NORTHWESTERN UNIVERSITY

Rethinking the Biology of IL-33 Outside of Disease:

Distinct Functions in Eosinophil Development and Microbiome Dysbiosis

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ABSTRACT

Allergic diseases, including asthma, atopic dermatitis, and food allergy, are a widespread health issue. The prevalence of these diseases has been increasing, but the mechanism behind this increase and how allergies develop is not well understood. Although the immune system is central to the pathology of allergy, recent work has begun to focus on the contributions of nonimmune processes, including the epithelial barrier, microbiome, and stem cells. IL-33 is a Type 2-associated cytokine that has been described as an “epithelial-derived cytokine”, along with TLSP and IL-25, and has been shown to act as an adjuvant to promote sensitization and elicit eosinophilic inflammation. My work demonstrates two novel functions for IL-33 that contribute to our understanding of allergic disease: supporting the development of eosinophils in the bone marrow and shaping the intestinal microbiome.

It was recently described that the receptor for IL-33, ST2, is expressed on hematopoietic stem cells, where its function remains unclear. Here I demonstrate that IL-33 regulates eosinophil development in bone marrow by supporting early lineage commitment. Initially, I observed that basal eosinophilopoiesis in naïve mice requires IL-33 and ST2. While both IL-33 and IL-5 can expand mature eosinophils (EoM), I found that IL-33 specifically expanded a pool of eosinophil precursors (EoPre) as well as induced upregulation of IL-5Rα on EoPre and expression of IL-5 by bone marrow cells. Serum levels of IL-5 were also increased under this treatment, and neutralizing IL-5 with a blocking antibody ablated the IL-33-induced EoM expansion. The homeostatic hypereosinophilia seen in IL-5–transgenic mice was significantly lower with ST2 deficiency. These findings establish a basal defect in eosinophilopoiesis in IL-33– and ST2-deficient mice and a mechanism whereby IL-33 supports mature eosinophils by driving both systemic IL-5 production and the expansion of IL-5Rα–expressing precursor cells.

In addition to the effects seen in the bone marrow, I found that the same IL-33 treatment is sufficient to significantly alter the cecal microbiome. At the phylum level, IL-33 increases the Bacteriodetes and decreases the Firmicutes. Overall, IL-33 significantly altered 69 operational taxon units (OTUs). To determine how IL-33 alters the microbiome, I used a microarray of mast cells treated with IL-33 and identified that the antimicrobial protein lipocalin 2 (Lcn2) is increased. Then I confirmed that IL-33 induces Lcn2 in the serum, lung, and small intestine in vivo as well as dendritic cells and mast cells, but

not neutrophils, in vitro. By examining the microbiome of IL-33-treated Lcn2 KO mice and comparing it to WT mice, I determined that there are 29 OTUs that appear to be significantly altered by IL-33 in a Lcn2-dependent fashion. Thus, Lcn2 appears to be one mechanism by which IL-33 alters the microbiome. These data provide a mechanism by which IL-33 disrupts homeostasis of the microbiome by decreasing potentially beneficial bacteria.

Collectively, my work defines two novel functions for IL-33 outside of any disease. In the bone marrow, IL-33 acts on eosinophil precursors to promote homeostatic development of eosinophils. In the intestine, IL-33 shapes the microbiome. Thus, IL-33 is central to two distinct processes that can both affect allergic disease. Future work is needed to determine if these novel functions are important in the development of allergy and maintenance of detrimental immune processes within disease.

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LIST OF ABBREVIATIONS

α	alpha
α-SMA	α-smooth muscle actin
AAM	alternatively activated macrophages
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AD	atopic dermatitis
Alum	aluminum hydroxide
ANOVA	analysis of variance
AP-1	activator protein-1
APC	antigen presenting cell
APRIL	a proliferation-inducing ligand
β	beta
BAL	bronchoalveolar lavage
BM	bone marrow
BMDC	bone marrow-derived dendritic cell
BMMC	bone marrow mast cell
BSA	bovine serum albumin
C/EBP	ccaat-enhancer-binding protein
CD	cluster of differentiation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
COPD	chronic obstructive pulmonary disease
CT	cholera toxin
DC	dendritic cell
DMEM	dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease 1

DSS	dextran sulfate sodium
ε	epsilon
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EoE	eosinophilic esophagitis
EoM	mature eosinophil
EoP	eosinophil progenitor
EoPre	eosinophil precursor
EPO	eosinophil peroxidase
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FMO	fluorescence minus one
Flt3L	fms like tyrosine kinase 3 ligand
Foxp3	forkhead box P3
FRC	fibroblastic reticular cells
γ	gamma
GF	germ free
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony-stimulating factor
GMP	granulocyte macrophage progenitor
GWAS	genome wide association studies
HBSS	hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HEV	high endothelial venule
i.n.	intranasal
i.p.	intraperitoneal
ICAM-1	intracellular adhesion molecule 1
IDO	indolamine 2,3-dioxygenase
IEC	intestinal epithelial cell
iEos	inflammatory eosinophils
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
JNK	c-Jun N-terminal kinase
KO	knockout
Lcn2	lipocalin 2
LPS	lipopolysaccharide
LT-HSC	long-term hematopoietic stem cells
LTA	lipoteichoic acid
μ	micro
MBP	major basic protein
MC	mast cell
MEP	megakaryocyte-erythroid progenitor
MFI	mean fluorescence intensity
MHC II	major histocompatibility complex class 2
MMP	matrix metalloproteinase
MPP	multipotent progenitor
NDC	non-digestible carbohydrates
NF-HEV	nuclear factor of high endothelial venules
NF-κB	nuclear factor “kappa-light-chain-enhancer” of activated B cells

Ngal	neutrophil gelatinase associated lipocalin
o.a.	oropharyngeal aspiration
OTU	operational taxon unit
OVA	ovalbumin
PAR-2	protease activated receptor-2
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PMA	phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
RA	retinoic acid
RBC	red blood cell
rEos	resident eosinophils
RNA	ribonucleic acid
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute-1640 media
RSV	respiratory syncytial virus
RT-PCR	reverse transcription-polymerase chain reaction
SCF	stem cell factor
SCFA	short chain fatty acids
SEB	staphylococcal entertoxin B
SIGIRR	single immunoglobulin IL-1-related receptor
ST-HSC	short-term hematopoietic stem cells
ST2	suppression of tumorigenicity 2
TGFβ	transforming growth factor beta
Th	helper T cell
TLR	toll-like receptor

TNF	tumor necrosis factor
Treg	regulatory T cell
TSLP	thymic stromal lymphopoietin
WT	wild type

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CHAPTER 1 – Introduction

IL-33

Over the last decade, significant interest in the contribution of three "epithelial-derived" cytokines, thymic stromal lymphopoietin (TSLP), interleukin 25 (IL-25), and interleukin 33 (IL-33), has developed. These cytokines have been strongly linked to the early events that occur during allergen exposure and research is focused on how they contribute to the subsequent Type 2 immune response. Of these three cytokines, IL-33 has proven particularly interesting because of the strong associations found between both it and its receptor, suppression of tumorigenicity 2 (ST2), in several genome wide association studies (GWAS) of allergic diseases (1-4). Further work has demonstrated clear mechanisms through which IL-33 might orchestrate allergic inflammation. This includes activation of several key effector cells that possess high levels of ST2, such as mast cells (MCs), basophils, innate lymphoid cells (ILCs) and eosinophils. However, controversies surround IL-33 that seem to indicate that the biology of this cytokine might not be as simple as the current dogmas suggest. These controversies include the relevant cellular sources of IL-33, the mechanistic contributions of nuclear localization versus secretion, and the mechanisms of secretion.

Molecular biology

The IL-1 family contains 11 members; IL-1α, IL-1β, IL-1Ra, IL-18, IL-33, IL-36ra, IL-36α, IL-36β, IL-36γ, IL-37, and IL-38. All members share a similar structure, which is made up of a C-terminal IL-1 homology domain and a N-terminal pro-domain of varying lengths (5). The IL-1 homology domain contains two key features: a β-trefoil structure made up of 12 β sheets and a A-X-D consensus sequence, where A is an aliphatic amino acid, X is any amino acid, and D is aspartic acid. The A-X-D sequence is important for the three-dimensional structure and in some proteins, it is 9 amino acids away from a cleavage site. While IL-1β, IL-1Ra, and IL-18 require cleavage of the pro-domain to produce the active cytokine, both the full length and cleaved forms of IL-1α and IL-33 are active.

IL-33 was originally found as a nuclear factor of high endothelial venules and termed NF-HEV (6). Interest was reignited when computational predictions discovered the characteristic IL-1 family β-trefoil domain, thus it became the eleventh family member also known as IL-1F11 (7). In humans, IL-33 is a 270 amino acid (aa) protein that has been described as having three domains: a nuclear domain (aa 1-65), an activation domain (aa 66-111), and an IL-1 homology domain (aa 112-270) (8). The nuclear domain contains a nuclear localization signal, a helix-turn-helix motif, and a chromatin-binding motif (located at aa40-58) (6, 9). The activation domain contains several cleavage sites, although there are also cleavage sites within the IL-1 homology domain (Figure 1B) (8). The mouse homologue of IL-33 is 266 aa and shares 55% aa sequence homology with human IL-33 (7).

The *Il33* gene has 8 exons, and the translational start site is within exon 2 (Figure 1A). Upstream of exon 2, there are two exons which are used alternatively (exon 1a and 1b) (10). The human *IL33* gene also contains a third option, exon 1a'. Despite this alternative usage of exon 1, the same protein is translated. Thus, it was proposed that exon 1 regulates IL-33 production. Further variants that lack a combination of exons 3, 4, and/or 5 have also been detected (11-13), and these *IL33* variants are translated into proteins that differ in length.

IL-33 can be post-translationally processed both intracellularly and extracellularly. The intracellular molecules caspase 1, caspase 3, caspase 7, and calpain can all cleave IL-33 within the IL-1 homology domain (14-18) (Figure 1B). However, caspase-driven cleavage leads to inactivation of IL-33, which is why IL-33 is said to be released by necrosis but not apoptosis. In contrast, extracellularly released MC proteases chymase, tryptase, and granzyme B as well as neutrophil proteases elastase and cathepsin G cleave IL-33 in the activation domain (8, 19) (Figure 1B). These cleavage products are significantly more potent at activating MCs and ILC2s than full length IL-33. The function of processed IL-33 in vivo is not fully understood, since most studies using exogenous IL-33 utilize the widely available "mature" form of recombinant IL-33 (aa109-266 for mouse and aa112-270 for human), which is closest to the granzyme B product (aa111-270). Interestingly, in asthmatics, lack of exons 3 and 4, which are translated to the nuclear localization signal and the activation domain, is associated with Type 2 inflammation (13). This would suggest that these patients have isoforms that

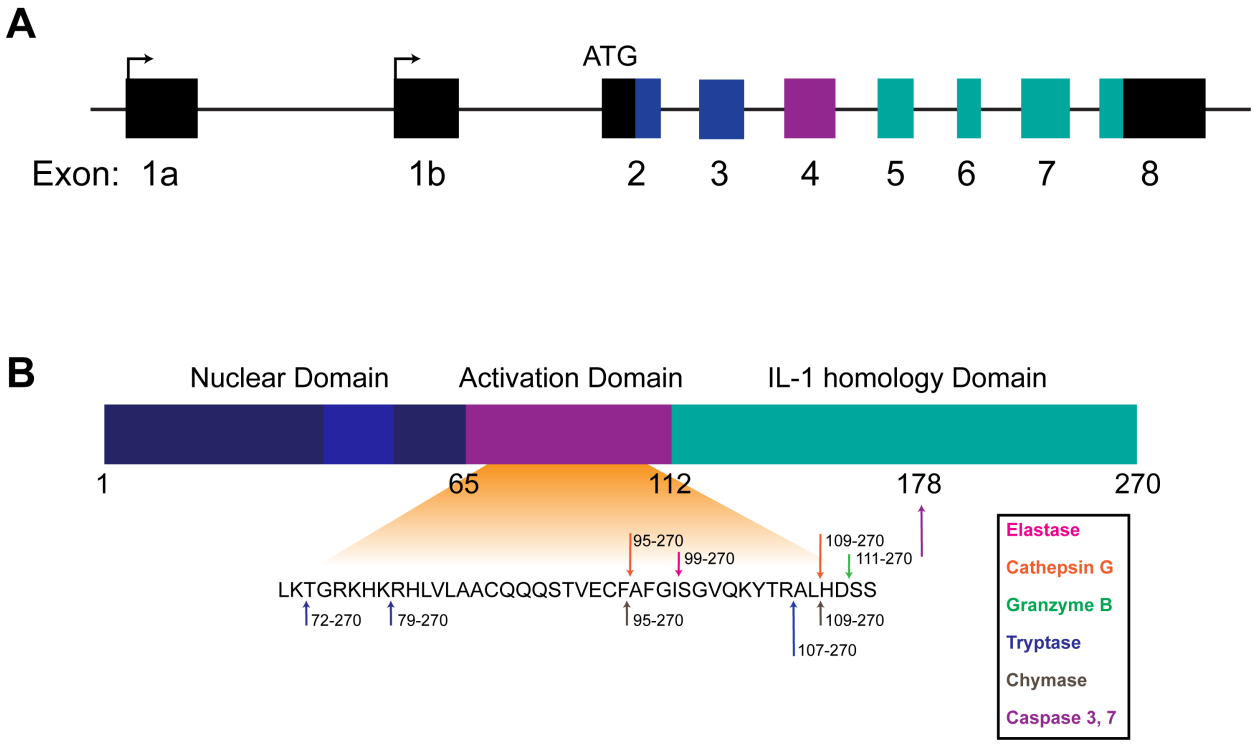

Figure 1. Molecular features of IL-33.

(**A**) Gene structure of *IL33*. (**B**) Protein structure and cleavage sites. Adapted from ref. (8, 20).

cannot be cleaved by MC or neutrophil proteases. Thus, alternative splicing and/or post-translational cleavage generates many isoforms of IL-33, but their function has yet to be determined.

ST2 receptor

When Schmitz et al. identified IL-33 as an IL-1 family member, they also discovered that it was the ligand for the previously orphan receptor ST2 (also called Interleukin 1 receptor-like 1 [IL1RL1]), which had already been associated with allergic disease (7). As a member of the Toll-like receptor-IL-1 receptor (TLR/IL-1) family, ST2 has three extracellular immunoglobulin domains and an intracellular Toll-IL-1R (TIR) domain (21), which allows it to signal using myeloid differentiation factor 88 (MyD88) and MyD88 adaptor-like (Mal) (22). Downstream signaling involves extracellular signal-regulated kinase (ERK), p38, p44/42, c-Jun N-terminal kinase (JNK), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and activator protein-1 (AP-1) (7, 23, 24). Importantly, because MyD88 is also utilized by the toll-like receptors (TLRs), ST2 negatively regulates TLR2, TLR4, and TLR9 by sequestering MyD99 and Mal (22). Once IL-33 binds ST2, ST2 forms a heterodimer with the co-receptor IL-1 receptor accessory protein (IL-1RAcP) (25), which is required for signaling. IL-1RAcP is part of the receptor complexes for all other IL-1 family members except IL-18 and IL-37 (26).

The ST2 gene, *IL1RL1*, is comprised of 11 exons, with two alternative promoters located in exon 1a and exon 1b (Figure 2). Three isoforms have been identified: a membrane-bound form (ST2 or ST2L), a soluble form (sST2), and a membrane-bound splice variant (ST2V) (27-30). While transcription of ST2L utilizes the distal promoter in exon 1a, transcription of sST2 utilizes the proximal promoter in exon 1b (30). Furthermore, the mRNA is alternatively spliced so that ST2L is translated from 11 exons and sST2 is translated from 8 exons. ST2V is another variant formed by alternative splicing and includes an additional exon between exons 5 and 6 (28). Tissue expression of these isoforms is quite distinct. Although it was originally described that ST2L is expressed in hematopoietic tissues (embryonic liver, spleen, and bone marrow) whereas sST2 is expressed in nonhematopoietic tissues (embryonic skin, bone, and retina)

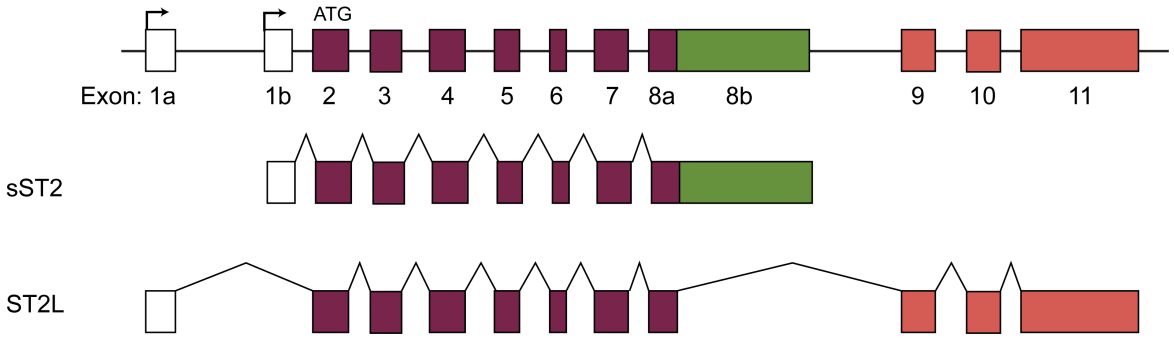

Figure 2. Gene structure of *IL1RL1*.

Adapted from ref. (30).

(31), it has since been shown that both structural and immune cells can express both forms of ST2 (32-34). ST2V appears to be mostly expressed in the gastrointestinal tract (29).

ST2 expression and signaling can be regulated by several mechanisms. It can be induced by several stimuli, including mechanical stress (35), bacterial infection (36), IL-3 (34), IL-13 (37), and IgE-crosslinking (38). Alternatively, ST2L can be ubiquitinated, internalized and degraded (36). Signaling can be prevented if ST2L dimerizes with single immunoglobulin IL-1-related receptor (SIGIRR) instead of IL-1RAcP (39). Furthermore, sST2 prevents IL-33 from binding to ST2L and thus blocks activation of any downstream signaling (40, 41).

Functions of IL-33

In vivo responses to exogenous IL-33

Initial studies utilized recombinant IL-33 to determine its function. Intraperitoneal injection of IL-33 into mice caused increases in spleen weight, serum IgE, serum IgA, Type 2 cytokines (IL-4, IL-5, and IL-13), mucus production by epithelial cells, and eosinophilia (7). Similarly, intranasal administration of IL-33 led to increases in Type 2 cytokines, eosinophilia, and airway hyperreactivity (42). Thus, IL-33 clearly has functions within the context of Type 2 immunity. However, IL-33 also has other functions outside of Type 2 immunity. Although it is well established that IL-33 can promote eosinophilia, IL-33 can also stimulate neutrophilic inflammation (43). It is unclear how IL-33 can play a seemingly contradictory role in both neutrophilic and eosinophilic inflammation, but the duration of IL-33 exposure seems important because one injection recruits neutrophils and multiple injections recruit eosinophils. However, mice with transgenic overexpression of IL-33 have significant increases in both neutrophils and eosinophils (44). Further research is needed to understand the role of IL-33 in these processes. IL-33 also increases vascular permeability, which likely contributes to the effects of IL-33 on inflammation (45). Similarly, it increases the permeability of the intestinal epithelium (46), demonstrating that IL-33 has effects on structural cells. Beyond inflammation, IL-33 plays a role in repair mechanisms. For example, subcutaneous administration of IL-33 promotes fibrosis (47) and can accelerate cutaneous wound healing (48, 49). Finally, IL-33 expands several recently discovered cell types, including Type 2 innate lymphoid

cells (ILC2s) (50), $ST2^+$ regulatory T cells (Tregs) (51), and IL-10-producing regulatory B cells (Bregs) (51).

Cellular responses to IL-33

ST2 is expressed on several "allergy-associated" cells, including Type 2 T helper (Th2) cells (33), ILC2s (50, 52), eosinophils (53), mast cells (54) and basophils (55), and these have been the major focus of IL-33 biology to date. A common response to IL-33 activation is cytokine production – notably, IL-33 induces Th2 cytokines in many cell types (Table 1). Survival, adhesion, and proliferation are also common responses to IL-33 (Table 1). In conjunction with other stimuli, IL-33 can have a synergistic effect. For example, IL-33 enhances lipopolysaccharide- (LPS) induced cytokine production from macrophages and IL-13-driven polarization (56, 57). It also enhances IgE-mediated degranulation of mast cells and basophils (58) and synergizes with c-Kit signaling (59).

Furthermore, IL-33 can function independently of ST2 since it has a nuclear localization signal. Using its chromatin-binging motif, IL-33 can bind chromatin and regulate chromatin compaction and gene expression (9, 60). IL-33 can also regulate gene expression by interacting with both the promoter and a protein subunit of NFκB (61, 62). While these studies showed that IL-33 suppressed gene expression, IL-33 can also promote gene expression. For example, nuclear IL-33 enhances IL-13 expression though *IL13* promoter binding (63) as well as amplifies IFNγ-induced gene expression (64).

IL-33 in disease

Allergic disease

IL-33 is elevated in asthma (65), atopic dermatitis (38), and chronic rhinosinusitis (66), and has been correlated with asthma severity (65, 67). Several roles for IL-33 have been identified within allergic disease. First, IL-33 can be used as an adjuvant (68) and it promotes allergic sensitization through multiple mechanisms. IL-33 enhances dendritic cell (DC) maturation through the upregulation of CD40, CD80, CD86, OX40L and IL-33-activated DCs can promote skewing of naïve T cells to Th2 cells (69, 70). However, IL-33 is not sufficient to skew naïve $CD4^+$ T cells. IL-33 and ST2 are necessary for the

Table 1. Cellular responses to IL-33.

Cell	Functions of IL-33	Molecules upregulated by IL-33	Stimuli enhanced by IL-33
Mast cell	survival, adhesion	GM-CSF, IL-1β, IL-6, IL-8, IL-13, TNF, CCL1, CCL2, PGD2	IgE crosslinking
Basophil	survival, adhesion	CD11b, IL-1β, IL-4, IL-5, IL-6, IL-8, IL-13, GM-CSF, CCL2, CCL3, CCL4	IgE crosslinking
Eosinophil	survival, adhesion	CD11b, ICAM-1, IL-6, IL-8, CCL2	
Th2	chemotaxis	IL-5, IL-13	
Treg	proliferation, suppression of CD4+ T cell proliferation	ST2, FoxP3, GATA-3. IL-5, IL-13	
ILC2	proliferation	IL-5, IL-13, amphiregulin	
Tc1		IFNγ	
NK cell			IL-12 or IL-23-dependent IFNγ
B-1 cell	proliferation	IL-5, IL-13, IgM	
NKT cell		IFNγ, IL-2, IL-4, IL-5, IL-13, TNF	
Macrophage		CCL3, CCL18, TLR2, TLR4	LPS, IL-13-driven alternative activation
Dendritic cell	maturation	IL-1β, IL-6, TNF, CCL17, MHC II, CD40, CD80, CD86, OX40L	
Microglia and astrocyte	proliferation	IL-1β, IL-6, IL-10, IL-13, TNF, CCL2, CCL3, CCL5, CXCL10	IFNγ-mediated NO production
Endothelial Cell		IL-6, IL-8, IL-17F, CCL2, NO, VCAM-1, ICAM-1, E-selectin	
Epithelial cell		IL-6, IL-8, CCL2	
Fibroblast		CCL11	

Adapted from ref. (71, 72).

development of antigen-specific IgE and IgG1 in several mouse models of allergy (73-75), however they are dispensable when a subcutaneous or epicutaneous sensitization route is used (76, 77). Second, administration of IL-33 increases ILC2s, which produce IL-5 and IL-13 and aid in activation of inflammatory cells (50, 52). Third, following sensitization, IL-33 has been suggested to be important for eosinophil recruitment, however this topic will be discussed further in Chapter 3. Finally, IL-33 and ST2 are necessary for anaphylaxis but not sensitization in epicutaneously sensitized mice (77, 78). Thus, IL-33 has roles in the effector stage as well as the sensitization phase.

Innate mechanisms

Although IL-33 is thought to participate in initiating responses, few studies have focused on the role of IL-33 in innate immunity. In vitro studies have established that LPS and other TLR agonists promote IL-33 expression in macrophages and DCs (10, 69, 79). In fact, it is possible that IL-33 induction could explain how low doses of LPS can act as an adjuvant to promote Type 2 responses (80, 81). However, LPS is more often thought to promote Type 1 responses. Several studies have proposed a role for IL-33 in Type 1 immunity, including models of sepsis (82) and viral and bacterial infections (83-86). As described previously, IL-33 can recruit neutrophils (43), which are associated with Type 1 immunity. However, further work is needed to fully understand the role of IL-33 in Type 1 immunity.

Injury, repair, and fibrosis

Recently, studies have begun to investigate the role for IL-33 in injury, repair, and fibrosis within allergy and other diseases. As an “alarmin” IL-33 is thought to be released in response to injury (87). Certainly, it appears as though IL-33 is released by protease activity of *Alternaria Alternata* (88), although other cell types can secrete IL-33 (discussed below). Unfortunately, several studies using models of colitis, which have extensive epithelial injury, have shown opposing roles for IL-33 (89-92). Despite this, IL-33 has a clear role in accelerating skin wound healing (48, 49) and exacerbating bleomycin-induced lung fibrosis (93, 94).

Controversies

Cellular source

Because IL-33 has been termed an "epithelial-derived" cytokine, it is often described as an alarmin that is released by necrotic epithelial cells (87). However, many cells express IL-33 in a constitutive and/or inducible fashion. To define the cellular sources of IL-33, two IL-33 reporter mice have been generated. Using the *Il-33-LacZ* gene trap reporter strain ($Il\text{-}33^{Gt/Gt}$), it was identified that IL-33 is constitutively expressed in the nuclei of α-smooth muscle actin positive fibroblastic reticular cells (α-SMA^{+}FRCs) in lymph nodes and spleen (95). This differed from human tissue, where IL-33 is expressed in the lymph node by both FRCs and HEVs. A second reporter mouse using a citrine fluorescent protein ($Il33^{Cit/+}$) further identified that mice basally express IL-33 in the Type II pneumocytes of the lung (96). Thus, IL-33 appears to be constitutively expressed in structural cells under homeostatic conditions.

Under inflammatory conditions, IL-33 is upregulated in several cell types. In vitro, macrophages and DCs upregulate IL-33 in response to various TLR ligands (69, 79). IL-33 is expressed in mast cells after stimulation with ionomycin and crosslinking of IgE receptors (97). After allergic airway inflammation, IL-33 was induced in both structural and hematopoietic cells, including Type II pneumocytes, neutrophils, eosinophils, macrophages, B1 cells and B2 cells (96). Sendai virus infection promoted IL-33 expression by Clara cells of the bronchial epithelium, which do not express IL-33 basally (98). A similar pattern of expression can be seen the small intestine, where $CD45^{-}$cytokeratin^{+} epithelial cells upregulate IL-33 in response to CPT-11, a topoisomerase I inhibitor cancer drug (99). Therefore, in response to stimuli, IL-33 can be upregulated both in structural cells with constitutive expression as well in immune and structural cells that have little to no basal expression.

A point of contention is the question of functional contributions of structural vs. immune cell–derived IL-33. In mouse studies addressing this question, IL-33 from macrophages (100), DCs (101), and monocytes (102) are sufficient to support the development of Th2 responses and eosinophilia. In contrast, one study showed that transferring IL-33 knockout (KO) bone marrow into irradiated wild type (WT) mice had no effect on allergic inflammation (103). Further studies are needed, especially given the significant caveat that several of these immune cells are highly radiation resistant. Taken together, while

current evidence shows clear roles for immune cell–derived IL-33, the relative importance of structural- vs immune cell–derived IL-33 remains to be determined.

Secretion

The mechanism of how cells release IL-33 is also a subject of debate. IL-33 has been described as residing exclusively in the nucleus of structural cells (104), yet evidence now suggests this conclusion is likely influenced by alterations in the IL-33 protein upon fusion with fluorescent tags used to track the protein. A more careful assessment of native IL-33 revealed both nuclear and cytoplasmic localization in endothelial cells and fibroblasts (105). Indeed, previous work in our lab demonstrated cytoplasmic location within mast cells (106). Unlike many other IL-1 family members, IL-33 does not utilize the inflammasome pathway (107). Although release of nuclear IL-33 upon necrotic cell death gave rise to the concept of IL-33 as an “alarmin” (87), mechanical stress can also induce secretion of full length IL-33 from fibroblasts in the absence of necrosis (105). Relevant to allergy, IL-33 release through necrosis was shown in response to the established adjuvant aluminum hydroxide (alum) (108). However, allergens do not typically cause epithelial necrosis. Instead, allergens interact with mucosal tissue surfaces through various receptors, including TLR2, TLR4, dectin-1, and protease activated receptor-2 (PAR-2) (109). While dectin-1 and PAR-2 are necessary for allergen-induced IL-33 in vivo (88, 110), TLR ligands are known to stimulate IL-33 in immune cells (69). Since allergens can modulate epithelial tight junctions, allowing them to enter the parenchyma where they can activate immune cells (109), allergens have the potential to activate IL-33-producing structural and/or immune cells independent of necrosis. Several studies have described IL-33 secretion from structural cells (105, 111-113), although mast cells (114), DCs (115), and human monocytes (116) can also express and release IL-33. Despite this, there is still a debate as to whether nuclear IL-33 is released through necrosis or cytoplasmic IL-33 is secreted, and which cells are sources of IL-33.

CHAPTER 2 – Materials and Methods

Mice

Wild-type C57BL/6J mice (WT) and Lcn2 KO mice were purchased from Jackson Laboratories (Bar Harbor, ME). ST2 KO mice were previously generated by Andrew McKenzie and backcrossed to C57BL/6J for 8 generations. IL-33 KO mice on the C57BL/6J background were provided by Dr. Dirk Smith (Amgen, Seattle, WA). IL-5–transgenic mice (strain NJ.1638, previously described (117)) were provided to Dr. Sergejs Berdnikovs by Dr. James Lee (Mayo Clinic, Phoenix, AZ) and crossed with ST2 KO mice. IL-33-eGFP reporter mice were generated previously in our lab. Depending on the experimental requirements, both male and female mice (aged 6–36 weeks) were used. Animals were housed under specific pathogen–free conditions at Northwestern University. All experiments were approved by the Northwestern University Animal Care and Use Committee.

Intraperitoneal injections

Mice were given IL-33 (eBioscience, San Diego, CA) daily by intraperitoneal injection at 0.4 μg in 200 μL 1X phosphate-buffered saline (PBS) per day for a total of 7 injections. For some experiments, mice were also given either anti–IL-5 (TRFK5; eBioscience) or isotype control (rat IgG1 κ; eBioscience) at a dose of 25 μg per mouse by intraperitoneal injection on days -1, 2, and 5. Samples were analyzed 18 hours after the last injection.

Intranasal instillations

Mice were anesthetized with isoflurane and 0.4 μg rIL-33 in 40 μL 1X PBS was pipetted onto the nostrils, where it was inhaled. Mice were administered IL-33 daily for a total of 3 times and harvested 18 hours following the last exposure.

Oropharyngeal instillations

Mice were anesthetized with isoflurane and the tongue was gently held outside of the mouth with forceps. As the mice were deeply inhaling through their mouth, 10 μg *Escherichia coli* LPS (0127:B8 Sigma L4516) in 50 μL 1X PBS was pipetted into the mouth. Mice were allowed to inhale 15 times before the tongue was released to ensure the LPS was fully delivered to the lungs. Mice were harvested 24 hours following the LPS exposure.

Broncheoalveolar lavage (BAL)

To lavage the lungs, a 20ga Angiocath IV Catheter (BD Biosciences) was first inserted into the trachea and tied in place with suture (Harvard Apparatus). Then 0.8 mL BAL fluid (10% FBS and 1 mM EDTA in 1X PBS) was inserted and recovered using a 1 mL syringe. Differential cell counts were obtained by cytospining 100 μL of BAL onto slides, staining the cells with Kwik-Diff stain (Thermo Scientific), and differentially counting macrophages/monocytes, neutrophils, lymphocytes and eosinophils for a total of 100 cells per slide. To obtain total counts (per mL) for each cell type, the total number of cells/mL was multiplied by the percentage of each cell type.

Chronic asthma model

Mice were sensitized with Grade V ovalbumin (OVA, Sigma, #A5503) as previously described (118, 119). Briefly, mice received three intraperitoneal injections of 50 μg of OVA on days 0, 3, and 6, followed by weekly intranasal installations (described above) of 20 μg of OVA starting on day 11 for a total of 9 challenges. Mice were harvested 24 hours following the final challenge.

Bone marrow extraction

To obtain bone marrow, femurs and tibias were extracted, cleaned using gauze to remove all skin and muscle, and placed in a tissue culture dish. After cleaning with ethanol followed by 1X PBS, both ends of the bones were cut using a razor blade. A hole was made in the bottom of a 0.7 mL Eppendorf tube with an 18-gauge needle. The bones from 1-2 legs were put into the 0.7 mL tube, which was then

placed inside a 1.5 mL Eppendorf tube and centrifuged at 6,000 rpm for 15 seconds to acquire a pellet of bone marrow cells in the 1.5 mL tube. Cells were resuspended in 1 mL RBC lysis buffer (eBiosciences) and incubated for 5 minutes.

Bone marrow analysis

Myeloid:erythroid ratios in bone marrow were assessed by histological inspection of nuclear and staining profiles on cytologic preparations (Shandon Cytospin; Thermo Fisher Scientific, Waltham, MA) stained with Kwik-Diff stain (Thermo Scientific) using established approaches (120); the relative proportions of granulocytic and erythrocytic cells were calculated after lymphocyte exclusion.

