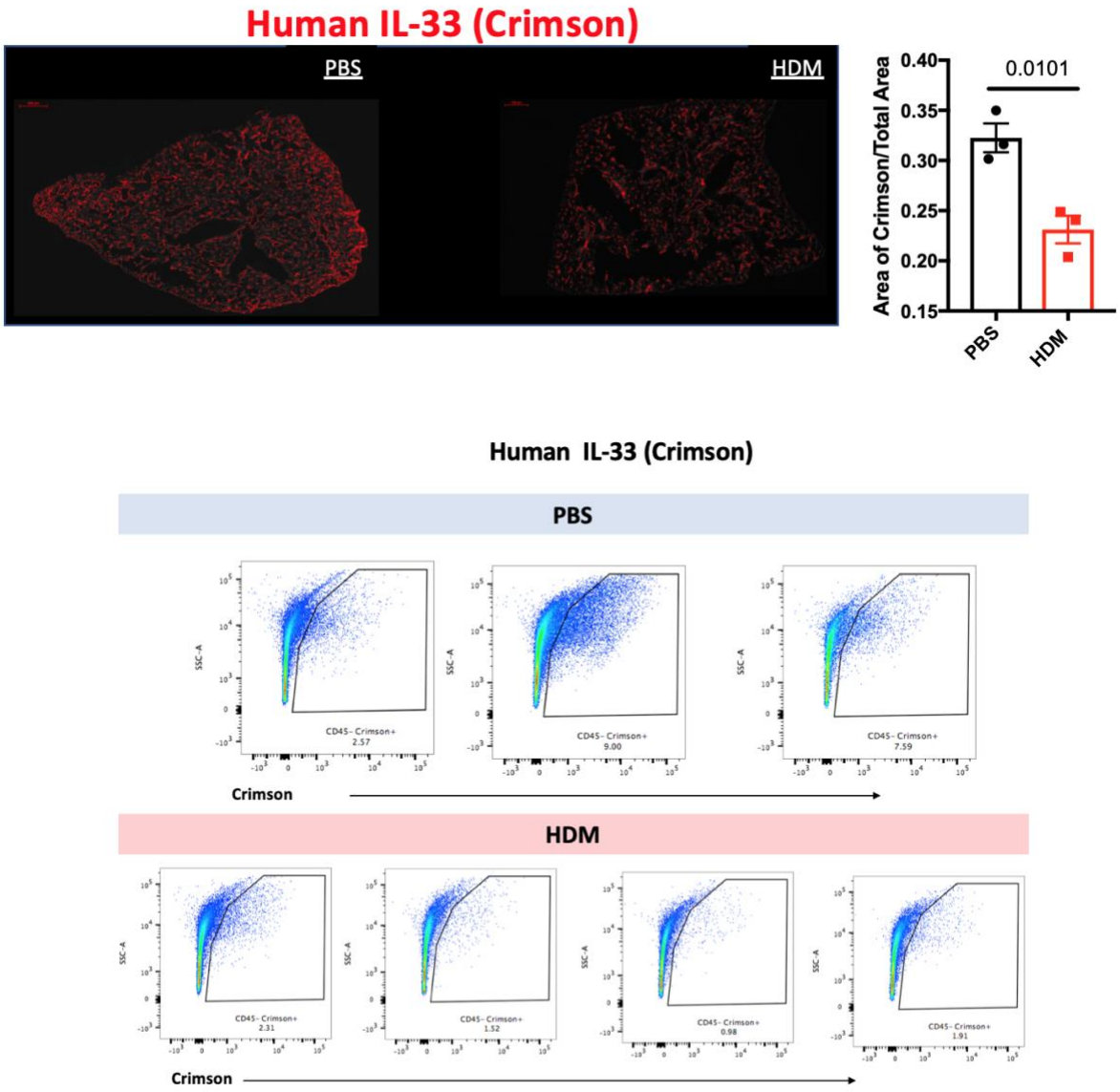

Figure 4.4 Expression of Crimson in BAC (+) lung endothelium following intratrachael administration of house dust mite. (Top) Quantification of Crimson and GFP fluorescence of the left lobe of BAC (+) and GFP$^{+/-}$ mice intratrachaeally sensitized with 50μL of PBS or 50μg/mouse of HDM and challenged four sequential times a week later with PBS or 25μg/mouse of HDM. (Bottom) Flow cytometric analysis of whole lung digest from HDM sensitized and challenged F1 mice. Data is representative of 3 or more experiments.

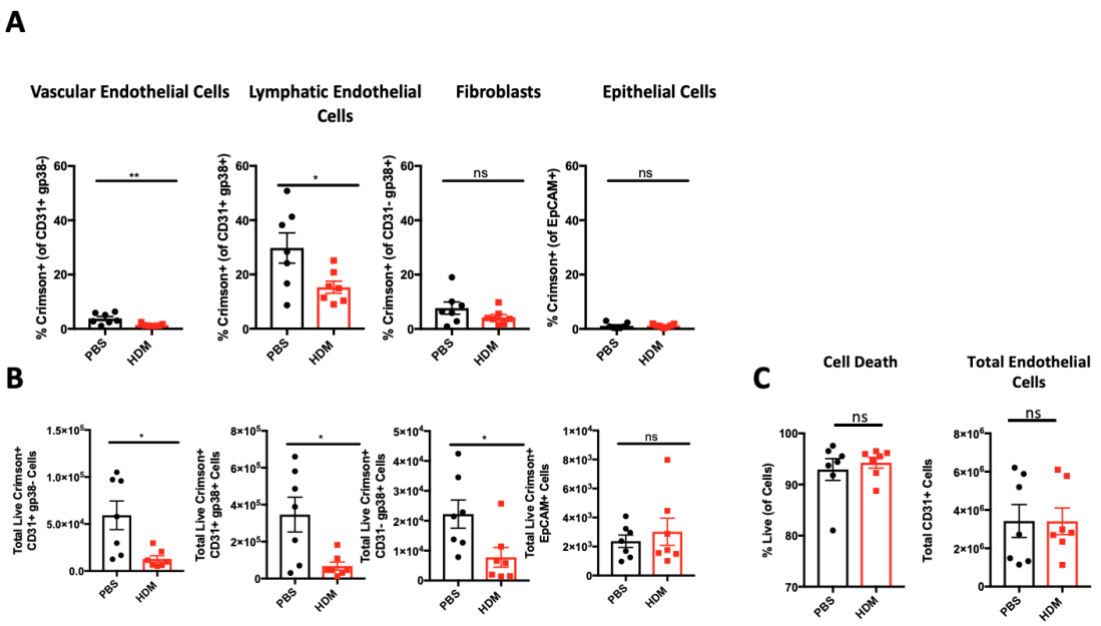

Figure 4.5 HDM significantly downregulates human *IL33* reporter expression in lung endothelium. (A) Flow cytometry analysis and (B) total cell numbers from whole lung digest from 6-8 week old BAC (+) mice intratrachaeally sensitized with 50mL of PBS or 50mg/mouse of HDM and challenged four sequential times a week later with PBS or 25mg/mouse of HDM. Data are representative of 3 or more experiments. (C) Viability quantification of all cells using flow cytometry of whole lung digest from 6-8 week old BAC (+) mice intratracheally sensitized with 50µL of PBS or 50µg/mL of HDM and challenged four sequential times a week later with PBS or 25µg/mL of HDM. Data are representative of 3 or more experiments. Viability was determined by Zombie Aqua™ Fixable Viability Kit.

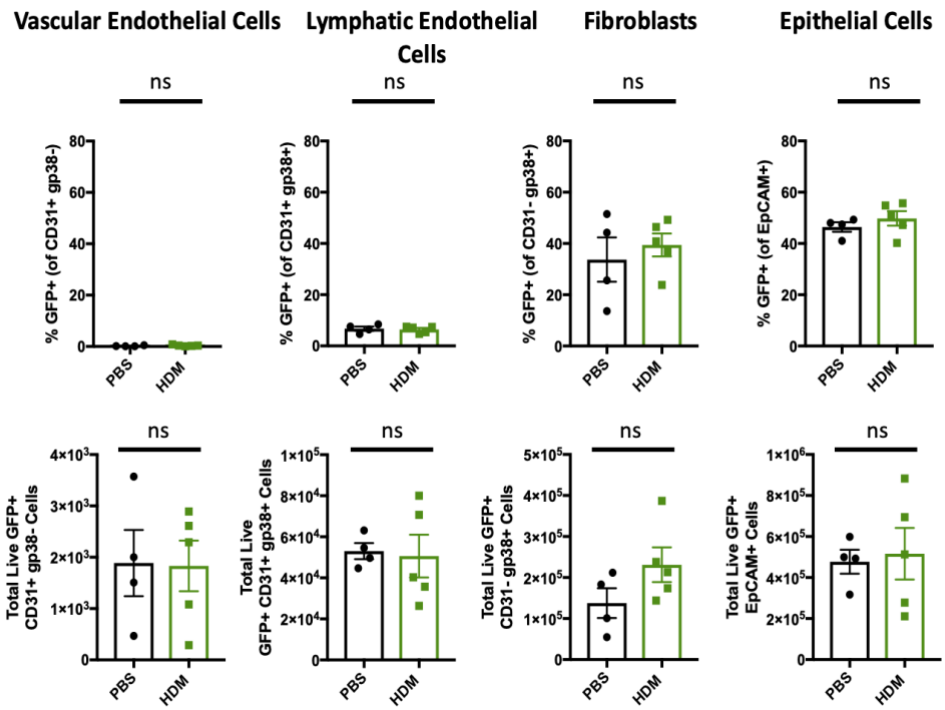

Figure 4.6 Murine *Il33* reporter expression in the lungs remain sunchanged after HDM sensitization and challenge. (Top) Flow cytometric analysis of percentage (bottom) and total cell numbers of whole lung digest from 6-8 week old GFP$^{+/-}$ mice intratracheally sensitized with 50μL of PBS or 50μg/mL of HDM and challenged four sequential times a week later with PBS or 25μg/mL of HDM. Data are representative of 3 or more experiments.

Novel regulatory region in human IL33 locus is required for endothelial cell specific response during allergic airway inflammation

Previously, we showed a regulatory region in the *IL33* locus that is necessary for Crimson expression in lung microvasculature. Because of this, we inquired whether it played a role in human *IL33* reporter expression in lung endothelium following airway exposure to bacterial and viral mimetics as well as allergen challenge. We observed no marked change in Crimson expression by either endothelial, epithelial, or lung fibroblasts to either LPS or Poly (I:C) (**Figure 4.7**). However, when we sensitized DEL-BAC (+) mice with 50μg/mL of HDM and challenged seven days later at 25μg/mL, Crimson expression was not significantly altered in either lung vascular endothelial cells or lymphatic endothelial cells despite comparable levels of

allergic airway inflammation with BAC (+) mice (**Figure 4.8**). This regulatory region, therefore, not only governs human *IL33* reporter expression at baseline but is necessary for its downregulation in lung endothelial cells following allergen challenge.

