Title
IL-33 and TSLP in chitin-induced lung inflammation

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IL-33 and TSLP in chitin-induced lung inflammation

by

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DISSERTATION

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of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

This dissertation is dedicated to my parents, Shyam and Subhra Mohapatra,

whose love, support, and encouragement have made it possible.

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Finally, I owe so much of what I am today to the love, support, and mentorship of my parents, Shyam and Subhra Mohapatra. As successful scientists in their own right, they spent countless hours nurturing my scientific interests. Earning a PhD has been a goal of mine since childhood, and they have made that possible. I aspire to achieve their success in science and in life.

Contributions to the Presented Work

Chapter 2 of this dissertation is based on work that has been prepared and submitted as a manuscript entitled “Group 2 innate lymphoid cells coordinate epithelial maintenance of lung homeostasis”. Co-authors of this work include Steven J. Van Dyken, Jesse C. Nussbaum, and Hong-Erh Liang. Steven J. Van Dyken contributed to experiments identifying cellular sources of IL-33 and TSLP. Jesse C. Nussbaum and Hong-Erh Liang generated the 9er reporter mouse and assisted with helminth infection studies. Richard M. Locksley supervised the entirety of this work. All authors are members of the Howard Hughes Medical Institute and the Departments of Medicine, Microbiology, and Immunology at the University of California, San Francisco.

Abstract

In humans, allergic disease bears striking resemblance to the host response to helminth infection. Thus, we studied *N. brasiliensis* infection in mice to identify factors that initiate host immunity and identified three that bind the environmental polysaccharide chitin. We further demonstrated that purified chitin acutely induces lung inflammation in mice, similar to that observed in human allergic disease, dependent on resident group 2 innate lymphoid cells (ILC2s). Given their number, distribution and function, lung ILC2s likely coordinate responses with resident epithelial cells that monitor barrier interactions with the environment. In this thesis, we demonstrate that alveolar type II (ATII) cells are the main source of interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) generated in response to chitin or *N. brasiliensis* challenge. IL-33 and TSLP synergistically activate ILC2s to produce IL-5, IL-13 and IL-9, and autocrine IL-9 promotes an ILC2 transcriptional program associated with rapid and robust cytokine production necessary to optimize epithelial gene responses in the conducting airways. Thus, ILC2s link a specialized, distal, epithelial population involved in alveolar function with more proximal epithelia that regulate airway flow, revealing a key role for ILC2s in tissue homeostasis that might underlie similar organizational hierarchies for innate lymphoid cells in other organs.

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Chapter 1: Introduction

Human Allergy

Allergic inflammation in humans spans a broad range of diseases, including a sub-type of asthma, conjunctivitis, eczema, and some gastrointestinal (GI) reactions to food (Yazdanbakhsh et al., 2002; Locksley, 2010). The tissue distribution of these manifestations, across the respiratory tract, the skin, and the GI tract, highlights the fact that "allergens", the inciting agents of inflammation, are uniformly non-infectious particles from the external environment (Palm et al., 2012). Atopy describes the observation that patients are often "sensitized" to certain allergens and develop acute and chronic symptoms upon re-exposure to those allergens (Bell, 1996). Broadly, these symptoms include: swelling due to local or systemic vasodilation; airway narrowing and diarrhea or vomiting in the respiratory and GI tracts, respectively, due to increased smooth muscle contractility; increased mucus production in the airways and intestines from goblet cell hyperplasia; and hives and pruritis in the skin via peripheral nerve activation (Locksley, 2010).

Despite the ubiquitous presence of many common allergens, the prevalence of allergic inflammation has a specific geographic distribution: higher in areas with sanitation, vaccination, and antibiotic use and in urban versus rural settings (Yazdanbakhsh et al., 2002). The inverse correlation between incidence of childhood infection and that of allergic inflammation and the direct correlation between environmental cleanliness and the prevalence of allergic inflammation are encapsulated in the Hygiene Hypothesis (Strachan, 1989). Thus, this type of inflammation is associated with the industrialization and modernization of societies worldwide, suggesting that

prevalence and morbidity will increase indefinitely and underscoring the need for better therapeutic and prophylactic tools.

Immune Dysregulation in Allergy

Although the Hygiene Hypothesis implies a role for the immune system in the pathogenesis of allergic inflammation, evidence of this arose from a study of patients with severe asthma that documented an increase in activated peripheral blood T cells during acute exacerbation (Corrigan et al., 1988). Insight into the mechanism underlying allergic inflammation was gained in subsequent studies that profiled protein expression in allergen-reactive T cell clones from the peripheral blood of asthmatics (Wierenga et al., 1990) and mRNA expression in T cells from the lungs of asthmatics (Robinson et al., 1992). Both studies characterized the cytokine expression as consistent with a T helper type 2 (Th2; Mosmann et al., 1986) pattern. Combined with prior observations that patients suffering from allergic inflammation often exhibited allergen-specific IgE antibody responses (atopy) and a pattern of granulocyte recruitment and activation that included mast cells, basophils, and eosinophils, the data implicating Th2 cells suggested that allergic inflammation is a programmed response driven by specific T cell cytokines (Locksley, 2010).

Parallel studies of parasitic helminth infection in humans and animal models revealed a strikingly similar host response (Urban et al., 1991). In particular, Th2-driven B cell production of IgE engages mast cell and basophil activation (Reinhardt et al., 2009), while Th2 expression of interleukin (IL)-13 drives epithelial and stromal cell differentiation in tissues, to generate the pruritis, smooth muscle contractility, and mucus

production that are thought to facilitate helminth expulsion (Liang et al., 2011). Indeed, the observation that incidence of helminth infection is inversely correlated with that of allergic inflammation in many parts of the world suggests that adaptations to co-evolution with helminths (and other macro-parasites) have become pathogenic in modern human environments (Bell, 1996). Thus, studies of helminth infection can inform our understanding of how Th2-driven inflammation is initiated in human allergic disease.