Blood analysis

Blood was collected into EDTA-coated tubes, and absolute eosinophil numbers were determined after staining with Discombe's fluid (0.05% eosin in 5% (vol/vol) acetone in distilled water).

In vitro culture of eosinophils

Bone marrow from the femur and tibia was recovered by brief centrifugation, and the pellet was resuspended in 10 mL BMMC complete media (RPMI 1640 with 2mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% non-essential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 0.05 mM β-mercaptoethanol). Cells were counted and seeded at 3×10^6 cells/ml in 1 ml complete media supplemented with one of the following conditions for 3 days: 100 ng/ml SCF and 100 ng/ml Flt3L; 10 ng/ml IL-5; or 10 ng/ml IL-33. Alternatively, cells were seeded at 0.5×10^6 cells/ml in 6 ml complete media supplemented with 100 ng/ml SCF and 100 ng/ml Flt3L with or without 10 ng/ml IL-33; on day 3 and day 7, non-adherent cells were collected, counted, and readjusted to 0.5×10^6 cells/ml with fresh medium containing 10 ng/ml IL-5.

Neutrophil isolation from bone marrow

Bone marrow was extracted as described above. A 82/82/51% discontinuous Percoll-HBSS gradient was made in a 15 mL tube by diluting 100% Percoll (45 mL Percoll with 5 mL 10X HBSS) with 2.5% FBS in 1X HBSS. The cells from one mouse were resuspended in 5 mL HBSS and layered on top of the gradient. The tubes were centrifuged as 100 xg for 30 minutes at room temperature with no break. The cells around the 62%/82% interface were collected and stained with anti-Ly6G MicroBead kit (Miltenyi Biotec) and sorted using the AutoMACS. The purity of neutrophils (>95%) was confirmed using flow cytometry against $Ly6C^{+}Gr\text{-}1^{+}Siglec\text{-}F^{-}$ cells.

Microbiome analysis

For microbiome analysis, WT and Lcn2 KO mice were separately housed and administered 7 i.p. injections of IL-33 or PBS as described above. 18 hours after the final injection, mice were euthanized and the cecal contents were collected for microbiome analysis. Instruments were washed with 70% ethanol between mice to ensure that microbes were not transferred between samples. Second Genome (South San Francisco, CA) extracted the DNA using the MoBio PowerMag Microbiome kit and quantified it with the Qubit Quant-IT dsDNA High Sensitivity Kit (Invitrogen). Second Genome then prepared the library by amplifying the DNA using primers surrounding the bacterial 16S V4 rDNA region. These primers also incorporated Illumina adapters and indexing barcodes. PCR products that met the post-PCR quantification minimum were concentrated using solid-phase reversible immobilization. These 16S V4 enriched, amplified, barcoded samples were sequenced using a MiSeq instrument. OTUs were generated from sequenced paired-end reads using USEARCH and UPARSE.

Microarray analysis

Microarray analysis on IL-33-stimulated bone marrow-derived mast cells was performed as previously described (121).

Culture of bone marrow-derived dendritic cells

Bone marrow was extracted as described above. On day 0, 10 x 10^6 cells in were plated on a 100 cm dish in 10 mL R10 media (RPMI with 10 % FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) supplemented with 15 ng/mL GM-CSF and 5 ng/mL IL-4. On day 3, 10 mL R10 with GM-CSF and IL-4 (Peprotech) was gently added to the cultures. On day 6 and 8, 10 mL of media was carefully removed from the top of the cultures to avoid disrupting the cells and centrifuged at 1250 rpm for 10 minutes. The pellet was resuspended in 10 mL fresh R10 media with GM-CSF and IL-4 and carefully added back to the original plate. On day 9, semi-adherent cells were detached by pipetting, counted, and plated for experiments.

Culture of peritoneal mast cells

Mice were euthanized and the peritoneal cavity was lavaged using 8-10 mL of sterile filtered lavage fluid (10% FBS and 1 mM EDTA in 1X PBS). Cells were centrifuged at 300 xg for 10 minutes and resuspended in 5 mL BMMC complete media supplemented with 10 ng/mL IL-3 and 30 ng/mL SCF (Peprotech) and plated in a T-25 flask. On day 2, media and nonadherant cells were discarded and 5 mL of fresh BMMC complete media with IL-3 and SCF was added to the flask. On day 5, 5 mL of BMMC complete media with IL-3 and SCF was added to the flask. On day 9, the media and nonadherant cells were collected and centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in 5 mL of BMMC complete media with IL-3 and SCF and returned to the original flask. On day 14, nonadherent cells were collected and plated for experiments.

In vitro stimulation of cells

Cells were plated at 1 x 10^6 cells/mL. For peritoneal mast cells, cells were first incubated with 1 µg/mL OVA-specific IgE for 1 hour, then stimulated with 0.5 µg/mL OVA. Cells were also stimulated with 2.5 µg/mL LPS or 10 ng/mL IL-33. For Lcn2 experiments, cells were collected for RNA and protein 24 hours after stimulation.

Cytokine analysis by ELISA

Cytokines were analyzed in serum, BAL fluid, tissue homogenate, and cell culture media. To obtain serum, blood was centrifuged in serum separator tubes at 8,000 rpm for 8 minutes. For tissue homogenate, tissue was homogenized in protease inhibitor (Sigma) in 1X PBS using a rotor-stator mechanical homogenizer. The homogenate was centrifuged at full speed for 3 minutes and the supernatant was used for cytokine analysis. For BAL fluid and cell culture media, samples were centrifuged to obtain cell-free supernatant. To detect IL-33 and Lcn2, the mouse DuoSet ELISA (R&D systems) was used following the manufacturers protocol. IL-5 was detected by sandwich ELISA. First, a 96-well ELISA plate was coated with 3 μg/mL of the primary antibody in carbonate buffer and incubated overnight at 4°C. After washing the plate three times with ELISA wash buffer (0.05% Tween-20 in 1X PBS), the plate was blocked in 3% BSA in 1X PBS for 2 hours at room temperature. The plate was washed three more times in ELISA wash buffer and 100 μL of the samples and standards were incubated overnight at 4°C. On the final day, the plate was washed three times with ELISA wash buffer, incubated with 3 μg/mL secondary antibody in 1X PBS for 2 hours at room temperature, washed three more times and incubated with streptavidin-HRP (R&D Systems) in 1X PBS for 30 minutes at room temperature before the final three plate washes. The ELISA was developed using ABTS (Invitrogen) and the intensity was read at 415 nm. The concentrations of the samples were calculated based on the standard curve.

Antibody analysis by ELISA

OVA-specific IgE and total IgA were determined by sandwich ELISA as described above. For the OVA-specific IgE ELISA, plates were coated with 3 μg/mL IgE primary antibody and OVA-biotin was used instead of a secondary antibody. For IgA, the concentration of the primary and secondary antibodies was 2 μg/mL.

RNA extraction and real time RT-PCR

RNA was isolated from tissues and cultured cells using the RNeasy Mini Kit (Qiagen) following the manufacturers protocol. Tissues were homogenized in RLT buffer with a rotor-stator mechanical

homogenizer and cells were homogenized using QIAshredder columns (Qiagen). For sorted cells, RNA was isolated using Trizol. Briefly, cells were suspended in 500 μL Trizol and incubated at room temperature for 5 minutes followed by -80°C overnight. After thawing the samples, 100 μL chloroform was added. Tubes were vigorously agitated for approximately 15 seconds, incubated for 2-3 minutes at room temperature, and then centrifuged at 12,000 xg for 15 minutes at 4°C. Roughly 250 μL of the aqueous phase was transferred to a new Eppendorf tube with 250 μL of isopropanol and incubated for >1 hour at -80°C. Thawed samples were centrifuged at 12,000 xg for 20 minutes at 4°C and the supernatant was discarded. The pellet was washed with 500 μL cold 75% ethanol and centrifuged at 7,400 xg for 15 minutes at 4°C. After discarding as much of the supernatant as possible, the pellet was air dried and then resuspended in 12.5 μL nuclease-free water. Using 500 ng RNA, cDNA was synthesized with the qScript cDNA synthesis kit (Quantabio) in a 20 μL reaction. Following the cDNA synthesis, samples were brought up to 100 μL with nuclease free water. Gene expression was determined by RT-PCR using an ABI 7500 Thermal cycler (Applied Biosystems). For each 20 μL reaction, 5 μL cDNA was added to 1 μL primer probes, 4 μL nuclease free water, and 10 μL PerfeCTa FastMix (Quantabio). Probes for *Il33*, *Il5*, *Lcn2*, *Csf2ra*, *Spi1*, *Csf3r*, *Epx*, *Prg2*, *Cebpa*, *Gata1*, *Gata2*, and *Actb* were purchased from Applied Biosystems.

Lung digestion for flow cytometry

Mice were euthanized and the abdominal and chest cavities were opened. After cutting the abdominal aorta, the right ventricle of the heart was perfused with a syringe containing 10 mL 1X PBS. A 20ga Angiocath IV Catheter (BD Biosciences) was inserted into the trachea and tied in place with suture (Harvard Apparatus). A 1 mL syringe was used to insert 1 mL of 0.25 mg/mL Liberase TM (Roche) in digestion buffer (0.5% BSA in 1X PBS, pH 7.2-7.4). The catheter was removed while the trachea was tied off, holding the digestion buffer in the lungs. The thymus and heart were discarded and the lungs were removed from the chest cavity and placed into 0.5 mL of digestion buffer with Liberase TM. The lungs were incubated at 37°C for 45 minutes, then 7 mL of DMEM containing 0.2 mg DNaseI was added. The

lungs were gently teased apart with forceps and then incubated in the Liberase TM/DNase I mixture for 10 minutes at room temperature with agitation. Cells were put through a 70 μm cell strainer (BD Biosciences) and centrifuged for 10 minutes at 1500 rpm at 4°C. Cells were lysed in 1 mL RBC lysis buffer for 5 minutes (eBiosciences) before counting and staining for flow cytometry.

Fecal analysis of IgA and Lcn2

Fecal and cecal extracts were generated following a previously described protocol (122). Cecal contents or one to three fecal pellets were collected in an Eppendorf and stored at -80°C before analysis. Feces and cecal contents were weighed and 250 μL 1X PBS containing 0.1% Tween 20 was added to each sample before vortexing for 20 minutes. Samples were centrifuged at 12,000 rpm for 10 minutes as 4°C. The entire supernatant was transferred to a new tube, which was centrifuged a second time to remove all debris. This debris-free supernatant was used for ELISAs. Lcn2 was detected by the murine Lcn2 DuoSet ELISA kit (R&D Systems). IgA was detected by sandwich ELISA as described above.

Flow cytometric analysis

Bone marrow cells, whole blood, or lung digest were lysed with RBC lysis buffer (eBioscience) following the manufacturer's protocol. Bone marrow and lung cells were counted, and 5×10^6 cells were used for staining. Cells were washed with PBS and stained with Invitrogen 0.25 μL LIVE/DEAD Fixable Aqua Dead Cell Stain (Thermo Fisher Scientific) in 500 μL PBS for 20 minutes at room temperature in the dark. In some experiments, cells were washed with FACS buffer (1% FBS in PBS) and incubated with APC-Cy7–labeled anti-CD16/32 for 30 minutes (Panel 5, Table 2). After washing in FACS buffer, cells were blocked with anti-CD16/CD32 (BD Biosciences, San Jose, CA) for 10 minutes and then stained in 100 μl antibody mixture in FACS buffer (as detailed in Table 2 and Table 3) for 30 minutes at 4°C in the dark. Cells were then washed in FACS buffer and fixed in 4% paraformaldehyde. Samples were run on a LSRII flow cytometer (BD Biosciences) or sorted on a FACSAria SORP system. Data were analyzed on FlowJo 10.7 (Tree Star, Ashland, OR). Compensation on samples collected by the LSRII was performed in FlowJo post-collection.

Table 2. Antibodies used for flow cytometry in Chapter 3.

Panel 1

Company	Clone	Specificity	Fluorophore
BioLegend	30-F11	CD45	Pacific Blue
BioLegend	M1/70	CD11b	AF488
BD	145-2C11	CD3	PE-Cy7
BD	1D3	CD19	PE-Cy7
BioLegend	1A8	Ly6G	APC-Cy7
BioLegend	HK1.4	Ly6C	AF700
BioLegend	AFS98	CD115	APC
BD	E50-2440	Siglec-F	PE
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 2

Company	Clone	Specificity	Fluorophore
BD	HL3	CD11c	AF700
BioLegend	1A8	Ly6G	APC-Cy7
BD	T21	IL-5Ra	AF488
BD	E50-2440	Siglec-F	PE
BD	M1/70	CD11b	PE-CF594
BioLegend	30-F11	CD45	PE-Cy7
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 3

BD	RM4-5	CD4	PerCP-Cy5.5
eBioscience	53-6.7	CD8a	PerCP-Cy5.5
eBioscience	M1/70	CD11b	PerCP-Cy5.5
eBioscience	eBio1D3 (1D3)	CD19	PerCP-Cy5.5
BD	RB6-8C5	GR1	PerCP-Cy5.5
BioLegend	D7	Sca-1	PECy7
BD	2B8 (RUO)	c-Kit	APC
eBioscience	RAM34	CD34	eFluor450
BD	T21	IL5Ra	AF488
BD	E50-2440	Siglec-F	PE
R&D	245707	ST2	AF700
BioLegend	30-F11	CD45	APC-Cy7
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 4

BioLegend	1-Mar	FceRI	Pacific Blue
BioLegend	M1/70	CD11b	AF488
eBioscience	RMST2-2	ST2	PE
BD	HMα2	CD49b	APC
BioLegend	C398.4A	ICOS	PE-Cy7
BD	1D3	CD19	APC-Cy7
BioLegend	30-F11	CD45	AF700
BD	RM4-5	CD4	PerCP-Cy5.5
eBioscience	53-6.7	CD8	PerCP-Cy5.5
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 5

BD	145-2C11	CD3	PECy7
BD	RM4-5	CD4	PECy7
BioLegend	53-6.7	CD8a	PECy7
BD	1D3	CD19	PECy7
BioLegend	RB6-8C5	GR1	PECy7
BioLegend	RA3-6B2	B220	PECy7
BD	2B8	c-Kit	PE
eBioscience	RAM34	CD34	eFlour660
eBioscience	D7	Sca-1	PerCP-Cy5.5
BD	T21	IL5Ra	AF488
BioLegend	93	CD16/CD32	APC-Cy7
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 6

BD	E50-2440	Siglec-F	BV421
eBioscience	RAM34	CD34	eFlour660
eBioscience	RMST2-2	ST2	PE
BioLegend	D7	Sca1	PECy7
BD	RM4-5	CD4	PerCP-Cy5.5
eBioscience	53-6.7	CD8a	PerCP-Cy5.5
eBioscience	M1/70	CD11b	PerCP-Cy5.5
eBioscience	eBio1D3 (1D3)	CD19	PerCP-Cy5.5
BD	RB6-8C5	GR1	PerCP-Cy5.5
BD	T21	IL5Ra	AF488
BioLegend	30-F11	CD45	AF700
BioLegend	2B8	c-Kit	APC-Cy7
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 7

BD	RB6-8C5	GR1	PerCP-Cy5.5
BD	M1/70	CD11b	APC-Cy7
BD	E50-2440	Siglec-F	BV421
BD	2B8	c-Kit	PE
eBioscience	RAM34	CD34	eFlour660
BioLegend	D7	Sca1	PECy7
BioLegend	30-F11	CD45	AF700
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 8

BioLegend	BM8	F4/80	APC
BioLegend	HK1.4	Ly6C	AF700
BD	1D3	CD19	APC-Cy7
BD	RB6-8C5	GR1	PerCP-Cy5.5
BD	2G9	MHC II	PE
BD	145-2C11	CD3	PE-Cy7
BioLegend	30-F11	CD45	Pacific Blue
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 1 was used for Figure 5. Panel 2 was used for Figure 6. Panel 3 was used for Figure 10, Figure 16, Figure 18, and Figure 25. Panel 4 was used for Figure 8. Panel 5 was used for Figure 12. Panel 6 was used for Figure 11. Panels 7 and 8 were used for Figure 26.

Table 3. Antibodies used for flow cytometry in Chapter 4.

Panel 9

Company	Clone	Specificity	Fluorophore
eBioscience	M1/70	CD11b	eFluor 450
BioLegend	N418	CD11c	Pacific Blue
BioLegend	RA3-6B2	B220	Pacific Blue
BD	500A2	CD3	Pacific Blue
BioLegend	53-7.3	CD5	eFluor 450
BioLegend	1-Mar	FceRI	Pacific Blue
BioLegend	PK136	NK1.1	Pacific Blue
BioLegend	53-2.1	CD90.2	APC
BioLegend	A7R34	CD127	AF488
BioLegend	2F1/KLRG1	KLRG	PE-Cy7
BioLegend	PC61	CD25	PE/Dazzle 594
BioLegend	30-F11	CD45	AF700
eBioscience	RMST2-2	ST2	PE
BioLegend	M5/114.15.2	MHC II	APC-Cy7
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 10

Company	Clone	Specificity	Fluorophore
BioLegend	30-F11	CD45	APC-Cy7
eBioscience	M1/70	CD11b	eFluor450
BD	HL3	CD11c	PE-Cy7
BioLegend	X54-5/7.1	CD64	PE
BioLegend	M5/114.15.2	MHC II	AF700
BioLegend	M1/69	CD24	APC
BD	E50-2440	Siglec-F	PerCP-Cy5.5
BioLegend	1A8	Ly6G	AF488
Life Technologies		Aqua (Live/Dead)	AmCyan

Panel 9 and 10 were used for Figure 39.

Statistical analysis

Data are represented at mean ± SEM. Data was analyzed using Student's t test, Mann-Whitney test, one-way ANOVA, or two-way ANOVA where appropriate (GraphPad Prism 6 software).

CHAPTER 3 - IL-33 precedes IL-5 in regulating eosinophil commitment and is required for eosinophil homeostasis

Introduction

Eosinophils

Eosinophils are granulocytes that make up 1-3% of the circulating blood leukocytes (123). While the half-life of blood eosinophils is 8-18 hours, resident eosinophils within the intestine can survive up to 14 days (124, 125). This increased survival is mediated by signaling through the common γ-chain (125), which is shared by receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, as well as ILC2-derived IL-5 and IL-13 (126). Although most eosinophils are found in the intestine, they also reside in the thymus, adipose tissue, mammary glands, and uterus (126, 127).

Eosinophils have similar morphology to neutrophils, except that they have bilobed nuclei and their granules are stained by the acid dye eosin (123). Four proteins are major constituents of eosinophil granules: major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP). All of these proteins have cytotoxic functions, and ECP and EDN are also ribonucleases.

Homeostatic functions

As tissue resident cells, eosinophils have numerous homeostatic functions. Within the intestine, eosinophils promote intestinal homeostasis, which is demonstrated by the fact that eosinophil deficient mice have impaired oral tolerance to food antigens (128). Several mechanisms have been identified to explain this phenotype. First, intestinal goblet cells produce less mucus in eosinophil deficient mice, which has been suggested to affect oral tolerance. Furthermore, eosinophils appear to regulate lymphocyte homeostasis. Eosinophil deficient mice have decreased $CD103^{+}Foxp3^{+}$ Tregs and increased Th17 cells within the lamina propria (129, 130). The Th17 cell numbers are controlled by eosinophils through the secretion of IL-1Ra, which antagonizes IL-1β for Th17 cell development (130). Furthermore, eosinophils

can also promote class switching and maintenance of IgA^+ plasma cells, likely through the secretion of IL-1β (128, 129). Eosinophils have been shown to promote lymphocyte homeostasis outside of the intestine as well. In the bone marrow, eosinophils promote plasma cell survival by secreting APRIL and IL-6 (131), whereas in the thymus, eosinophils participate in thymocyte negative selection (132).

In addition to regulation of lymphocytes, eosinophils have been shown to regulate macrophages. As producers of IL-4 and IL13, eosinophils can promote alternative activation of macrophages (133). Alternatively activated macrophages (AAMs) are known to be important for glucose metabolism in adipose tissue. Since eosinophils are necessary for AAM development and hypereosinophilia improves the response to a glucose challenge, eosinophils regulate metabolic homeostasis through AAMs (133).

Recently, significant contributions were made in understanding the homeostatic functions of eosinophils when it was determined that resident eosinophils, now termed rEos, have a distinct phenotype apart from the well characterized inflammatory eosinophils, known as iEos (Table 4)(125, 134-136). One critical difference between rEos and iEos is that treatment with an anti-IL-5 antibody significantly diminished the number of iEos recruited to the lung following allergen challenge, however rEos numbers remained the same (134). Furthermore, gene and cell surface receptor expression by rEos were largely unaffected by allergen challenge. Instead, rEos have immunosuppressive effects on DCs and appear to play a role in suppressing allergic sensitization. In summary, resident eosinophils have the ability to modulate several immune cells to promote normal thymic T cell development and bone marrow plasma cell survival, as well as glucose, intestinal, and lung homeostasis.

Roles in disease

As mentioned previously, the role for inflammatory eosinophils in inflammation has been extensively studied. Several diseases have been shown to be highly associated with eosinophils, including helminth infection, respiratory syncytial virus (RSV) infection, asthma, allergic rhinitis, eosinophilic esophagitis (EoE), and atopic dermatitis (127, 137-139). Within these diseases, eosinophils have been shown to possess innate immune functions as well as promote the development of adaptive immunity.

Table 4. Eosinophil phenotypes in various tissues.

	Resident						Inflammatory
	Intestine	Thymus	Uterus	Spleen	Blood	Lung	Lung
Siglec-F	high	high	high	int	int	int	high
IL-5Ra	int	ND	ND	ND	ND	int	ND
CCR3	+	+	+	+	+	+	+
CD11b	+	ND	ND	+	+	+	ND
CD11c	+	+	+	–	–	–	
GR-1	+/–	ND	ND	+	ND	+	ND
F480	–	ND	ND	+	ND	+	+
MHC II	–	ND	ND	–	–	–	ND
SSC	high	high	high	high	high	high	high
CD62L	–	ND	ND	ND	high	+	–
CD101	–	ND	ND	ND	–	low	high
Nucleus shape	bilobed	bilobed	bilobed	bilobed	bilobed	donut/bilobed	segmented

+ indicates expression; − indicates no expression; ND indicates expression not determined.

Eosinophils have several innate functions which are important for the clearance of viral, bacterial, and helminth infections. While eosinophils express TLR1, TLR4, TLR7, TLR9 and TLR10 (140), TLR7 expression on eosinophils was specifically shown to be important for clearance of RSV (141). Furthermore, TLR7 activation led to superoxide generation, which was required for viral clearance (140, 141). Superoxide production by eosinophils has also been shown to kill *E. coli* in vitro (142), though it is unclear if it was triggered by TLR activation or another stimulus. In addition to superoxide, bacteria can be killed by eosinophils through the release of MBP and ECP, which cause permeabilization of bacterial membranes (143). Antibacterial properties of EPO and EDN have also been shown (142, 144). The bactericidal activity of these granule proteins functions both intracellularly in phagocytic vacuoles and extracellularly, with the release of granule protein-containing extracellular nets (143, 145). Finally, unlike viruses and bacteria, the involvement of eosinophils in helminth infection is complex and not fully understood. Despite the fact that eosinophils are increased in helminth infections, eosinophil deficient mice have been utilized to demonstrate that eosinophils can be beneficial, detrimental, or have no effect on parasite clearance (137). The necessity for eosinophils differs depending on the parasite, location of infection, lifecycle stage, and whether it is a primary or secondary infection. Because of this, eosinophils have been shown to function in many ways, including presenting antigen to T cells, recruiting T cells, modulating macrophages and dendritic cells through IL-10 production, and promoting tissue repair by IL-4 production (137).

In allergic disease, eosinophils have been extensively studied as effector cells that respond to allergen challenge. Despite the new evidence that defines regulatory eosinophils that limit sensitization as described above, eosinophils are known to support allergic sensitization by stimulating DCs to promote Th2 development. This can be accomplished through mediators such as EPO, EDN and indolamine 2,3-dioxygenase (IDO) (146-148). Eosinophils can also act as antigen presenting cells (APC), although they do not express MHC II until they are stimulated with IFNγ, IL-4, or granulocyte macrophage colony-stimulating factor (GM-CSF) (149, 150). After sensitization, eosinophils can become APCs and acquire antigen from the intestinal lumen using antigen-specific IgG (151). Furthermore, eosinophils have been

shown to effect airway hyperresponsiveness, mucus production, collagen deposition, and Th2 cytokine levels (123).

Activation of mature eosinophils by cytokines

IL-3, IL-5, and GM-CSF functions on mature eosinophils

Some of these described functions of eosinophils are a result of activation by cytokines. Eosinophils can be activated by a variety of cytokines, but the most commonly studied eosinophil-associated cytokines are IL-3, IL-5, and GM-CSF. These three cytokines are mainly known to prime eosinophils, making them more responsive to a secondary stimulus (152-156). However, they also are stimuli on their own. IL-5 and GM-CSF can induce degranulation, although they are not as potent as other stimuli such as *Alternaria alternata* or phorbol 12-myristate 13-acetate (PMA) (157, 158). GM-CSF may have differential effects on granule release, because it was shown to promote release of EDN but not ECP (158, 159). Both IL-5 and GM-CSF can promote eosinophil survival (160-163). However, IL-5, IL-3 and GM-CSF all downregulate the IL-5R and upregulate the IL-3R (164).

IL-33 functions on mature eosinophils

Beyond the functions of these three cytokines classically associated with eosinophils, recent interest in the “epithelial-derived” cytokines TSLP, IL-25, and IL-33 have prompted new studies of cytokine-activated eosinophils. Little is known about the activation of eosinophils by TSLP and IL-25, likely because eosinophils have low expression of the TLSP and IL-25 receptors prior to priming (165, 166). After priming with IL-3 and TNFα, TSLP can promote release of EDN and increase eosinophil survival (165). Alternatively, eosinophils that were both primed and stimulated with IL-25 produced many cytokines and upregulated ICAM-1 (166). IL-33 is strongly linked to eosinophilic inflammation, but until recently it was assumed that these effects were indirectly caused by the induction of IL-5. Several recent studies have uncovered that IL-33 can directly act on eosinophils to regulate their survival, death, activation, and adhesion (Figure 3B).

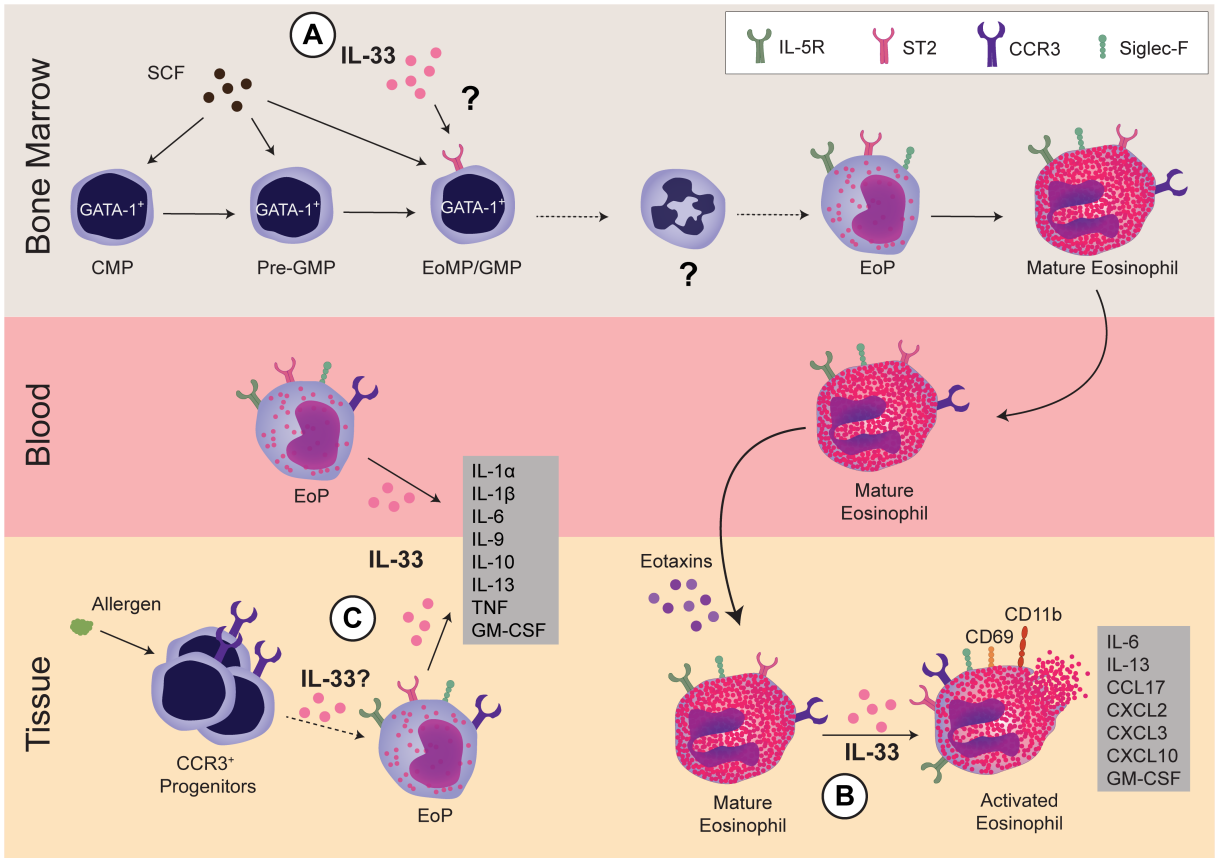

Figure 3. Regulation of eosinophils by IL-33.

(A) In the bone marrow, GATA-1$^+$ CMP differentiate into GATA-1$^+$-pre-GMP (Lin$^-$Sca-1$^-$c-kit$^+$CD41$^-$ CD16/32$^-$CD105$^-$CD150$^-$GATA-1$^+$), then to GATA-1$^+$-GMP, also known as eosinophil/mast cell progenitors (EoMP, Lin$^-$Sca-1$^-$c-kit$^+$CD41$^+$CD16/32$^+$GATA-1$^+$). At this early stage, IL-33 has the potential to regulate ST2$^+$EoMP/GMP. **(B)** In the tissue, IL-33 can activate mature eosinophils, leading to cytokine production and upregulation of CCR3, CD69, and CD11b. Notably, IL-33–driven production of GM-CSF and IL-13 promote eosinophil survival and differentiation of alternatively activated macrophages, respectively. **(C)** Finally, IL-33 can regulate EoP outside of the bone marrow. IL-33 increases the number of EoP in blood as well as activates EoP to produce many cytokines. Although allergens increase CCR3$^+$ progenitors in tissue, it is unclear if increases in EoP in asthma patients are due to EoP leaving the bone marrow or extramedullary eosinophilopoiesis. It has yet to be determined if IL-33 also regulates eosinophilopoiesis within the tissue.

Administration of IL-33 is sufficient to drive in vivo eosinophilia in various tissues (7). While IL-33 does not act as an eosinophil chemoattractant (53), several studies show that IL-33 regulates eosinophil survival. For example, transferring ST2 KO eosinophils into recipient mice led to significantly fewer lung eosinophils after allergen challenge than WT eosinophils despite normal migratory functions, implying impaired survival (167). IL-33 also induces GM-CSF that acts in an autocrine fashion to promote survival by inducing the anti-apoptotic protein Bcl-x_L (163), a response that is negatively regulated by dual specificity phosphatase 5 (Dusp5) (168). Beyond these positive effects of IL-33 on eosinophil survival, IL-33–primed human eosinophils are more susceptible to Siglec-8–induced death; while this priming effect is less effective than IL-5, the two cytokines may function synergistically (169). Thus, the effects of IL-33 on eosinophil survival support a role on both survival and death, most likely in a context-specific fashion.

IL-33 is a potent activator of eosinophils, even more so than IL-5 in terms of triggering degranulation and superoxide release from human eosinophils (170). In mice, IL-33 stimulation alters over 500 genes, many of which are immune related, including IL-6, IL-13, CCL17, CXCL2, CXCL3, and CXCL10 (171). IL-33 can also upregulate several cell-surface markers, including the adhesion molecule CD11b (53), the eotaxin receptor CCR3 (57), and the activation marker CD69 (168).

The functional nature of IL-33–activated eosinophils has been addressed. Transfer of eosinophils activated with GM-CSF, IL-4, and IL-33 into eosinophil-deficient mice during the challenge phase of an asthma model drove IL-13–dependent mucus production and accumulation of AAMs (172). In a complementary approach, increased IL-13 and AAMs were again observed after intranasal IL-33 administration to ST2 KO mice after adoptive transfer of WT eosinophils; recruitment of several cell types, including macrophages, neutrophils, lymphocytes, and the recipient's own eosinophils were also observed in this model (57). In the skin, IL-33 has been proposed to directly act on eosinophils to promote fibrosis in an IL-4– and IL-13–dependent manner (47).

Eosinophil Development

Stages of homeostatic eosinophil development in bone marrow

Initially, eosinophil development was characterized into 4 classes (I-IV) based on nuclear morphology, granular morphology, and Wright-Giemsa staining (173). While Class I cells were described as granulocytic but not eosinophilic, Class II cells had small numbers of granules and appeared to have committed to the eosinophil lineage. Prior to terminal differentiation, Class III cells have the characteristic donut-shaped nucleus. However, Class IV cells were the only eosin positive cells and maintained the ring-shaped nucleus, which often twists into a figure 8-like structure.