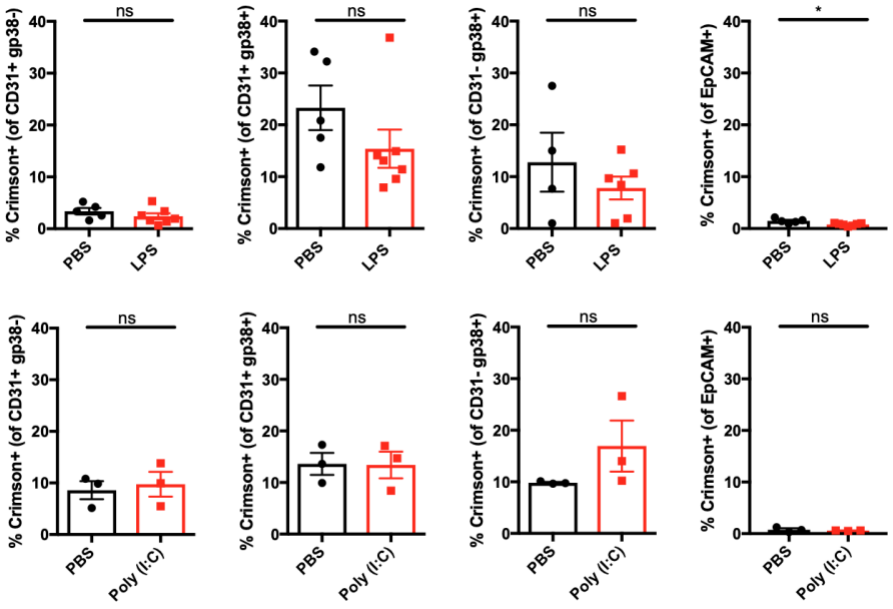

Figure 4.7 Expression of Crimson in DEL-BAC (+) lung following intratrachael administration of LPS and Poly (I:C). (Top) Flow cytometric analysis of whole lung digest from 6-8 week old BAC (+) mice intratracheally challenged with 0.63mg/kg of LPS or 50µL of PBS 24 hours before sacrifice. (Bottom) Flow cytometric analysis of whole lung digest from 6-8 week old BAC (+) mice intratracheally challenged three times sequentially with 1mg/kg of Poly (I:C) or 50µL of PBS 9 hours before sacrifice. Data are representative of 3 or more experiments.

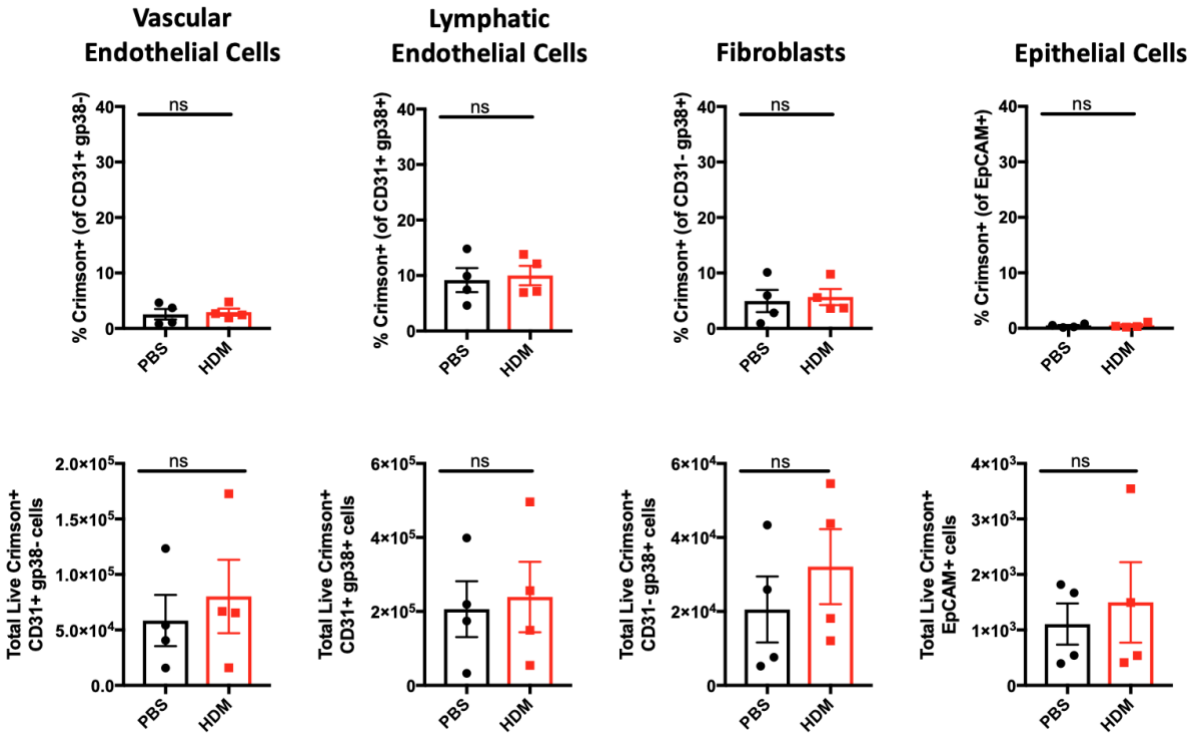

Figure 4.8 Pulmonary vasculature from DEL-BAC (+) mice fails to downregulate Crimson expression after HDM administration. (Top) Flow cytometric analysis of whole lung digest of 6-8 week old DEL-BAC (+) intratrachaelly sensitized with 50µL of PBS or 50µg/mL of HDM and challenged four sequential times a week later with PBS or 25µg/mL of HDM. Data are representative of 3 or more experiments. (Bottom) Total cell numbers of whole lung digest from 6-8 week old DEL-BAC (+) mice intratrachaelly sensitized with 50µL of PBS or 50µg/mL of HDM and challenged 4 sequential times a week later with PBS or 25µg/mL of HDM. Data are representative of 3 or more experiments.

IL-33 administration is sufficient to downregulate Crimson expression in BAC (+) mice.

In our labs and others, HDM sensitization and challenge of mice have demonstrated downstream IL-33 production in the lung. We thus inquired if endogenous murine IL-33 production downstream of HDM administration could alter human *IL33* reporter expression in endothelium. To test this, we administered recombinant IL-33 down the airways of BAC (+) mice and examined Crimson levels at both nine and twenty-four hours later. Remarkably, administration of a single dose of IL-33 was able to reproduce- endothelial downregulation of Crimson observed following HDM sensitization and challenge **(Figure 4.9**). This downregulation

occurred at the transcriptional level, as *Crimson* mRNA was significantly diminished twenty-four hours following IL-33 administration while GFP expression, again, remained unaffected by IL-33 (**Figure 4.10**). Importantly, recombinant IL-33 administration at 50ng/mouse down the airways of DEL-BAC (+) mice failed to downregulate Crimson expression in lung endothelial cells, suggesting that the upstream regulatory region important in human *IL33* reporter expression at baseline also governed human *IL33* reporter responses in lung endothelium during inflammatory challenge (**Figure 4.11**).

Given the downregulation of Crimson by recombinant IL-33, we hypothesized that IL-33 protein was acting directly on lung endothelial cells that express ST2. When we examined expression of ST2 on endothelial cells, however, we found that that lung endothelial cells from BAC (+) mice did not express ST2 during homeostasis (**Figure 4.12**). Furthermore, while hematopoietic cells responsed to IL-33 administration by upregulating ST2 expression, endothelial expression of ST2 remained low after IL-33 administration and was and comparable to ST2$^{-/-}$ mice. Therefore, Crimson downregulation by lung endothelium may not be due to direct IL-33/ST2 signaling on endothelial cells.

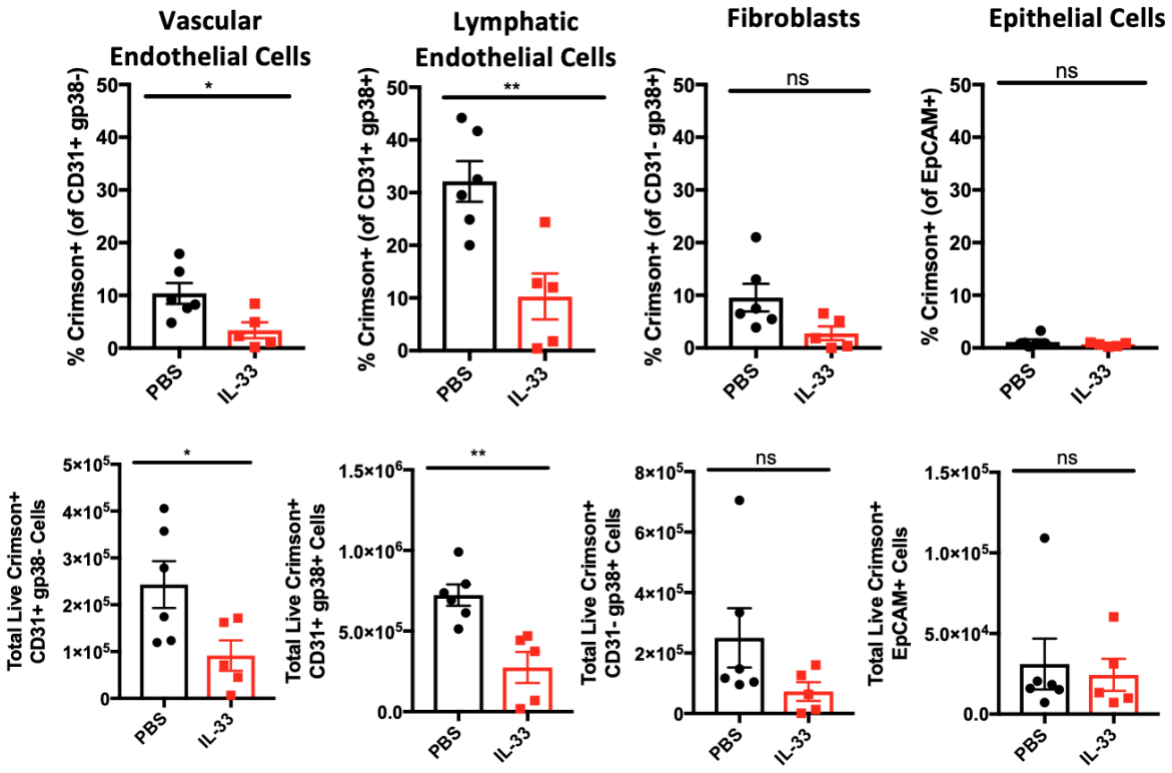

Figure 4.9 IL-33 administration downregulates Crimson expression *in vivo*. (A) Flow cytometric analysis and (B) total cell numbers from whole lung digest from 6-8 week old BAC (+) mice intratracheally treated with 100ng of recombinant murine IL-33 and sacrificed 9 hours later. Data are representative of 3 or more experiments.

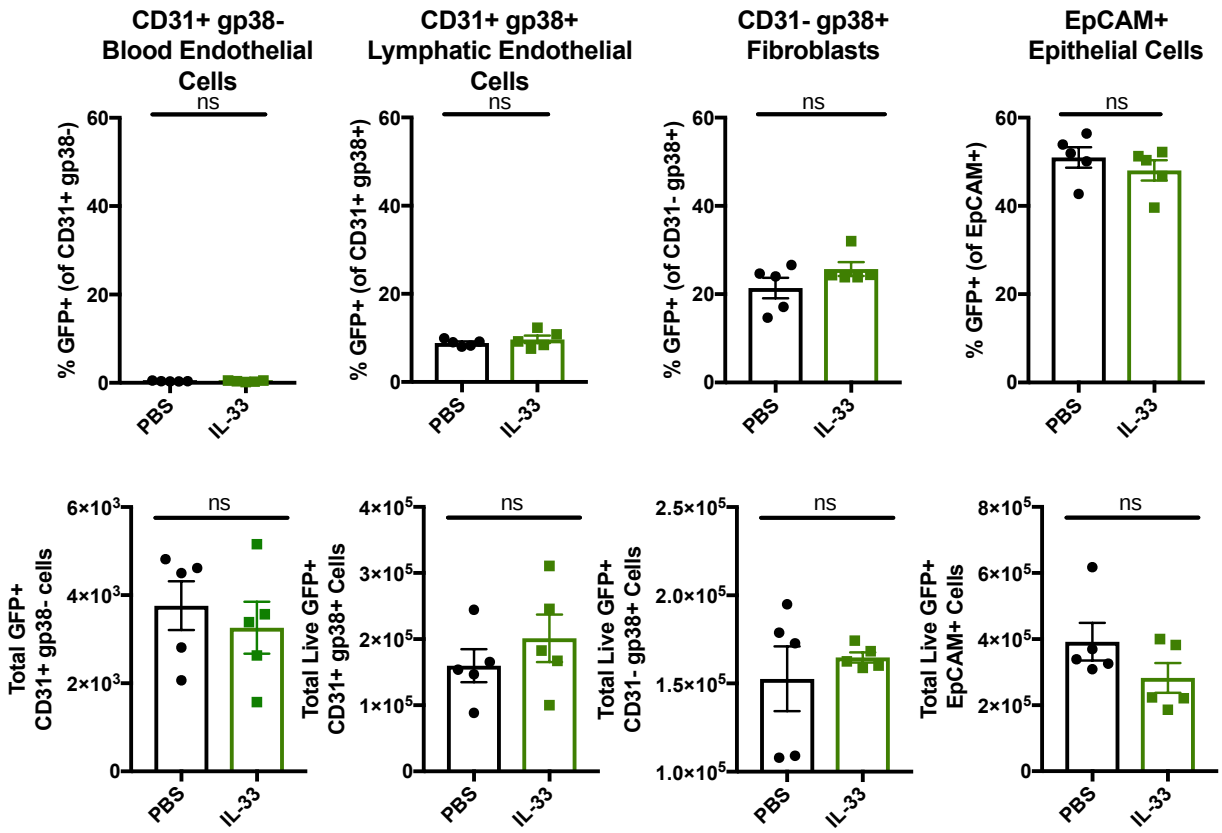

Figure 4.10 IL-33 administration does not affect murine *Il33* reporter expression *in vivo*. (Top) Flow cytometric analysis and (bottom) total cell numbers of whole lung digest from 6-8 week old GFP$^{+/-}$ mice intratracheally treated with 100ng of recombinant murine IL-33 and sacrificed 9 hours later. Data are representative of 3 or more experiments.