More recently, stages in eosinophilopoiesis have been phenotyped using cell surface markers (Table 5). When the eosinophil lineage–committed progenitor (EoP) was initially identified in mice (174), it was proposed that eosinophils developed in four defined stages within the myeloid pathway. Originating from common myeloid progenitors (CMPs) that differentiate to granulocyte and macrophage progenitors (GMPs), a lineage decision into EoPs then occurs before terminal differentiation into eosinophils. Importantly, although EoP stain with eosin, eosin-negative precursors have been reported (173), suggesting a precursor stage prior to the granulation events occurring in EoP.

From all of the markers defining eosinophils, three appear to be important for defining stages of eosinophil development: IL-5Rα, Siglec-F, and CCR3. IL-5Rα is an indicator of commitment to the eosinophil lineage, as it is a key differentiator between the EoP and earlier stages of development. Although Siglec-F was originally thought to only mark mature mouse eosinophils and alveolar macrophages outside of the bone marrow, it has been shown that the EoP also expresses Siglec-F (175). Furthermore, colony forming assays comparing $Lin^{-}CD34^{+}CD117^{int}$IL-5R$\alpha^{+}$ (EoP-IL-Rα) vs Lin^{-} $CD34^{+}CD117^{int}$Siglec-F^{+} (EoP-Siglec-F) showed that only EoP-IL-5Rα gave rise to pure eosinophils. EoP-Siglec-F cultures generated a mixture of eosinophils and macrophages. Thus, Siglec-F appears to mark eosinophil potential in the bone marrow, whereas IL-5Rα indicates commitment to the eosinophil lineage. Finally, CCR3 is a late marker, as it allows eosinophils to exit the bone marrow in response to eotaxin (176).

Table 5. Cell surface markers of cells involved in murine eosinophilopoiesis.

	CMP	GMP	EoPre	EoP	Mature Eo
Lineage	–	–	high[a]	–	ND
Sca-1	–	–	–	–	–
c-Kit	+	high	–	low	–
CD34	+	+	–	+	–
FcγRII/III	low	high	ND	ND	ND
IL-5Rα	–	–	+	+	+
IL-3R	ND	+	ND	ND	+
IL-4Rα	ND	ND	ND	ND	+
GM-CSFR	ND	+	ND	ND	+
Siglec-F	ND	ND	low	+	+
CCR3	ND	ND	ND	ND	+
Granularity (SSC)	low	low	low	high	high
ST2	+/–	+/–	–	+	+

+ indicates expression; − indicates no expression; ND indicates expression not determined.

[a]Unlike other studies, this study included CD11b in the lineage cocktail and demonstrated that the EoPre is CD11b^{hi}.

In humans, the hEoP (IL-5Rα^{+}CD34^{+}CD38^{+}IL-3Rα^{+}CD45RA^{-}) differentiates directly from the hCMP (Lin^{-}CD34^{+}CD38^{+}IL-3Rα^{+}CD45RA^{-}IL-5Rα^{-})(177). Furthermore, the hGMP (Lin^{-}CD34^{+}CD38^{+}IL-3Rα^{+}CD45RA^{+}) is capable of generating neutrophils, monocytes, and basophils. Other stages of human eosinophil progenitors have yet to be determined. Although IL-5Rα^{+} progenitors only generated eosinophils, IL-5Rα was found to be expressed in the blood on both mature eosinophils and mature basophils (177). Thus, it is unclear if IL-5Rα may be used to identify commitment to the eosinophil lineage as it does in mice. Furthermore, Siglec-8, the human functional paralog of Siglec-F, does not mark eosinophil potential in progenitors as Siglec-F does in mice (178, 179). Instead, Siglec-8 is expressed only at later stages of development of eosinophils, mast cells, and basophils.

ST2 expression on these progenitors has been debated. Two studies generally examining bone marrow stem cells showed opposing results: while Le et al. found ST2 on Lineage^{-}c-kit^{+}Sca-1^{+} cells, CMP, GMP, megakaryocyte-erythroid progenitors (MEP), and common lymphocyte progenitors (CLP) (180), Mager et al. did not find ST2^{+} long-term or short-term hematopoietic stem cells (LT-HSC, ST-HSC), multipotent progenitors (MPP1, MPP2, MPP3), MEP, CMP, or GMP (181). More recently, Tsuzuki et al. demonstrated that ST2 is expressed on CMP, MEP, and EoP, but not GMP (182). These differences in ST2 expression may be partially resolved by new research that redefines the early stages in eosinophil development (183). Using single cell RNA sequencing of pre-granulocyte macrophage progenitors (Pre-GMP, Lin^{-}c-Kit^{+}Sca-1^{-}CD41^{-}CD16/32^{-}CD105^{-}CD150^{-}), Pre-GMP clustered into two groups: GATA-1^{+}Flt3^{-} and GATA-1^{-}Flt3^{+}. By sorting cells from a GATA-1–EGFP reporter and culturing them in eosinophil-promoting conditions, GATA-1^{+} Pre-GMPs generate eosinophils, whereas GATA-1^{-} Pre-GMPs generate neutrophils and monocytes. Drissen et al. proposed that GATA-1^{+}GMPs be renamed eosinophil/mast cell progenitors, and GATA-1^{-} GMPs retain their name. Thus, instead of the classical model (CMP, GMP, EoP, mature eosinophil), the EoP population can develop independently of the GMP (Figure 3A). This aligns with the description of the hEoP arising from the hCMP and not the hGMP (177). Notably, gene expression of ST2 differentiated the GATA-1^{+} Pre-GMP and GATA-1^{-} Pre-GMP populations (183). Thus, despite continuing debate over ST2 on CMPs and GMPs, eosinophils likely arise from ST2-expressing progenitors.

Transcriptional regulation of eosinophil development

In addition to cell surface markers, eosinophil lineage commitment has also been defined by transcription factors. GATA-1, a member of the GATA family of transcription factors, is known to be critical for eosinophil development. In agreement with the potential of GATA-1$^+$GMPs to produce eosinophils (183), human CD34$^+$ stem cells transduced to express GATA-1 develop into eosinophils (184). Mice that lack a GATA binding site in the promoter of GATA-1, known as Δdbl-GATA mice, have no eosinophils (185). GATA-2 also instructs eosinophil commitment and may compensate for a deficiency in GATA-1, although GATA-2 deficiency reduces hematopoiesis generally and is not specific to eosinophils (184).

Several members of the CCAAT/enhancer-binding protein family have also been implicated in eosinophil development. Both C/EBPα and C/EBPβ have been shown to promote eosinophilopoiesis, but only mice lacking C/EBPα have lower eosinophils (186). Functionally, C/EBPβ and GATA-1 act together to activate transcription of MBP. MBP expression can also be induced when PU.1, C/EBPε32 and GATA-1 are simultaneously expressed.

Transcriptome analysis comparing GMPs to EoPs and mature eosinophils identified 56 transcription factors specific to the eosinophil lineage, including GATA-1, C/EBPε, NFκB, NFAT2, STAT1, STAT3, STAT6, IRF1, IRF2, Helios, and Aiolos (175). However, it has yet to be determined if and how all of these transcription factors play a role in eosinophil development. The eosinophilic potential of GATA-1$^+$–GMPs certainly indicates that GATA-1 plays an important role early in eosinophil development. After commitment to the eosinophil lineage, XBP1 is required for eosinophil development and promotes survival of EoP (187). It is unclear how IL-33 and ST2 may be regulated by and modulate these transcription factors. It is known that ST2 signaling leads to NFκB activation in mature cells (7), though whether this occurs in hematopoietic stem cells and how it affects eosinophilopoiesis remains to be determined. Since ST2 has two GATA binding sites upstream of its promoter, it was determined that ST2 expression can be regulated by GATA-1 and GATA-2 (188). The fact that GATA-1$^+$GMPs express ST2 mRNA but not protein would hint that GATA-1 may regulate ST2 in eosinophil development, but this requires further study.

Cytokine regulation of eosinophil development

Several cytokines have been shown be important for the differentiation and maturation of eosinophils in the bone marrow. Notably, IL-5 is the hallmark eosinophil-associated cytokine (189). IL-5-overexpressing transgenic mice (NJ.1638) have an excessive number of eosinophils in the blood, bone marrow, and several tissues (173). Examination of the bone marrow revealed that the NJ.1638 mice had significantly more Class III and Class IV cells, which, in conjunction with the fact that IL-5Rα marks eosinophil lineage commitment, indicates that IL-5 acts on the later stages of development. Indeed, IL-5 promotes terminal eosinophil differentiation by upregulating CCR3 (190). It has been demonstrated that IL-5 upregulates its own receptor on human $CD34^+$ cells, but it is unclear if this occurs in vivo and how it would affect eosinophil development (191).

IL-5, IL-3, and GM-CSF all signal through a shared β-chain (CD131, CSF2RB), which dimerizes with the corresponding cytokine-specific α-chain. IL-3 and GM-CSF were originally thought to be important for eosinophil development because they stimulated eosinophils in colony forming assays, but eosinophils were only seen with high concentrations. At lower concentrations they promote the development of other myeloid cells (189). IL-3 is critical for mast cell and basophil development and affects mature eosinophils by globally promoting protein translation (192). As previously mentioned, GM-CSF promotes survival of mature eosinophils, however it has not been determined if this pro-survival signal is utilized in developing eosinophils. Instead, when GM-CSF is added to bone marrow cultures that promote the growth of highly pure eosinophils within ten days, the resulting cultures only contain 24% eosinophils (193). GM-CSF appears to antagonize eosinophil development, though the mechanism has yet to be determined. One interpretation of this data is that perhaps due to the shared β-chain, signaling through the GM-CSFR competes with the IL-5R and limits IL-5 signaling. Thus despite initial studies demonstrating the ability of IL-3 and GM-CSF to promote eosinophil development through colony forming assays, their exact function on specific stages of progenitors remains elusive.

The current protocol for culturing eosinophils from bone marrow utilizes stem cell factor (SCF) and fms like tyrosine kinase 3 ligand (Flt3L) for three to four days before switching to IL-5 (194). Recent

work has demonstrated that Flt3L is not required for eosinophil development (183, 195). C-Kit, the receptor for SCF, is expressed early on many stem cells and is lost in the later stages of eosinophil development, so it is expected that the effects of SCF on eosinophilopoiesis precede IL-5. SCF likely supports cells not committed to any specific lineage, but it is unclear if SCF promotes lineage commitment. In the following data, I define a role for IL-33 in promoting commitment to the eosinophil lineage.

Alternative eosinophilopoiesis mechanisms within tissues

There is increasing evidence that progenitors can circulate in the blood and that local hematopoiesis may occur in tissues (reviewed here (196), Figure 3C). Eosinophil progenitors are increased in the blood and sputum of asthmatic patients (197, 198), but their role in disease is not fully understood. Intravenous IL-5 increased not only circulating eosinophil progenitors but also CCR3 expression on $CD34^+$ progenitors (199). Similarly, IL-33 increased peripheral blood EoP (182). In response to allergen, $CD34^+CCR3^+$ and $Sca\text{-}1^+CCR3^+$ cells proliferated within the lung tissue, demonstrating expansion of local eosinophil lineage–committed stem cells (200). Whether these lung stem cells express ST2 and how IL-33 may affect these cells is unclear. In vitro, IL-33 activated EoP to produce chemokines, Th2 cytokines, and pro-inflammatory cytokines, with more IL-9, IL-10, IL-13, IL-1α, IL-1β, IL-6, TNFα, and GM-CSF than mature eosinophils (182); thus, these data implicate EoP as potential regulators over inflammation. Further research is certainly required to determine how eosinophil progenitors contribute to tissue eosinophilia in disease and if IL-33 serves to initiate their responses.

Summary

Eosinophils are immune cells that circulate in the blood and also reside in several tissues, including the intestine, thymus, and adipose tissue (123). In addition to their roles in homeostatic processes, eosinophils contribute to the pathology of many Type 2–mediated diseases, such as asthma, eosinophilic esophagitis, and atopic dermatitis (201). Many studies have established the important effector functions of eosinophils and their ability to modulate inflammation through the release of granule

contents and cytokines. However, the development of eosinophils in the bone marrow is less understood. It has been established that granulocyte–macrophage progenitors (GMP) give rise to eosinophil lineage–committed progenitors (EoP), which then develop into fully granulated mature eosinophils (EoM) (174). While IL-3, GM-CSF, and IL-5 can drive this eosinophilopoiesis process in vitro (123), IL-5 appears to be the critical cytokine specific to eosinophil development (202-204) and acts mechanistically to drive expansion and survival of EoM within the bone marrow (205). In contrast, the factors involved in driving the initial commitment of GMP into the eosinophil lineage are less clear.

IL-33 is the most recently discovered member of the IL-1 family of cytokines. In its initial description by Schmitz et al., recombinant IL-33 was shown to promote several Type 2-associated responses, including Type 2 cytokine expression (IL-4, IL-5, and IL-13) and IgE production (7). Furthermore, ST2, the IL-33 receptor, is expressed on many cell types involved in Type 2 effector responses, including Th2 cells (33), mast cells, basophils, eosinophils (55), and Type 2 innate lymphoid cells (ILC2s) (50). Subsequently, IL-33 has been extensively studied in the setting of helminth infections and allergic diseases. Studies in mouse models of asthma (103, 115, 206), food allergy (73), and hookworm infection (207) have reported the presence of reduced eosinophilic inflammation in IL-33– or ST2-deficient mice, suggesting a positive interplay between IL-33 and eosinophils. Indeed, the initial description of IL-33 demonstrated that in vivo administration of recombinant IL-33 was sufficient to increase peripheral blood eosinophil numbers (7). Similarly, in vitro IL-33 was proposed to support eosinophil differentiation from bone marrow (57). In sharp contrast, Dyer et al. examined the effects of IL-33 on eosinophil development using in vitro differentiation approaches and concluded that IL-33 antagonized IL-5–dependent eosinophilopoiesis and supported monocyte development (193). Macrophage activation has also been implicated in driving IL-33–induced lung eosinophilia (208).

In the present study, I sought to reconcile these conflicting results by examining the role of IL-33 in mouse eosinophil development in vivo and in vitro. I demonstrate that IL-33– and ST2-KO mice show homeostatic dysregulation of granulocyte responses in both the blood and bone marrow compartments. Furthermore, my data not only show that IL-33 is a potent stimulus for expansion of the Siglec-F^{+} eosinophil pool, but also that the functional influence of IL-33 lies in expansion of an eosinophil precursor

(EoPre) population as well as in upregulation of the IL-5 receptor α (IL-5Rα) on this population. As already established, IL-33 also strongly induces IL-5, which further fuels the development of EoPre cells into an EoM phenotype. Consequently, I propose that IL-33 and IL-5 are cooperative cytokines for eosinophilopoiesis and that IL-33 precedes the need for IL-5 support in the progression towards eosinophil maturity.

Results

Granulocytes are reduced in ST2-deficient mice

Since it had recently been shown that ST2 was expressed on hematopoietic stem cells (180), we initially asked if ST2 was necessary for competent hematopoiesis. Naïve ST2 KO mice had lower spleen weight/body weight ratios and total cell numbers in bone marrow than WT mice (Figure 4), suggesting a defect in the hematopoietic compartment. Upon further cytological examination of hematopoietic-derived cell populations in peripheral blood, ST2 KO mice had fewer peripheral blood eosinophils than WT mice as determined by staining with Discombe's fluid (Figure 5A). As IL-33 had been proposed to promote a macrophage-like phenotype in vitro (193) and neutrophilic inflammation in vivo (43), the frequency of lymphocytes, neutrophils, eosinophils, and monocytes in the blood of ST2 KO and WT mice was also assessed using flow cytometry (Figure 5B, C). In addition to eosinophils, ST2 KO mice had fewer neutrophils than WT mice (Figure 5C). Notably, there were no differences in total monocytes, $CD115^{+}Ly6C^{+}$ monocyte progenitors, or $CD115^{+}Ly6C^{-}$ mature monocytes. Similar to ST2 KO mice, IL-33 KO mice also showed lower numbers and frequency of both eosinophils and neutrophils in peripheral blood than WT mice (Figure 6 and data not shown). Thus, IL-33 and ST2 are necessary for neutrophil and eosinophil homeostasis in the periphery.

IL-33 expands eosinophils in vivo

Next, I wanted to determine if exogenous IL-33 was sufficient to induce hematopoiesis in vivo and if such an effect was dependent on ST2. Following the approach used by Schmitz et al., WT, IL-33 KO, and ST2 KO mice were injected with 0.4 µg recombinant IL-33 or PBS for 7 days and analyzed 18 hours after the last injection (7). As predicted, exogenous IL-33 increased splenic weight (Figure 7) and peripheral blood eosinophils (Figure 6A) in WT and IL-33 KO mice but not ST2 KO mice. This increase in eosinophils by cytology was also confirmed by flow cytometry, with eosinophils being defined as $CD45^{+}SSC^{hi}Ly6G^{neg\text{-}lo}CD11b^{+}Siglec\text{-}F^{+}$ cells (Figure 6B, C). While the levels of eosinophils observed after IL-33 treatment were lower in IL-33 KO mice than in WT, this was not significantly different, and the

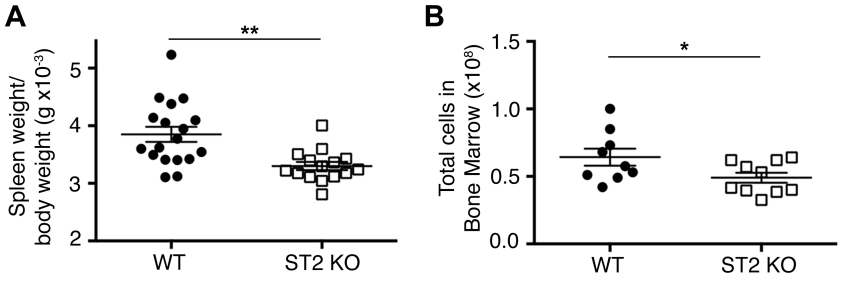

Figure 4. ST2 KO mice have reduced spleen/body weight and total BM cells.

WT and ST2 KO mice were analyzed for (**A**) spleen weight/body weight, (**B**) total cells in bone marrow. Data represent mean ± SEM (n = 8–18 from 3 independent experiments). *$p \leq 0.05$, **$p \leq 0.01$, compared to WT by two-tailed Student t test.

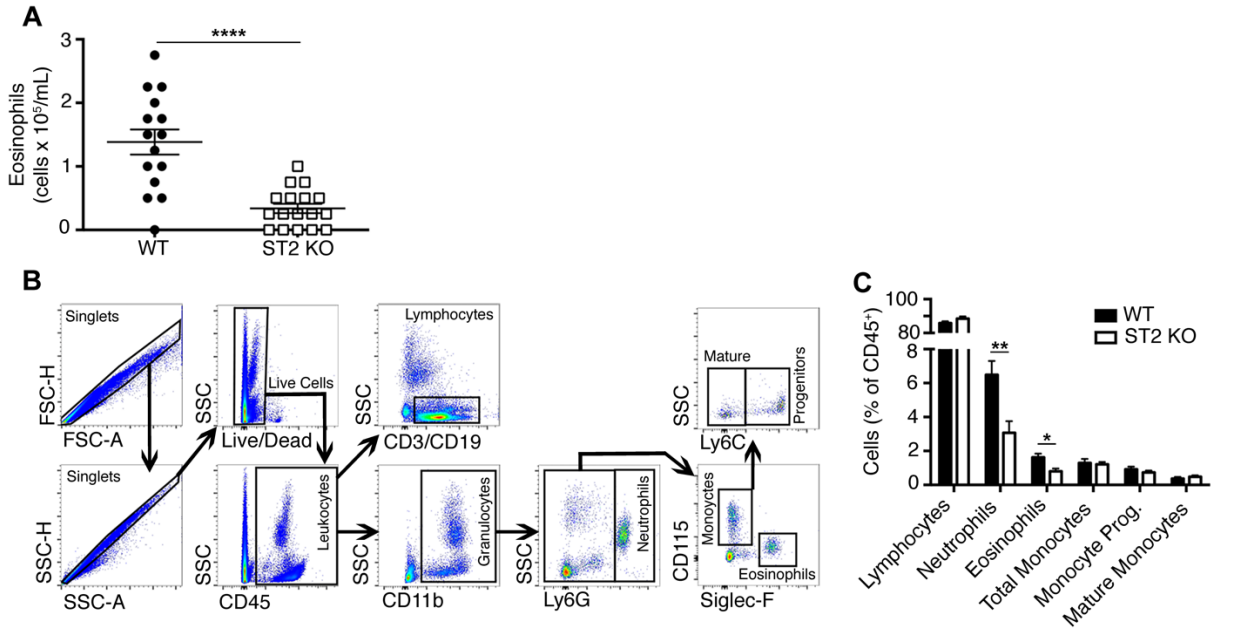

Figure 5. ST2 KO mice have reduced blood eosinophils and neutrophils.

Blood from WT and ST2 KO mice were analyzed for (**A**) eosinophil numbers by staining with Discombe's fluid. (**B**, **C**) Using the flow cytometry gating strategy shown in (**B**), blood leukocytes were analyzed (**C**). Data represent mean ± SEM (n = 8–18 from 3 independent experiments). *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$ compared to WT by two-tailed Student t test.

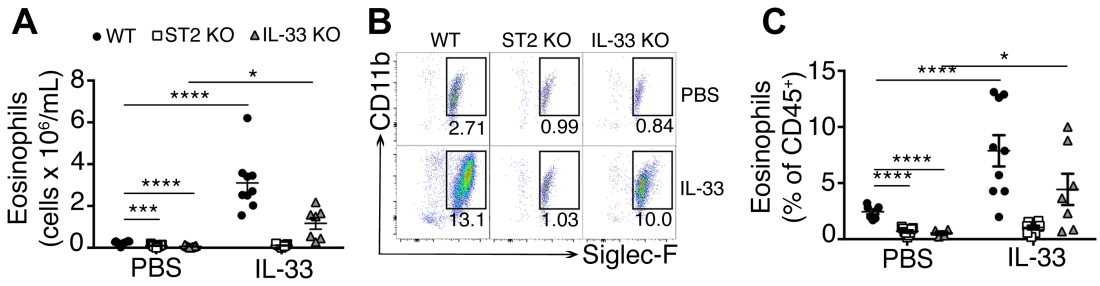

Figure 6. IL-33 is sufficient to drive eosinophil expansion in vivo.

WT, ST2 KO, and IL-33 KO mice were given 0.4 µg IL-33 or PBS intraperitoneally for 7 days and analyzed 18 hours after the last injection. Blood eosinophils were counted by (**A**) Discombe's fluid and (**B, C**) flow cytometry. Data represent mean ± SEM (n = 4–12 from 3 independent experiments). *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$ by two-way ANOVA.

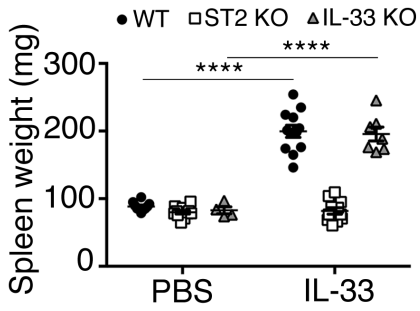

Figure 7. IL-33 increases spleen weight.

WT, ST2 KO, and IL-33 KO mice were given 0.4 μg IL-33 or PBS intraperitoneally for 7 days and spleens were weighed 18 hours after the last injection. Data represent mean ± SEM (n = 4–12 from 3 independent experiments). ****$p \leq 0.0001$ by two-way ANOVA.

relative increases were similar when the differences in basal numbers were considered. In terms of the effect of exogenous IL-33 on other cell populations in the blood, IL-33 did not increase the frequency of neutrophils in the blood (Figure 8) despite ST2 KO mice displaying impaired neutrophil numbers in the basal state (Figure 5C); it did, however, increase the numbers of ILC2s in agreement with previous literature (50). Since I observed differences in total bone marrow cell numbers (Figure 4B) and eosinophil numbers in the peripheral blood between ST2 KO and WT mice (Figure 5), I queried whether the reduced numbers in ST2 KO mice might be due to an effect on the bone marrow itself (and perhaps eosinophilopoiesis) rather than just the known effect on the periphery. Indeed, in addition to the changes seen in the blood, the bones of the IL-33–treated WT mice were noticeably lighter in color than in PBS-treated controls, and this color change was ST2 dependent (Figure 9A). Upon further examination, IL-33 treatment increased the myeloid:erythroid ratio in the bone marrow, indicating an expansion in the myeloid compartment within the bone marrow compartment and that IL-33 might exert direct function at this important eosinophil development site (Figure 9B). Taken together, these data suggest that, within granulocyte populations, IL-33 can expand mature eosinophils in the blood and alter myeloid cells in the bone marrow.

IL-33 expands mature eosinophils in the bone marrow

The increased myeloid:erythroid ratio led us to further examine eosinophil development specifically in the bone marrow using flow cytometry. Since I observed an increase in the pool of Siglec-F$^+$ eosinophils in peripheral blood after IL-33 treatment, I used this Siglec-F marker to initially examine the cells in the bone marrow. Indeed, the total Siglec-F$^+$ cell population in the bone marrow was similarly increased in WT and IL-33 KO, but not ST2 KO, mice after IL-33 treatment (Figure 10A, B). Interestingly, strict gating based on the fluorescence minus one (FMO) controls incorporated a range of Siglec-F expressing cells and higher overall percentages than have been previously reported for mature eosinophils (209). To further examine eosinophil related populations within this Siglec-F$^+$ pool, I defined a population of GMP-like cells (Lin$^-$Sca1$^-$Siglec-F$^+$IL-5Rα$^-$SSC^{lo}ckit^{hi}CD34$^+$ST2$^+$), eosinophil precursors

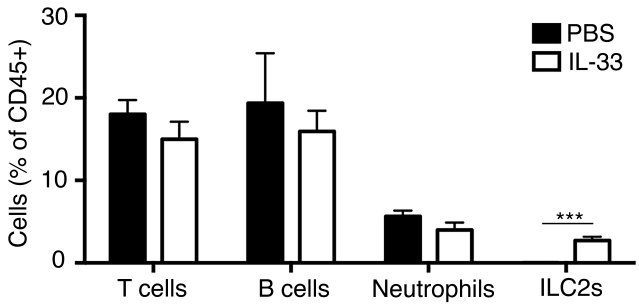

Figure 8. IL-33 increases ILC2s in the blood.

Frequencies of T cells, B cells, neutrophils, and ILC2s were determined in WT mice by flow cytometry. Data represent mean ± SEM. ***p≤0.001, compared to PBS by two-tailed Student's *t*-test.

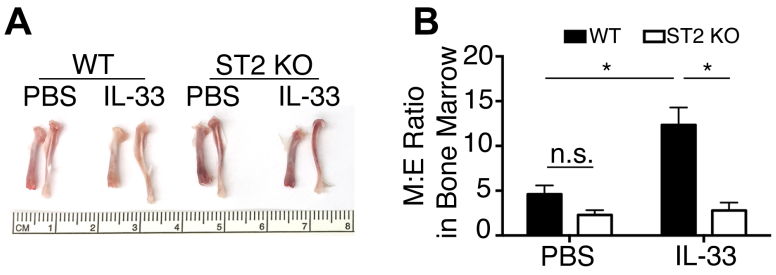

Figure 9. IL-33 increases myeloid cells in the bone marrow.

WT and ST2 KO mice were given 0.4 µg IL-33 or PBS intraperitoneally for 7 days and analyzed 18 hours after the last injection. (**A**) Representative photograph of color change of bone marrow. (**B**) Quantification of the myeloid:erythroid ratio in bone marrow. Data represent mean ± SEM (*n* = 4–12 from 3 independent experiments). *$p \leq 0.05$ by two-way ANOVA.

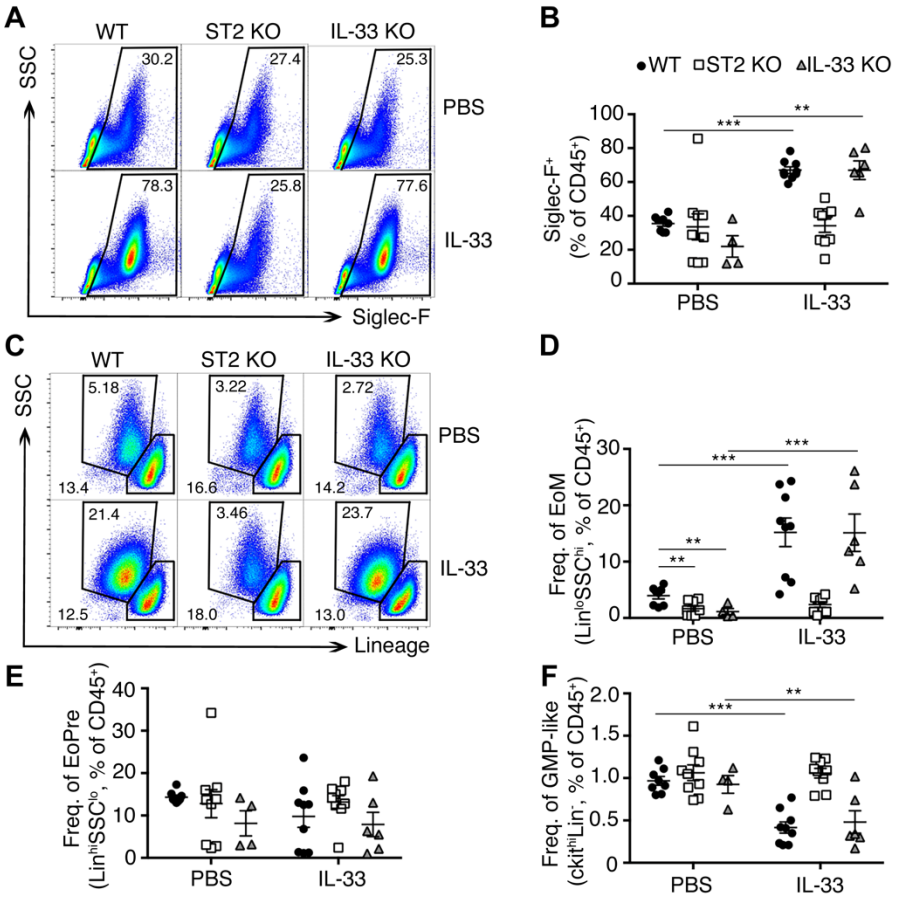

Figure 10. In vivo IL-33 expands mature eosinophils.

WT, ST2 KO, and IL-33 KO mice were given 0.4 µg IL-33 intraperitoneally for 7 days and analyzed 18 hours after the last injection. (**A**) Representative flow plots of the expansion of the Siglec-F$^+$ population in bone marrow. (**B**) Frequency of the Siglec-F$^+$ population. (**C**) Representative flow plots of eosinophil populations in bone marrow. (**D**) Frequency of EoM (Siglec-F$^+$Lin^{lo}SSC^{hi}). (**E**) Frequency of EoPre (Siglec-F$^+$Lin^{hi}SSC^{lo}). (**F**) Frequency of GMP-like (Siglec-F$^+$Lin$^-$ckit^{hi}). All frequencies are shown as the percent of CD45$^+$ cells. Data represent mean ± SEM (n = 3–9 from 3 [A–D] or 2 [E–F] independent experiments). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$ by two-way ANOVA.

(EoPre) ($Lin^{hi}Sca1^{-}Siglec\text{-}F^{lo}IL\text{-}5R\alpha^{+}SSC^{lo}ckit^{-}CD34^{-}ST2^{-}$), and EoM cells ($Lin^{lo}Sca1^{-}Siglec\text{-}F^{hi}IL\text{-}5R\alpha^{+}SSC^{hi}ckit^{-}CD34^{-}ST2^{lo}$) (characterization and FMO control staining shown in Figure 11). Since these GMP-like and EoPre cells expressed slightly different markers than the classically defined GMP and EoP cells, I also used traditional staining regimens to examine the GMP ($Lin^{-}Sca1^{-}ckit^{hi}CD34^{+}CD16/CD32^{hi}$), EoP ($Lin^{-}Sca1^{-}IL5R\alpha^{+}ckit^{lo}CD34^{+}$), and common myeloid progenitor (CMP) ($Lin^{-}Sca1^{-}ckit^{hi}CD34^{+}CD16/CD32^{hi}$), which precedes the GMP (Figure 12) (174). All of these populations express Siglec-F, so they were not excluded from my Siglec-F^{+} gate (Figure 13). Importantly, EoM frequency in the bone marrow was significantly lower in PBS-treated ST2 KO and IL-33 KO mice than in WT mice (Figure 10C, D), which is in agreement with my analysis of EoM in the peripheral blood (Figure 6). Similar frequencies of EoPre, GMP-like, and GMP cells were observed at baseline for all genotypes (Figure 10C, E, F, and Figure 12). ST2 KO mice had significantly lower EoP at baseline (Figure 12). After treatment with IL-33 for 7 days, WT and IL-33 KO mice had dramatically increased frequency of EoM (Figure 10C, D), lower GMP-like cells (Figure 10F), and similar numbers of EoPre (Figure 10C, E). Similar results were observed when CD11b was used instead of the full lineage cocktail (Figure 14). Since IL-33 was previously shown to induce IL-5 (7), I hypothesized that the expanded EoM pool might be due to the influence of IL-5. Indeed, IL-33 treatment significantly increased IL-5 mRNA levels in bone marrow (Figure 15A) and IL-5 protein levels in serum (Figure 15B). Taken together, these data define that IL-33 supports both the expansion of the EoM pool as well as the elevation of IL-5 in the bone marrow and periphery.

IL-33–driven eosinophilopoiesis is IL-5 dependent

To address the contribution of this elevated IL-5 in IL-33–driven eosinophil expansion, mice were treated with intraperitoneal injection of 0.4 μg IL-33 for 7 days in the presence of a neutralizing anti–IL-5 (αIL-5) antibody or its isotype control (Figure 16A). In this particular experiment, I noticed that αIL-5 treatment dramatically altered IL-5R expression levels on cells from those treated mice, most likely due feedback from IL-5R being internalized upon binding to IL-5 (210). I therefore felt it was inappropriate to define the EoPre and EoM populations here, since proper identification of these populations relied on IL-

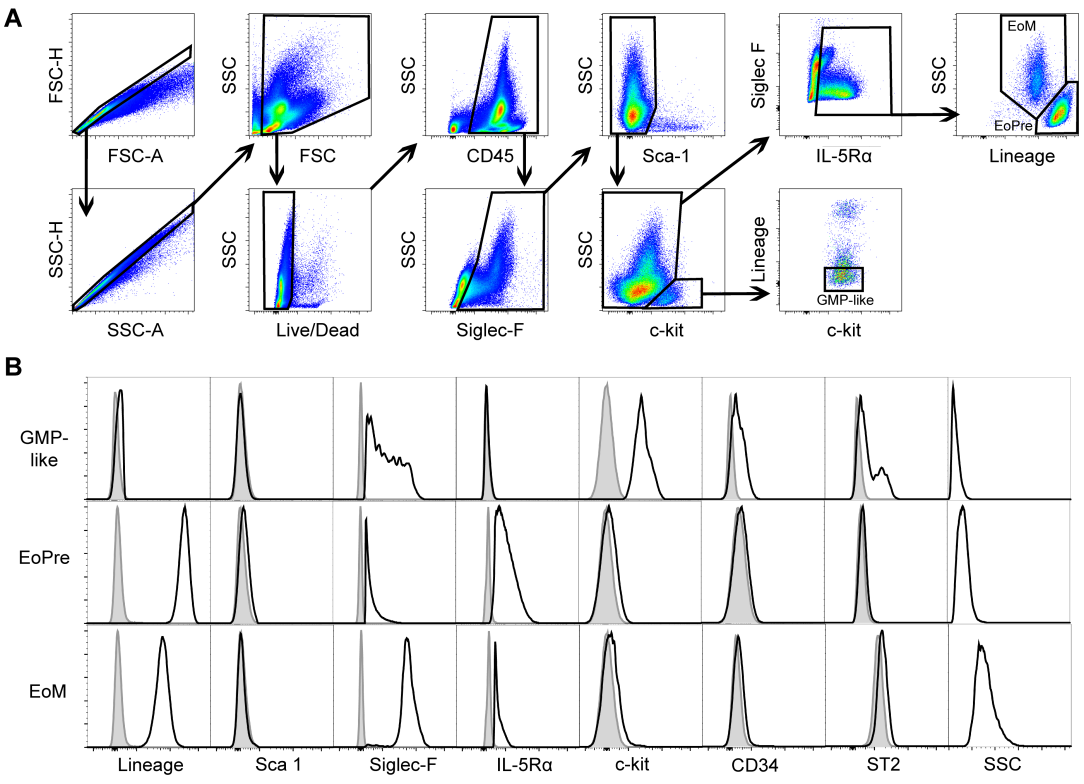

Figure 11. Eosinophil progenitor populations in bone marrow.

(**A**) gating strategy. (**B**) Representative GMP, EoPre, and EoM surface markers based off of the gating strategy in (A). Shaded represents FMO, open represents stained population.

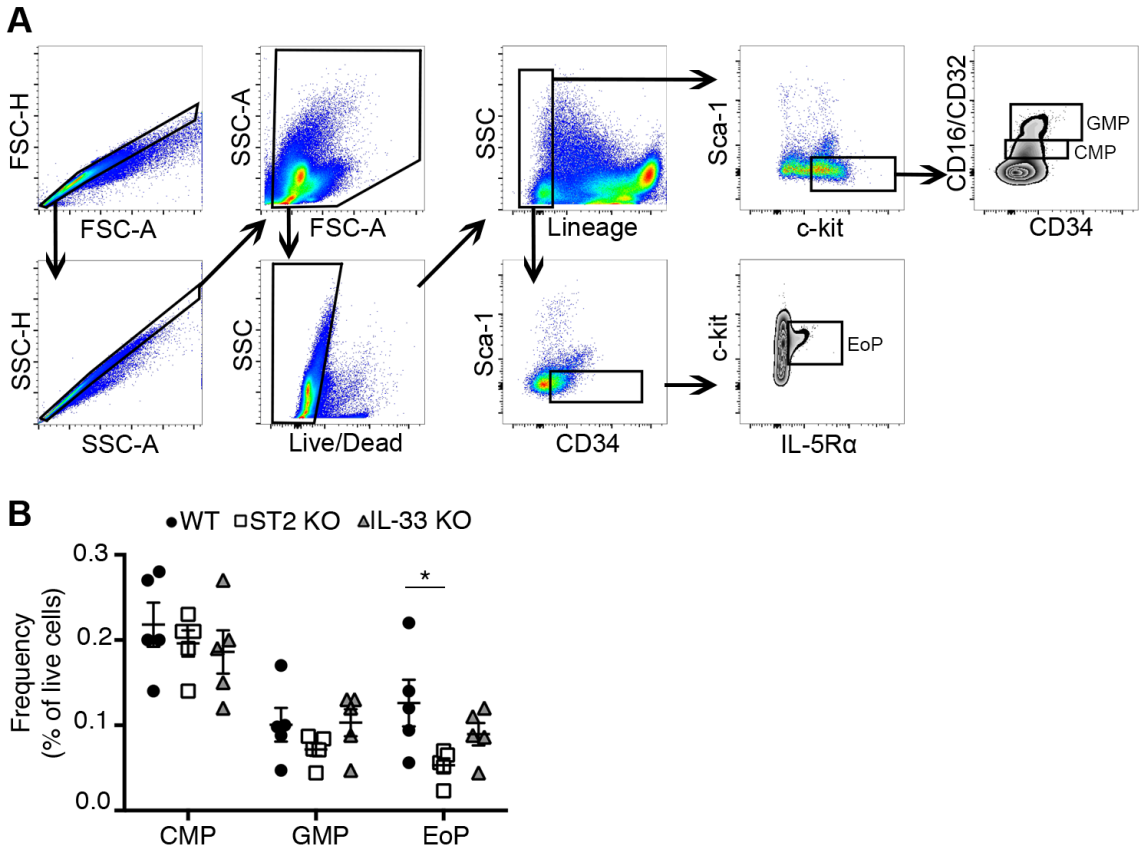

Figure 12. IL-33 and ST2 are not necessary for hematopoietic homeostasis prior to eosinophil lineage commitment.

(A) Gating strategy for CMP ($Lin^-Sca\text{-}1^-c\text{-}kit^+CD34^+CD16/CD32^{int}$), GMP ($Lin^-Sca\text{-}1^-c\text{-}kit^+CD34^+CD16/CD32^{hi}$), and EoP($Lin^-Sca\text{-}1^-c\text{-}kit^{lo}CD34^+IL\text{-}5R\alpha^+$) in the bone marrow. **(B)** Quantification of (A) in naïve WT, ST2KO, and IL33 KO mice.

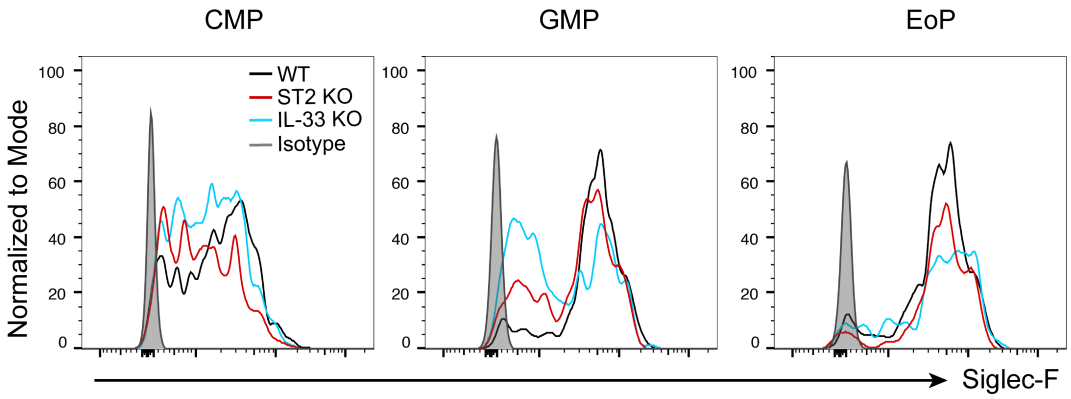

Figure 13. Stem cells express Siglec-F.

Siglec-F expression was determined for the cells defined in Figure 12. Data are representative of 5 mice per group.

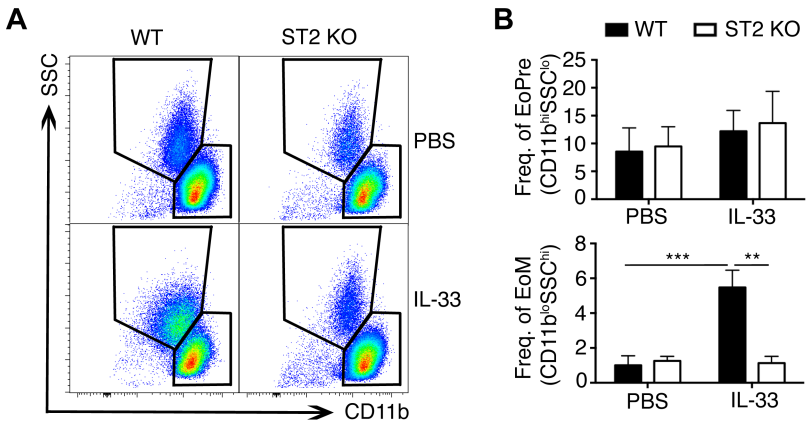

Figure 14. IL-33 expands CD11b-expressing eosinophil populations in vivo.

WT and ST2 KO mice were given 0.4 µg IL-33 i.p. for seven days and analyzed 18 hours after the last injection. (**A**) Representative flow plots of CD11b+ expansion in bone marrow. (**B**) Frequency of $CD11b^{+}$ cell expansion.

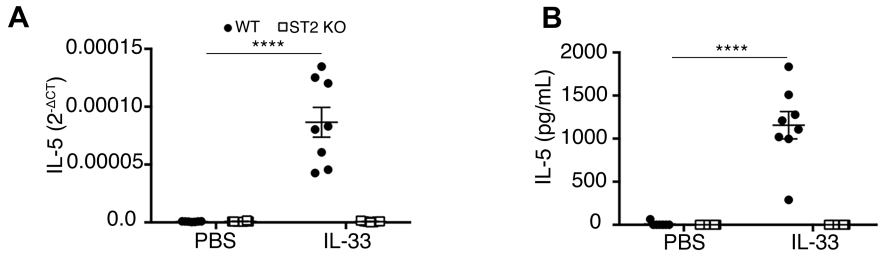

Figure 15. IL-33 induces IL-5 in the bone marrow and serum.

WT, ST2 KO, and IL-33 KO mice were given 0.4 μg IL-33 intraperitoneally for 7 days and analyzed 18 hours after the last injection. (**A**) IL-5 mRNA expression in bone marrow. (**H**) Serum IL-5 concentration. Data represent mean ± SEM (*n* = 3–9 from 2 independent experiments). ****$p \leq 0.0001$ by two-way ANOVA.

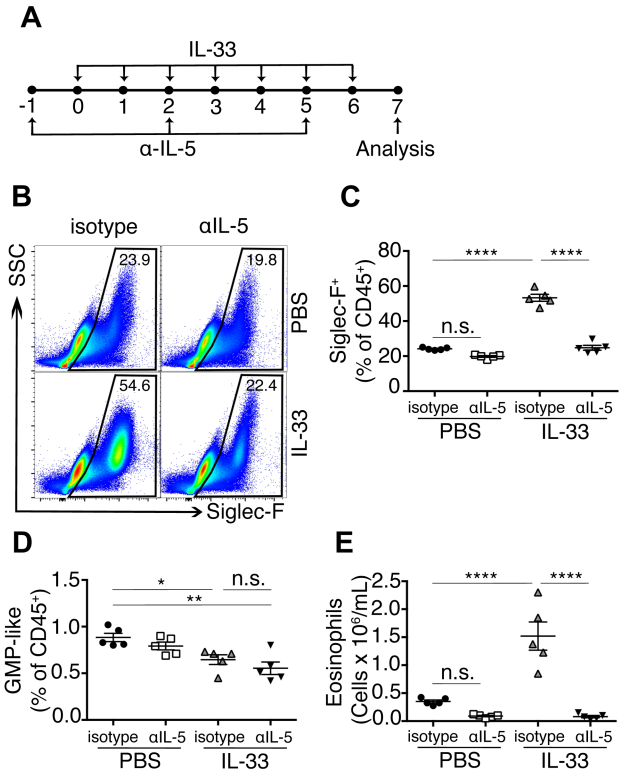

Figure 16. IL-33-driven eosinophil development is IL-5 dependent.

(**A**) Injection scheme. (**B**) Representative flow plots of the expansion of the Siglec-F$^+$ population in bone marrow. (**C**) Frequency of the Siglec-F$^+$ population shown in B. (**D**) Frequency of GMP-like (Siglec-F$^+$Sca-1$^-$Lin$^-$ckit^{hi}). (**E**) Blood eosinophil number determined by staining with Discombe's fluid. Data represent mean ± SEM (*n* = 5–14 from 1 experiment). n.s. = not significant. ***p* ≤ 0.01, *****p* ≤ 0.0001 by one-way ANOVA.

5R staining as a defining marker. Instead, I focused on the significant increase in Siglec-F^+ cells, as seen in Figure 10B and the significant decrease in GMP-like cells, as seen in Figure 10F. In mice that received PBS, αIL-5 treatment did not have any significant effect on the frequency of Siglec-F+ cells (Figure 16B-C) or GMP-like cells (Figure 16D) in the bone marrow; moreover, despite a trend toward being lower, no significant difference was observed in the number of eosinophils in the peripheral blood after αIL-5 treatment (Figure 16E). As before, the addition of IL-33 significantly increased the Siglec-F+ population and this was prevented by αIL-5 treatment (Figure 16C), suggesting that this response was regulated by the elevated IL-5 levels upon IL-33 treatment. αIL-5 treatment was also sufficient to completely block the increases in peripheral mature eosinophils (Figure 16E). In contrast, the significant decrease in GMP-like cells upon IL-33 treatment was unaffected by blockade of IL-5. Collectively, these data suggest that the IL-33–driven expansion of mature eosinophils I observed is dependent on IL-5. In contrast, the decrease in the GMP-like population is IL-5 independent and demonstrates that IL-33 exerts its influence on less mature populations that are separable from the influences of elevated systemic IL-5.

IL-5–driven eosinophilopoiesis is ST2 dependent

I next sought to better define whether the requirement for IL-33 on basal eosinophil homeostasis lay upstream or downstream of IL-5. We utilized the NJ.1638 strain of mice, which possess transgenic overexpression of IL-5 and develop a profound, age-dependent hypereosinophilia in the blood (173), and examined the effects of ST2 deficiency on this response. Evaluating the homeostatic peripheral blood eosinophil numbers over time, we observed that the substantial elevations in eosinophils seen in the NJ.1638 mice were significantly diminished in the absence of IL-33 signaling in the NJ.1638/ST2KO mice (Figure 17A). While we observed a trend towards higher serum IL-5 at early timepoints in the NJ.1638/ST2KO mice versus NJ.1638, this was not statistically significant and there were no significant differences as we tracked these animals during aging (Figure 17B). Interestingly, NJ.1638/ST2KO mice still had more eosinophils than WT and ST2 KO mice, implying that ST2 is not an essential checkpoint for eosinophilopoiesis and that ST2-independent mechanisms exist to support IL-5–responsive eosinophil

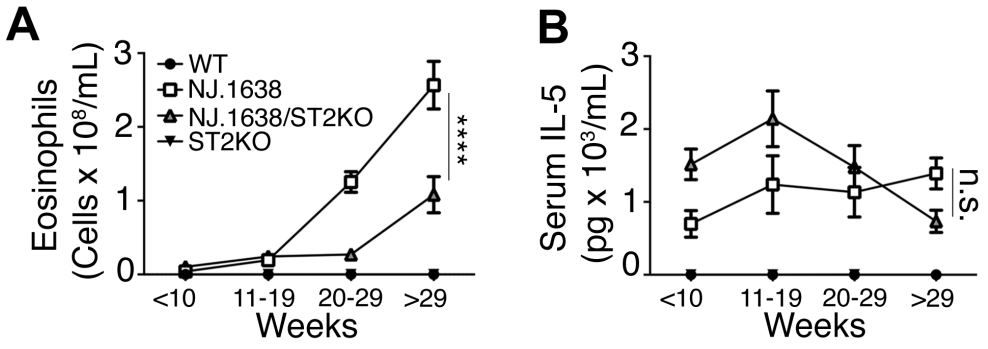

Figure 17. Eosinophilopoiesis is ST2-dependent.

IL-5-overexpressing mice (NJ.1638) mice were crossed with ST2 KO mice. (**A**) Blood eosinophil numbers over time as determined by staining with Discombe's fluid. (**B**) Serum IL-5 concentration. Data represent mean ± SEM (*n* = 5–14 from 1 [B–E] or 4 [F–G] independent experiments). n.s. = not significant. ****$p \leq 0.0001$ by two-way ANOVA.

development. Regardless, these studies establish that ST2 clearly regulates IL-5–driven eosinophil homeostasis and that IL-33 signaling lies upstream of the effect of IL-5 on eosinophils.

IL-33 expands EoPre and upregulates IL-5R in vitro

With my in vivo data showing that IL-33 and IL-5 both play a role in increasing eosinophils, I next turned to in vitro culture systems to assess the mechanisms by which IL-33 and IL-5 cooperate to promote development of EoM. Previously, Dyer et al. utilized a protocol for generating eosinophils in vitro in which bone marrow cells were cultured in SCF and Flt3L for the first 4 days to expand the progenitor cells, followed by a switch to IL-5 in the culture to promote eosinophil development; the effect of IL-33 was assessed during these later stages of culture (193, 194). Since my data suggests that IL-33 is upstream of IL-5, I sought to examine the effects of IL-33 during the initial progenitor expansion phase. I cultured freshly isolated bone marrow cells with a) SCF and Flt3L, b) IL-5, or c) IL-33 for 3 days and assessed for changes in EoPre and EoM populations by flow cytometry (Figure 18). As shown in Figure 18A and quantified in Figure 18B, both populations were maintained after 3 days when cultured in SCF and Flt3L or IL-5. In contrast, IL-33 drastically increased the EoPre population in WT and IL-33 KO cultures but not in ST2 KO cultures. Assessment of cytology after cell sorting of the EoPre and EoM populations showed clear multi-lobed nuclear morphology consistent with eosinophils but poor granule staining in the EoPre, while the EoM population possessed clear eosin-stained granules (Figure 19). Moreover, bone marrow cultured with IL-33, but not SCF and Flt3L or IL-5, led to a substantial increase in IL-5Rα mean fluorescence intensity (MFI) on the EoPre (Figure 20), further indicating their commitment to an eosinophil lineage. Although there were no basal differences in IL-5Rα MFI on the EoPre, the low levels of IL-5Rα detected on the GMP required IL-33 and ST2 (Figure 20, Figure 21). When CD11b was specifically utilized instead of the full lineage cocktail, a similar decline in EoM and expansion of EoPre was observed, as well as an upregulation of IL-5Rα on the EoPre population (Figure 22). Furthermore, when WT bone marrow was cultured with IL-33, Flt3L and SCF for three days, I found a significant increase in EoP, but not the classically defined CMP or GMP (Figure 23).

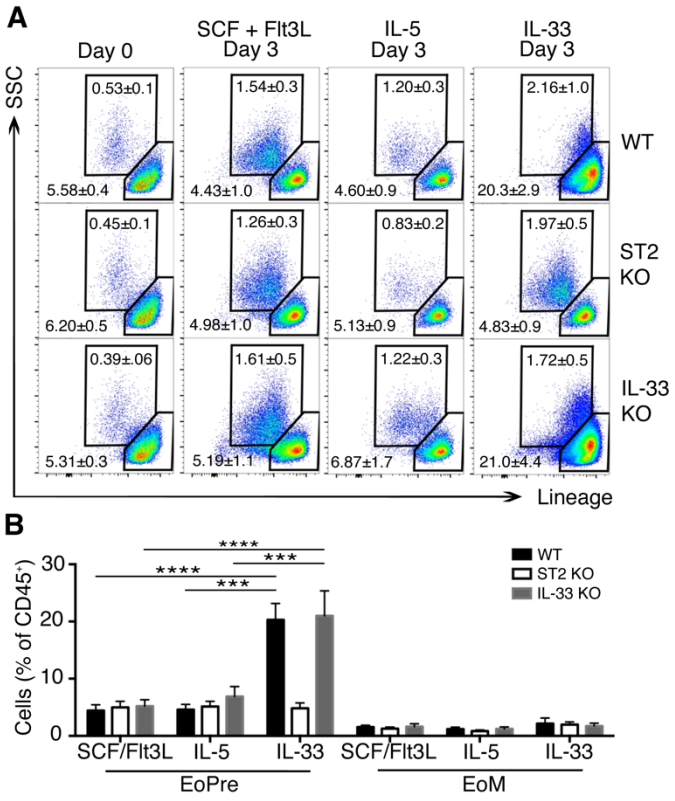

Figure 18. IL-33 increases EoPre in vitro.

Bone marrow cells were cultured with SCF and Flt3L, IL-5, or IL-33 for 3 days and eosinophil populations were assessed by flow cytometry. (**A**) Representative flow cytometry. (**B**) Quantification of (A). Data represent the mean ± SEM. (*n* = 3–5 from 4 independent experiments). ***$p \leq 0.001$, ****$p \leq 0.0001$ by two-way ANOVA.

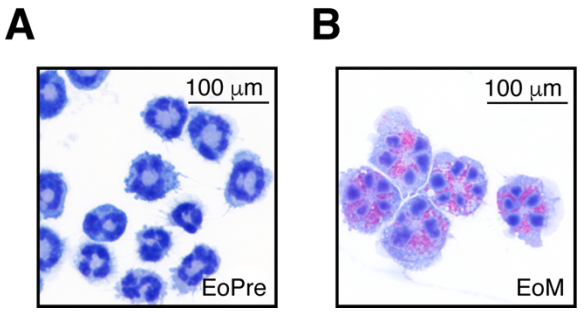

Figure 19. EoPre lack eosin staining.

Bone marrow cells were cultured with SCF, Flt3L, and IL-33 for 3 days. (**A**) EoPre and (**B**) EoM were sorted, cytospun, and stained with Kwik-Diff.

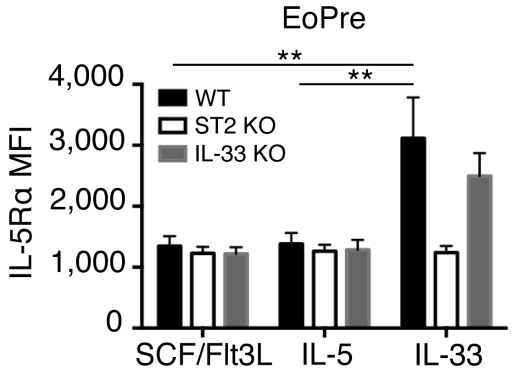

Figure 20. IL-33 increases IL-5Rα on EoPre.

IL-5Rα expression was assessed by flow cytometry on the EoPre population defined in Figure 18. Data represent the mean ± SEM. (n = 3–5 from 4 independent experiments). **p ≤ 0.01 by two-way ANOVA.

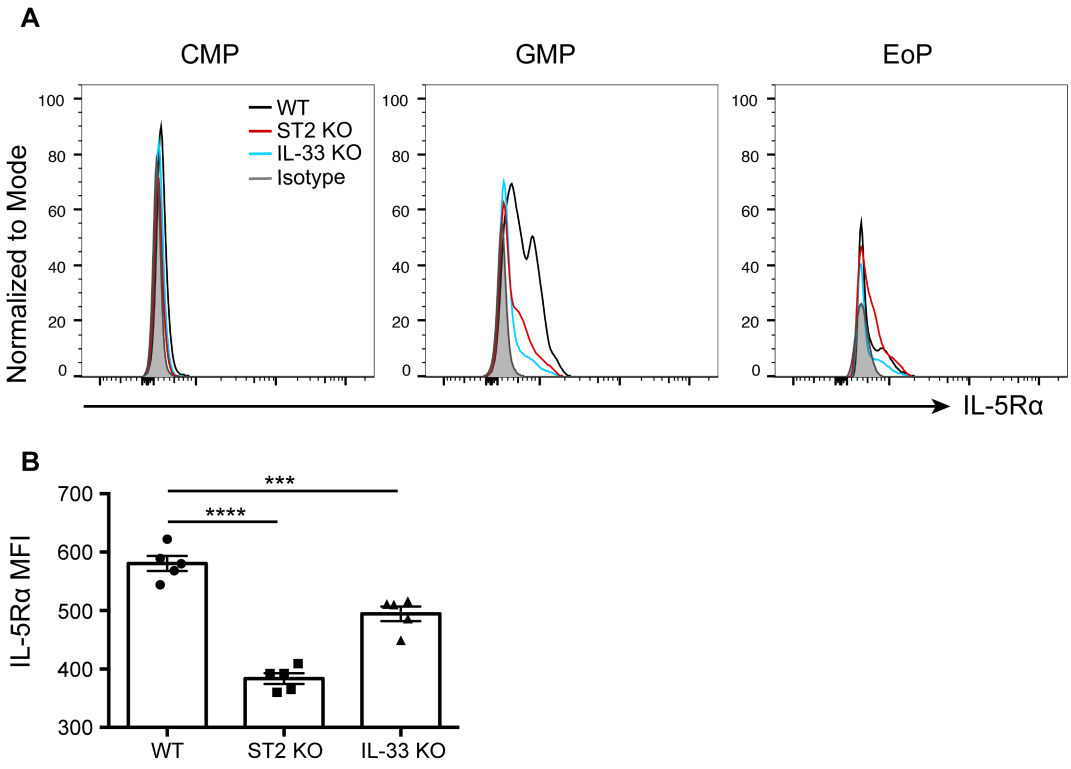

Figure 21. IL-33 and ST2 are necessary for IL-5Rα expression on GMP.

IL-5Rα expression was determined on the CMP, GMP, and EoP (defined in Figure 12). (**A**) Representative histograms of IL-5Rα. (**B**) IL-5Rα MFI of GMP. Data represent the mean ± SEM. (*n* = 5 from one experiment). ***$p \leq 0.001$, ****$p \leq 0.0001$ by one-way ANOVA.

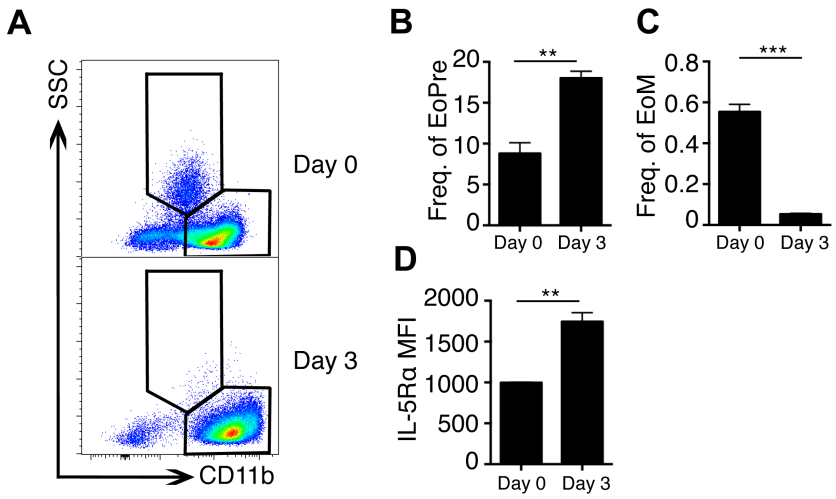

Figure 22. IL-33 expands CD11b-expressing eosinophil populations in vitro.

(**A**) Representative flow plots of eosinophil populations in in vitro cultures with IL-33. (**B**) Frequency of EoPre (Siglec-F^{+}CD11b^{+}SSC^{lo}) or (**C**) EoM (SiglecF^{+}CD11b^{lo}SSC^{hi}) at day 0 or after 3 days of IL-33 exposure. (**D**) Quantification of IL-5Rα levels on the EoPre population at day 3 of IL-33 exposure. Data represent the mean ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ by two-tailed Student *t* test.

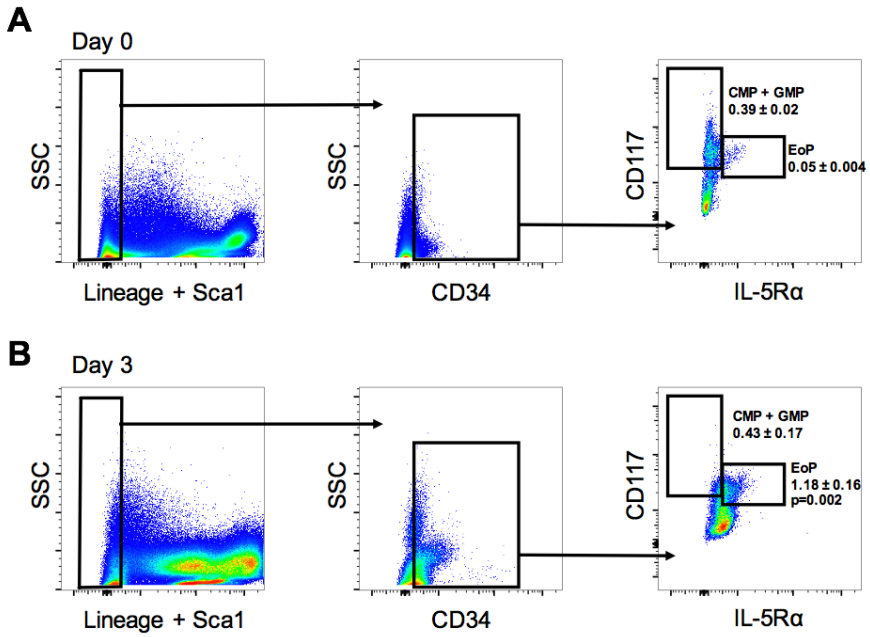

Figure 23. IL-33 expands EoP in vitro.

(**A**) Classical CMP + GMP and EoP in naïve bone marrow. (**B**) CMP + GMP and EoP after three days of culture with SCF, Flt3L, and IL-33. Data represent mean frequency of live cells ± SEM.

While the difference in granularity I see by cytology is consistent with the differences in side scatter I observe in my flow cytometry analysis, it contrasts to the previously described eosinophil precursors, which have been reported to possess granule proteins (175). Moreover, IL-5Rα can also be expressed on neutrophils under certain conditions (211). Therefore, to further confirm that this population I considered to be EoPre cells was truly within the eosinophil lineage, I interrogated the gene expression profiles of these EoPre cells to assess if they expressed eosinophil-associated genes. In this experiment, I used the EoM population as a positive control and bone marrow neutrophils as a negative control lying still within the granulocyte lineage. While bone marrow neutrophils, EoPre, and EoM had similar expression of granulocyte-associated genes Csf2ra (GM-CSFRα) and Spi1 (PU.1), EoPre and EoM had significantly lower expression of the neutrophil associated gene Csf3r (G-CSFR) than neutrophils (Figure 24). Instead, the EoM and EoPre populations expressed significantly higher levels of the eosinophil-associated genes Epx (eosinophil peroxidase), Prg2 (major basic protein), Cebpa (C/EBPα), Gata1 (GATA-1), and Gata2 (GATA-2) than neutrophils (Figure 24). Furthermore, I noted that the EoPre population expressed intermediate levels of these eosinophil-associated genes, suggesting that they possessed an immature eosinophil phenotype. Together, these findings suggest that IL-33 may function as a growth factor for the early commitment towards the EoPre population and/or drive their expansion.

To test this idea further, bone marrow cells were cultured in SCF and Flt3L with or without IL-33 for 3 days before re-culturing them in IL-5 for another 7 days; cell populations were assessed by flow cytometry at various stages of the culture. While no difference was seen in the GMP populations, WT cultures grown with IL-33 exhibited high numbers of EoPre populations at early stages of culture as well as a rapid increase in total EoM that was sustained throughout the entire culture period (Figure 25). In contrast, the WT culture without early IL-33 treatment showed little change in EoPre cells, and increases in EoM appeared only after day 10 of culture. ST2 KO cells showed no expanded EoPre on IL-33 treatment and generated even fewer EoM over the course of culture with IL-5. Taken together, these data suggest that IL-33 precedes the need for IL-5 and functions mainly to promote not only the expansion of eosinophil precursor cells but also IL-5Rα upregulation, which then sustains the mature eosinophil pool if IL-5 is provided.

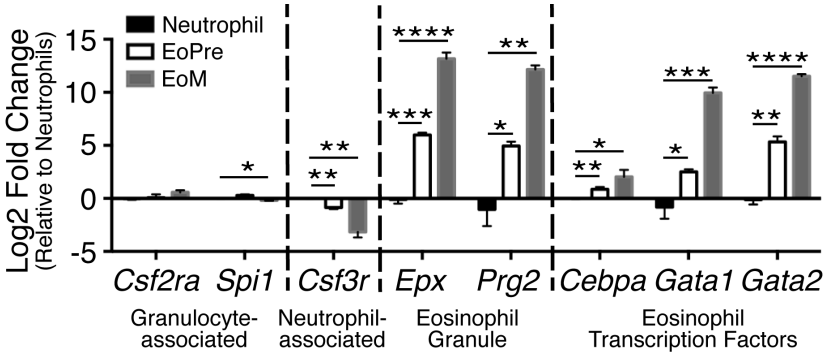

Figure 24. Expression of neutrophil- and eosinophil-associated genes in neutrophils, EoPre, and EoM.