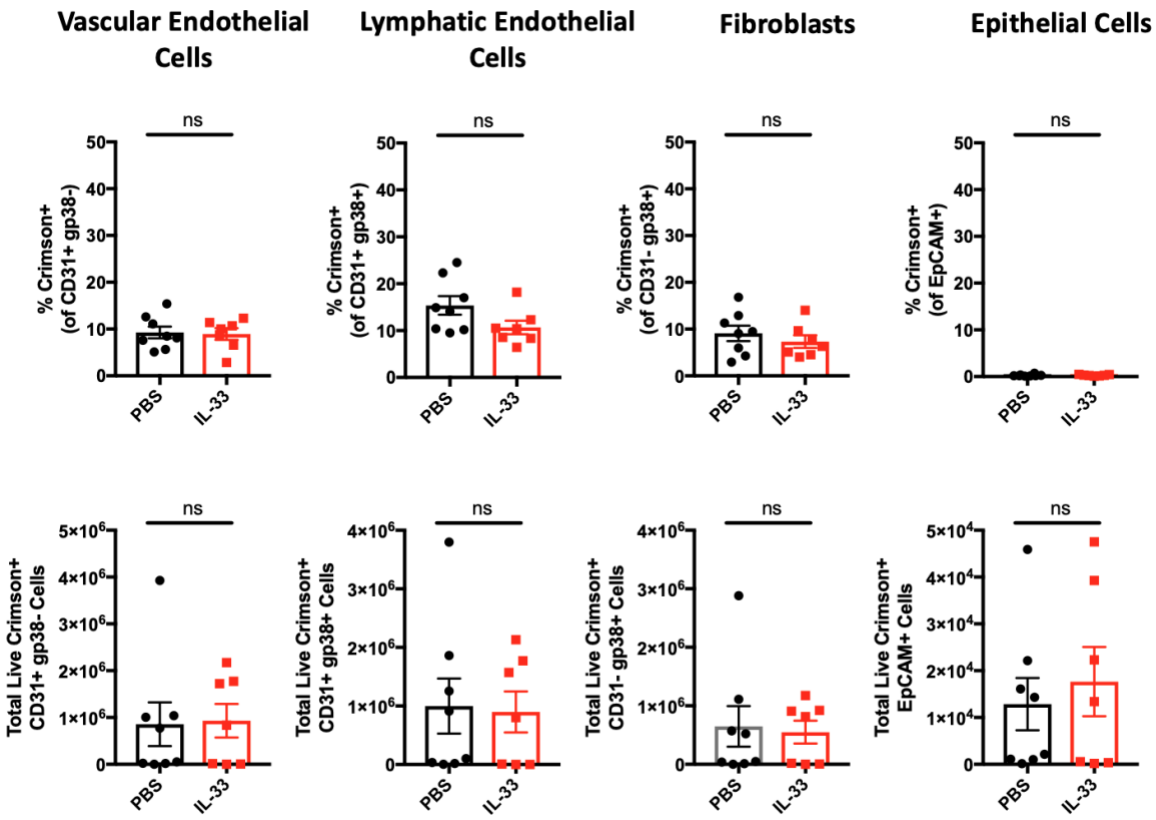

Figure 4.11 Pulmonary vasculature in DEL-BAC (+) mice does not respond to IL-33 administration *in vivo*. (Top) Flow cytometric analysis and (Bottom) total cell numbers from whole lung digest from 6-8 week old DEL-BAC (+) mice intratracheally treated with 100ng of recombinant murine IL-33 and sacrificed 9 hours later. Data are representative of 3 or more experiments.

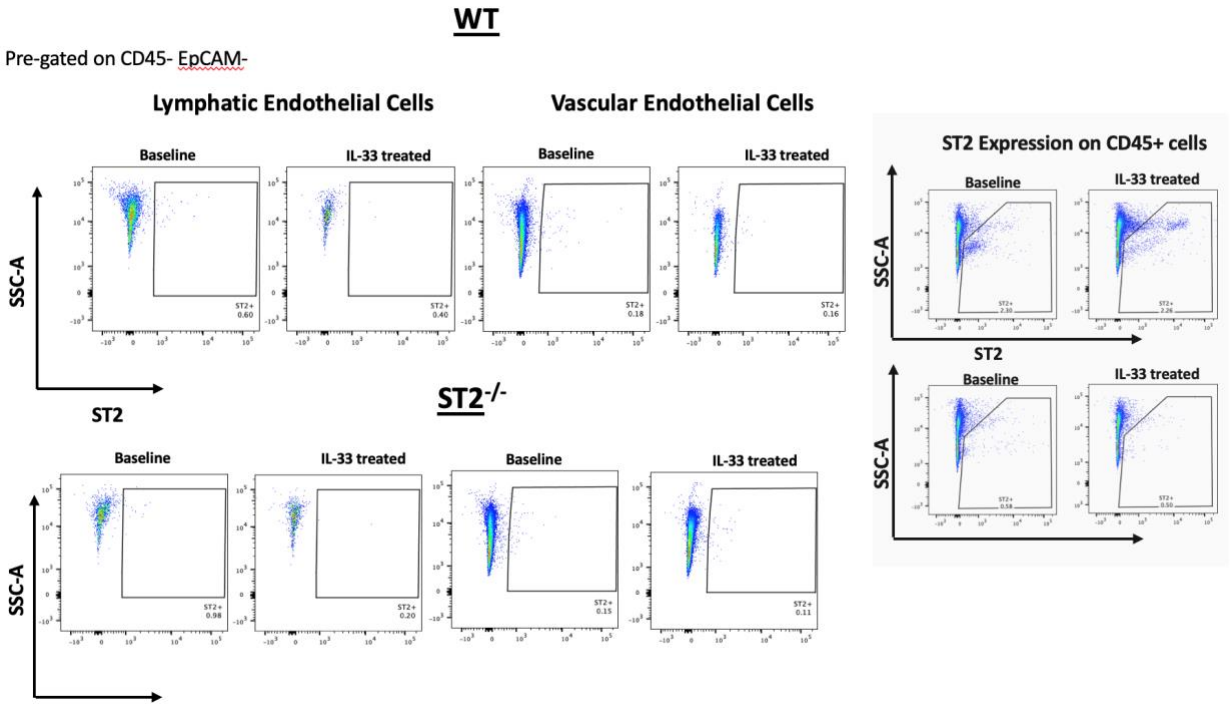

Figure 4.12 ST2 is not expressed in the lung vasculature of BAC (+) mice. Flow cytometric analysis of whole lung digest from GFP$^{-/-}$ and ST2$^{-/-}$ mice intratracheally treated with 100ng of recombinant murine IL-33.

IL-33 administration is sufficient to downregulate IL33 expression in primary human lung endothelium.

To assess whether the downregulation of *IL33* mRNA by IL-33 protein occurred in human endothelium, we turned toward *in vitro* cultures of various human endothelial cells which include HUVECs (**Figure 4.13A**), human lymphatic endothelial cells (**Figure 4.13B**), and primary endothelial cells from human lungs (**Figure 4.13C**). In all three cell types, recombinant IL-33 treatment led to a significant decrease in human *IL33* mRNA levels. Furthermore, when we examined ST2 expression on human endothelial cells, we found that ST2 was expressed highly by human endothelial cells. The soluble transcript sST2, was expressed preferentially compared to membrane bound ST2L which fell below the limit of detection (**Figure 4.14**). While this is in stark contrast to ST2 expression on murine endothelium, which was negative for ST2 expression, these data show that human endothelial cells are capable of directly responding to

extracellular IL-33 and downregulating *IL33* transcripts in response.

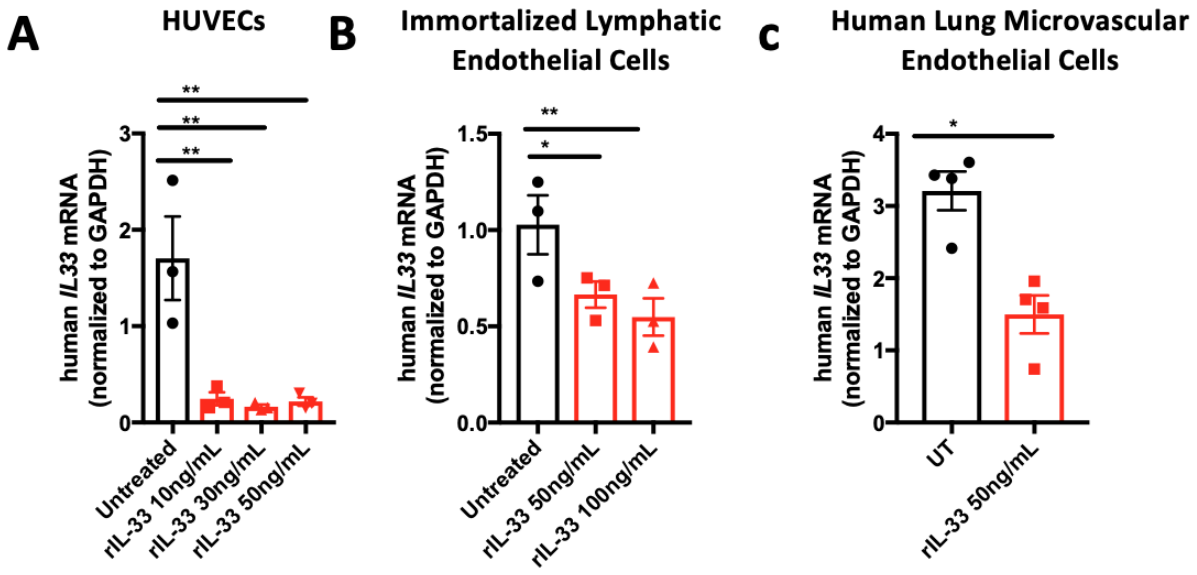

Figure 4.13 Exogenous IL-33 downregulates endogenous *IL33* transcript levels in human endothelium. (A) Human *IL33* message was detected by qPCR in 2x10$_4$ pooled Human Umbilical Vein Endothelial Cells (HUVEC) purchased from Lonza and treated 9 hours *in vitro* with recombinant IL-33. (B) Human *IL33* message was detected by qPCR in 2x10$_4$ conditionally immortalized dermal LECs treated 9 hours *in vitro* with recombinant IL-33. (C) Human *IL33* message was detected by qPCR in 2x10$_4$ Human Lung Microvascular Endothelial Cells (HMVEC-L) purchased from Lonza and treated 9 hours *in vitro* with recombinant IL-33.

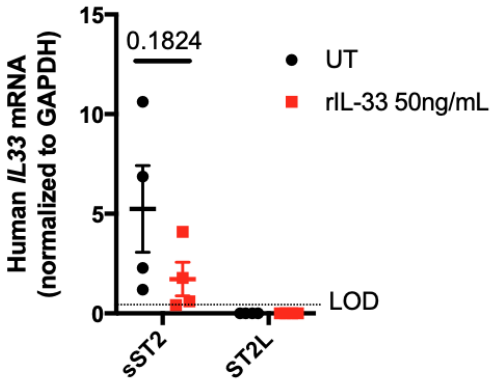

Figure 4.14 Human Umbilical Vein Endothelial Cells express soluble ST2. Human *ST2* message was detected by qPCR in 2x10$_4$ pooled Human Umbilical Vein Endothelial Cells (HUVEC) and treated 9 hours *in vitro* with recombinant IL-33.

Exogenous IL-33 treatment modulates nuclear IL33 mRNA stability in human endothelial cells.

To understand how the *IL33* locus is altered by exogenous IL-33, Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) was used to define changes in global genome accessibility. To our surprise, examination of chromatin accessibility following IL-33 treatment revealed little change at the endogenous *IL3*3 locus. Quantitative analysis of genome accessibility resulted in 609 peaks that were significantly upregulated and 286 peaks downregulated out of 137,281 total accessible regions following IL-33 treatment (FDR<0.05) (**Figure 4.15A**). IL-33 treatment led to a reduction in global accessibility, with more peaks downregulated after treatment than upregulated (**Figure 4.15B and 4.15C**). However, accessibility at the *IL33* locus was not significantly regulated by IL-33 treatment (**Figure 4.15D**). While accessibility was similar between untreated and treated samples at the *IL33* locus, there were differences in nucleosome positioning. As seen in **Figure 4.16A**, nucleosomes were present in the untreated group at a region upstream of Exon 1 (denoted region A) that were absent following IL-33 treatment. The second region (denoted region B) is close to a known promoter within the *IL33* gene (**Figure 4.16B**). To determine the potential significance of these IL-33 treatment-associated nucleosome-free sites, we analyzed these regions for putative TF motifs. In region A, the most significant TF motifs observed were OCT1 (POU2F1) and GATA-3. Interestingly, we previously demonstrated that OCT1 binds to the *IL33* locus and may be a player in the regulation of the cytokine (Swanson and Decker et al., manuscript in preparation). Region B contained the motifs for YY1 and XBP1, transcription factors that have been implicated in chromatin remodeling complexes. However, our ATAC-Seq results demonstrate that extracellular IL-33 does not alter the accessibility of the endogenous *IL33* locus and as such the chromatin landscape remains largely unaffected by treatment.

Since dramatic changes were found in *IL33* expression levels in HUVECs after IL-33 treatment, but no significant alterations to the chromatin were observed by ATAC-seq, we posited that extracellular IL-33 could alter mRNA transcript stability. To test *IL33* mRNA stability, we compared message that was directly being transcribed in the nucleus with other nuclear compartments and the cytoplasm. HUVECs were biochemically fractionated with a forcing buffer to separate the soluble and loosely bound material from the chromatin pellet. The chromatin pellet contained nascent RNA still tethered to the chromatin while the soluble nuclear extract is enriched for newly synthesized mRNA transcripts within the nuclear compartment not yet exported into the cytoplasm. Upon IL-33 treatment, cytoplasmic *IL33* mRNA was dramatically reduced while the chromatin associated transcripts remained comparable to untreated cells (**Figure 4.17**). Furthermore, the soluble nuclear extract, which contains *IL33* mRNA that is not bound to the chromatin but contained within the nuclear envelope, also demonstrated a reduction in *IL33* message. Despite dramatic reductions in transcript levels in the soluble nuclear extract and cytoplasmic fractions, no changes were observed in chromatin-associated transcript levels. Taken together, our data demonstrate that exogenous IL-33 alters mRNA stability post-transcriptionally in the soluble nuclear extract and in the cytoplasm, but transcripts that remain tethered to the chromatin after treatment are unaffected by treatment. These data are in accordance with our ATAC-Seq results where no significant changes to accessibility were found in the *IL33* locus after exogenous IL-33 treatment.

Because the effects of IL-33 treatment were observed post-transcriptionally, but no effect occurred at the *IL33* locus, we hypothesized that another protein, which was involved in *IL33* messages stability, was affected by IL-33 treatment. To test this hypothesis, HUVECs were treated with the translational inhibitor cycloheximide, which abrogates the translocation step in

protein synthesis, before the addition of recombinant IL-33 to the culture. As before, there was a decrease in *IL33* mRNA expression with exogenous IL-33 treatment (**Figure 4.18**), but surprisingly, we saw a similar response with just cycloheximide treatment alone. Pretreatment with cycloheximide before the addition of IL-33 did not have an additive effect on *IL33* message, indicating that new protein synthesis was required for *IL33* mRNA levels. Moreover, because treatment with just cycloheximide or IL-33 alone yielded similar results without an added effect together, it is likely these two work in a similar pathway to downregulate *IL33*.

Taken together, these data demonstrate that IL-33 treatment does not affect nascent transcription of *IL33* since chromatin-associated transcripts were unaffected by treatment and that the ATAC-seq data found no significant changes at the *IL33* locus. Rather, the IL-33 treatment decreases the level of endogenous *IL33* transcripts that are found in the cytoplasm and nuclear envelope. The effect on the nuclear *IL33* stability may be due to enhancement of the degradation machinery found within the nuclear envelope and cytoplasm since IL-33 treatment while blocking protein synthesis failed to change mRNA levels compared to IL-33 treatment alone.

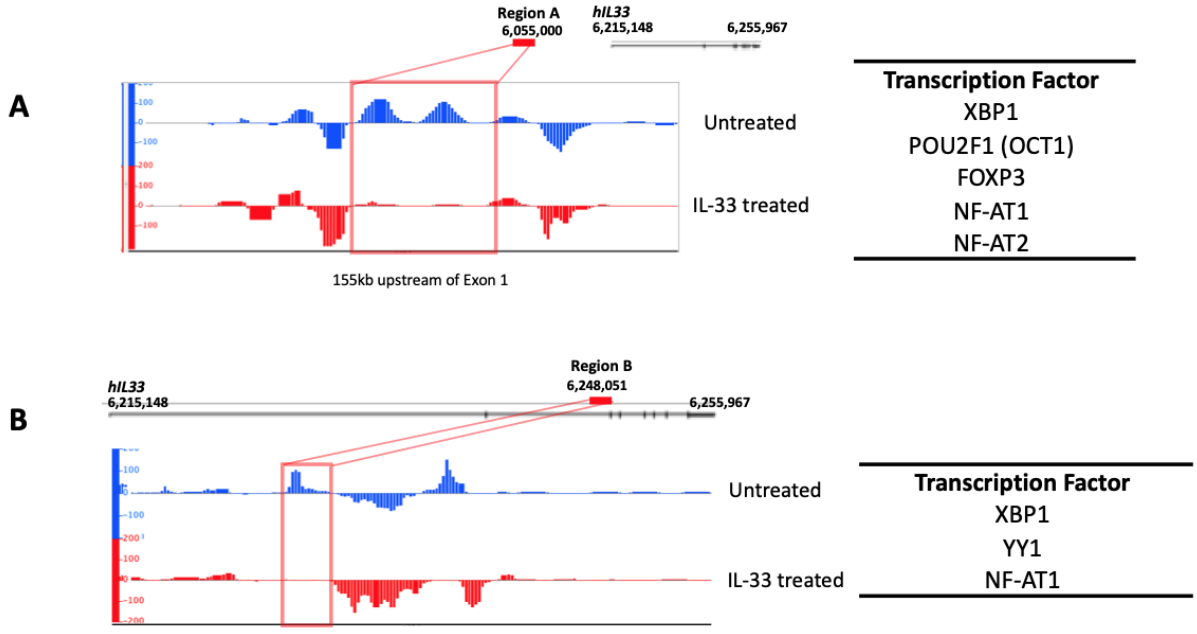

Figure 4.15 Identification of differential nucleosome positioning in the *IL33* locus with ATAC-seq. (A) Region of open nucleosome positioning 5' of the *IL33* locus after stimulation *in vitro* with recombinant IL-33 and (right) enrichment of known transcription factor motifs in region A as predicted by using the motif analysis package HOMER (B) Region of open nucleosome positioning within the *IL33* locus after stimulation *in vitro* with recombinant IL-33 and (right) enrichment of known transcription factor motifs in region B as predicted by using the motif analysis package HOMER.

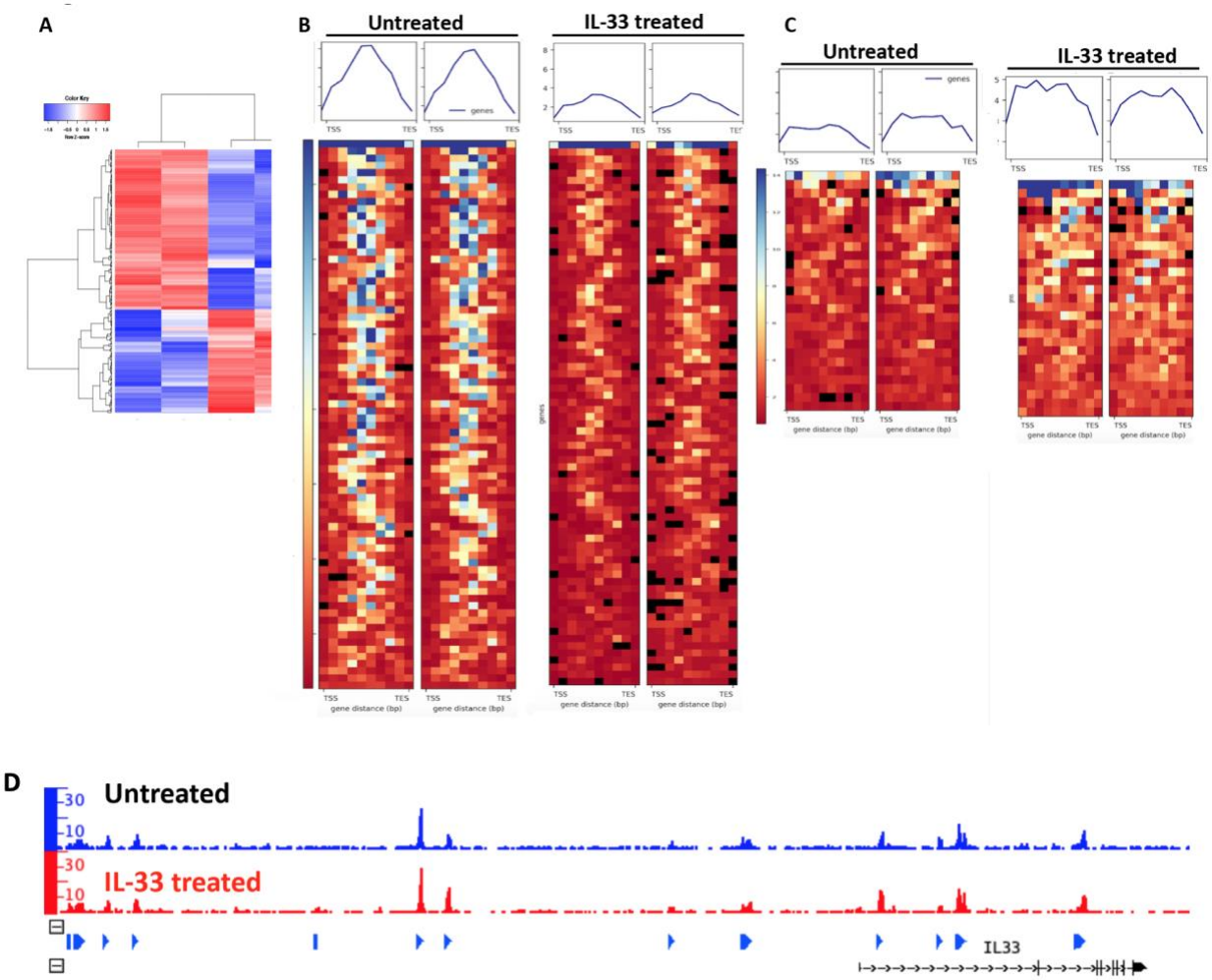

Figure 4.16 Extracellular IL-33 does not alter accessibility at the *IL33* locus. (A) Number of differential chromatin accessibility peaks (log2(FC) > 2, false discovery rate [FDR] <0.05) after *in vitro* IL-33 treatment. (B) Heatmap of genome wide differential chromatin accessibility peaks significantly (log2(FC) > 2, false discovery rate [FDR] <0.05) downregulated after *in vitro* IL-33 treatment. (C) Heatmap of genome wide differential chromatin accessibility peaks significantly (log2(FC) > 2, false discovery rate [FDR] <0.05) upregulated after *in vitro* IL-33 treatment. (D) Chromatin accessibility peaks in the endogenous *IL33* locus after *in vitro*. IL-33 treatment.