Bone marrow cells were cultured with SCF, Flt3L, and IL-33 for 3 days and eosinophil populations were sorted for RNA. Gene expression was determined by RT-PCR and normalized to that of neutrophils isolated from bone marrow. Data represent the mean ± SEM. (n = 3–5 from 3 independent experiments). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by two-tailed Student t test.

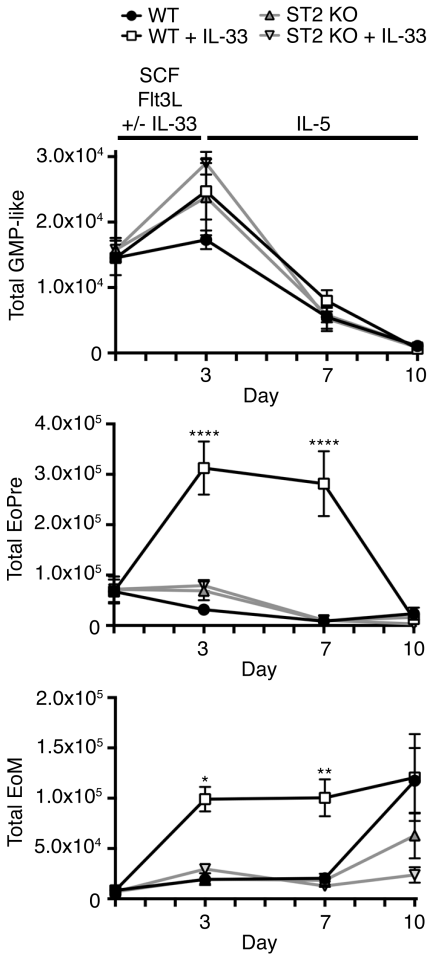

Figure 25. IL-33 rapidly increases EoPre and EoM in bone marrow cultures.

Bone marrow from WT and ST2 KO mice was cultured in SCF and Flt3L with or without IL-33 for three days followed by IL-5 for seven days. (**A**) GMP-like, (**B**) Total EoPre, and (**C**) total EoM were determined at day 0, 3, 7, and 10 by flow cytometry. Data represent the mean ± SEM. (n = 3–5 from 3 independent experiments). *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001 by two-way ANOVA.

The source of IL-33 in the bone marrow is a CD45$^+$CD19$^+$MHC II$^+$ cell

Since I have shown that IL-33 is both necessary and sufficient for eosinophil development, I wanted to determine if there were cells within the bone marrow that expressed IL-33. To identify IL-33-expressing cells, I used a IL-33-eGFP reporter mouse. First, I identified that there was a GFP$^+$ population in the bone marrow and determined that it could be found in the CD45$^+$ but not the CD45$^-$ fraction (Figure 26A). I then determined that this CD45$^+$GFP$^+$ cell had low side scatter and stained positive for CD19 and MHC II (Figure 26B). This cell had low to no expression of CD34, c-Kit, GR-1, F/480, and CD11b and had no expression of CD3, Sca-1, Ly6C, or Siglec-F. It is possible that there are two populations of CD45$^+$GFP$^+$ cells – a major population that is CD19$^+$MHC II$^+$GR-1^{lo} and a minor population that is GR-1^{hi}CD19$^{+/-}$ (Figure 26C). Since the minor population is GFP^{lo}, it is unclear if this population is truly GFP$^+$ or if it is autofluorescence. Regardless, the major IL-33-expressing population in the bone marrow appears to be B cells.

Eosinophil progenitors are not altered in a model of chronic asthma

Our data demonstrate a role for IL-33 in homeostatic eosinophil development, so I next wondered if eosinophil development was altered following an asthma model. WT and IL-33 KO mice received three intraperitoneal injections of 50 µg of OVA on days 0, 3, and 6, followed by weekly intranasal installations of 20 µg of OVA starting on day 11 for a total of 9 challenges (Figure 27). Controls received PBS. After the final challenge, bone marrow was collected and CMP, GMP, and EoP populations were assessed by flow cytometry (Figure 27C-E). The OVA treatment did not alter any of the populations in WT or IL-33 KO mice. Similar to what I saw in Figure 12, IL-33 KO mice had significantly lower EoP than WT mice, but the EoP frequency was unaffected by OVA treatment.

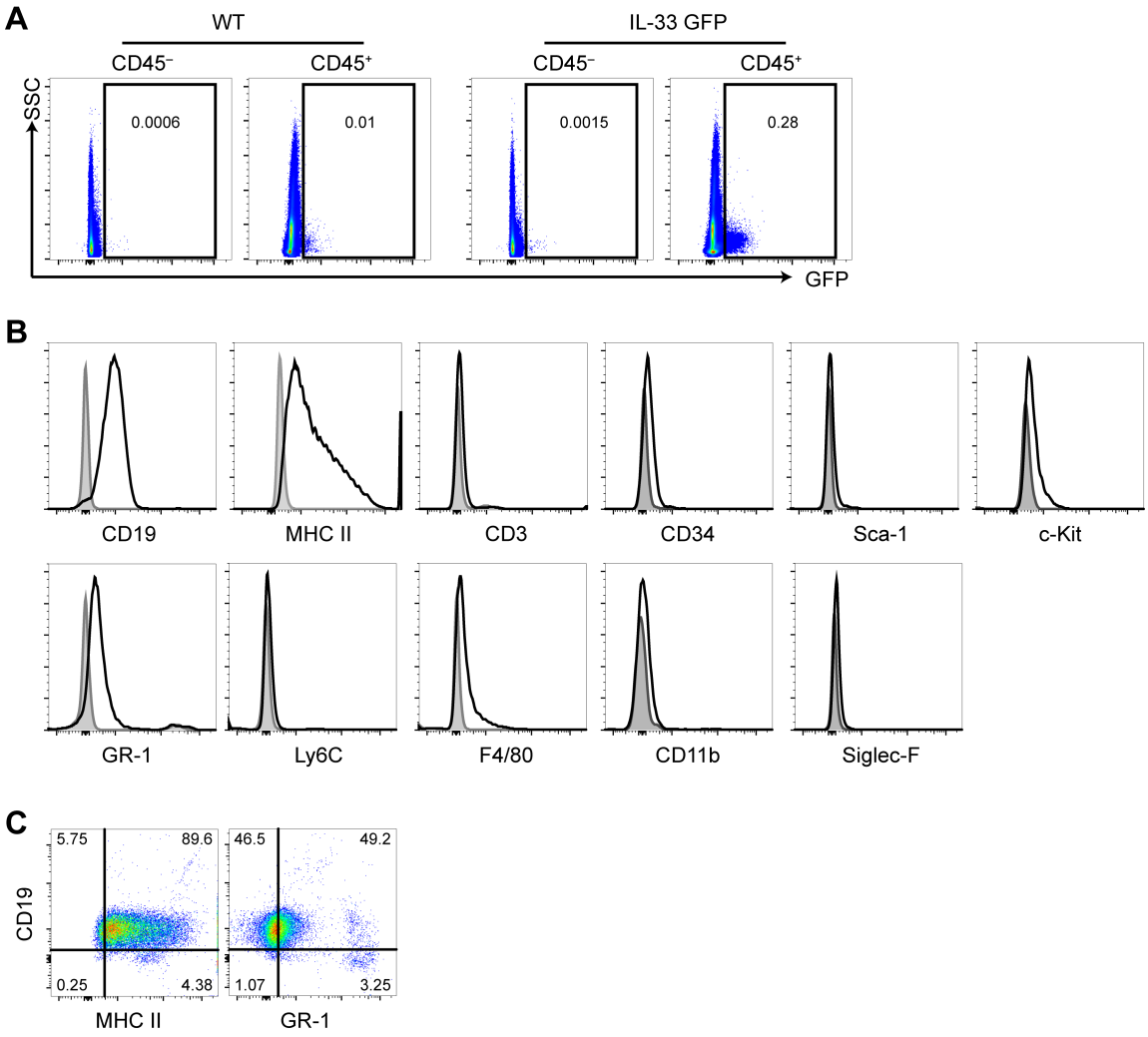

Figure 26. IL-33 is expressed in the bone marrow by a CD45$^+$CD19$^+$MHC II$^+$ cell.

A IL-33-eGFP reporter mouse was used to identify IL-33-expressing cells in the bone marrow. (**A**) GFP+ expression within the CD45$^+$ and CD45$^-$ gates in WT and IL-33-eGFP mice. Frequencies were determined as a percent of the live cells. (**B**) Cell surface markers on CD45$^+$GFP$^+$ cells in the IL-33-eGFP mouse. (**C**) CD19, MHC II, and GR-1 expression of the CD45$^+$GFP$^+$ population. n=1 from two independent experiments.

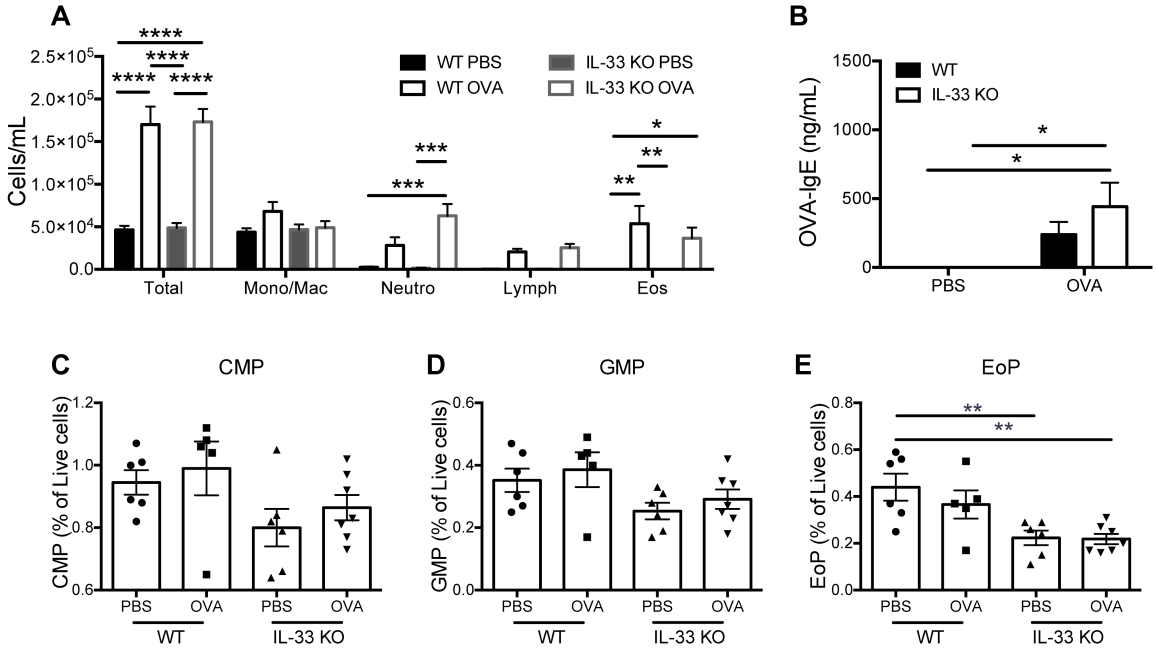

Figure 27. Chronic asthma does not alter eosinophil progenitor numbers in bone marrow. WT and IL-33 KO mice were given three i.p. injections of OVA followed by 9 i.n. challenges with OVA. (**A**) Differential cell counts in the BAL. (**B**) Serum OVA-IgE. Bone marrow was analyzed by flow cytometry for (**C**) CMP, (**D**) GMP, and (**E**) EoP. Data represent the mean ± SEM. (n =5-7 from 1 experiment). **p ≤ 0.01by two-way ANOVA.

Discussion

IL-33 has emerged as an important cytokine in allergic diseases, largely because of its potential to activate cells that are hallmarks of allergy, including eosinophils, mast cells, and basophils (212). Outside of allergy, IL-33 has also been proposed to be involved in bacterial and viral infections, tumorigenesis, autoimmunity, fibrosis (58), and more recently, hematopoiesis (180, 213). Here, I define a previously unappreciated mechanism for IL-33 in regulating eosinophil lineage commitment.

Our data demonstrates that IL-33 directs the eosinophil compartment by expanding the eosinophil precursor frequency and upregulating IL-5Rα to license the responsiveness of these precursors to IL-5 within the bone marrow (Figure 28). Importantly, the defects in basal eosinophil populations we identified in the IL-33 KO and ST2 KO mice strongly implicate a homeostatic contribution of this cytokine that functions outside of a disease-pathogenesis setting. Indeed, the previously defined function of IL-33 as an alarmin released upon tissue damage or injury (104) seems unlikely to explain such homeostatic regulation in healthy animals. Thus, the underlying alteration in eosinophil homeostasis in IL-33 KO and ST2 KO mice I describe here may impact the numerous interpretations others have made from studying the IL-33 KO and ST2 KO mice in disease models; consequently, reconsideration of some conclusions made from data using eosinophil numbers as a key response readout in previous studies may be warranted.

While my data clearly show a role for IL-33 in the bone marrow, other studies examining ST2 expression on hematopoietic stem and progenitor cells in the bone marrow have shown conflicting findings. Initial reports claimed that ST2 was present on multiple subsets of $Lin^{-}ckit^{+}$ progenitor cells, including GMP cells (214). In contrast, recent work using a spontaneous mutant mouse model of myeloproliferative neoplastic tumorigenesis failed to detect ST2 expression on a variety of hematopoietic stem cell and progenitor lineages (181). However, this work did demonstrate a functional role for IL-33 in regulating myeloid cell fate in this model. From my own characterization of the Siglec-F–expressing compartment (Figure 11) in which I define the stages of eosinophil development based on surface markers as well as size and granularity, ST2 does appear to be expressed on GMP-like cells and mature eosinophils, but its expression is lost at the EoPre stage. One possibility for this diminished ST2

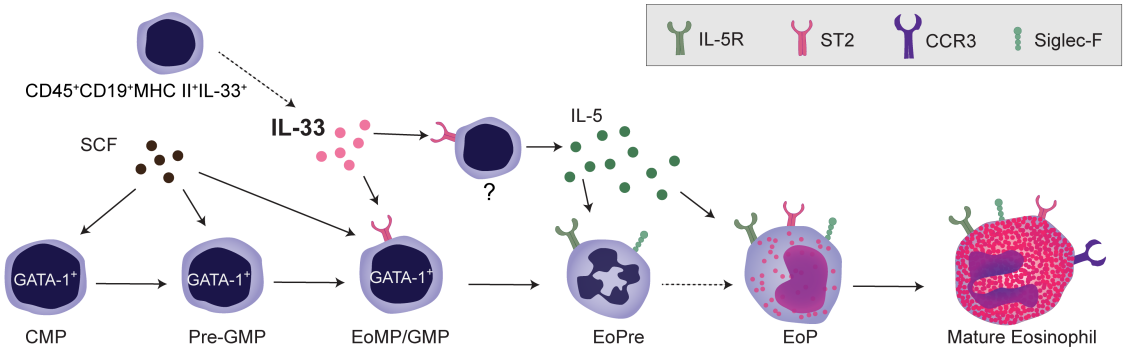

Figure 28. IL-33 affects eosinophilopoiesis in the bone marrow.

In the bone marrow, IL-33 acts on a GMP-like cell to promote expansion of EoPre. Since IL-33 also upregulates IL-5Rα on EoPre, it regulates commitment to the eosinophil lineage. Simultaneously, IL-33 induces another currently unidentified cell within the bone marrow to make IL-5, which promotes final eosinophil maturation.

expression on the EoPre population is that IL-33 binding leads to receptor internalization, as occurs when IL-5 binds to IL-5R (210). ST2 can also be shed from the surface of cells after activation (97). Alternatively, differences in gating strategy in terms of how to define GMP cells has influenced my conclusions; certainly, the expression of low Siglec-F levels on a progenitor cell, which I am choosing to term GMP-like because of the other marker profiles ($Lin^-Sca1^-IL\text{-}5R\alpha^-c\text{-}kit^+CD34^+$), has not been previously described to the best of my knowledge. Interestingly, in agreement with my data (Figure 13), publicly available gene expression microarray datasets do support the potential for expression of Siglec-F on GMP cells as well as on other progenitor subsets (http://biogps.org/gene/233186).

Recently, significant increases in mature eosinophils, $CD34^+$ hematopoietic progenitor cells, and committed eosinophil progenitors were reported within the lungs of asthmatic patients; this report further showed that IL-33 treatment primed the $CD34^+$ population for migration towards stromal cell-derived factor 1α (CXCL12) (215). It has also been shown that $CD34^+$ cells circulating in the blood express ST2, and activation of these cells with IL-33 leads to significant production of Th2-type cytokines, including IL-5 (216). While my data defines the responses to IL-33 that occur within the bone marrow compartment, the recruitment of progenitor populations after IL-33 exposure might support similar eosinophil developmental processes that occur within peripheral tissues; however, whether this might occur under homeostasis, as has been suggested for eosinophil maintenance (126), or only under disease conditions remains to be determined.

In agreement with others, I show that administration of IL-33 is capable of inducing significant elevation of IL-5, both systemically and within the bone marrow itself (Figure 15). However, the source of this IL-5 remains to be determined. Since the mice in this study were not primed towards a Th2 adaptive response, it seems reasonable to predict that innate cells, such as ILC2s, might be a significant source. ST2-expressing ILC2 populations that produce IL-5 were previously been shown to regulate eosinophil homeostasis in the intestine and lung, but this production was spontaneous; moreover, these cells were shown to be largely absent from the bone marrow compartment (126). Interestingly, a $Sca\text{-}1^+$ precursor population within the bone marrow that expresses ST2 and produces IL-5 in response to IL-33 has been reported (217), suggesting that both the expansion of the $IL\text{-}5R\alpha^+$ EoPre population and the elevation of

IL-5 itself could occur locally within the bone marrow and be driven by IL-33. In my in vitro cultures, IL-5 was detected in the media after 3 days of culture with SCF, Flt3L, and IL-33 (54.6 ± 6.7 pg/ml) but was undetectable (less than 31.25 pg/ml) with SCF and Flt3L alone. This might explain the rapid transition from precursors towards EoM that I observed. Importantly, through the use of the IL-5–transgenic mouse, our studies clearly define a role for IL-33 signaling in eosinophil frequency that is independent of the IL-33–induced IL-5 response and establishes that the contribution of IL-33 lies upstream of IL-5–driven eosinophilopoiesis. Similarly, it has previously been shown that IL-33 cannot elicit eosinophilia in IL-5 KO mice, helping to define that IL-5 lies downstream of IL-33–driven responses (193). Furthermore, blocking IL-5 in vivo prevented the increased mature blood eosinophil expansion that occurs in response to IL-33 but failed to prevent the observed decreases in the GMP-like cells in the bone marrow. Currently, I postulate that the GMP-like cells, shown as expressing ST2 in Figure 11, are being driven towards the EoPre phenotype by IL-33 and that the high expression of IL-5R on these cells combined with the elevated IL-5 (from currently unknown cells) results in rapid increases in mature eosinophils. This model is supported by my studies in which I neutralized IL-5 and only affected the IL-33 induced changes in mature eosinophils, but this concept requires further studies in order to be fully defined.

Previous studies using in vitro culture approaches to eosinophilopoiesis have led to confusing findings. While Stoarski et al. claimed that culture with IL-33 alone for 5 or 8 days was sufficient to induce eosinophil differentiation (57), Dyer et al. concluded that IL-33 did not support eosinophilopoiesis and, instead, antagonized the effects of IL-5 and promoted monocyte differentiation (193). Data from my initial in vitro studies were in agreement with Dyer et al. (193) in that IL-33 failed to sustain the cell cultures to day 5 or beyond, with cells dying (Figure 29). I also observed that the addition of IL-33 to SCF and Flt3L during the first 3 days of culture led to significant expansion of total cell numbers at day 3 compared to cultures with SCF and Flt3L alone and that this was not seen with ST2KO cells. As shown in Figure 19 and Figure 24, these cells, while clearly not mature eosinophils, possessed hallmark characteristics indicative of eosinophil precursors. Interestingly, they expressed mRNA for eosinophil granule proteins and eosinophil-associated transcription factors, but lacked eosin-positive granules. Because of this and the clearly defined nuclear morphology of an eosinophil, I postulate that these cells represent an

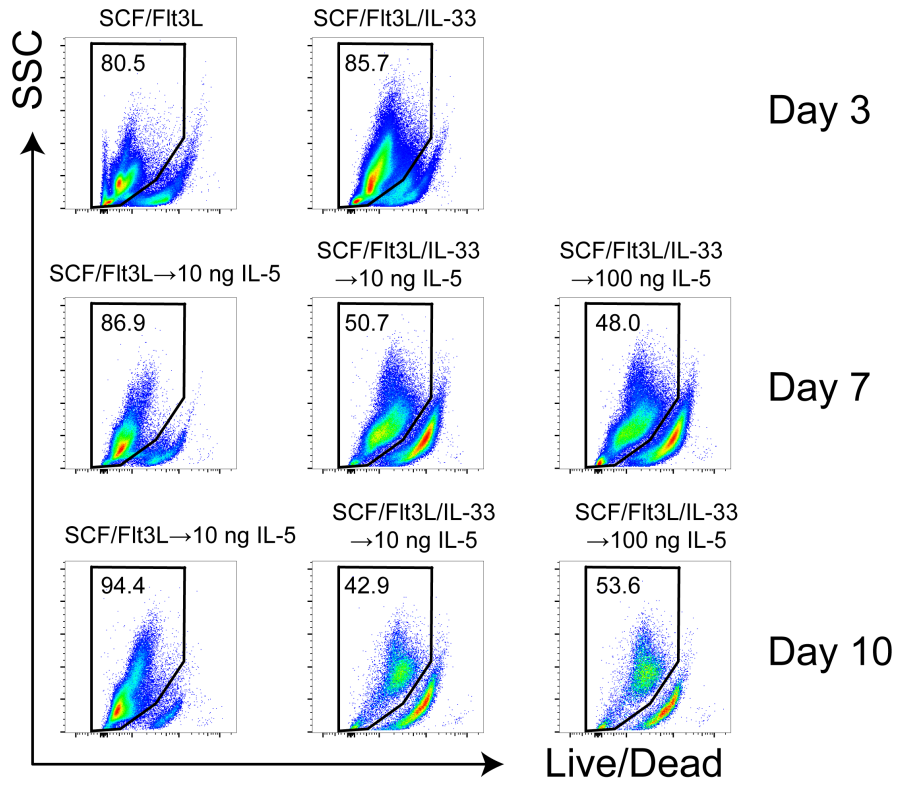

Figure 29. Live cells in eosinophil cultures.

Bone marrow cells were cultured as in Figure 25, but when the medium was changed on days 3 and 7 the cell density was not adjusted. Numbers represent the frequency of the single cells.

alternative precursor state than those defined from long-term IL-5 culture approaches (175). Initially, when these cultures were switched into IL-5, many of these expanded cells (>50%) were dying at day 7, even when a 10-fold higher concentration of IL-5 was used (Figure 29). I subsequently found that readjusting the cell density with each medium change helped facilitate survival of these cells, and these cultures were used as the source of the data shown in Figure 25. Potentially, since the IL-33–treated cultures have significantly more IL-5Rα^{+} EoPre cells, consumption of the available IL-5 and outgrowth of other cell types might explain the elevated monocyte numbers seen by Dyer et al. (193). An interesting aspect of my data in Figure 25 is that I observed a rapid expansion of both the EoPre and EoM populations within the first few days but that the overall numbers of EoM plateaued, rather than continued to increase after removal of IL-33. While further work is required to understand this fully, one possibility is that the pool of precursors became a limiting factor and that the balance between development of mature EoM and the apoptotic death I observed in the expanded IL-33 cultures simply maintained the EoM populations during the IL-5 treatment. Nonetheless, the overall numbers of EoM generated by IL-33 treatment during the early days of bone marrow cultures significantly outpaced those cultures without IL-33 and is consistent with the concept that early expansion of precursors supports a more rapid establishment of eosinophil populations.

Our findings showing that NJ.1638/ST2KO mice have diminished eosinophil numbers (Figure 17) also provide in vivo evidence that IL-33 signaling positively regulates eosinophil differentiation rather than antagonizing it. Furthermore, I did not find basal differences in blood monocyte subsets between IL-33 KO and ST2 KO mice or WT mice (Figure 5C). Instead, I did observe a diminished frequency of blood neutrophils that, unlike the eosinophil response, was not altered by exogenous IL-33 treatment (Figure 5C and Figure 8). Sustaining eosinophil and neutrophil populations requires a competent GMP population, but I did not see any significant effect on the homeostatic frequency of GMP or GMP-like cells in the bone marrow of either ST2 KO or IL-33 KO (Figure 12 and Figure 10F), although exogenous IL-33 treatment did diminish the GMP-like pool (Figure 10F and Figure 16D). Intriguingly, ST2KO bone marrow did seem to generate fewer EoM cells by Day 10 of culture. While I saw no obvious difference in either the frequency of EoPre (Figure 18B) or basal IL-5R levels (Figure 20) between ST2KO and WT that did not

receive IL-33 treatment, subtle differences in the basal numbers of GMP-like cells or their basal levels of IL-5R expression could explain this. Alternatively, the $CD45^{+}CD19^{+}MHC\ II^{+}IL\text{-}33^{+}$ cell in the bone marrow (Figure 26) may support eosinophil development during the course of culture. These ideas require further investigation to define properly. Previous reports suggested that IL-33 might directly influence neutrophils during inflammation (218), and mice overexpressing IL-33 under the CMV promoter exhibited elevated eosinophilia and neutrophilia (44). I have been unable to observe ST2 expression on neutrophils (Figure 36), and my data show that the doses of IL-33 required to increase eosinophils are separable from any effect on neutrophils. Interestingly, subsequent studies related to IL-33–induced neutrophil responses have proposed an indirect regulation via activation of mast cells (43). In contrast to the neutrophils, the ILC2 population was not increased by IL-33 treatment in my study, indicating that the doses of IL-33 needed to influence these lymphoid cells were likely similar to that for eosinophils.

Eosinophils are involved in many diseases, including asthma, eosinophilic esophagitis, atopic dermatitis, and hypereosinophilic syndromes. Since IL-5 is needed to support eosinophil development and survival, two antibodies targeting IL-5 (Mepolizumab and Reslizumab) and one targeting the IL-5R (Benralizumab) have been therapeutically tested. Particularly in the setting of severe eosinophilic asthma, these therapies have shown success in reducing symptoms and dependence on oral glucocorticoids (219). Targeting IL-33 and/or ST2 has already been extensively suggested for allergic diseases (220); importantly, my findings suggesting that IL-33 participates in maintaining the eosinophil pool upstream of IL-5 further predict the usefulness of targeting IL-33 and/or ST2 in disease settings similar to those targeted by IL-5–based therapies. Although I did not find any differences in eosinophil progenitors in the bone marrow, the IL-33 KO mice were also capable of eosinophilic lung inflammation (Figure 27). However, IL-33 has been shown to be necessary for eosinophilic inflammation in other models (73, 76, 92, 115, 221). Further work is needed to determine any affects of IL-33 in the bone marrow in a disease setting.

In conclusion, my data demonstrate a previously unappreciated role for IL-33 in supporting eosinophil development. Since basal homeostasis is affected in both ST2 KO and IL-33 KO mice, this role seems to represent a homeostatic function for IL-33. Furthermore, my findings suggest that an elevation

in IL-33 levels can induce eosinophil development. Mechanistically, I show that IL-33 is vital for promoting IL-5Rα upregulation on eosinophil precursors to facilitate their responsiveness to IL-5. Moreover, while IL-33 signaling does not appear to be a necessary checkpoint in eosinophil development, my data demonstrates that it functions as an important regulator over the numbers of EoPre as well as the output of eosinophils from the bone marrow.

CHAPTER 4 – IL-33 alters the microbiome through the antimicrobial protein Lipocalin 2

Introduction

The Microbiome

The microbiome is a community of 10-100 trillion microorganisms that inhabit animals in a relationship that may be commensal, symbiotic, or pathogenic. In addition to bacteria, which are the most well studied component of the microbiome, the microbial community also includes archaea, protozoans, fungi, and viruses (222). At birth, microbiota colonize the surfaces of the human body that are exposed to the environment, including the mouth, nose, lungs, stomach, intestine, vagina, and skin (223, 224). At each location, the microbiome has a distinct composition. For example, the skin is dominated by Actinobacteria, Firmicutes, Proteobacteria, and eukaryotic viruses, whereas the intestine is dominated by Bacteriodetes, Firmicutes, and bacteriophages (224). The majority of bacteria reside in the gastrointestinal tract, with the highest density of bacteria in the colon (225). Since most of the intestinal bacteria are anaerobic and difficult to culture, it has been challenging to study many of these bacteria in a laboratory setting. Sequencing has greatly improved our understanding of the bacterial species within the intestine (223). To date, the intestinal microbiome is the most well studied and has been shown to have effects both within the intestine as well as in other organ systems. Recent research on the intestinal microbiome has been focused on how the microbiome may be beneficial or detrimental for the host. This includes understanding the mechanisms by which the microbiome and host interact, in both homeostasis and disease, and what factors influence the microbiome to promote or inhibit inflammation and/or disease.

Maintenance of intestinal homeostasis by the host

Intestinal homeostasis is achieved through tightly regulated crosstalk between the microbiome, the intestinal epithelium, and the immune system (226). In a healthy gut, each of these three components

occupies a specific location: the microbiome is in the gut lumen, the immune cells are in the lamina propria of the intestinal tissue, and the epithelium is a barrier that separates the lumen from the lamina propria (Figure 30). Although the microbiome is not pathogenic, it is still capable of activating the immune system and intestinal epithelial cells (IEC) through toll like receptors (TLRs) and other pattern recognition receptors (PRRs) (227). Because of this, the microbiome lives mostly in the outer mucus layer, whereas the inner mucus layer between the outer mucus layer and the IECs is mostly devoid of microbes (226). Thus, the mucus significantly minimizes the contact between the microbes and host cells. In addition to this physical separation, the host has several mechanisms in place to control the size of the microbiome: decreased pH, secretion of IgA, and production of antimicrobial molecules.

Within the stomach, gastric acid significantly limits bacterial density by creating a highly acidic environment (228). Gastric acid can be reduced both naturally (as occurs in infants and the elderly) or by medical intervention, such as acid secretion–blocking drugs (229-231). This reduction leads to a higher risk of infection. Furthermore, increased bacterial density in the intestine can lead to malabsorption (232). Therefore, the low pH of the stomach is important for restricting pathogenic bacteria from entering and colonizing the intestine and maintaining a low density of microbes.

Although direct contact between the immune system and the microbiome is inhibited by the intestinal epithelium and mucus layers, crosstalk occurs through secreted molecules that can be transported throughout the layers of the intestine. A notable molecule from the immune system is IgA, which is the most abundant antibody isotype in the body. It is produced by plasma cells in the lamina propria and transported by the epithelium to the gut lumen (Figure 30A). The precise mechanisms of how IgA controls the microbiome are not fully understood, but IgA is important for inhibiting bacterial adherence to IECs (233), preventing systemic infection (234), and preventing dysbiosis (235).