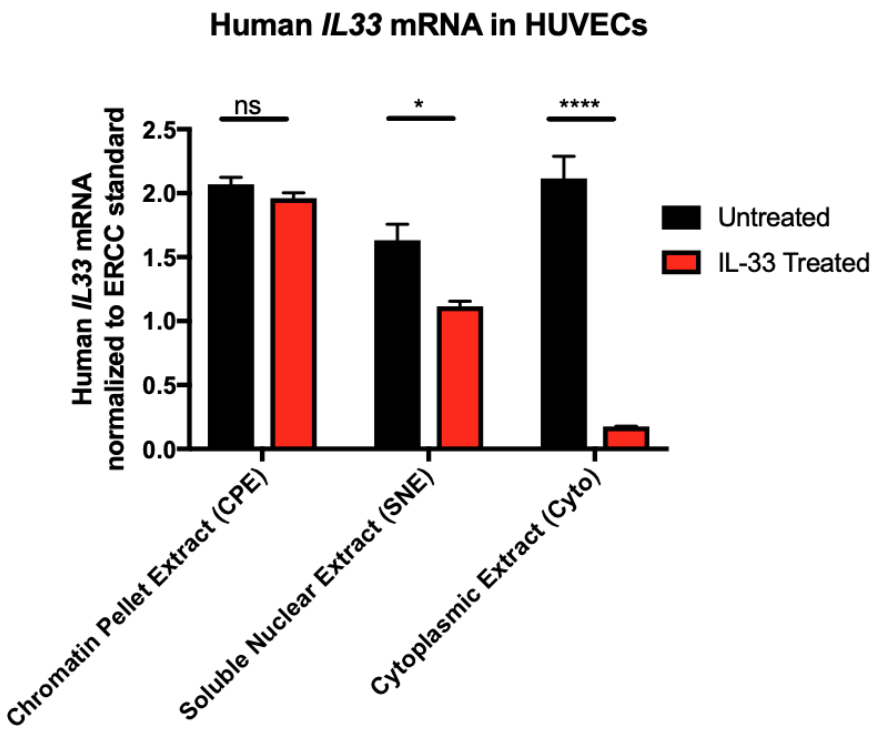

Figure 4.17 Recombinant IL-33 alters *IL33* transcript stability in the soluble nuclear extract and in the cytoplasmic compartment of the HUVECs. Briefly, $2x10_4$ pooled Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza and treated 9 hours *in vitro* with recombinant IL-33. Cell pellets were rinsed in 1× PBS/1 mM EDTA and fractionated into the chromatin pellet extract (CPE), the soluble nuclear extract (SNE) or the cytoplasmic extract (cyto).

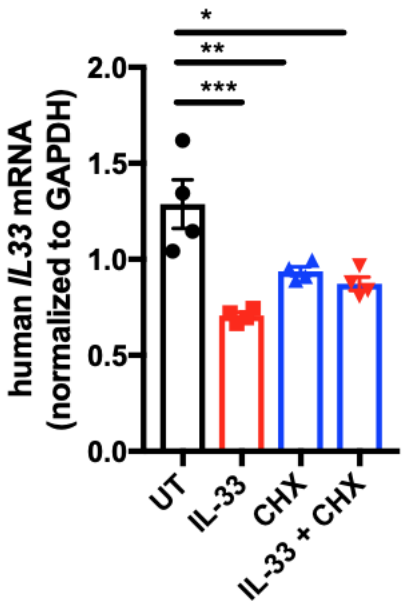

Figure 4.18 Cycloheximide treatment downregulates *IL33* transcript levels in HUVECs. Human *IL33* message was detected by qPCR in 2x104 pooled Human Umbilical Vein Endothelial Cells (HUVEC) purchased from Lonza and treated for 30 minutes in vitro with 10µg of cycloheximide (CHX) before the addition of 100ng of recombinant IL-33 to culture for 6 hours.

Discussion

House dust mite extract is a potent allergen in atopic patients, but how the allergen modulates IL-33 responses in the human lung remains unknown. Here, we show human *IL33* reporter levels are dramatically reduced in pulmonary endothelial cells following house dust mite exposure while murine *Il33* reporter levels remain unaffected. Exogenous IL-33 administration alone is sufficient to recapitulate this phenotype in the lungs of BAC (+) mice, as well as primary human endothelial cells, demonstrating that IL-33 can regulate its own expression. Analysis of human *IL33* transcript levels following extracellular IL-33 demonstrate that reduced transcript levels are due to *IL33* mRNA stability. Further, cycloheximide pretreatment of human endothelial cells before the addition of IL-33 also reduced endogenous *IL33* mRNA implicating *de novo* protein synthesis of a tertiary protein in the regulation of *IL33* mRNA. Together, our data indicate that extracellular IL-33 protein is able to autoregulate intracellular *IL33* levels in endothelial cells by decreasing mRNA stability.

Given that house dust mite protease allergens have been shown to penetrate the airway mucosa and directly activate endothelial cells in mice, it is possible the HDM proteases are key to IL-33 responses. Cayrol *et al*. demonstrated that full length IL-33 is rapidly cleaved in response to allergen proteases (Cayrol et al., 2018). The cleaved form of IL-33 can be 10-fold more potent in activating ILC2 than the full-length protein. Experiments preventing cleavage of full-length protein via protease inhibitors fail to induce Th2 inflammation, suggesting that the protease activity of the HDM is a key step in the development of inflammation. Moreover, full-length IL-33 functions as a biochemical sensor of allergen proteases, as an antibody against the central protease 'sensor' domain of human IL-33 reduced IL-33 activation. *In vivo*, blocking the sensor domain reduced the activation of ILC2s, eosinophilia, and mucus production (Cayrol et

al., 2018). Similarly, in *Alternaria* sensitization, it is the intrinsic serine protease activity that elicits rapid release of IL-33 into the airways of mice (Snelgrove et al., 2014). Therefore, cleavage by allergen proteases is an important molecular mechanism for the induction of allergic inflammation. Loss of function mutations in *IL33* that result in significantly reduced levels of *IL33* mRNA transcript lead to lower blood eosinophil counts and asthma protection (Smith et al., 2017). Perhaps genetic modifications in the abundance of full-length IL-33 and sensitivity to allergen proteases determine the initiation and severity of asthma. In our human *IL33* reporter mice, it is possible that the protease activity is important for the effect of HDM on endothelial cells.

In our hands, IL-33 reporter levels remained unchanged by LPS and Poly (I:C) instillation, ligands of TLR receptors that do not contain proteases. In fact, TLR4 signaling may counter the inflammatory Type 2 effects of IL-33 signaling. Previous studies have demonstrated that children who grow up in an endotoxin-high environment are protected from allergy, hay fever, and asthma (Schuijs et al., 2015; Stein et al., 2016). Mice exposed to low doses of LPS before HDM sensitization and challenge had a reduction in IL-5 and IL-13 levels as well as decreased airway eosinophilia compared to HDM treated mice alone. Further analysis of epithelial lung tissue from these mice showed that this protection of increased TLR4 signaling led to an increase in the ubiquitin-modifying enzyme A20, which counteracts the inflammatory effects of NF-κB (Schuijs et al., 2015). In the case of Poly (I:C), many studies have examined the effects of viral mimetics on lung epithelium but have largely ignore the endothelial barrier. In fact, viral infections are often accompanied by pulmonary microvascular dysfunction which may predispose individuals to subsequent allergic inflammation.

Because these data adhere to the traditional paradigms of pulmonary epithelium as the driver of lung immune responses, they fail to examine the changes in lung vasculature during endotoxin exposure and HDM sensitization. Based on our data that HDM is able to elicit responses from lung endothelium but not LPS and Poly (I:C), it would be important to examine the effects of coexposure on overall Crimson expression. Prior exposure to Poly (I:C) may lead to vascular remodeling in BAC (+) mice that may further drive HDM-induced Crimson downregulation. Moreover, if downregulation of *IL33* is a way for the cytokine to regulate its own inflammatory effects, it would be of great therapeutic interest to see if pretreatment with LPS offers protection to endothelial cells that leads to an even greater significant reduction in *IL33* message following subsequent exposure to HDM.

In our hands, we do not see a change in IL-33 reporter levels following LPS instillation. In line with this, studies examining the effects of aerosolized endotoxin demonstrate that vascular pressures and permeability are unaltered after exposure (Ghofrani et al., 1996). However, the literature examining the effects of endotoxin on endothelium has been conflicting (Alves-Filho et al., 2009; Michel, 2003; Nagata et al., 2012; Nascimento et al., 2017; Zhang et al., 2011). In the lung, endotoxin is often used to induce hyperpermeability as a model for barrier dysfunction observed in gram-negative sepsis (Alves-Filho et al., 2009; Michel, 2003; Nascimento et al., 2017; Zhang et al., 2011). Interestingly, ST2 has been implicated in LPS tolerance as the receptor attenuates the effects of TLR4 signaling by sequestrating the adaptors MyD88 and Mal (Espinassous et al., 2009). ST2-deficient mice failed to develop endotoxin tolerance and have increased levels of IL-6 and IL-12 (Nascimento et al., 2017). Recombinant IL-33 administration has been shown to both exacerbate the inflammatory effects of LPS and attenuate the LPS/TLR4 signaling (Alves-Filho et al., 2009; Nascimento et al., 2017). The fact

that we do not see observable changes in human *IL33* reporter levels does not rule out a role for LPS in the airways. It may be that the effects of endotoxin are limited to higher doses over multiple exposures and ages. Understanding if and how endotoxin modulates IL-33 in the vascular system may provide a key pathway to maintenance of tissue homeostasis. Extracellular IL-33 has been previously described to induce expression of multiple cell adhesion receptors involved in leukocyte/endothelium interactions during inflammation (Gautier et al., 2016). In contrast, knockdown of nuclear IL-33 using RNA silencing strategies does not seem to affect endothelial cell proteome. Therefore, it appears that extracellular IL-33 protein and not nuclear IL-33, affects endothelial cell responses. In support of this, we observe a decrease in endogenous *IL33* transcripts in HUVECs after exogenous treatment with recombinant IL-33. Three important mechanisms could explain the dramatic reduction in *IL33* levels after abrogating translation and reduced *IL33* levels after the addition of recombinant IL-33. First, it is possible that another protein, protein "X", outside of IL-33 increases the stability of *IL33* mRNA. When the translation of this protein is blocked by cycloheximide, *IL33* transcript levels decline. Second, extracellular IL-33 may exert its effects on the newly synthesized *IL33* transcript, either by enhancing the activity of exonucleases, decreasing mRNA packaging, or exporting into the cytoplasm. There are multiple checkpoints of RNA stability throughout transcription in the nucleus. Turnover of mRNAs plays a key role in the overall regulation of gene expression. Pre-mRNAs in the nucleus must undergo several processing events including removal of introns, 3' cleavage and addition of a poly(A) tail (Houseley and Tollervey, 2009). At all these stages, exonucleases can degrade the nascent transcript. Failure of newly-transcribed mRNA to be packaged and exported to the cytoplasm leads to decapping and degradation of the mRNA by exonucleases (Houseley and Tollervey, 2009). Lastly, both of these mechanisms may be at play:

extracellular IL-33 increases exonuclease activity in the cytoplasm and decreases the activity of "protein X" to lead to an overall reduction in *IL33* mRNA (**Figure 4.19**). It would be worthwhile to determine which pathway is at play, as how IL-33 is sensed by endothelial cells and the signaling pathways involved to enhance degradation machinery remain to be elucidated.

In conclusion, our results demonstrate that HDM-induced IL-33 is capable of regulating *IL33* mRNA levels by enhancing mRNA degradation machinery. This effect was unique to pulmonary endothelium, as IL-33 reporter expression in epithelium and in fibroblasts was unaltered by allergen and IL-33 exposure. Given that SNPs at the *IL33* locus have been implicated in asthmatic disease, it would be worthwhile to examine the extent to which allergen exposure in asthmatics leads to a reduction in vascular IL-33. Perhaps it is the inability of lung vasculature to sense and downregulate *IL33* mRNA that results in the perpetuation of inflammation and Th2 responses.

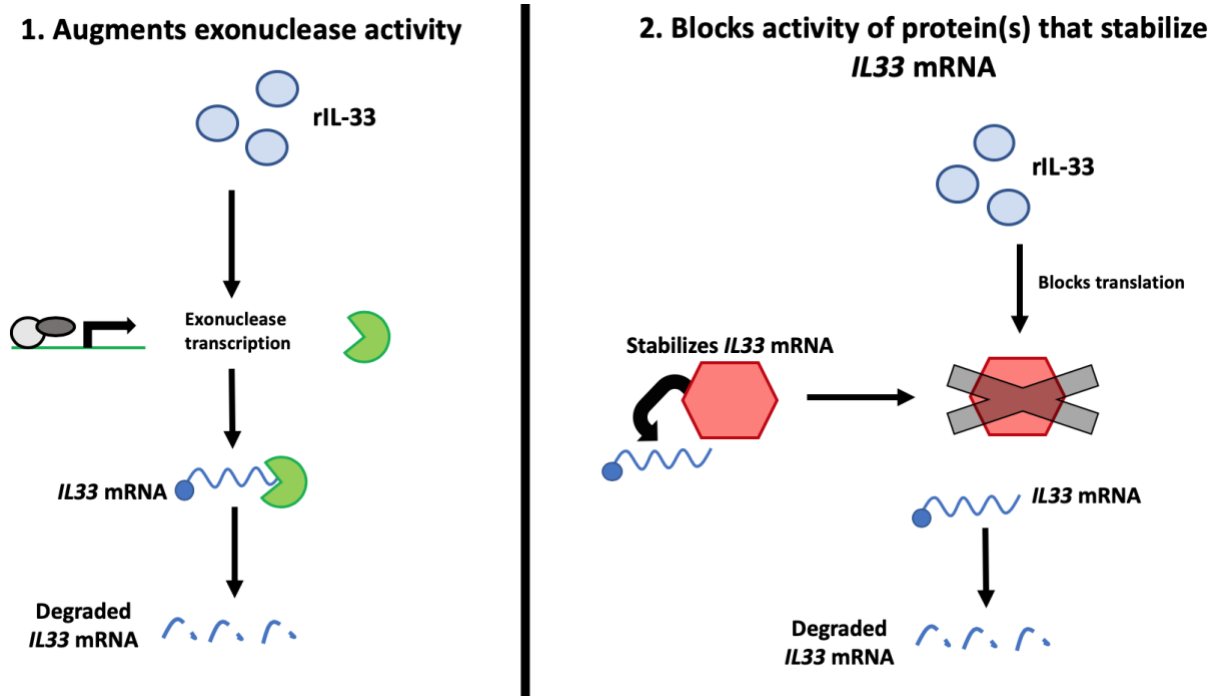

Figure 4.19 Schematic for how extracellular IL-33 may work to downregulate intracellular *IL33*. (Left) Exogenous IL-33 increases the transcription of mRNA degradation machinery. (Right) Exogenous IL-33 blocks the activity of a protein that stabilizes *IL33* mRNA.

Chapter 5 Discussion

Development of a novel murine model to reflect human IL33 expression.

In these studies, we investigate human *IL33* expression using a novel reporter system that uniquely identifies transcription from the human *IL33* locus using a fluorescent marker. This mouse model reflects the expression profile of human *IL33* as measured by RNAscope providing a platform to interrogate the reporter expression, as a surrogate for human *IL33* expression, *in vivo*. Breeding our human *IL33* reporter mice to murine *Il33* reporter mice allowed us to investigate the differential expression of *IL33* between mice and humans in primary murine lung cells. Thus, we have developed a reporter model, which is the first of its kind, that accurately mirrors the expression of human *IL33* in a manipulatable *in vivo* system.

While animal models prove valuable for dissecting human disease, the degree to which they are able to capture the full cellular profile and immune network of their human counterparts remains limited by differences in their genomic landscape. In fact, vast differences exist in the noncoding regions of the human and murine *Il33* loci, and human and murine IL-33 shares only 52% amino acid sequence homology (Baekkevold et al., 2003). Our previous study identified an important upstream regulatory region within the human *IL33* locus, but absent from the murine locus, containing asthma-associated SNPs. Therefore, a key advantage of our BAC transgenic system is the inclusion of all regulatory components found in the human *IL33* locus. Thus, our BAC (+) reporter model offers a reliable *in vivo* snapshot of IL-33 expression as it would be found in the human lung.

Using the *IL33* reporter mouse and primary human donors demonstrated that human *IL33* is expressed predominantly by two important sources in the lung: the vascular network and basal cells that line the basal lamina. In contrast, murine *Il33* is found in type 2 pneumocytes and

largely absent from the lung vascular network (Hardman et al., 2013; Pichery et al., 2012a). Human *IL33* studies have relied on findings from mouse models to provide the basic foundation for possible reservoirs of lung IL-33. Because murine IL-33 is found abundantly in the lung epithelium, most *IL33* studies in humans have isolated and examined *IL33* expression in bronchial alveolar lavages (BAL), nasal epithelial brushings or using epithelial cell lines (Hammad and Lambrecht, 2015; Pichery et al., 2012b; Préfontaine et al., 2010). However, there are three key problems with these types of human analyses for IL-33 expression. BAL and epithelial brushings often cause physiological damage to the tissue barrier which could influence levels of *IL33*. Second, these epithelial brushings are often contaminated with basal cells, which we have shown express *IL33*. Since there is no way to account for heterogeneity, it is difficult to parse out whether the epithelial cells collected express *IL33*. Lastly, cell lines are often derived from cancerous lines or passaged multiple times and thus do not resemble actual human pulmonary epithelium. With so many confounding factors in isolating epithelial human *IL33* mRNA, it is difficult to derive meaningful interpretations from *in vitro* cultures. Moreover, due to the influence of murine IL-33 findings, human *IL33* studies have failed to focus on the vascular network. While some epithelial cell lines and cell cultures can express low levels of *IL33* mRNA, we have shown using our BAC-reporters and cell lines that epithelial expression pales when compared to endothelial derived *IL33*. Our studies address the ratio of endothelial versus epithelial-derived *IL33* and clearly demonstrate that is it the lung endothelium in humans that is responsible for primary production of the cytokine. Through queries of single-cell gene expression and quantitative RNA expression of IL-33 in each lung cell type from both human and mouse lungs from the Lung Gene Expression Analysis (LGEA) web portal, we found that human *IL33* was expressed almost exclusively by CD31+, EpCAM-negative lung endothelium.

Little expression was detected in cells that were positive for EpCAM. In contrast, murine *Il33* was found most abundantly in lung epithelial cells. These data indicate that existing murine models fail to accurately profile *IL33* expression as it were found in the human lung. Future studies examining when and to what extent tissue barrier *IL33* plays a role in airway inflammation should compare expression levels in both the vasculature and epithelium.

Our data demonstrate *IL33* is found in human lung basal stem cells, which are capable of differentiating into both ciliary and secretory epithelial cells. Interestingly, while basal cells are found just below the epithelium of human bronchi and large bronchioles, they are anatomically confined to the trachea in mice. This difference in anatomical location of *IL33* expressing cells may explain why mice do not reflect all the pathogenetic mechanisms of human asthma and the cytokines involved. For example, intra-epithelial recruitment of eosinophils, a hallmark of human asthma, and subepithelial fibrosis found in severe asthmatics are notably absent in murine models (Bousquet et al., 1990; Korsgren et al., 1997; Kumar and Foster, 2001; Vargaftig, 1999). Because one of the downstream effects of IL-33 production is the recruitment of eosinophils to the lung, it is possible that *IL33*-expressing basal cells that line the periphery of epithelium are involved in the recruitment of intra-epithelial eosinophils observed in humans. Further, the presence of *IL33*-expressing basal cells near the basal lamina could explain subepithelial fibrosis found in humans. Dahlgren et al. demonstrated in mice that ILC2s localize near fibroblast-like adventitial stromal cells (ASCs) because ASC produce IL-33 and TSLP in response to IL-13 signaling (Dahlgren et al., 2019). These ASCs, absent from airway lumens but found in the perivascular adventitial cuffs, are mesenchymal fibroblast-like in nature and sufficient to support ILC2 survival, proliferation, and cytokine production as well as Treg recruitment. Likewise, in humans *IL33*-expressing basal cells could define a population of stromal cells that is important in

maintaining the homeostasis of tissue resident cells and may also be involved in the initiation of the immune response.

Limitations of BAC transgenic technology.

There are limitations with using BAC transgenic technology to study human *IL33* expression. First, unlabeled cells could result from the transgene failing to reach a detectable threshold. However, results obtained from our BAC (+) strain show similar expression patterns in primary human lung cell types by both the LungGENs database and our explant donor lungs. Second, the transcriptional effects of long-range chromosomal interactions that extend beyond the region captured in the BAC are lost in our BAC transgenic strains. Third, it is possible that the transcriptional machinery in murine cells differs from that found in humans. In BAC-transgenic mice, the BAC integration into murine genome is non-specific and therefore chromosomal location may affect the expression of the reporter in the BAC. However, consistent patterns of Crimson expression in specific cell types and tissue sites across multiple founders allowed us to conclude these phenotypic profiles were unambiguously attributed to our human *IL33* transgene. Lastly, while our murine model may reflect the phenotypic profile of human *IL33*, there are stark differences between known expression patterns of human *ST2* and murine *St2*. Interrogation of the LungGens database reveals that endothelial cells both express *IL33* and *ST2*. In contrast, our *in vivo* interrogation of ST2 shows that leukocytes, not endothelium, from the murine lung are the predominant cell type that express ST2 under naïve conditions. While we can detect high levels of soluble *ST2* transcript expression from our *in vitro* cultures of human endothelium, murine endothelial cells in the lung are absent for the receptor. Thus, while our transgenic model appears to recapitulate human IL-33 expression, it does not phenotypically profile human receptor expression. Given the discrepancy in endothelial

expression of *ST2*, one approach might be to generate a new BAC (+) containing the both the human *ST2* locus and *IL33* locus into a murine *St2* and *Il33* knockout mouse. Despite caveats to our system, we believe our human *IL33* BAC transgenic mouse is the first of its kind to incorporate the entirety of the human *IL33* locus and all its functional regulatory elements into a manipulatable, in vivo platform.

The vasculature is an important reservoir for human IL-33 and may play a role in T cell migration through HEV.

In these studies, analysis of the human *IL33* reporter at homeostasis revealed that the lung vasculature was a large reservoir for human *IL33*. Indeed, the endothelium may constitute the biggest source of IL-33 in the human body, as it is expressed in both large and small vascular and lymphatic vessels (Baekkevold et al., 2003; Feltis et al., 2006; Moussion et al., 2008; Pichery et al., 2012a; Sundlisæter et al., 2012). Other studies using primary human endothelial cells and cell lines have demonstrated that human endothelium expresses and can alter *IL33* during proliferation (Feltis et al., 2006; Hoshino et al., 2001a; Hoshino et al., 2001b; Sundlisæter et al., 2012). Murine IL-33, however, is absent from vascular networks at steady-state. In fact, high endothelial venules where human IL-33 was originally discovered do not express IL-33 in the mouse (Hardman et al., 2013; Pichery et al., 2012a).