The epithelium also plays an active role in maintaining the size of the microbiome by secreting antimicrobial molecules (Figure 30A). These molecules can be classified into several groups: antimicrobial peptides, S100 proteins, elastase inhibitors, peptidoglycan-recognition proteins (PGLYRPs), C-type lectins, and iron metabolism proteins (236). The most well-studied antimicrobial molecules are the defensins and cathelicidins, which are categorized as antimicrobial peptides. The mechanism of these

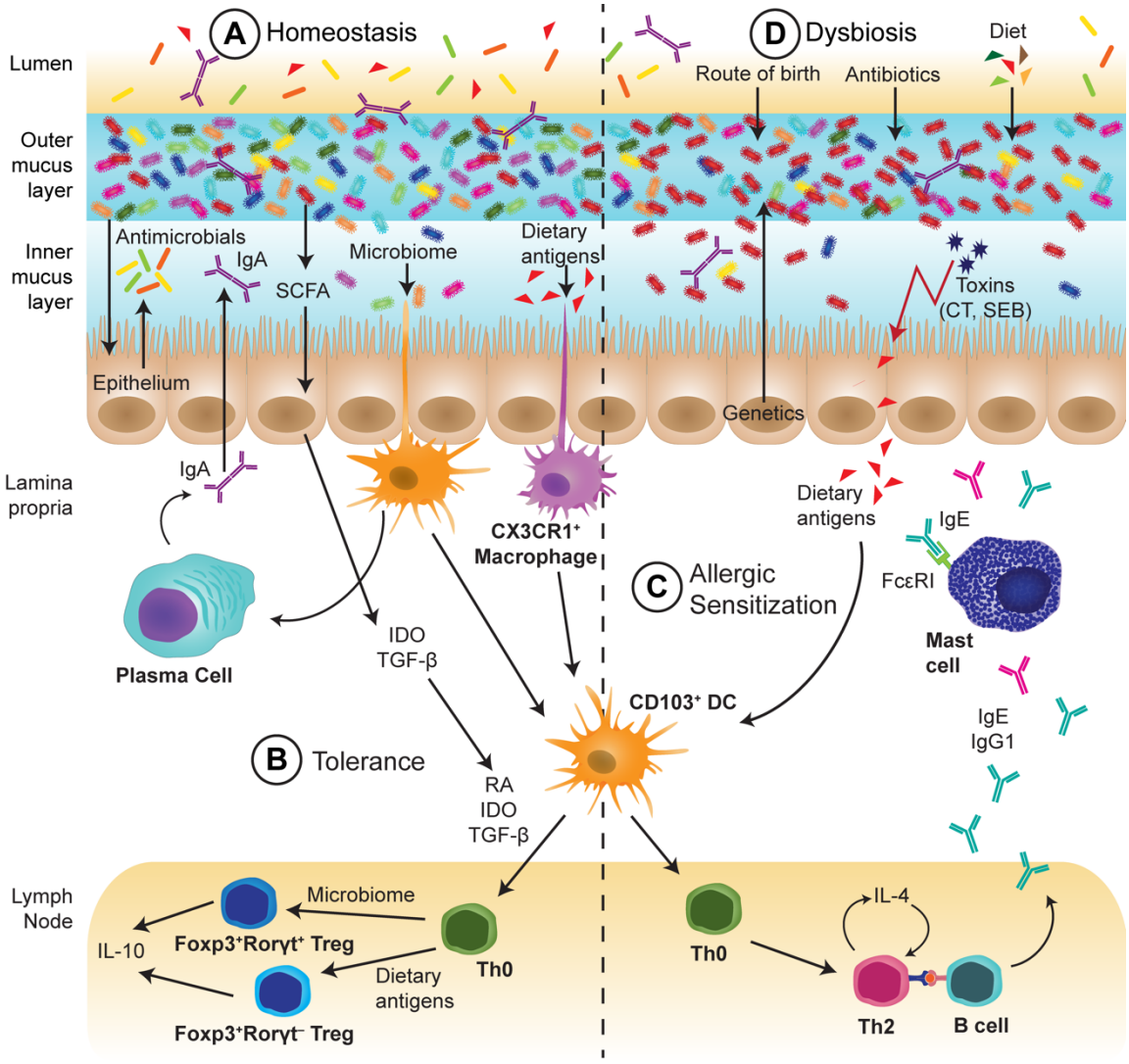
A Homeostasis
D Dysbiosis
Diet
Route of birth
Antibiotics
Lumen
Outer mucus layer
Inner mucus layer
Antimicrobials
IgA
SCFA
Microbiome
Dietary antigens
Toxins (CT, SEB)
Epithelium
Genetics
Lamina propria
IgA
CX3CR1+ Macrophage
Dietary antigens
IgE
FcεRI
C Allergic Sensitization
Mast cell
Plasma Cell
IDO
TGF-β
CD103+ DC
IgE
IgG1
B Tolerance
RA
IDO
TGF-β
Lymph Node
Microbiome
IL-10
Foxp3+Roryt+ Treg
Th0
Dietary antigens
Foxp3+Roryt− Treg
Th0
IL-4
Th2
B cell

Figure 30. Mechanisms of intestinal homeostasis, dysbiosis, and allergy.

(**A**) Within the intestine, the microbiome, epithelium, and immune system maintain homeostasis. The microbiome is kept in check by antimicrobials and IgA. Conversely, the microbiome promotes production of these molecules. (**B**) Oral tolerance is achieved by $CX3CR1^{+}$ macrophages and $CD103^{+}$ DCs which drive development of IL-10–producing Tregs and IgA-secreting B cells. Critical signals for tolerance are provided by retinoic acid (RA), indoleamine 2, 3-dioxygenase (IDO), and TGF-β. The microbiome can promote tolerance through interactions with $CD103^{+}$ DCs and epithelial cells, through SCFAs, and is required for development of $Foxp3^{+}ROR\gamma t^{+}$Tregs. Dietary antigens are taken up by $CX3CR1^{+}$ macrophages and are required for $Foxp3^{+}ROR\gamma t^{-}$Tregs. (**C**) Perturbation in these cells or mediators through signals including but not limited to CT and SEB breaks tolerance. Lack of tolerance can lead to allergic sensitization, which is characterized by dominant Th2-biased responses and class-switching towards IgG1 and IgE. (**D**) Dysbiosis can be caused by external factors, such as route of birth, antibiotics, and diet, and host factors, such as genetics.

cationic peptides is to bind negatively charged bacteria, leading to membrane permeabilization and death; this is a common mechanism for antimicrobial proteins. Other mechanisms include digestion of bacterial peptidoglycan by PGLYRPs and sequestration of iron from the bacteria by iron metabolism proteins. In summary, the gastrointestinal system has several epithelial and immune mechanisms by which it keeps the microbiome in check.

Maintenance of intestinal homeostasis by the microbiome

While the epithelium and immune system produce antimicrobial proteins and IgA in order to manage the microbiome, the microbiome also plays a critical role in conversely regulating these processes. Furthermore, the microbiome helps maintain barrier function, metabolize food that cannot be digested by the host, and promote oral tolerance to food.

Much of the work in understanding the role of the microbiome in intestinal homeostasis has been achieved through the use of germ-free (GF) mice, which are born into and live in a sterile environment. GF mice have numerous immune defects that involve both immune and epithelial cells, demonstrating that the microbiome plays a critical role in the development of the immune system (237). For example, the structure of the intestinal tissue is altered. The immune structures, including Peyers patches, mesenteric lymph nodes, isolated lymphoid follicles and cryptopatches, are under developed, whereas the IECs have reduced turnover and altered microvilli structure (238). Furthermore, the microbiome is important for the expression of some antimicrobial proteins, including the C-type lectin RegIIIγ and the ribonuclease angiogenin-4, which are significantly reduced in GF mice (237). However, since many antimicrobial proteins are constitutively expressed by the epithelium, the microbiome is not absolutely required for their expression. GF mice also have significantly lower IgA and plasma cells within the germinal centers of the small intestine and mesenteric lymph nodes than conventionally housed mice (237), demonstrating that bacterial signals are required to promote the generation of IgA. Indeed, DCs sample the bacteria in the lumen and promote the differentiation of B cells into IgA-producing plasma cells (Figure 30A). Therefore, the microbiome provides signals to both educate the immune system in producing IgA against commensal bacteria as well as produce antimicrobial molecules.

Another major role of the microbiome is to assist in food digestion. The digestion process first occurs as food travels through the mouth, stomach and duodenum, where host-derived digestive enzymes break down food into simple sugars, amino acids, and fatty acids. After these are absorbed by the small intestine, non-digestible carbohydrates (NDC) remain (239). When the NDC reach the colon, they are fermented by the microbiome to produce short chain fatty acids (SCFA). These SCFA, which mainly include formate, acetate, propionate, and butyrate, can be utilized by the host. At the phylum level, the Bacteriodetes (gram negative) mainly produce acetate and propionate, whereas the Firmicutes (gram positive) mainly produce butyrate (240). The effects of the microbiome through SCFA production are diverse and include maintenance of the colonic epithelial barrier, glucose homeostasis, lipid metabolism, appetite regulation, and immune function (239).

Microbes can also participate in educating the immune system to not react to food that enters the intestine, a process called oral tolerance. In order to achieve oral tolerance, the immune system must sample the food in the intestinal lumen and then develop a mechanism to suppress responses to subsequent encounters with that food (Figure 30B). One critical step in maintaining oral tolerance is ensuring that the immune system has limited exposure to food proteins. As mentioned previously, the intestinal epithelium is an important barrier that not only prevents the microbiota or pathogens from interacting with the immune system, but it also regulates food and fluid intake (241). Therefore it is critical that the intestinal epithelium maintain its barrier function. This is largely achieved by the junctional complexes between IECs, including tight junctions, adherans junctions, and desmosomes (242). TLR2 ligands have been shown to increase intestinal barrier function both in vitro and in vivo (243, 244). However, microbial components are not required for barrier function because mice that lack MyD88 or TRIF, two molecules involved in TLR signaling, do not have increased intestinal permeability (245). Therefore, the microbiome modulates the intestinal epithelial barrier but is not required for normal barrier integrity.

Two immune cells have been shown to be critical in establishing oral tolerance: $CD103^+$ DCs and $CD4^+$ regulatory T cells that express the transcription factor Forkhead box protein 3 ($Foxp3^+$ Tregs) (246-248) (Figure 30B). $CD103^+$ DCs reside in the intestinal lamina propria, but migrate to the mesenteric

lymph nodes after taking up food antigens. In the lymph node, these $CD103^+$ DCs activate naïve T cells to promote $Foxp3^+$ Tregs through TGF-β, retinoic acid, and indolamine 2,3-dioxygenase (IDO). Retinoic acid is critical for the induction of the gut-homing receptors CCR9 and $\alpha_4\beta_7$ integrin expression. Once they have reached the gut, $Foxp3^+$ Tregs are thought to stimulate tolerance through the production of IL-10 and TGF-β and suppress the generation of antigen-specific effector lymphocytes (249). The microbiome modulates the development of oral tolerance through several mechanisms. First, it was shown that TLR4-deficient CD103+ DCs have a significant impairment in generating $Foxp3^+$ Tregs from naïve T cells (250). SCFAs have also been shown to promote Treg differentiation and proliferation through multiple mechanisms, including inducing TGFβ and IDO expression from IECs and inhibiting histone deacetylases (HDAC)(251). Recently, it was shown that intestinal $Foxp3^+$ Tregs could be further subdivided into $ROR\gamma t^+$ and $ROR\gamma t^-$ Tregs, and that the microbiome was specifically necessary for $ROR\gamma t^+$ Treg development (252, 253). Alternatively, $ROR\gamma t^-$ Tregs require dietary antigens (252). Overall, the microbiome promotes gut health by promoting the generation of host-derived molecules that control the microbiome itself, increasing epithelial barrier, encouraging oral tolerance, and generating SCFAs that aid in digestion and oral tolerance (Figure 30A, B).

Microbiome and allergic disease

In the absence of oral tolerance, mechanisms of allergic sensitization may occur. Specifically, exposure to certain food antigens can promote the generation of pro-allergic Th2 responses (Figure 30C). The microbiome has been suggested to play a role in sensitization because germ-free and antibiotic-treated mice are more susceptible to food allergy (254, 255). Although GF mice appear to have fewer intestinal plasma and mast cells, they have increased serum IgE and more IgE is bound to each mast cell (256). Several bacteria have been shown to protect against the development of food allergy, including Bifidobacteria, Lactobacillus, and Clostridia (255, 257, 258). Thus, the loss of these beneficial bacteria may allow the development of allergy.

In conjunction with the fact that mechanisms to develop oral tolerance occur early on, there appears to be a window early in life where it is easier to break oral tolerance and for allergic sensitization

to occur. At birth, the microbiome is unstable and highly variable between individuals, and it continues to mature before stabilizing around three to five years of age (259). While GF mice have increased IgE, a previous study shows that there is a critical window in the first four weeks of life in which colonization of GF mice can prevent the increase in IgE later in life (256). Furthermore, the GF mice were only protected when they were colonized with a cocktail of 40 bacterial species but not two or eight species. This suggests that increased bacterial diversity and/or the presence of specific protective bacteria prevent the development of IgE, but these protective mechanisms only work if they are established early in life.

One mechanism that is known to break oral tolerance and promote sensitization involves bacterial-derived toxins (Figure 30C). In the lab, toxins are commonly used in mouse models of food allergy. Cholera toxin (CT) is the most commonly used adjuvant in food allergy models. Although the precise mechanism is not known, it has been suggested to break tolerance through an innate mechanism by inducing MHC II and costimulatory molecule expression on DCs as well as increasing IL-1 in the intestinal tissue (260). Another toxin, staphylococcal enterotoxin B (SEB), is more clinically relevant than CT, since food allergy has been shown to correlate with atopic dermatitis (AD) and it has been suggested that up to 65% of patients with AD have skin colonization of SEB-secreting *Staphylococcus aureus* (261). One caveat when using CT is that it elicits a mixed Th1 and Th2 response (262, 263), whereas SEB promotes a strong Th2 response (264). SEB has been suggested to function by inducing TIM4 expression on DCs, which promotes Th2 skewing, or deletion of Tregs (264, 265). Therefore, bacterial toxins are one way by which bacteria can prevent oral tolerance.

In addition to food allergy, the gut microbiome plays a role in other allergic diseases, including asthma and AD. Several studies have examined the fecal microbiome of children with allergies. Infants with AD and/or positive skin prick tests have a high correlation with increased *S. aureus* and decreased Bacteroides and Bifidobacteria (266). Another study found that infants with eczema had reduced diversity within the Bacteriodetes and a lower abundance of Proteobacteria within the gut (267). Interestingly, one study examined children that grow out of milk allergies and found that the children who became tolerant to milk had more Clostridia and Firmicutes early in life (268). Thus, increases in these bacteria may be predictive in identifying children who become tolerant later in life. In mice, oral administration of probiotic

bacteria have protected against allergic inflammation (269-272). Although asthma and AD are not diseases of the intestine, alterations in the gut microbiome have strong associations with allergy, indicating that the intestinal microbiome can impact other organs.

Beyond the intestine, the microbiota in other parts of the body also play a role in disease. In the skin, patients with AD have higher colonization of *S. aureus*, which has been shown to lower the skin microbiome diversity (273). Furthermore, *S. aureus* produces several molecules, including *Staphylococcal* α-hemolysin, which can disrupt the epithelial barrier (274), and *Staphylococcal* δ-toxin, which can activate mast cells (275). There are fewer studies examining the lung microbiome because it is less stable and is sparsely populated compared to the skin and intestine (224). The small number of human studies on the lung microbiome have collectively shown that asthmatics have more microbial diversity, likely due to the increased pathogenic bacteria, and more Proteobacteria than healthy controls (276-279). Therefore, the microbiome of the skin and lung may also affect disease.

A large portion of the research on the microbiome has been to characterize the differences between allergic patients and controls. Although this approach has been helpful in identifying specific bacteria that may play a role in disease, it is still unclear whether the altered microbiome is the cause of disease, a response to disease, or both. More recently, microbiome research has focused on determining the mechanisms by which the microbiome can become dysbiotic.

Causes of dysbiosis in allergy

The microbiota can become dysbiotic in response to many factors. Early in life, the microbiome is affected by three factors: route of delivery, feeding, and antibiotic use (Figure 30D). The first major colonization of the microbiome occurs at birth. Babies that undergo vaginal delivery are colonized with microbes in the vaginal tract, whereas those that are born via cesarean section are colonized with microbes from the skin (259). Children born via cesarean section have a higher risk of allergic disease (280). It has been proposed that this is caused by a failure to promote a healthy immune system by the skin-derived microbiome. After birth, the source of feeding also impacts the microbiome (280). Not only does formula differ from breast milk in nutrients, it also lacks important growth factors, cytokines,

antibodies, digestion enzymes, and microbes from the breast skin that may shape the child's microbiota. Finally, early antibiotic use is also linked to increased risk for allergic disease. Short-term doses of antibiotics have been shown to have long lasting effects on the microbiome and promote allergy (281), thus antibiotics may tip the balance from a healthy microbiome to an inflammatory microbiome.

In older children and adults, diet is a major modifier of the microbiome. The westernized diet, which is high in fat and low in fiber, is known to promote dysbiosis (282). Alternatively, a high fiber diet has been shown to be protective against allergy (283). The studies to date on diet-induced shifts in the microbiome focus on how the diet influences SCFAs. Certainly, because SCFAs are fermented from NDCs in the diet, fiber seems an obvious choice for understanding how SCFAs are altered. Alternatively, it is possible that SCFAs can be altered by the relative contributions of bacteria that are capable of producing SCFAs. Perhaps other factors that change the microbiome affect SCFA production by altering these SCFA-producing bacteria.

Since most of the common factors that are known to change the microbiome are external, a general view within the field is that these external factors alter the microbiome and lead to disease. However, it is unclear if the microbiome is the cause or the result of disease. Just as the host can shape the microbiome during homeostasis, it can also shape the microbiome during disease. Recently, GWAS have identified SNPs that associate with altered microbiomes (284, 285), demonstrating that genetic factors can predispose a host to microbial signatures (Figure 30D). Furthermore, several cytokines have been shown to modulate the microbiota. One key study found that mice engineered to have a common SNP mutation in the IL-4 receptor that increases IL-4 signaling have a dysregulated microbiome and are more susceptible to developing disease in a murine food allergy model (286). Furthermore, transfer of this altered microbiome was sufficient to obtain the enhanced allergic response. IL-15–overexpressing, IL-22 KO, and IL-33 KO mice all have dysregulated microbiota (89, 287, 288). In the IL-33 KO mouse, it was shown that the dysbiosis was due to higher IgA and *Akkermansia muciniphila*. Although diet, route of birth, antibiotic use, genetics, and cytokines have all been shown to alter the microbiome, further research is needed to fully understand how host factors like genetics and cytokines modulate the microbiome.

Lipocalin 2

The Lipocalin family

The lipocalin family has many members and is made up of small (160–230 amino acids) glycoproteins that share a highly conserved tertiary structure, but do not necessarily share sequential similarities (289). This three-dimensional structure, called the lipocalin fold, is a symmetrical β-barrel composed of eight anti-parallel β-strands. The lipocalin fold contains a binding site for small molecules. Because of this binding site and the fact that they are secreted molecules, lipocalins often act as transporters of retinol, odorants, steroids, lipids, pheromones, and other small molecules.

Between the β-strands within the lipocalin fold, there are three structural conserved regions (SCRs) that have significant sequence and structural conservation (289). These SCRs can be used to identify kernel lipocalins, which have three SCRs, and outlier lipocalins, which have 2 SCRs. Members of the kernel lipocalins include β-lactoglobulin (Blg), prostaglandin D2 synthase (PGD2), bilin-binding protein (BBP), major urinary protein (MUP), retinol binding protein (RBP), α_{2u}-globulin, and lipocalin 2 (LCN2). The lipocalins are also part of the calycin protein superfamily, which includes three other protein families with similar three-dimensional structure: the fatty-acid-binding proteins (FABPs), avidins, and metalloproteinase inhibitors (290).

One particularly interesting feature of the lipocalin family is that many of the animal-derived allergens are lipocalins. For example, Blg is a milk allergen. It is unclear why some proteins make good allergens. Given that many allergens are part of this structurally similar lipocalin family, the structure seems like a likely explanation. For example, lipocalins are known to bind lipids; since dietary lipids can act as adjuvants, the lipid-binding properties of lipocalins may explain their allergenicity (291).

Molecular biology of Lcn2

Human LCN2 is also known as neutrophil gelatinase associated lipocalin (NGAL), 24p3, oncogene 24p3, p25, siderocalin, uterocalin, human neutrophil lipocalin (HNL), migration stimulating factor inhibitor (MSFI), and α1–microglobulin related protein (289). The *LCN2* gene is located on chromosome 9 and has seven exons. The five functional mRNA transcripts encode proteins between

198–200 amino acids in length, but 198 amino acid–length protein is most common. The mouse homologue of LCN2 is Lcn2, and the gene is found on chromosome two. The six-exon *Lcn2* gene encodes two functional transcripts, which are translated in to a protein that is 200 or 284 amino acids long. These transcripts are regulated by NFκB, NFAT1, STAT1, STAT5, and several transcription factors in the C/EBP family (292-295). Although the amino acid sequence for human and mouse Lcn2 is only 62% similar, sequences in the lipocalin fold appear to be similar, suggesting that they bind similar ligands (289).

Lcn2 can bind small hydrophobic ligands, such as retinol, oleic acid, and cholesterol oleate (289). However, the most well-studied ligands for Lcn2 are proteins called siderophores, which are iron-carrier proteins. Thus, Lcn2 can transport iron by binding iron-bound siderophores, but it cannot bind iron directly (296).

Receptors, cellular sources and stimuli of Lcn2

Lcn2 has two described receptors. The first, megalin, also known as low density lipoprotein–related protein 2 (LRP2), is expressed on the apical side of epithelial cells and is utilized to endocytose various ligands (297). Megalin is utilized in endocytosis and does not appear to be specific to LCN2. While the binding of human LCN2 to megalin has been shown, murine Lcn2 has been shown to bind to 24p3R, also known as Solute Carrier Family 22 Member 17 (Slc22a17) and neutrophil gelatinase associated lipocalin receptor (NGALR). This 24p3R receptor appears to be specific for Lcn2 and is expressed in many tissues throughout the body, including the small intestine, heart, lung, liver, spleen, kidney, and thymus (298). Monocytes, macrophages, and neutrophils have been shown to express 24p3R (299, 300). In neutrophils, signaling through 24p3R activates the Erk1/2 and p38 MAPK pathways, inducing expression of IL-6. IL-8, IL-1α, and TNFα (301).

As the name “neutrophil gelatinase associated lipocalin” suggests, Lcn2 was originally discovered in neutrophils, where it was found to be stored within the granules (302). It is also expressed in macrophages (303), DCs (304), T cells (305), adipocytes, and epithelial cells (301, 306). Tissues that express Lcn2 include ileum, colon, lung, spleen, white adipose tissue, subcutaneous fat, and bone (307-

309). Bone marrow chimeric mice have been utilized to determine the relative contributions of radioresistant and radiosensitive cells to serum Lcn2 levels (308). While both groups make contributions, the radioresistant cells, which are mostly structural cells, have a larger involvement. Another study generated cell-specific Lcn2 KO mice and found that osteoblasts and adipocytes make significant contributions to serum Lcn2 levels (309).

Many molecules can stimulate Lcn2, including LPS, lipoteichoic acid (LTA), IL-1, IL-17, IL-22, TGF-α, IFNγ, TNF, and IL-9 (289, 293). Furthermore, GF mice have reduced Lcn2 in the serum and feces, indicating that the microbiome drives Lcn2 expression (308).

Lcn2 function

In patients, LCN2 is extensively used as a biomarker in metabolic and inflammatory diseases. It has been proposed as a biomarker for diseases such as obesity (310), acute kidney injury (311), inflammatory bowel diseases (312), cardiovascular diseases (313-315), and the overlap of asthma and chronic obstructive pulmonary disease (COPD) (316). Despite the fact that LCN2 is increased in disease, its function in the pathogenesis of these disorders is not well understood.

Studies using murine systems have provided insight into the diverse functions of Lcn2. Originally, Lcn2 was discovered to bind to and stabilize matrix metalloproteinase-9 (MMP-9, gelatinase), leading to higher MMP9 activity due to slower degradation (317). The interaction of MMP9 and Lcn2 was shown to be important in *Salmonella enterica* serovar Typhimurium infection, where Lcn2 KO mice are protected from any signs of inflammation or damage (318).

As mentioned previously, numerous studies have focused on the interactions of Lcn2 with bacterial catecholate-type siderophores. Bacteria acquire iron from the host by secreting siderophores, which have a high affinity for the ferric (Fe^{3+}) form of iron. Generally, siderophores can be classified into catecholates and hydroxamates based on their chemical structure (296). Once bound, the bacteria subsequently reabsorb the siderophore-iron complex. Lcn2-bound siderophores result in sequestration of iron away from the bacteria and produce a bacteriostatic effect (289, 296). Thus, Lcn2 is critical for the clearance of bacteria that utilize catecholate-type siderophores, such as *Klebsiella pneumoniae*, *E. coli*,

and *S. enterica* serovar Typhimurium (319-322). However, bacteria can also produce other types of siderophores that do not bind Lcn2, so not all bacteria are affected by the presence of Lcn2. Interestingly, bacteria can also acquire iron from the mammalian siderophore 2,5-dihydroxybenzoic acid (2,5-DHBA), which binds Lcn2 and functions similar to bacterial siderophores (323, 324).

In addition to its antimicrobial properties, Lcn2–siderophore complexes are also used for iron transport, which leads to apoptosis. Lcn2 can bind siderophores in two states, iron-loaded (holo-Lcn2) or iron-lacking (apo-Lcn2), which leads to opposite outcomes (298). When holo-Lcn2 is internalized through 24p3R, the intracellular iron concentration is increased and apoptosis does not occur. However, internalization of apo-Lcn2 decreases intracellular iron by exporting it outside of the cell. This decrease in iron also triggers apoptosis through the proapoptotic protein Bim. Many cells are susceptible to this Lcn2-mediated apoptosis, including thymocytes, splenocytes, bone marrow cells, neutrophils, mast cells, erythrocytes, peripheral blood lymphocytes, and several B cell immortalized cell lines (325, 326). While the mammalian siderophore 2,5-DHBA binds apo-Lcn2, the in vivo function is unclear (323).

Studies using recombinant Lcn2 have identified many other functions. Lcn2 is sufficient to cause pain, trigger itching, promote neutrophil migration, induce keratinocyte migration, inhibit alternative activation of astrocytes, and enhance wound healing (300, 327-330). However, despite the fact that Lcn2 is crucial for the clearance of several pathogenic bacteria, it is not yet known whether recombinant Lcn2 is sufficient to alter commensal bacteria.

Many functions of Lcn2 have also been defined using Lcn2 KO mice. Although Lcn2 deficiency is not lethal, these mice have extensive defects. Metabolically, Lcn2 KO mice have increased body weight, increased food intake, and decreased glucose tolerance (309). Within the hematopoietic compartment, Lcn2 KO mice have increased bone marrow cells (326). With the exception of basophils, all cell types are increased. For neutrophils, mast cells, thymocytes, and erythrocytes, the increased number is due to a defect in apoptosis. In addition to apoptotic defects, Lcn2 deficient neutrophils are impaired in chemotaxis, bacterial phagocytosis, and adhesion (327, 331). In regards to the microbiome, two studies have examined microbial differences between WT and Lcn2 KO mice and obtained opposing results. Toyonaga et al. found no differences in the microbiome between WT and Lcn2 KO mice, whereas Singh

et al. found significant differences between genotypes (308, 332). One difference in these studies is that Toyonaga et al. examined 4-week-old mice whereas Singh et al. examined 12-week-old mice. Therefore, many defects have been identified in Lcn2 KO mice, but further research is needed regarding how Lcn2 impacts the microbiome.

Summary

Allergic diseases are increasing worldwide, yet the mechanisms behind loss of tolerance and generation of allergic immune responses remain poorly understood. It has been suggested that changes in the microbiome contribute to the increase in the prevalence of allergy (259). The microbiome plays a critical role in maintaining intestinal homeostasis and educating the immune system to develop oral tolerance. Several factors have been identified as modulators of the microbiota: route of birth, feeding with breast milk versus formula, and antibiotic usage (280). While alterations in these factors may contribute to the increase in allergic diseases, it is still unclear if dysbiosis is the cause or the result of disease. Genetics have been suggested as another factor that can alter the microbiome (284, 285), but less is known about how host factors can alter the microbiota. Several recent studies have shown that cytokines play a role in the microbial composition of the intestine, including IL-4, IL-15, IL-22, and IL-33 (89, 286-288).

GWAS have established identified a strong association between SNPs in IL-33 and its receptor ST2 and allergic disease (1). Although a substantial amount of research has linked IL-33 and ST2 to Type 2–mediated allergic sensitization and inflammation (71), there is increasing evidence that IL-33 has functions outside of this scope, including promoting wound repair (48, 49), fibrosis (93, 333, 334), and neutrophilic inflammation (43). Recently, one study identified a mechanism by which IL-33 modulated the microbiome. Malik et al. determined that IL-33 KO mice have a dysregulated microbiome because they have fewer IgA^+ plasma cells in the intestine and increased *A. muciniphila*. In a dextran sulfate sodium (DSS) model of colitis, the IL-33 KO mice had increased inflammation and disease severity, demonstrating that IL-33 plays a protective role in colitis.

While Malik et al. utilized a phenotype in IL-33 KO mice, allergic diseases are associated with high levels of IL-33. Thus, I wondered if increasing IL-33 was sufficient to alter the microbiome. In Chapter 4, I determined that IL-33 altered the microbiome and identified that it decreased the Bacteriodetes, Erysipelotrichi, and Bacteroidia and increased the Firmicutes and Verrucomicrobiae. Furthermore, I found that IL-33 increased the antimicrobial protein Lcn2 in several cell types and tissues, including the intestine and lung. Finally, I examined the role of IL-33–driven Lcn2 in inflammation and shaping the microbiome.

Results

IL-33 alters the microbiome

Since it was recently shown that IL-33 KO mice have dysbiotic microbiota, I wondered if IL-33 was sufficient to alter the intestinal microbiome. I injected 0.4 μg rIL-33 or PBS into wild type mice for seven days. To examine the microbiome, V4 16s rRNA gene sequencing was performed on the cecal contents. A total of 523 operational taxon units (OTUs) were detected and used for analysis. Hierarchal clustering analysis using Ward's method revealed that the PBS-treated and IL-33-treated microbiomes were distinct from each other (Figure 31A). Alpha diversity was measured within each sample using the observed and Shannon diversity measurements (Figure 31B). There was no significant difference in alpha diversity between PBS- and IL-33-treated samples, indicating that richness and evenness were similar. At the phylum level, Firmicutes and Bacteriodetes were the most abundant taxa. IL-33 treatment caused a significant increase in Bacteriodetes and a significant decrease in Firmicutes (Figure 31C). The most abundant taxa at the class level were Clostridia, Erysipelotrichi, Bacteroidia, and Bacilli, which comprised over 90% of the bacteria (Figure 31D). In response to the IL-33 treatment, Erysipelotrichi and unclassificed Firmicutes were significantly diminished whereas Bacteroidia and Verrucomicrobiae were significantly increased (Table 6). Examination of individual operational taxon units (OTUs) revealed that 69 were significantly altered by IL-33 treatment (Figure 31E). The majority of significantly altered OTUs were in the order Clostridiales. Overall, these data indicate that IL-33 significantly alters the microbiome.

IL-33 induces several antimicrobial molecules

Since IL-33 changes the microbiome, I next wondered how these changes occurred. It was shown that IL-33 KO mice had altered microbiome through increased IgA (89), so I wondered if this mechanism was occurring in response to recombinant IL-33. However, I found that fecal IgA was not significantly increased after IL-33 treatment (Figure 32). IL-33 had been shown to promote expression of antimicrobial proteins (335), so I next wondered what antimicrobial proteins were increased by IL-33. Using data from a microarray analysis on bone marrow mast cells (BMMC) stimulated with IL-33 that our lab has recently published (GSE96696), I identified highly expressed antimicrobial molecules that were

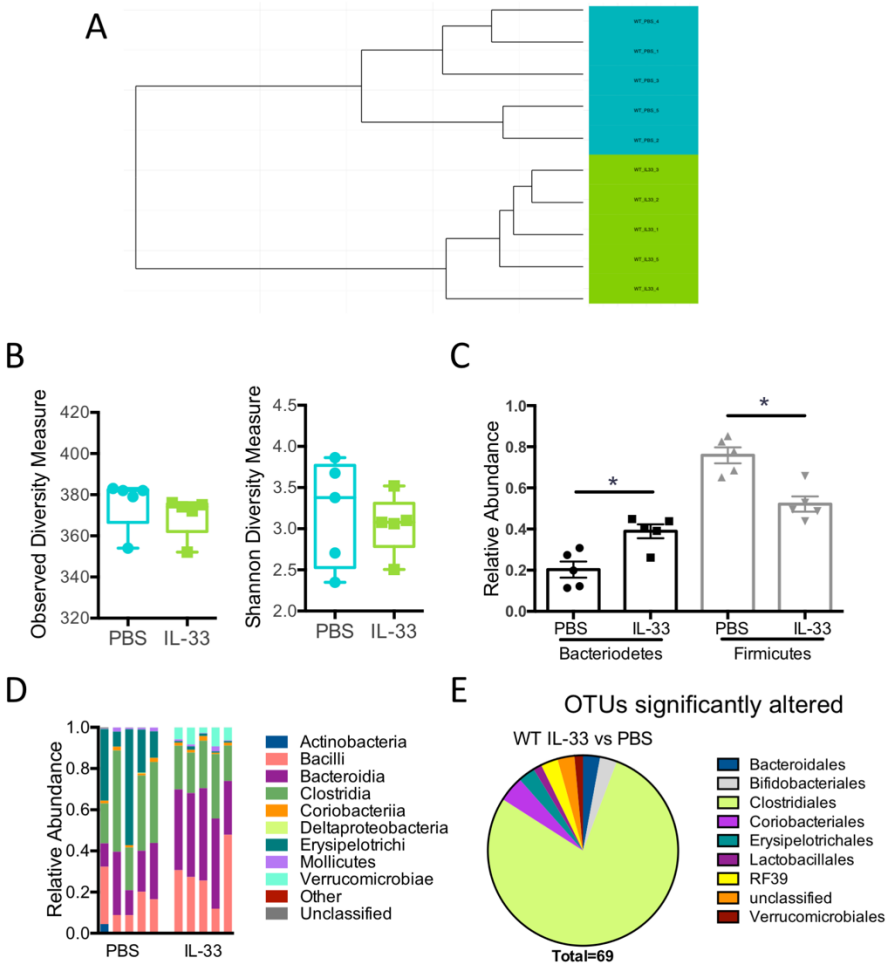

Figure 31. IL-33 is sufficient to alter the microbiome.

WT mice were injected intraperitoneally with 0.4 μg of IL-33 or PBS for seven days and cecal contents were collected for microbiome analysis. (**A**) Hierarchal clustering analysis of WT PBS (blue) and WT IL-33 (green). (**B**) Alpha diversity analysis. (**C**, **D**) Relative abundance of (C) phyla and (D) classes. (**E**) OTUs that were significantly altered by IL-33 as determined by Log2 fold change analysis. Results represent 5 mice per group. *$p \leq 0.05$ by Mann-Whitney *U* test.

Table 6. Mean relative abundance of PBS- and IL-33–treated WT mice by class.