In our hands, the human *IL33* reporter, but not murine *Il33* reporter, is found in the HEV of mice. Strikingly, we show that *IL33* expression is abrogated by removal of an upstream regulatory region. An open question to these findings is what role does the expression of *IL33* by the lymph nodes play in the overall immune response. Studies have demonstrated that HEV endothelial cells express specific genes that are not expressed by endothelial cells from other blood vessels such as sialomucins and selectins (Girard et al., 2012). In our hands, however, we

see *IL33* is lost in small lung microvasculature structures but retained in larger vessels demonstrating that our upstream regulatory *IL33* region is not HEV specific. Perhaps HEV *IL33* has an important role in lymphocyte migration through HEVs. For example, autotaxin, a secreted enzyme important in the chemotaxis and wound healing, has been shown to facilitate HEV entry (Girard et al., 2012). In a manner similar to autotaxin, HEV IL-33 may be one signal to facilitate retention of T cells in the lymph node.

The lung endothelium downregulates IL33 expression by decreasing mRNA stability.

Remodeling of airway epithelial structures during chronic inflammation, as in the case of asthma, has been extensively studied (Jeffery, 2001; Rydell-Törmänen et al., 2008). Structural remodeling of both large and small airways are characteristic of asthmatic pathology (Amin et al., 2000; Jeffery, 2001). However, dilatation of bronchial blood vessels and edema often accompany changes in epithelial strata, pinpointing a role for pulmonary vasculature in the asthmatic inflammatory response (Carroll et al., 1997; Kuwano et al., 1993; Lambert et al., 1993; Vrugt et al., 2000). Indeed, the number of bronchial vessels is increased in severe corticosteroid-dependent asthma (Vrugt et al., 2000). The endothelium acts as a web that coordinates proper regulation, activation, and function of immune cells by mediating their recruitment and retention during inflammatory challenge. It is therefore unsurprising that the network of pulmonary vessels must be malleable to changes in the tissue microenvironment.

When there is increased permeability of the pulmonary vasculature, it is often due to the influence of VEGF, a growth factor that promotes angiogenesis of endothelium (Feltis et al., 2006; Hoshino et al., 2001a; Hoshino et al., 2001b; Lee et al., 2004). Previous reports have demonstrated that protease allergens from HDM are capable of breaching the lung endothelial barrier and eliciting an angiogenic response (Asosingh et al., 2018). Indeed, lung endothelium

downregulates the human *IL33* reporter in response to allergen sensitization and challenge in our model, and was significantly reduced in lung endothelial cells following recombinant IL-33 administration. Further, *in vitro* cultures of primary human lung endothelium treated with recombinant IL-33 resulted in a remarkable decline of intracellular *IL33*. Mechanistically, we show that extracellular IL-33 acts to decrease the stability of intracellular *IL33* mRNA, either by enhancing exonuclease machinery that may be involved in degradation or in the export of new transcripts into the cytoplasm.

The stability of mRNA fluctuates vastly in eukaryotes, with turnover anywhere from minutes to 24 hours. Tight post-transcriptional regulation of cytokines ensures that once transcription is turned off, the mRNA pool does not build up. Interactions between cis-acting elements and trans-acting RNA binding proteins are important in mRNA decay and mRNA stability. For example, a highly conserved sequence AU-rich elements (ARE) are cis-acting elements often found within the 3' UTR of mRNA. This sequence is essential for mRNA decay in cytokines such as IL-2, GM-CSF, and IL-1 as ARE-containing mRNAs provide a signal for trans-acting RNA binding proteins (TTP) such as exosomes to bind and degrade the mRNAs. ARE-containing mRNAs are often targeted for degradation, but some ARE sequences also permit other proteins to compete or displace TTP (Caput et al., 1986; Seko et al., 2006). In the case of IL-2 in humans, NF90 is reported to bind the ARE in its 3′-UTR and retard ARE-dependent mRNA decay (Saravia et al., 2015; Seko et al., 2006). In our studies, we show that cycloheximide alone is able to downregulate *IL33* message, indicating that protein production is important. One hypothesis is that there is a protein, similar to NF90, that binds the *IL33* mRNA and slows decay. Perhaps this intermediate protein, "X", functions by blocking recruitment of *IL33* mRNA to the exosome for degradation. In this case, in the absence of protein X, *IL33*

mRNA levels would plummet. As extracellular addition of recombinant IL-33 also results in a dramatic loss of *IL33* mRNA, it is possible that ST2 signaling inhibits protein X transcription and renders *IL33* mRNA more unstable. In fact, our ATAC-seq data demonstrates that IL-33 treatment leads to global chromatin accessibility changes, so it is likely that protein X may decrease as a result of these changes. Taken together, regulation of mRNA turnover plays an important role in the control of cytokine gene expression. Given that IL-33 acts on a wide range of cells during the initiation of inflammation, sensing extracellular levels of IL-33 is one method where lung endothelium is able to limit the cytokine's deleterious effects.

Allergen challenge reshapes the lung endothelial barrier.

While allergen proteases may directly contribute to airway remodeling by modifying vascular permeability, the contribution of the innate vascular network to asthma is often ignored. Yet pulmonary endothelium expresses several pattern recognition receptors as well as cytokine and chemokine receptors. Naïve lung endothelial cells have been shown to express the HDM sensing pattern recognition receptor PAR-2, which indicates that they sense and respond directly to allergen (Asosingh et al., 2018). Moreover, activation of lung endothelium and subsequent angiogenesis are closely linked to eosinophilic infiltration in asthma, as hematopoietic progenitor cells are recruited to the lung following allergen exposure (Asosingh et al., 2016). One of the most important findings in this study is that pulmonary vessels, not airway epithelium, express *IL33* at homeostasis and downregulate the cytokine during ongoing allergic airway inflammation. Thus, the lung vascular network has a central role in innate immunity similar to the airway epithelium.

It is worth considering the possibility that any given immune response in a mouse model may not occur precisely the same way as found in humans. It is well established that almost all mice sensitized and challenged with allergens develop lung inflammation characteristic of asthma. However, humans are constantly exposed to environmental allergens and most do not mount an inflammatory response to these benign particles. One possibility is that mice mount a robust inflammatory response to harmless antigens because IL-33 is found abundantly in the epithelium. We know that allergens are capable of directly reaching the lung vascular network in humans, but the lung vasculature may be more protected from constant changes in the environment when compared to the epithelium. IL-33 is carefully sequestered away in the basal

cells and the human vasculature and only released upon cellular stress, damage, or death. Another possibility is that mice mount robust inflammatory responses to inhaled allergens because the murine *Il33* locus necessitates fewer regulatory elements compared to the human *IL33* locus. Our *in silico* interrogation of the human *IL33* locus revealed multiple levels of regulation, including two CTCF sites near asthma associated SNPs. Not only does the murine *Il33* locus lack these two CTCF sites, but the genetic landscape surrounding these regulatory marks is nonhomologous altogether. The paucity of regulatory elements in the murine *Il33* locus compared to the human *IL33* locus may explain the kinetic difference of *IL33* between mice and humans. Incubation of full-length IL-33 with increasing amounts of *A. alternata* resulted in rapid degradation of recombinant mouse full-length IL-33 after cleavage, whereas recombinant human full-length IL-33 was converted into more stable mature forms (Cayrol et al., 2018).

Anatomical directionality of airways and lung vasculature suggest crosstalk between the two networks.

Anatomically speaking, how is the endothelium organized so that it can sense allergen, recruit inflammatory infiltrates, and affect bronchial epithelium? Arterial supply of the lungs occurs via two pathways. Bronchial arteries from the aorta supply blood to the upper airways and tracheobronchial lymph nodes while the pulmonary arteries branch with the airways and supply the distal lung (Hislop, 2002). Many of the small bronchial veins within the airway drain into the pulmonary veins. A close relationship exists between blood vessels and airways. From the trachea to the lower airways, the number of airways increases. At the alveolar level, gas exchange occurs but this is only efficient if there is blood flow to the area. Thus, pulmonary arteries run along the airways, enwrapping and branch with them, with the pulmonary airways draining to the capillary bed (Hislop, 2002). During lung development, nascent CD31 endothelial

cells surround airways, and VEGF is found abundantly in the branching points of the peripheral airways.

When there is a disturbance to the lung vascular network, as observed in pulmonary hypertension and hypoxic/hyperoxic conditions, there are significant changes to the bronchial smooth muscle surrounding the endothelium that affect airway hyperresponsiveness. In healthy lungs, the airway smooth muscle contracts and luminal walls of the airway are able to expand and collapse into folds accordingly. However, when there is increased vascularity and dilation of capillary blood vessels as observed in chronic lung diseases such as asthma, such folds are reduced and the airway is morphologically hindered from adequate air flow (Li and Wilson, 1997; Reichard and Asosingh, 2019). Given our data that the lung endothelium downregulates *IL33* in response to extracellular IL-33, it is possible that IL-33 is released into the lung by endothelial cells that line encircle the alveoli. If this extracellular IL-33 is not sensed by adjacent endothelium, uncontrolled angiogenesis may drive vascular engorgement and subsequent bronchoconstriction of the airway lumen. While there has been a push in more recent years to elucidate the relationship between the airways and vascular network, the signals that govern angiogenesis and airway disease still remain poorly understood.

Th2 dysregulation during resolution of inflammation leads pulmonary fibrosis.

Cytokines play a crucial role in the initiation and perpetuation of an immune response by modulating expression of other cytokines. With regards to IL-33, TNF, IL-1β, IL-3, IL-4 have all been shown to positively regulate IL-33 expression while IFN-γ destabilize IL-33 protein (Kopach et al., 2014). Additionally, cytokines are able to mediate their own activities via positive or negative feedback loops. IL-4 and IL-13 exert their functions by activating a heterodimeric receptor complex consisting of the IL-4 receptor α-subunit and the IL-13 receptor α1-subunit.

However, IL-13 activity is inhibited when it binds to the IL-13 receptor α2 chain which does not interact with any other receptor subunit or signaling pathway. This latter pathway pinpoints a clear mechanism for how IL-13 can dampen its own potent effects along with sST2. Based on our data that exogenous IL-33 destabilizes *IL33* transcripts, it is entirely likely that this level of regulation is crucial in restoring homeostasis following asthma exacerbation and/or vascular remodeling. The question that remains, however, is what are signals that drive lung endothelial cells to sense this shift in their cytokine milieu? At what point in an inflammatory response do lung endothelial cells start to downregulate *IL33* message? While direct exposure to IL-33 results in mucus accumulation in bronchial structures and recruitment of Th2 cells, the role of IL-33 in the lung may be more complex than just the stimulation of type 2 inflammatory cell production.

IL-33 has been shown to play two important roles in tissue repair. First, IL-33 effectuates ILC production of amphiregulin, a ligand for the epidermal growth factor receptor and pro-survival signal. Following viral infection or *Nippostrongylus brasiliensis* infection, ILC2s aid in lung epithelial cell repair. A subset of tissue-resident regulatory CD4+ T cells also express amphiregulin and aid in stem cell proliferation (Kuswanto et al., 2016). While amphiregulin promotes proliferation of the stem cell niche and tissue barrier regeneration, overproduction and activation of mesenchymal cells may lead to fibrosis in situations where inflammatory conditions fail to resolve. Fibrosis is thought to represent the end stage of the repair process during tissue damage, wherein inflammatory cell infiltration and resident fibroblast activation persists, while the reparative ability of mesenchymal stem cells is diminished. IL-33 has been shown to be key to myofibroblasts differentiation, and elevated IL-33 levels are observed in fibroblasts with radiologically established fibrosis compared to non-fibrotic controls (Kuswanto et al., 2016). Second, IL-33 facilitates wound healing by increasing collagen deposition, as observed both in

the repair process following acute injury and in the resolution of bacterial infection (Luzina et al., 2013; Zaiss et al., 2015). Therefore, if left unchecked, IL-33 has the ability to both initiate and propagate a type 2 immune response and subsequent progression to fibrosis. Control of the innate IL-33/ST2 axis may depend on the endothelial network's ability to sense a shift in the levels of extracellular IL-33 and dampen *IL33* transcript levels. The threshold at which extracellular IL-33 is able to drive endothelial downregulation of *IL33* and when this occurs along the resolution of the inflammatory process remains to be elucidated.

Asthmatic response may involve inability of endothelial cells to return to quiescence.

The vascular network in the human body forms a critical component of regulation in health and disease. While inflammatory cells like eosinophils and neutrophils have been recognized as essential components of asthma pathophysiology, the endothelium through which these cells traverse have largely been ignored. Here, our data demonstrate that innate host responses by the lung endothelium may be critically important in the origin of Th2 cytokine-driven inflammation. In our hands, pulmonary endothelial cells respond to extracellular IL-33 by downregulating *IL33* transcript levels. This negative regulation could be an important mechanism for endothelium to dampen the inflammatory effects of IL-33, shifting the immune system toward a resolution of inflammation.

Two key questions arise from our studies. First, what is the source of IL-33 during allergic inflammation that acts promotes endothelial downregulation of *IL33*? It is clear form our data that the human vasculature both responds to and expresses *IL33*. More recently, house dust mite allergen from the airway lumen has been found breaching lung-resident endothelial cells which respond by releasing angiogeneic factors (Asosingh et al., 2018; Postma and Timens, 2006; Reichard and Asosingh, 2019). Blood vessels have traditionally been thought to exhibit a

biphasic response to inflammation: first, vessel activation occurs to promote leukocyte traffic and is subsequently followed by angiogenesis to promote blood and nutrient delivery to tissue sites. Is thus entirely possible that IL-33 is released by endothelium upon contact with allergen proteases, with the cytokine initially acting as an alarm to signal tissue stress and/or damage. Subsequently, inflammatory cells are recruited from the blood to the site of damage during the angiogenesis phase. Upon resolution of inflammation, vascular quiescence is returned when the pro-angiogenic state is turned off. Whether downregulation of *IL33* is the signal that drives the pro-angiogenic state to become anti-angiogenic or a byproduct of another signal in the resolution of inflammation remains to be understood.

The second question that arises is what are the functional consequences when downregulation of endothelial *IL33* is impaired? Endothelial cells are able to robustly activate T lymphocytes, yet neovascularization of the airway often correlates with asthma disease severity (Feltis et al., 2006; Li and Wilson, 1997; Reichard and Asosingh, 2019; Salvato, 2001). Therefore, a switch toward the resolution of angiogenesis must exist to restore homeostasis. An "off" button, sensed by the vasculature, would be paramount to controlling vascular growth. In support of uncontrolled angiogenesis, engorgement of the vasculature and stiffening of the airway wall are often observed in asthmatics. Asthmatics who die of acute exacerbations have enlarged capillary beds lining airway walls, and levels of proangiogenic VEGF are significantly higher than antiangiogeneic mediator endostatin (Feltis et al., 2006; Reichard and Asosingh, 2019). Clinical studies of exhaled nitric oxide (FeNO) as a measure of lung function demonstrate activation of the lung vascular network prior to any changes in airway epithelium. Circulating NO is derived from the vasculature, while exhaled NO stems from the airway epithelium. Whole-lung allergen challenge of atopic asthma patients show significant increases in serum NO,

VEGF levels, and a concurrent decrease in forced expiratory volume (FEV_1). However, exhaled NO did not increase upon exposure to allergen (Asosingh et al., 2013). These data demonstrate that alterations in airway endothelium precedes changes to lung epithelium, and blocking changes to the vascular endothelium provide an important therapeutic target.

Therapeutic impact of our studies.

Our findings beg the question: what is the functional consequence of the dichotomous sources between murine and human IL-33. Further, to what extent do these differences matter if the cytokine produced elicits similar downstream responses? By assuming that the shared downstream cell types such as ILC2s, eosinophils and mast cells dictate responses and are thus more important than the difference observed in upstream sources between endothelium and epithelium, one runs the risk of overlooking aspects of human immunology that are difficult to model. As therapies of human diseases become more sophisticated, it is prudent to understand the limitations of extrapolating data from mice. In the case of IL-33, human vasculature and basal stem cells not only express this asthma-associated cytokine but also play important roles in maintaining homeostasis. Potential therapies for asthma may exist in targeting these two reservoirs during inflammation.

Mouse models have contributed substantially to our understanding of disease, serving as an *in vivo* system for certain aspects of disease and as a platform to explore the efficacy of candidate drugs. A better murine model of human asthma should appropriately represent its relevant cytokine networks and barrier aspects. However, no single murine model is able to accurately recapitulates the human lung. For correct interpretation of findings in mouse models, the aim is to integrate data gained from clinical, human disease pathophysiology with mechanistic murine models. Given that the literature is littered with therapies that are efficacious

in mice but fail in humans, we can better optimize our murine models of human disease. In conclusion, for correct interpretation of findings in mouse models of asthma, human clinical and immunological profiles should be carefully analyzed alongside murine findings.

Future Directions

Our studies show that HDM-induced allergic inflammation, not LPS or Poly (I:C) signaling, downregulates *IL33* reporter expression in endothelium. However, there may be additional conditions under which human IL-33 is induced, given that species-specific differences in cytokine induction have been observed in other contexts. Future studies should focus on parsing out the various stimuli that augment IL-33 production or decrease its expression. While we see endothelial downregulation of our reporter after allergen sensitization and challenge similar to human *IL33* in primary endothelial cells, the effects of common viruses such as influenza on reporter expression remain unknown. Furthermore, given that exposure to respiratory viruses in early childhood has been linked to asthma later in life, it would be important to determine if and how IL-33 is related to these responses (Le Goffic et al., 2011; Saravia et al., 2015; Werder et al., 2018). Co-infection models in BAC (+) mice either with viruses or with viral mimetics such as Poly (I:C) before subsequent challenge would address what cell types are implicated in asthma induced by viral infection.

One open question that remains is how the nature of the protease allergen influences expression and half-life of IL-33. The types of proteases present in HDM vary drastically from those of fungal allergens such as *Alternaria*. We show that HDM drives down human *IL33* reporter expression in endothelium, but it is entirely possible that other protease allergens may drive expression up or have no effect on endothelial IL-33.

As no human IL-33 protein is produced, our BAC (+) system cannot be used for

functional studies of human IL-33. Our model allows us to interrogate the cellular sources of the human *IL33* reporter, the spatial and temporal expression patterns of the human *IL33* reporter, but it does not allow us to interrogate how various proteases might limit or elongate the half-life of human *IL33* transcripts. Moreover, it does not allow us to interrogate how regulation affects IL-33 function or how IL-33 derived from basal cells could recruit a different set of cells than IL-33 derived from lung endothelium.

To address how the human *IL33* locus affects IL-33 function, a new model must be developed whereby the human *IL33* exons are maintained with a reporter after the final (8_{th}) exon. We could use hCD2 or a marker that does not inhibit function attached with a p2A site that will cause cleavage after translation. Since human IL-33 cross reacts with murine ST2, we should be able to measure function in this model. Generation of this system will allow us to determine whether *Il33*, when regulated the way it is in humans, alters the initiation and perpetuation of airway inflammation by lung endothelium and other cell subsets.

Using this model, one could test how the role of various protease activity on IL-33 by challenging these mice in the airways with stimuli known to possess very different protease activity such as HDM compared to *Alternaria* and examining IL-33 protein levels via hCD2 as a readout. Second, this model would allow us to examine the effects of blunting IL-33 in HEV and in microvessels by removal of the upstream regulatory region using the DEL-BAC (+) schematic. It may be entirely possible that HEV IL-33 in humans is a gatekeeper to inflammation, by recruiting leukocytes to the lymph nodes. Accurate and efficient tissue-specific trafficking between tissue site and the lymph node is fundamental for effector T cells in mounting an immune response. Additionally, during the initial priming of naïve T cells, some T cells are “imprinted” with a preferential tissue-specific inflammatory program that allow them to

home to their tissue site (Islam and Luster, 2012). Perhaps contact with dendritic cells and with IL-33 in the HEV is one method in which particular T cells are imprinted to traffic to the lung. Outside of recruitment, another hypothesis is that IL-33 is involved in proliferation of antigen-specific T cells and/or their retention in the lung. In the absence of lymph node or tissue-associated IL-33, the proliferation of T cells following inflammation may be reduced. Further, enhanced turnover of lung T cells, due to a defect in their retention, may be observed in the absence lack of IL-33 in the tissue microvasculature. We would investigate T cell profiles of DEL-BAC (+) mice both during homeostasis and after inflammation, to assess whether IL-33 removed from lymph nodes and microvessels would result in a functional or phenotypic alteration to T cells both in the lymph node and in the lung.

An open question remains from our studies—to what extent does the physical location of type 2 immune response determine the type of response. For example, what is the contribution of the endothelial network which is found throughout the human lung compared to basal cells that microanatomically localize below epithelium? Though lung endothelial cells and basal cells both express *IL33*, do these cellular subsets act synergistically to drive the Th2 response or is one cell type more pertinent to the overall immune response? One could imagine that the different anatomical locations would necessitate crosstalk with nearby cells. Basal cells may also communicate with vessels in close proximity. It would be important to determine whether abrogation of *IL33*-expressing basal cells would blunt the Th2 cytokine milieu during inflammatory challenge. Ultimately, a precise anatomic understanding of the regulation of various *IL33*-expressing niches, the cells they act on and the stimuli that alter their expression of *IL33* may have profound implications on what cells to target during lung inflammation. Using our new BAC (+) with a functional IL-33 with the hCD2 reporter, we could interrogate the cell

types that lie in close proximity and may respond to endothelial IL-33. However, because basal cells are not expressed in murine lung bronchioles, analysis of the cell types that respond uniquely to human basal cells during homeostasis and are responsive during *ex vivo* stimulation would require microscopy of direct human lung tissue or the mouse trachea. Moreover, variability of basal cell *IL33* may exist between donors, and this would be prudent to examine multiple human lung donors. As basal cells are associated with tissue remodeling, perhaps there is a difference between basal cell expression in asthmatics where cellular stress and tissue remodeling is often observed versus non-asthmatic donors. It would be important to note if the cell types, ILC2s and other leukocytes, that localize near basal cells are different between asthmatics and non-asthmatics. If so, levels of ILC2s or location of these ILC2s may be a potential biomarker of asthma.

Lastly, while the murine model may reflect the phenotypic profile of human IL-33, it does not accurately recapitulate known expression patterns of human ST2. Our *in vivo* interrogation of ST2 shows that immune cells from the murine lung are the predominant cell type that express *St2* under naïve conditions. In contrast, the LungGENS database shows that human endothelial cells express high levels of *ST2* while immune cells are negative for receptor expression at baseline. Furthermore, we can detect high levels of soluble ST2 expression from our *in vitro* cultures of human endothelium. Thus, while our transgenic model appears to recapitulate human *IL33* reporter expression, it does not phenotypically profile human IL-33 receptor expression. These data suggest that human lung vasculature could participate directly in IL-33/ST2 associated inflammation and perpetuate downstream Th2 responses while murine lung endothelium would likely need an intermediate ST2 expressing cell to respond to exogenous IL-33 administration. To account for differences in the human IL-33/ST2 pathway in

a murine model, one approach might be to generate a new BAC (+) containing the both the human *ST2* locus and *IL33* locus into a murine *St2* and *Il33* knockout mouse.

The process of developing a murine model that best mirrors human asthmatics is dependent upon identifying sources that drive disease and uncovering novel genetic factors that influence disease severity. As new SNPs are identified and potential haplotype blocks in the *IL33* and ST2 locus emerge along with their functional consequences on signaling and processing, incorporation of these findings into new mouse models will help us to develop a better working model to understanding disease. The debate about murine models as a platform for human disease continues, with opponents arguing that it is simply not the human. However, as new insight into both IL-33 and its role in asthma pathogenesis develops, generation of an efficient and effective model to interrogate various aspects of disease initiation and perpetuation is likely to offer new areas for asthma therapeutics.

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