	PBS		IL-33		
	Mean	**SEM**	**Mean**	**SEM**	**p value**
Clostridia	0.3302395	0.0568803	0.2251571	0.0239431	0.3095
Erysipelotrichi	0.2646925	0.0877347	0.0100774	0.0018094	**0.0079**
Bacteroidia	0.2031442	0.0391224	0.3893325	0.0336629	**0.0317**
Bacilli	0.1617293	0.0370958	0.2860497	0.0577505	0.1508
Coriobacteriia	0.0145485	0.0020423	0.0142982	0.0025396	0.8413
Actinobacteria	0.0117641	0.0082864	0.0010085	0.0007215	0.0556
Mollicutes	0.0112520	0.0032334	0.0095112	0.0038907	0.8413
unclassified firmicutes	0.0017067	0.0008218	0.0000558	0.0000312	**0.0159**
Deltaproteobacteria	0.0006784	0.0006584	0.0000155	0.0000062	0.2222
unclassified	0.0001934	0.0000099	0.0001760	0.0000115	0.2222
Verrucomicrobiae	0.0000368	0.0000061	0.0642954	0.0112788	**0.0079**
Gammaproteobacteria	0.0000057	0.0000024	0.0000041	0.0000012	> 0.9999
Betaproteobacteria	0.0000048	0.0000015	0.0000046	0.0000010	> 0.9999
Alphaproteobacteria	0.0000023	0.0000010	0.0000021	0.0000013	> 0.9999
4C0d-2	0.0000011	0.0000007	0.0000029	0.0000014	0.2857
Epsilonproteobacteria	0.0000006	0.0000006	0.0000047	0.0000008	0.0159
TM7-3	0.0000004	0.0000004	0.0000038	0.0000038	> 0.9999
Deferribacteres	0.0000000	0.0000000	0.0000007	0.0000007	> 0.9999
Methanobacteria	0.0000000	0.0000000	0.0000000	0.0000000	> 0.9999

Statistical analysis was performed using the Mann-Whitney *U* test. Bold values indicate significance.

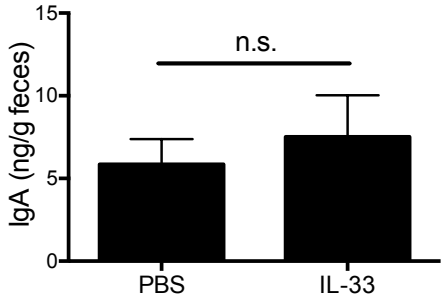

Figure 32. Fecal IgA is not altered by IL-33 treatment in WT mice.

Fecal IgA was measured by ELISA following seven days of IL-33 treatment in WT mice. Data represent mean ± SEM (*n*=6 from two independent experiments).

altered by IL-33 (Figure 33A). From this analysis, I identified Lcn2 as an antimicrobial molecule that is upregulated by IL-33 but not IgE crosslinking (Figure 33B). While Lcn2 has been extensively studied in clearing infections by pathogenic bacteria, the role for Lcn2 in shaping commensal bacteria is not well understood.

Lcn2 is upregulated in multiple tissues in response to systemic IL-33

After identifying Lcn2 as a potential mechanism for changing the microbiome in response to IL-33, I wanted to confirm its expression in vivo. IL-33 appears to drive systemic expression of Lcn2, as Lcn2 was increased in the serum and lung (Figure 34A-C). Furthermore, I detected Lcn2 in the small intestine, colon and feces (Figure 34D-F). Lcn2 levels were significantly increased with IL-33 treatment throughout the small intestine, although these differences were not significant in the feces or colon. As expected, the ST2 KO mice did not respond to the IL-33 treatment. There were no significant differences between PBS-treated WT and ST2 KO mice, indicating that ST2 is not necessary for Lcn2 expression.

IL-33 upregulates Lcn2 in dendritic cells and mast cells, but not neutrophils

Since IL-33 can directly induce Lcn2 in several tissues, I next wondered what cells could produce Lcn2 in response to IL-33. DCs are known to respond to IL-33 and are capable of making Lcn2 in response to LPS (336), so I cultured bone marrow derived DCs (BMDCs) with LPS or IL-33. IL-33 induced a dose-dependent increase of Lcn2 (Figure 35A, B). Notably, the levels of Lcn2 induced by IL-33 were comparable to those induced by LPS. Next, I examined peritoneal mast cells, which are known to respond to IL-33 but have not been described as sources of Lcn2. Although crosslinking of IgE with antigen did not induce Lcn2, IL-33 did significantly induce Lcn2 (Figure 35C, D). Finally, I examined bone marrow neutrophils because they are major producers of Lcn2. Although LPS induced high levels of Lcn2, IL-33 had no effect on Lcn2 expression or release (Figure 35E, F). This lack of response was likely due to their inability to respond to IL-33 since ST2 was not detected on the surface of these neutrophils (Figure 36).

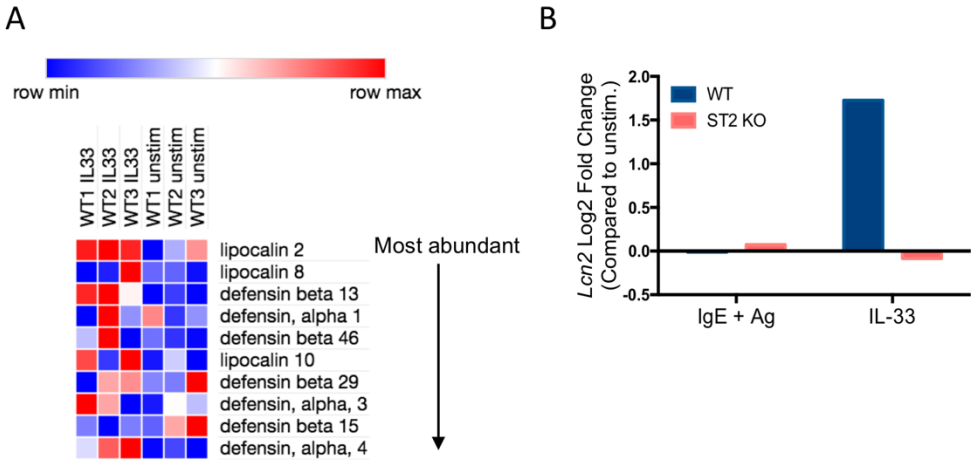

Figure 33. Microarray analysis of mast cells treated with IL-33.

BMMC were stimulated with IL-33 and analyzed by microarray. (**A**) Heatmap of the top 10 most abundant antimicrobial molecules. (**B**) Log2 fold change for Lcn2 of IgE/Ag- or IL-33-stimulated BMMC.

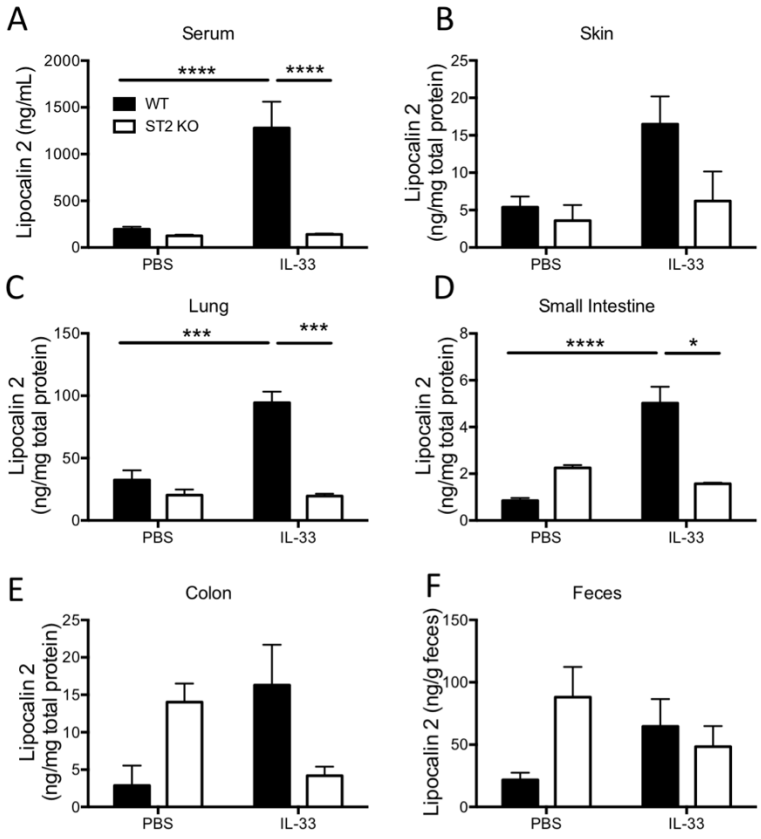

Figure 34. IL-33 induces Lcn2 in vivo.

WT and ST2 KO mice were treated with 0.4 μg IL-33 or PBS intraperitoneally seven times. Lcn2 was measured by ELISA in the (**A**) serum, (**B**) skin, (**C**) lung homogenate, (**D**) small intestine homogenate, (**E**) colon homogenate, and (**F**) feces. Data represent mean ± SEM (*n*=3-12 from two independent experiments). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by two-way ANOVA.

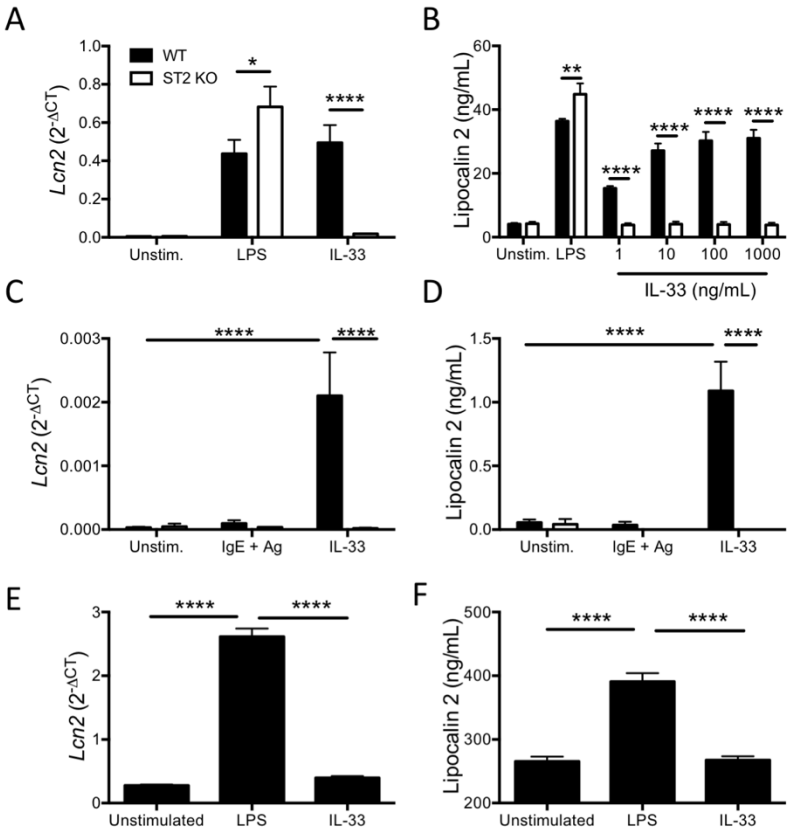

Figure 35. IL-33 induces Lcn2 in DCs and MCs but not Neutrophils.

(**A**, **B**) Bone marrow–derived DCs were cultured and stimulated with 2.5 μg/mL LPS, 10 ng/mL IL-33 (A), or 1-1000 ng/mL IL-33 (B). After 24 hours, Lcn2 mRNA (A) and protein (B) were assessed. (**C**, **D**) Peritoneal MCs were cultured and stimulated with either OVA (following incubation with anti-OVA IgE) or 10 ng/mL IL-33. After 24 hours, Lcn2 mRNA (C) and protein (D) were assessed. (**E**, **F**) Neutrophils were isolated and purified from the bone marrow and stimulated with 2.5 μg/mL LPS or 10 ng/mL IL-33. Lcn2 mRNA (E) and protein (F) was assessed after 24 hours. Data represent mean ± SEM (*n*=3-9 from 2-3 independent experiments). *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001 by two-way ANOVA (A-D) or one way ANOVA (E,F).

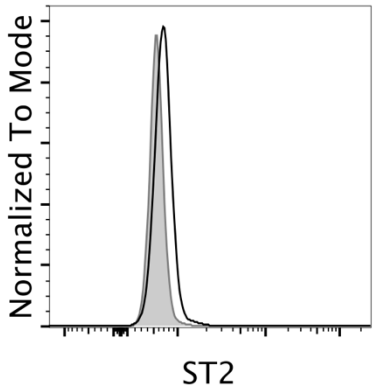

Figure 36. Neutrophils do not express ST2.

ST2 expression was determined by flow cytometry. Data are representative of three mice.

Lcn2 is not required for IL-33-driven lung inflammation

Because IL-33 and Lcn2 had both been shown to promote inflammation, I wondered if Lcn2 was required for IL-33–driven inflammation. In Figure 34, I noticed that the lungs possessed the highest amount of Lcn2. I chose to examine inflammation in the lung because it had been well characterized and I could focus the inflammation to one organ as opposed to systemic inflammation. First, I confirmed that Lcn2 was increased in the lung following three intranasal (i.n.) doses of 0.4 μg rIL-33 compared to PBS (Figure 37A). This treatment also increased serum Lcn2 (Figure 37B). Flow cytometry was used to assess populations of immune cells following i.n. rIL-33 in WT and Lcn2 KO mice as a measure of inflammation (Figure 38). In the PBS-treated mice, I found no significant differences between WT and Lcn2 KO mice in $CD103^+$ DCs, CD11b+ DCs, neutrophils, monocytes, interstitial macrophages, eosinophils, and ILC2s (Figure 39). Alveolar macrophages were slightly higher in Lcn2 KO mice compared to WT mice (Figure 39F). Following the IL-33 treatment, I saw an increase in interstitial macrophages, eosinophils, and ILC2s as well as a decrease in monocytes and alveolar macrophages (Figure 39D-H). IL-33 treatment did not significantly alter $CD103^+$ DCs, $CD11b^+$ DCs or neutrophils (Figure 39A-C). The only impact of Lcn2 deficiency in IL-33–driven inflammation was that the increase in interstitial macrophages was significantly lower than the IL-33-treated WT mice (Figure 39E). Overall, although IL-33 treatment alters the frequency of many cells, Lcn2 plays a specific and minor role only in the increase of interstitial macrophages.

Since Lcn2 is increased by LPS (Figure 35) and LPS also increases IL-33 in vivo (Figure 40), LPS induction of Lcn2 may also be dependent of IL-33. I noticed that in response to LPS, IL-33 reached maximal levels after 4 hours, whereas Lcn2 peaked around 24 hours (Figure 40 and Figure 41A, B). Thus, the timing of these two molecules suggests that it could be possible for LPS to induce IL-33, which then increases Lcn2. To study this in vivo, I examined Lcn2 in the lungs 24 hours after LPS exposure in WT and IL-33 KO mice. IL-33 KO mice make similar levels of Lcn2 in response to LPS (Figure 41C, D). Therefore, LPS can induce Lcn2 independent of IL-33.

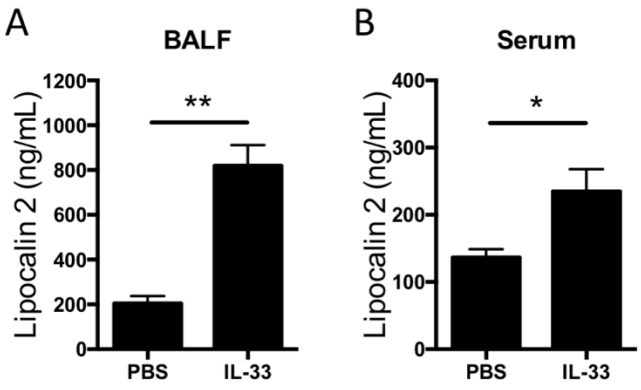

Figure 37. Intranasal IL-33 increases Lcn2 in the BALF and serum.

WT mice were given 0.4 μg IL-33 or PBS three times intranasally. Lcn2 was determined by ELISA in the (**A**) BAL and (**B**) serum. Data represent mean ± SEM (*n*=3 from one experiment). *p ≤ 0.05, **p ≤ 0.01, by two-tailed Student *t* test.

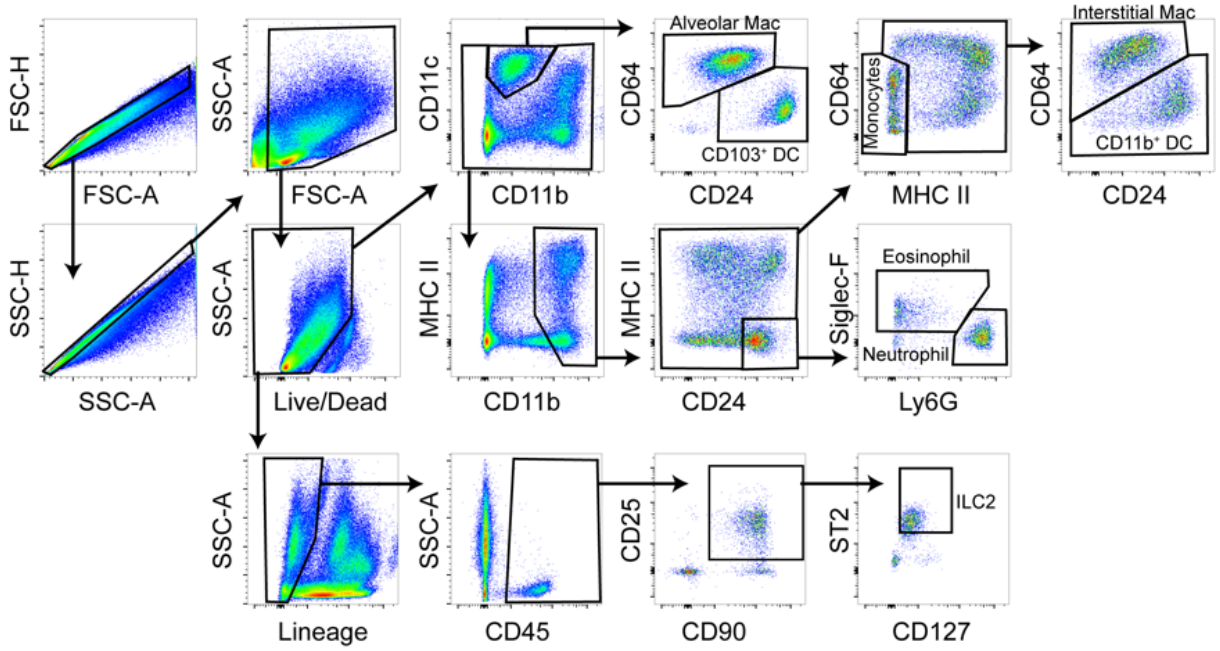

Figure 38. Gating strategy for immune cells in digested lung.

Gating strategy for the cells quantified in Figure 39.

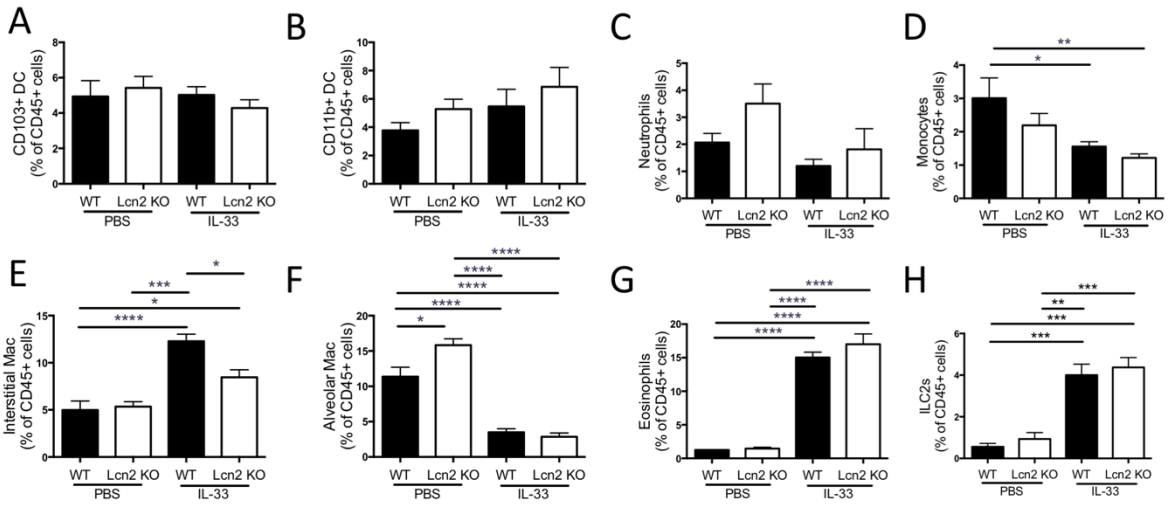

Figure 39. Lcn2 is not required for IL-33–driven inflammation.

WT and ST2 KO mice were administered 0.4 μg IL-33 or PBS i.n. for three days. The lungs were digested and flow cytometry was used to assess (**A**) $CD103^+$ DCs, (**B**) $CD11b^+$ DCs, (**C**) neutrophils, (**D**) monocytes, (**E**) interstitial macrophages, (**F**) alveolar macrophages, (**G**) eosinophils, and (**H**) ILC2s. Data represent mean ± SEM (*n*=3-5 from two independent experiments). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by two-way ANOVA.

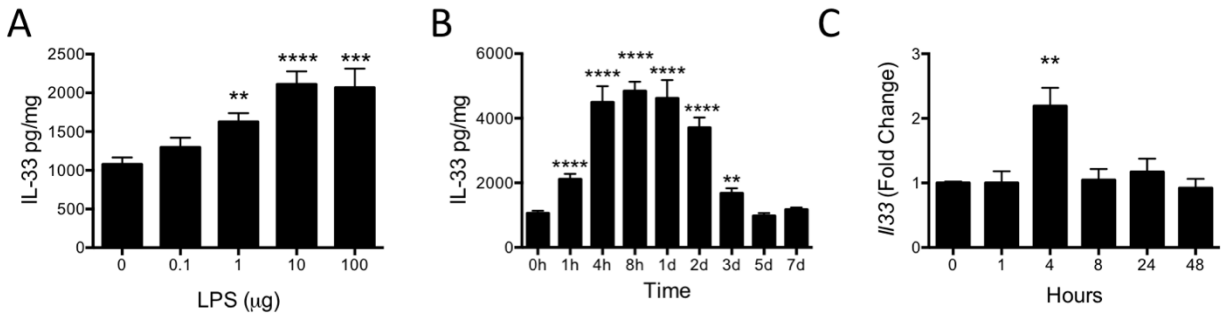

Figure 40. LPS induces IL-33 in vivo.

IL-33 protein and mRNA was measured in the lung tissue by ELISA after different doses of LPS at 1 hour (**A**) and over several timepoints after 10 μg LPS (**B**). *Il33* mRNA was measured by RT-PCR at multiple timepoints after 10 μg LPS (**C**). Data represent mean ± SEM (*n*=3-12 from two independent experiments). **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by one-way ANOVA.

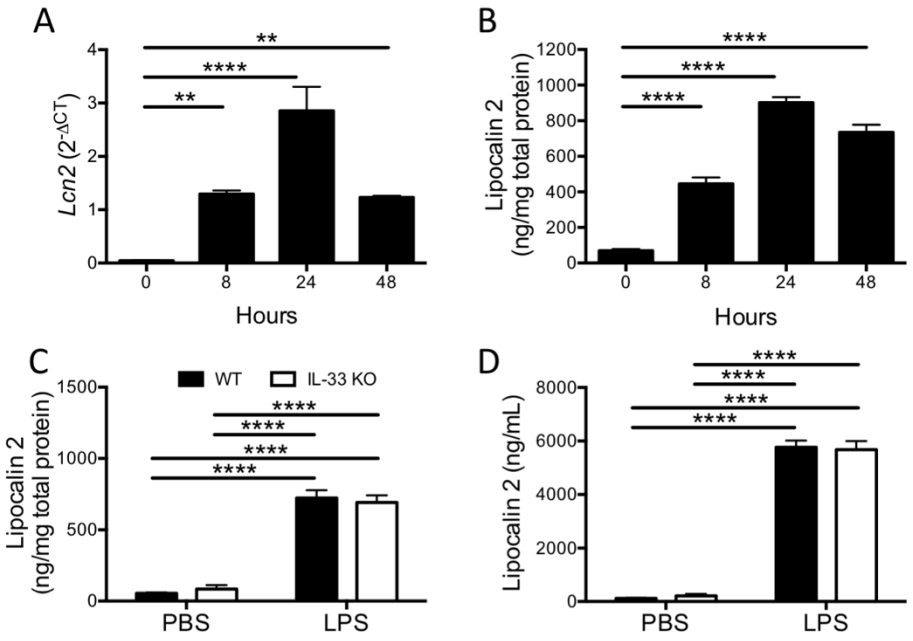

Figure 41. LPS induces Lcn2 independent of IL-33.

WT and IL-33 KO mice were administered 10 μg LPS i.n. (**A**, **B**) Lcn2 mRNA (A) and protein (B) were assessed in lung homogenates at 8, 24, and 48 hours following LPS challenge. (**C**, **D**) Lcn2 was determined by ELISA in lung homogenate (C) and BALF (D) 24 hours after LPS treatment. Data represent mean ± SEM (*n*=4-6 from two independent experiments). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 by one-way ANOVA (A,B) and two-way ANOVA (C,D).

IL-33 alters the microbiome in Lcn2-deficient mice

Because the absence of Lcn2 did not drastically affect IL-33-driven lung inflammation, I turned to examine other functions of Lcn2. Since Lcn2 is most often studied as an antimicrobial molecule, I wondered if Lcn2 participated in IL-33–driven changes in the microbiome. I approached this question by treating Lcn2 KO mice with PBS or 0.4 μg IL-33 daily for one week and then analyzing the cecal microbiome by V4 16s rRNA gene sequencing after the final treatment. A total of 526 OTUs were detected and used for analysis. Samples from each treatment group clustered together using hierarchical clustering analysis (Figure 42A), indicating that the PBS-treated and IL-33–treated microbiota were distinct. Analysis of the observed and Shannon indices of alpha diversity showed no significant differences between PBS- and IL-33–treated groups (Figure 42B). Bacteriodetes and Firmicutes were the dominant phyla. IL-33 treatment caused an increase in Bacteriodetes and decrease in Firmicutes (Figure 42C), similar to the IL-33 treatment of WT mice in Figure 31C. Over 90% of the microbiota was dominated by four classes of bacteria: Clostridia, Erysipelotrichi, Bacteroidia, and Bacilli (Figure 42D). Following IL-33–treatment, Deltaproteobacteria and Betaproteobacteria were decreased whereas Bacteroidia and Gammaproteobacteria were increased (Table 7). Looking at specific OTUs, 62 OTUs were significantly altered by IL-33 treatment (Figure 42E). Similar to the WT mice, the majority of altered OTUs were in the order Clostridiales.

Some of the IL-33-induced alterations in the microbiome are Lcn2-dependent

Although IL-33 clearly altered the microbiome in Lcn2 KO mice, I wanted to determine if any of these alterations were Lcn2 dependent by comparing the WT and Lcn2 KO mice after IL-33 treatment. To set up this experimental series, I first considered cohousing the mice because microbiome studies are often performed this way. However, I noticed in Figure 34 that Lcn2 is detectable in the feces. Mice are coprophagic, so I wondered if Lcn2 could be transferred from WT mice to cohoused Lcn2 KO mice. Indeed, when cohoused with WT mice, Lcn2 KO mice acquired detectable Lcn2 in their feces (Figure 43). Because of this, I chose to separately house the mice for these experiments. To identify Lcn2-dependent changes, I initially looked at the Log2 fold change of each OTU for three comparisons. First,

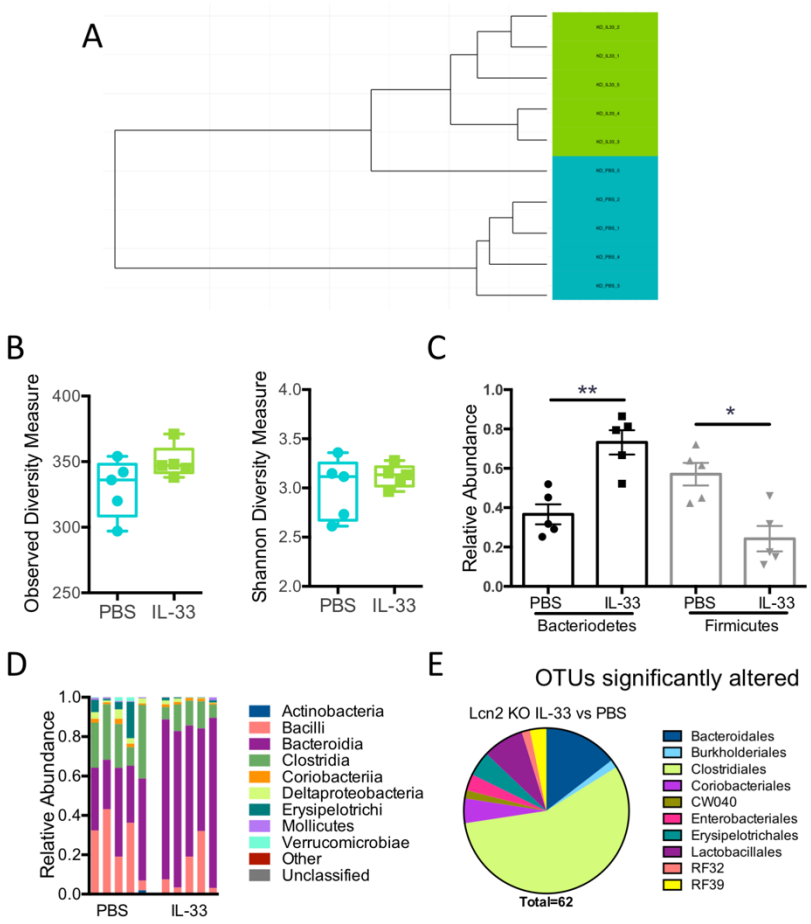

Figure 42. IL-33 alters the microbiome in Lcn2 KO mice.

Lcn2 KO mice were treated with IL-33 or PBS for seven days, and cecal contents were collected for microbiome analysis. (**A**) Hierarchal clustering analysis of Lcn2 KO PBS (blue) and Lcn2 KO IL-33 (green). (**B**) Alpha diversity analysis. (**C**, **D**) Relative abundance of (C) phyla and (D) classes. (**E**) OTUs that were significantly altered by IL-33 as determined by Log2 fold change analysis. Results represent 5 mice per group. *p ≤ 0.05, **p ≤ 0.01, by Mann-Whitney *U* test.

Table 7. Mean relative abundance of PBS- and IL-33-treated Lcn2 KO mice by class.

	PBS		IL-33		
	Mean	SEM	Mean	SEM	**p value**
Clostridia	0.2399802	0.0456033	0.1057328	0.0167974	0.0556
Erysipelotrichi	0.0588779	0.0333224	0.0065701	0.0026163	0.3095
Bacteroidia	0.3665113	0.0509204	0.7323197	0.0618080	**0.0079**
Bacilli	0.2713610	0.0680386	0.1299643	0.0556316	0.0952
Coriobacteriia	0.0162120	0.0034790	0.0120482	0.0012768	0.5476
Actinobacteria	0.0039495	0.0039247	0.0000926	0.0000627	> 0.9999
Mollicutes	0.0053103	0.0010604	0.0032090	0.0026102	0.1508
unclassified firmicutes	0.0000012	0.0000007	0.0000012	0.0000012	> 0.9999
Deltaproteobacteria	0.0290098	0.0062224	0.0092686	0.0038188	**0.0317**
unclassified	0.0001953	0.0000225	0.0001826	0.0000082	> 0.9999
Verrucomicrobiae	0.0083211	0.0043383	0.0000363	0.0000053	0.0952
Gammaproteobacteria	0.0000420	0.0000245	0.0005534	0.0002304	**0.0317**
Betaproteobacteria	0.0000712	0.0000241	0.0000035	0.0000007	**0.0159**
Alphaproteobacteria	0.0000183	0.0000121	0.0000021	0.0000015	0.1746
4C0d-2	0.0000006	0.0000006	0.0000006	0.0000006	> 0.9999
Epsilonproteobacteria	0.0000015	0.0000015	0.0000018	0.0000012	> 0.9999
TM7-3	0.0001360	0.0000652	0.0000114	0.0000016	0.0952
Deferribacteres	0.0000000	0.0000000	0.0000005	0.0000005	> 0.9999
Methanobacteria	0.0000008	0.0000008	0.0000012	0.0000012	> 0.9999

Statistical analysis was performed using the Mann-Whitney *U* test. Bold values indicate significance.

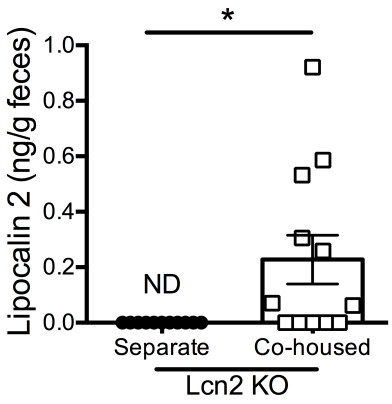

Figure 43. Lcn2 is detectable in the feces of Lcn2 KO mice cohoused with WT mice.

Feces were collected from Lcn2 KO mice that were either housed with other Lcn2 KO mice or with WT mice. Data represent mean ± SEM (*n*=11-12 from two independent experiments). ND indicates not detectable. *p ≤ 0.05 by two-tailed Student *t* test.

we compared PBS–treated WT mice to PBS–treated Lcn2 KO mice to examine which OTUs differed at baseline. Second, I compared PBS–treated to IL-33-treated WT mice to determine which OTUs changed in response to IL-33 treatment in WT mice. Third, I compared PBS-treated to IL-33-treated Lcn2 KO mice to identify the IL-33–driven alterations in Lcn2 KO mice. In Figure 44A, I enumerated the number of OTUs that were significantly up, significantly down, and not significantly altered. For each of the three comparisons, the majority of the OTUs were unaltered. However, I wanted to focus on what was significantly altered. Specifically, I wanted to know how the significantly altered OTUs overlapped between each of the three comparisons (Figure 44B). Looking at the significant differences between WT and Lcn2 KO mice at baseline, 100 of these significantly different OTUs at baseline were not altered by IL-33–treatment. Because it is difficult to interpret OTUs that were affected by both basal genotype differences and IL-33-treatment, I excluded all OTUs that were significantly different at baseline (grey circle). This left 39 OTUs altered by IL-33 in WT mice, 40 OTUs altered by IL-33 in Lcn2 KO mice, and 10 OTUs altered in both. I believe the most interesting group contains the 29 OTUs (highlighted in white) that are altered by IL-33 in WT mice but not Lcn2 KO mice, as this subset describes the OTUs that are altered by IL-33 and dependent on Lcn2. The individual OTUs that make up this IL-33–altered Lcn2-dependent group are defined in Figure 45.

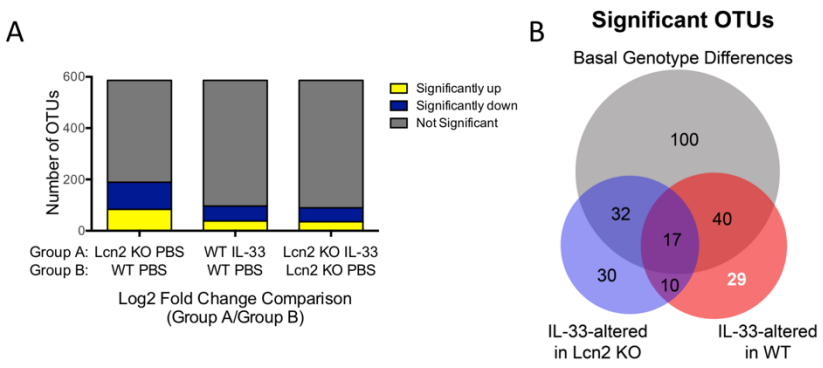

Figure 44. Identification of Lcn2-dependent and Lcn2-independent changes in the microbiome following IL-33 treatment.

(**A**) The Log2 fold change was calculated for three comparisons: WT PBS vs. Lcn2 KO PBS, WT PBS vs. WT IL-33, and Lcn2 KO PBS vs. Lcn2 KO IL-33. Significance was determined for adjusted $p \leq 0.05$. The number of OTUs that were significantly up, significantly down, or not significant are graphed for each comparison. (**B**) Focusing only on the significant OTUs, the three groups were compared to determine similarities and differences.

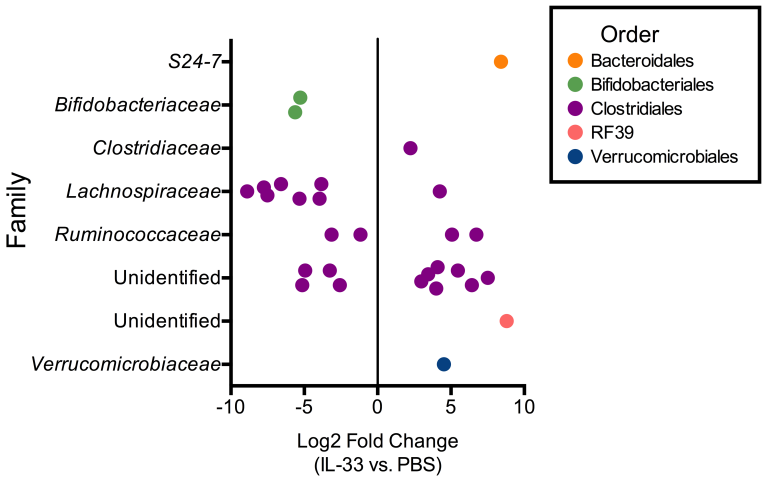

Figure 45. 29 OTUs are significantly altered by IL-33 in a Lcn2-dependent fashion.

Each dot represents a single OTU. Significance was determined by an adjusted $p \leq 0.05$.

Discussion

Understanding the role that the microbiome plays in the development of allergic disease has gained significant interest in recent years. Normally, intestinal homeostasis requires tightly regulated cross talk between the microbiota and the host. Disease occurs when this cross talk becomes dysregulated, although it remains unclear if the dysbiotic microbiota is the cause or the result of disease. Several external factors, including diet, route of birth, and antibiotic usage, are known to influence the microbiome (280, 283). However, host factors that alter the microbiome are less well understood. Here I establish a role for IL-33 in shaping the microbiome and determine that Lcn2 is one mechanism by which IL-33 can exert this change (Figure 46).

IL-33 causes a distinct shift in the microbiome and appears to decrease beneficial bacteria. The distinct signature I observe after IL-33 treatment is quite intriguing because many of the bacteria altered by IL-33 have been previously connected to allergic disease. At the phylum level, IL-33 increases the Bacteriodetes and decreases the Firmicutes. This is similar to what was seen in children who outgrew their allergy to cow's milk; they had a higher abundance of Firmicutes (268). The Firmicutes phylum encompasses several taxa that have been shown to protect against allergy. For example, *Clostridia* protect against the development of food allergy (255). Although I did not see a significant difference in *Clostridia* at the class level, most of the individual OTUs that were significantly altered in response to IL-33 treatment are in the class *Clostridia*. Mice with increased IL-4R signaling (*Il4raF709*) possess a microbiome that was sufficient to promote the development of food allergy. After sensitization, *Il4raF709* mice have significantly decreased *Erysipelotrichaceae* and *Lachnospiracae* but increased *Enterbacteriacae.* Even though I also found similar decreases with IL-33 treatment, I did not detect *Enterbacteriacae* in my mice.

My data also showed that IL-33 increased Verrucomicrobiae, which in my dataset is entirely comprised of OTUs that correspond to *A. muciniphila*. Alternatively, the IL-33 KO mice also possess increased *A. muciniphila* and microbial dysbiosis so it seems likely that these similar phenotypes are a result of two different mechanisms. The IL-33 KO mice have lower IgA, which allows the expansion of *A. muciniphila.* However, I did not find that IgA was altered by IL-33 treatment (Figure 32), indicating that

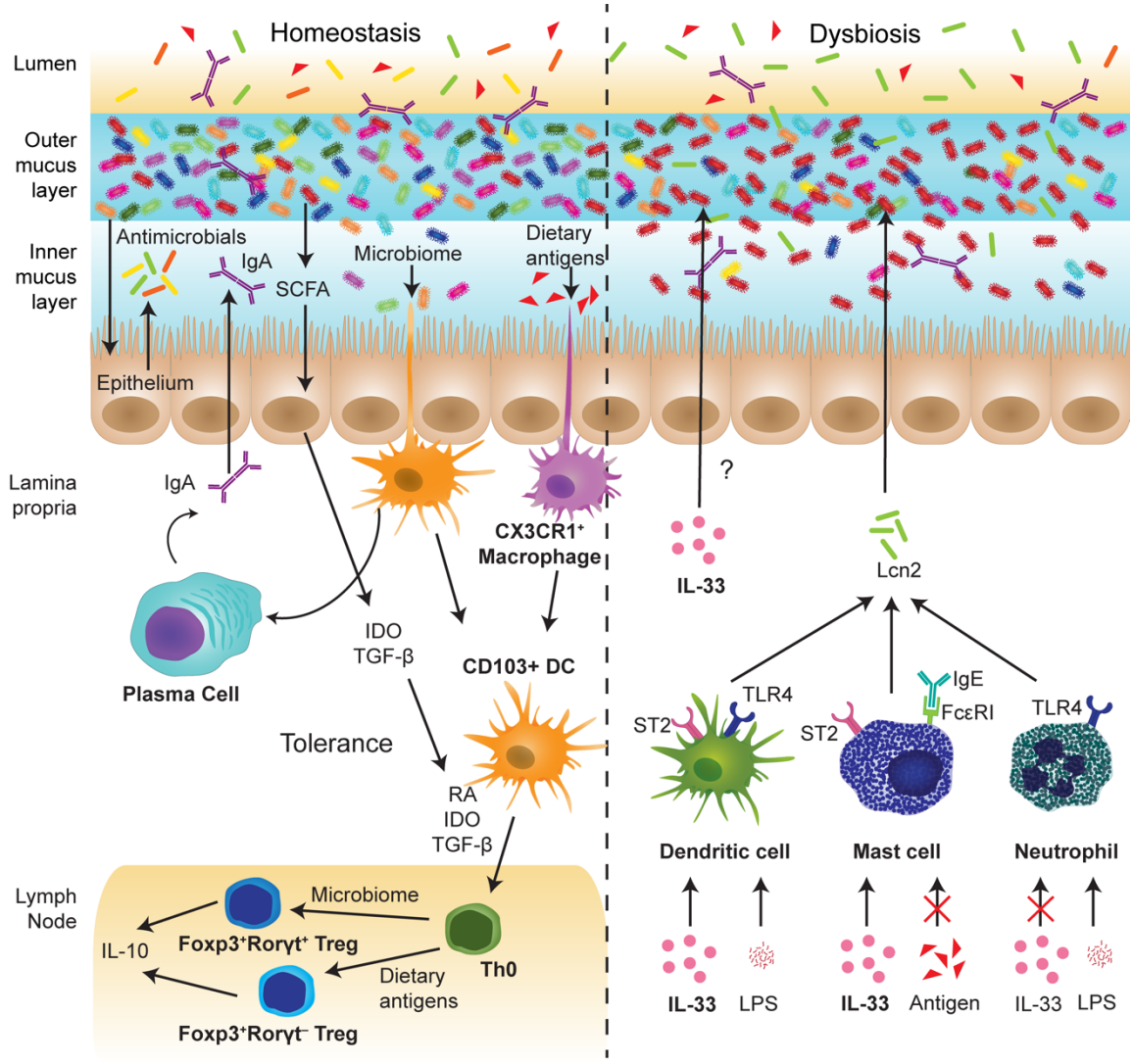

Figure 46. IL-33 induces Lcn2 and intestinal dysbiosis.

IL-33 can induce dysbiosis of the intestinal microbiome through Lcn2–dependent and –independent mechanisms. IL-33 can directly induce Lcn2 from DCs and MCs but not neutrophils. Instead, LPS can induce Lcn2 from neutrophils as well as DCs. Other mechanisms of IL-33–driven dysbiosis remain to be determined.

modulation of *A. muciniphila* by IgA unlikely to be the mechanism occurring in my system. Instead, I found that IL-33 increases the antimicrobial protein Lcn2. Thus, one possibility is that Lcn2 is directly inhibiting bacteria, such as those within the Firmicutes phylum, which provides an opportunity for *A. muciniphila* to expand.

We chose to focus on Lcn2 as one potential mechanism for how IL-33 can alter the microbiome. To examine Lcn2-dependent changes, I identified OTUs that were significantly altered by IL-33 in WT mice but not in Lcn2 KO mice. I also excluded OTUs that were different in PBS treated- WT and Lcn2 KO mice to account for basal differences. It is possible that some of the OTUs within the basal genotype differences are altered by IL-33 in an Lcn2-dependent fashion, but I was unable to determine this from my experimental setup. Ideally, I would have eliminated the basal genotype differences by cohousing the mice. However, Lcn2 can be transferred to Lcn2 KO mice that are cohoused with WT mice, so addressing my question with cohoused mice would be confounded by the fact that the microbiota in Lcn2 KO mice would be exposed to Lcn2. Despite my stringent methods, I identified 29 OTUs that appear to be IL-33 driven as well as Lcn2 dependent. Since Lcn2 is generally considered to be bactericidal, I concluded that the decreased OTUs were directly affected by Lcn2 and that the increased OTUs were a response to this change. Of the decreased OTUs, the two *Bifidobacteriaceae* are particularly interesting because *Bifidobacteria* have established roles in maintaining intestinal homeostasis and protecting against allergic responses. For example, *Bifidobacterium infantis* can prevent intestinal permeability and internalization of tight junction proteins in a model of necrotizing enterocolitis (337). In food allergy, probiotic supplementation with *Bifidobacteria* reduced intestinal permeability and OVA-specific IgE as well as restored normal Treg levels (338). Furthermore, *Bifidobacterium longum* releases extracellular vesicles that induce mast cell apoptosis, which limits diarrhea symptoms in a model of food allergy (339). Thus, it would be interesting to determine how the IL-33–driven loss of *Bifidobacteria* would impact the development of allergy.

In addition to *Bifidobacteriaceae*, IL-33 impacts several OTUs within *Lachnospiraceae* and *Ruminococcaceae* in an Lcn2-dependent fashion. These two families are known to be major producers of the SCFA butyrate (340). Butyrate has been correlated with children who outgrow food allergy (341) and

is known to promote epithelial barrier integrity and limit inflammation through modulation of histone deacetylases and NFκB (342). In mice, butyrate, propionate, and acetate have all been shown to be protective in models of food allergy and asthma (283, 343). However, the levels of SCFAs have mainly been studied as an outcome of diet. Perhaps instead of focusing on specific bacteria that are altered by IL-33, it would be helpful to investigate how IL-33 alters SCFA levels. This mechanism has been demonstrated with IL-15–overexpressing mice, which have decreased butyrate and are more susceptible to colitis (287).

Although Lcn2 is best known as an antimicrobial protein, it is clear that it has other functions beyond bacterial death. Thus, IL-33–induced Lcn2 may have other roles outside of modulating the microbiome. I addressed one of these functions by examining the inflammatory infiltrate in WT and Lcn2 KO mice following i.n. IL-33 exposure (Figure 39). However, I did not find that the IL-33–driven inflammation was overtly Lcn2 dependent. Of the many cell types that I examined, I only saw that the increase in interstitial macrophages in response to IL-33 was partially Lcn2 dependent. This would suggest that Lcn2 can recruit macrophages to the lung. A second function that has been shown for Lcn2 is apoptosis. While Lcn2 can have an apoptotic effect on many cell types, it is particularly effective in cells that are dependent on IL-3 (325). Thus, mast cells and plasma cells, which reside in the intestine, could be potential targets of Lcn2-driven apoptosis. Alternatively, there may be a scenario where IL-33 recruits inflammatory cells and subsequently induces Lcn2 to assist in clearance of those cells through apoptosis. Finally, IL-33 and Lcn2 have independently been shown to promote wound healing (49, 328). However, it is unclear if Lcn2 is downstream of IL-33 or if both molecules can promote the same outcome through separate mechanisms.

Despite my interest in Lcn2-dependent functions of IL-33, it is undeniable that Lcn2 is not the sole mechanism by which IL-33 alters the microbiome. Indeed, there are several potential mechanisms by which IL-33 could alter the microbiome independent of Lcn2. IL-33 has been shown to induce multiple antimicrobial molecules (335), so it would not be surprising if the IL-33–microbiota signature was a result of multiple antimicrobial mechanisms. Furthermore, IL-33 has several known effects on the epithelium. It is known to increase intestinal permeability (46), which may increase the exposure of TLR ligands and

trigger the immune system for a stronger antimicrobial response. IL-33 also promotes mucus production (7), which could affect the microbiome because mucus is a critical component of intestinal homeostasis. Lastly, there is a subset of Tregs in the intestine that are $ST2^+$ (251), but it is unclear how or if these $ST2^+$ Tregs modulate the microbiome.

The use of recombinant IL-33 has allowed us to better understand its functions and mechanism through Lcn2; however, it is only a tool for studying human disease. In humans, there are several situations in which high levels of IL-33 can occur. For example, several diseases have been shown to have increased IL-33, including parasite infection (207), bacterial infection (86), viral infection (98, 344), allergic diseases (345), inflammatory bowel disease (IBD) (346), and rheumatoid arthritis (347). In infectious diseases, the pathogen initially encounters the microbiome before breeching the mucosal barrier and infecting the host. Thus, most if not all of the alterations in the microbiome likely originate from the pathogen. It seems unlikely that the function of IL-33 in infectious disease is to alter the microbiome. Instead, it is possible that IL-33 induces Lcn2 during infection for purposes unrelated to the microbiome, but it will be quite challenging to identify an IL-33–driven effect because pathogens, through TLR activation, are potent inducers of Lcn2 (289). Despite this, this mechanism may drive any of several potential functions, including apoptosis, neutrophil migration, or epithelial repair. In contrast to infectious diseases, IL-33–driven Lcn2 may have a greater impact in inflammatory diseases in which TLR activation is less robust. In these diseases, it is unclear if the microbiome is the result or cause of disease. While allergy, IBD, and rheumatoid arthritis have all been associated with alterations in the microbiome (266, 348, 349), the mechanism of how this occurs is not fully understood. Thus, one possibility is that IL-33 is increased once these diseases are established, which induces Lcn2 and changes the microbiome. In this scenario, IL-33 would either create dysbiosis once disease is established, or it would maintain or enhance the dysbiosis that initiated the disease. Alternatively, IL-33 may induce Lcn2 for another purpose outside of its antimicrobial functions. Therefore, because IL-33 is elevated in numerous diseases, there are many situations in which IL-33–driven Lcn2 could feasibly play an important role.

In contrast to IL-33 being elevated as a response to a stimulus within disease, high IL-33 could also be a result of genetic predisposition. GWAS have established a strong connection between asthma

and SNPs in IL-33 and its receptor ST2 (1-3). Importantly, *IL1RL1*, the ST2 gene, is one of the few susceptibility genes that is associated with several GWAS that span allergy/allergic sensitization, asthma, and AD (4). Importantly, one study generated cell lines expressing WT ST2L and several *IL1RL1* variants and assessed the functionality of these SNPs (350). Of the six *IL1RL1* variants, five had increased IL-33 expression. Furthermore, two of the variants, A433T (rs4988956) and Q501R (rs10204137), had enhanced responsiveness to IL-33, with increased phosphorylation of NFκB and c-Jun. These data indicate that *IL1RL1* variants can both modulate IL-33 expression and have an enhanced response to IL-33. Given the fact that IL-33 correlates with disease severity (67), it is possible that patients with A433T or Q501R variants have the most severe forms of disease because of enhanced IL-33 responsiveness. Perhaps this enhanced signaling also further increases Lcn2 and maintains dysbiosis in the intestine. Alternatively, these SNPs may affect the development of allergy. Developing fetuses express IL-33 (95) and the neonatal immune system is initially Th2 biased (344). Perhaps neonates with the A433T or Q501R variants have higher IL-33 and/or IL-33 responsiveness at the critical early stage in development where oral tolerance can fail and allergy can develop. Furthermore, these potential “high IL-33–expressing” neonates could have high levels of Lcn2 and therefore a microbiome lacking protective bacteria. Further research is needed to determine if these *IL1RL1* variants correlate with increased IL-33, increased Lcn2 and/or an IL-33–altered microbiota signature in neonates.

In summary, my data demonstrate a novel role for IL-33 in modulating the intestinal microbiota. Specifically, IL-33 appears to reduce beneficial bacteria, including *Bifidobacteria*, Bacteroidia, and *Erysipelotrichaceae*, and increase inflammatory commensal bacteria, like *A. muciniphila*. In addition, I am the first to demonstrate that IL-33 induces the antimicrobial protein Lcn2 in several tissues as well as DCs and MCs. Although IL-33–induced inflammation does not appear to be overtly Lcn2 dependent, some of the IL-33–driven changes in the microbiota are dependent on Lcn2. These findings expand our knowledge of the current functions of IL-33 as well as generate new potential mechanisms for how IL-33 may participate in the development of or enhance the severity of allergic disease.

CHAPTER 5 – Overall Summary and Discussion

IL-33 is multifunctional molecule that affects numerous diseases. Although initial research focused on its role in allergic disease, IL-33 has since been shown to have numerous roles in inflammatory diseases, fibrotic diseases, and cancer (8, 20). In allergy, several controversies still surround IL-33, including how it is released from cells and the relevance of immune- versus structural-derived IL-33. Thus, IL-33 appears to be quite a complex molecule that is far from well understood. My work demonstrates two novel homeostatic functions for IL-33, thus defining roles for IL-33 outside of disease.

IL-33 acts on stem cells

In Chapter 3, I identified that IL-33 acts on eosinophil precursors in the bone marrow. When IL-33 is added to bone marrow cells, EoPre and mature eosinophils expand (Figure 25) and IL-5Rα is significantly upregulated on EoPre (Figure 20), enhancing their responsiveness to IL-5. Simultaneously, IL-33 induces IL-5 in the bone marrow (Figure 15). Therefore, I propose that IL-33 acts directly on eosinophil precursors while simultaneously inducing IL-5 from another bone marrow resident cell to drive eosinophil development. NJ.1638 mice crossed with ST2 KO mice had diminished eosinophils (Figure 17), indicating that there is a role for IL-33 in IL-5-driven eosinophilopoiesis, but eosinophils can develop in the absence of IL-33. In agreement with my data, Stolarski et al. found that culture of sorted c-Kit$^+$ bone marrow cells with IL-33 generates mature eosinophils in an IL-5-dependent manner (57). Furthermore, ST2 KO cells cultured with IL-5 produced similar numbers of eosinophils, demonstrating that IL-5-driven eosinophilopoiesis can occur in vitro in the absence of IL-33. This finding is not surprising given that IL-5 KO mice, as well as ST2 KO and IL-33 KO mice, have significantly reduced – but not absent – eosinophils. This indicates that unlike GATA-1 and XBP1, which are absolutely required for eosinophil development, the absence of one cytokine may be compensated by others. Perhaps a cross between the ST2 KO and the IL-5 KO or the CD131 (common β-chain) KO would prevent eosinophil development.

Prior to my work, it was established that mouse eosinophils developed through four stages after myeloid commitment: CMP, GMP, EoP, and EoM. This was recently challenged by a study that

demonstrated the GMP population could be divided into GATA-1^{+}GMP and GATA-1^{-}GMP, and that only GATA-1^{+}GMP developed into eosinophils (183). Thus, Drissen et al. propose that GATA-1^{+}GMP be renamed EoMP. My work also defines a novel cell, the EoPre, which expresses eosinophil-associated genes but lacks granules. Taken together, this suggests that eosinophils develop through five stages: CMP, EoMP, EoPre, EoP, and EoM (Figure 3). My work further addresses that IL-33 may be the missing signal that directs stem cells to commit to the eosinophil lineage.

Although my work demonstrates a role for IL-33 specifically in eosinophil development, IL-33 functions in the development of other stem cells. The expression of ST2 on bone marrow stem cells upstream of eosinophil progenitors is controversial, but ST2 expression is found on epithelial stem cells within the gut (335). IL-33 promotes the differentiation of these intestinal stem cells into secretory epithelial cells. Another potential stem cell target for IL-33 is ILC2 progenitors, which express ST2 (351). However, the development of ILCs is not fully understood and the precise involvement of IL-33 remains to be determined.

Eosinophil precursors have been identified in tissues, but their function remains unclear. While eosinophils are a major immune cell type resident to the intestine, it is unknown if eosinophil precursors maintain the pool of resident eosinophils through extramedullary eosinophilopoiesis. In the intestine, IL-33–driven differentiation of resident stem cells becomes important in response to epithelial injury, where the intestinal stem cells are needed to repair the gut epithelium (335). Since it has not been shown that IL-33 KO mice have structural defects in the intestine, IL-33 does not appear to be necessary for normal structural integrity. Thus, IL-33 may be important for in situ stem cell development. Recently, it was also shown that IL-33 causes eosinophil progenitors to produce higher levels of cytokines than mature eosinophils (182). This suggests that eosinophil precursors may directly participate in an immune response.

IL-33 alters the microbiome

In Chapter 4, I found that IL-33 is sufficient to alter the microbiome. IL-33 treatment caused reductions in Firmicutes, *Erysipelotrichaceae*, and *Lachnospiracae*, and increases in Bacteriodetes,

Bacteroidia and *Verrucomicrobiaceae* (Figure 31 and Table 6). I also determined that IL-33 promotes expression of the antimicrobial protein Lcn2 (Figure 34), and that this may be one mechanism by which IL-33 alters the microbiome. Lcn2 was required for changes the relative abundance of *Bifidobacteriaceae, Lachnospiraceae*, and *Ruminococcaceae* by IL-33 (Figure 45). A previous study showed that IL-33 KO mice have an altered microbiome (89), but my work is the first to show that IL-33 is sufficient to alter the microbiome. Furthermore, although mice that transgenically overexpress IL-33 have increased antimicrobial proteins (335), my work is the first to demonstrate that IL-33 directly stimulates expression of an antimicrobial protein to the best of my knowledge. This work provides a novel mechanism by which IL-33 can directly affect bacterial survival.

Since TLR ligands can also induce Lcn2 (289, 293), I chose to focus on the homeostatic microbiome where exogenous TLR ligands like LPS did not affect Lcn2 levels. This allowed me to focus on IL-33–driven Lcn2 effects, as opposed to Lcn2 from other stimuli. However, Lcn2 is known for its role in bacterial infection (320-322) and roles for IL-33 have also been demonstrated in TLR-driven models (92), which led me to question the involvement of IL-33–driven Lcn2 in infection. I did examine the necessity of IL-33 in LPS-driven Lcn2 (Figure 41) but found that IL-33 was dispensable for whole tissue Lcn2 expression. Thus, LPS and IL-33 independently drive Lcn2 expression. Since IL-33 is known to prime cells or act synergistically with other stimuli, perhaps on a cellular level IL-33 enhances LPS stimulation of Lcn2. This mechanism has been demonstrated in macrophages, where IL-33 enhances LPS-driven cytokine production (56).

While my research focused on Lcn2 as a mechanism for how IL-33 alters the microbiome, there are certainly other mechanisms for IL-33-driven microbial changes, including other antimicrobial molecules and alterations in epithelial barrier function and mucus production. Given my work on eosinophils, it is interesting that eosinophil deficient mice have an altered microbiome (129). Perhaps alterations in IL-33 may affect the microbiome through alterations in eosinophil numbers.

Other potential functions for IL-33

IL-33, eosinophils, and B cells

Interestingly, several studies closely related to my work highlight an important connection between eosinophils, IL-33, and B cells in both the bone marrow and the intestine. In the bone marrow, I found that the IL-33-expressing cell is a B cell (Figure 26), suggesting that B cells may promote eosinophil development. Conversely, eosinophils are important for plasma cell survival within the bone marrow (131). It is not known if IL-33 is important for plasma cell survival, but there seems to be crosstalk between eosinophils and B cells in the bone marrow, and IL-33 may be involved. In the intestine, both IL-33 KO and eosinophil deficient mice have fewer IgA^+ plasma cells and microbial dysbiosis (89, 129). Since I found that IL-33 KO mice have reduced bone marrow and blood eosinophils (Figure 5 and Figure 10) and eosinophils are important for plasma cell survival in the intestine (129), it seems likely that the low numbers of IgA^+ plasma cells are a result of reduced intestinal eosinophils, though it has yet to be shown that IL-33 KO mice are deficient in tissue eosinophils. I did not find IL-33 to be sufficient to increase fecal IgA (Figure 32), indicating that exogenous IL-33 does not expand IgA^+ plasma cells and/or does not promote increased secretion of IgA into the intestinal lumen. Despite this, the sum of my work and others suggests that a IL-33-eosinophil-B cell axis may be important in the intestine. Because these factors have also been shown to affect the microbiome, perhaps the function of this mechanism is to modulate the microbiome.

Functions of IL-33-driven Lcn2

Outside of the scope of my work, there may be other functions for IL-33–driven Lcn2. Perhaps the most promising option is wound repair. In models of skin wounds, IL-33 and Lcn2 have been separately shown to accelerate closure of the wound (48, 328). In the intestine, IL-33 KO mice have more severe tissue damage upon *Salmonella Typhimurium* infection and promote epithelial stem cell differentiation (335), whereas Lcn2 promotes mucosal repair through increased cell migration of colonic epithelial cells (352). Thus, the functions of IL-33 and Lcn2 in wound repair may be connected.

Lcn2 is also known to interact with and enhance the activity of MMP9, which is important for wound repair (353), fibrosis (354, 355), and transepithelial migration (356). IL-33 and MMP9 have been linked because IL-33 is required for MMP9 induction in response to *Alternaria alternata* (88). In the bleomycin model of fibrosis, IL-33 enhances fibrosis (93, 94). Thus, one potential mechanism is that IL-33 can induce MMP9 as well as Lcn2, which enhances the MMP9 activity and thus exacerbates fibrosis. In a model of allergic airway inflammation, MMP9 KO mice have reduced transepithelial migration in response to allergen (356). Although I did examine the requirement of Lcn2 in IL-33-driven inflammation, I examined total numbers of immune cells and did not distinguish between the alveolar and tissue compartments. Thus, perhaps Lcn2 and MMP9 are required for the transepithelial migration of immune cells in response to IL-33.

Future directions

Functions of IL-33 isoforms

Several studies have described multiple isoforms of IL-33 (8, 11-13, 19). While the full length protein can be secreted from cells (105), it can also be cleaved into a shorter, more active form by proteases (Figure 1). The functions of these isoforms are not well understood. Luziana et al. demonstrated that while both full-length mouse IL-33 (flmIL-33) and mature IL-33 (aa109-266, mmIL-33) caused inflammation, only mmIL-33 increased eosinophils, Type 2 cytokines, and mucus production (357). My work relies heavily on mmIL-33, thus it is not known if flmIL-33 can also promote eosinophil development, modulate the microbiome, or induce Lcn2. Different functions for IL-33 isoforms may provide an answer to why IL-33 has such diverse effects. For example, IL-33 is known to promote both eosinophilic and neutrophilic inflammation (7, 43), but it is not known how it affects these distinct cells. This discrepancy could be explained by the effect of different isoforms of IL-33. I found that ST2 KO mice had significantly reduced eosinophils and neutrophils (Figure 5), but mmIL-33 treatment was only sufficient to increase eosinophils (Figure 8). Treatment with flmIL-33 increases neutrophils (357), so perhaps flmIL-33 has a greater effect on neutrophils and is responsible for the neutrophil deficiency in IL-

33 KO mice. Since mature bone marrow neutrophils have low to no expression of ST2 (Figure 36), flmIL-33 would either act on neutrophil precursors or function independently of ST2 in the nucleus.

Furthermore, because I used mmIL-33, it would be beneficial to understand how IL-33 is released in the bone marrow. If flIL-33 is secreted in the bone marrow, proteases would be required to generate the mature form that promotes eosinophil development. Since B cells are a major source of IL-33 in the bone marrow (Figure 26) and they are capable of secreting a serine protease (358), it would be interesting if B cells secrete and cleave IL-33.

Relevant sources of IL-33 and Lcn2

Within the IL-33 field, controversy surrounds the importance of cell-specific IL-33. While structural cells appear to be the dominant source of IL-33 at homeostasis (95, 96), several studies have shown roles for immune cell-derived IL-33 in disease (100-102). In the bone marrow, I have identified that B cells express IL-33 (Figure 26). However, it has also been shown that chondrocytes and osteoblasts express IL-33 (359, 360). In the intestine, IL-33 was found to be expressed by pericryptal fibroblasts (335). Since I have defined two distinct functions for IL-33 in two separate tissues, it would not be surprising if the cellular source required for each function was different.

Neutrophils are a major source of Lcn2, but it was determined that osteoblasts and adipocytes are major contributors to serum Lcn2 (309). Since the intestinal epithelium is known to produce many antimicrobial proteins and express ST2 (335), I hypothesize that this is the most likely source of Lcn2 that functions in shaping the microbiome. I did also find that DCs and MCs express Lcn2 in response to IL-33 (Figure 35), suggesting that these cells could either contribute to Lcn2 levels or DC- or MC-derived Lcn2 could have a specific function. Lcn2 has been shown to regulate apoptosis in IL-3-dependent cells (325), so the function of MC-derived Lcn2 may involve apoptosis. Since Lcn2 interacts with iron-bound siderophores, it functions in transporting iron (298). It was recently shown that changes in intracellular iron regulate the inflammatory phenotype of DCs (361), so it is possible that DC-derived Lcn2 could alter the DC phenotype through iron transport.

Overall impact of IL-33 in driving eosinophil development and microbiome dysbiosis

Although these functions for IL-33 are distinct from previously described functions, my work contributes to two well established but not fully understood concepts that IL-33 contributes to the development of allergic disease and is correlated with disease severity. In Chapter 3, I showed that IL-33 acts on eosinophil progenitors in the bone marrow to increase the output of mature eosinophils into the blood. While this demonstrates a homeostatic role for IL-33, its role in eosinophil development in disease is not fully understood. It has been suggested that rEos in the lung play a role in allergic sensitization by suppressing Th2 development (134). Although ΔdblGATA mice lack both rEos and iEos, it is unclear how these populations develop and if IL-33 plays a role in both or one of these populations. Thus there is a hint that IL-33 and eosinophils may somehow regulate allergic sensitization, but certainly further research is needed to understand this. Instead, it seems more likely that IL-33 regulates eosinophils after allergic sensitization. Upon allergen challenge, it is unclear if eosinophils simply migrate from the blood into tissues or if eosinophilopoiesis occurs in the bone marrow or tissues to increase eosinophil numbers. Perhaps IL-33 drives eosinophilopoiesis in response to allergen exposure to exacerbate and/or prolong the response, and blocking IL-33 upon allergen exposure would lessen the severity of response to allergen. In Chapter 4, I identified that IL-33 shifts the intestinal microbiome in such a way that beneficial and/or protective bacteria are lost. This could potentially lead to loss of oral tolerance and development of allergy or it could maintain the dysbiotic microbiota in disease and thus enhance severity. In summary, although these two functions of IL-33 in eosinophilopoiesis and microbiome dysbiosis are seemingly distinct, they could each provide a mechanism to the overall function of IL-33 in the development and severity of allergy.

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