A Polysaccharide that Induces Allergic Inflammation

With this approach in mind, we focused on novel proteins acutely induced in the lungs of mice following infection with the soil-dwelling helminth *Nippostrongylus brasiliensis* (Reese et al., 2007). We identified three that bind chitin (Reese et al., 2007), a polysaccharide of *N*-acetyl-β-D-glucosamine that provides structural support in bacteria, fungi, insects, crustaceans, and helminths (Funkhouser and Aronson, 2007). These phyla express conserved enzymes for chitin synthesis and degradation (Funkhouser and Aronson, 2007). Chitin has no known roles in vertebrate biology, but chitin degrading enzymes, chitinases, have been retained in vertebrate genomes, suggesting that these enzymes play a role in digestion and defense (Bussink et al., 2007). Surprisingly, we observed that administration of chitin to the lungs of mice induced acute accumulation of eosinophils and basophils and alternative activation of macrophages, reminiscent of the response to helminth infection and allergens (Reese et al., 2007). Further, the induced expression of a specific chitinase, acidic mammalian chitinase (AMCase), in response to administration was shown to be necessary and sufficient for chitin degradation, which resolved inflammation (Reese et al., 2007). We observed a

similar relationship between induction of lung AMCase expression and resolution of inflammation in response to the fungus *Aspergillus niger* (Van Dyken et al., 2011).

Although the expression of chitin-degrading enzymes may be relevant to host defense against chitin-containing macro-parasites (Choi et al., 2001), these data also suggest that chitin may be a recognition element, or pathogen-associated molecular pattern, for the immune system and a relevant model allergen. In at least some environments, lipopolysaccharide load is inversely correlated with chitin load (Van Dyken et al., 2011), potentially due to competition between bacteria and fungi within the same niche (Locksley, 2010). By extension, chitin may be more prevalent in the cleaner, more developed, and more urban environments associated with allergic inflammation. Indeed, chitin is a common constituent of house dust (Van Dyken et al., 2011) and may have a role in the high incidence of new onset asthma among workers in the crab and oyster processing industries (Wada et al., 1967). Further, a protective association has been appreciated between some AMCase polymorphisms and the development of asthma in humans (Seibold et al., 2009). Thus, elucidation of chitin recognition and the acute tissue response to chitin may have important implications for human allergic inflammation.

A Novel Cell that Regulates Allergic Inflammation

Using the same model of intranasal chitin administration to the lungs of mice, we defined cells and molecules required for the reactive accumulation of eosinophils and alternatively activated macrophages. Eosinophil influx was dependent on the Th2-associated cytokine IL-5 and partially dependent on IL-13, whereas alternative activation

was independent of IL-5 but dependent on IL-13 (Van Dyken et al., 2014). Strikingly, neither event required another Th2-associated cytokine, IL-4. Further, known cellular sources of IL-5 and IL-13 did not appear to be involved, including basophils (unpublished data), mast cells, and T and B cells (Reese et al., 2007). This paradox was resolved with the discovery of a novel cell, the group 2 innate lymphoid cell (ILC2). First identified in the intestine (Moro et al., 2010; Neill et al., 2010), these cells have now been observed at other mucosal sites, including the lung (Price et al., 2010; Halim et al., 2012) and skin (Kim et al., 2013). Like other recently characterized innate lymphoid populations, they are dependent on the transcription factor ID2 and the common γ chain receptor, IL2RG, and have general lymphoid morphology (Spits et al., 2013). Further, these cells all lack recombination-activating gene (RAG)-dependent antigen receptors and markers associated with the myeloid and dendritic blood lineages (Spits et al., 2013). Specifically, ILC2s, like their Th2 counterparts, express GATA3, which confers the ability to produce IL-5 and IL-13 (Liang et al., 2011) and is required for their development (Klein Wolterink et al., 2013; Mjosberg et al., 2012). Using *Il5*$^{red5/red5}$ (Nussbaum et al., 2013) and *Il13*$^{Smart13/Smart13}$ (Liang et al., 2011) reporter mice to track cells expressing these cytokines, we established that ILC2s are the major source of IL-5 and IL-13 following chitin administration (Van Dyken et al., 2014).

The IL-1 family member IL-33, the IL-17 family member IL-25, and the IL-7-like cytokine thymic stromal lymphopoietin (TSLP) have all been implicated as signals that activate ILC2s to produce cytokine. Originally identified as a novel IL-1-related protein *in silico*, IL-33 binds a heterodimer of IL1RL1 and IL1RAP and induces significant expression of IL-5 and IL-13 when administered to mice (Schmitz et al., 2005). Although

polarized Th2 cells respond to IL-33 (Schmitz et al., 2005), the cytokine was linked to ILC2s when they were shown to express IL1RL1 and when RAG-deficient mice expressed IL-5 and IL-13 following IL-33 administration (Moro et al., 2010). IL-25 was similarly identified in a computational search for genes homologous to IL-17A (Lee et al., 2001; Fort et al., 2001) and binds a heterodimer of IL17RA and IL17RB (Lee et al., 2001; Rickel et al., 2008). Transgenic expression (Pan et al., 2001) and exogenous administration (Fort et al., 2001) of IL-25 both resulted in elevated IL-5 and IL-13. In the latter experiment, cytokine expression was attributed to a lineage-negative cell that persisted in RAG-deficient mice (Fort et al., 2001), and this cell was later confirmed to be the same ILC2 that produces IL-5 and IL-13 in response to IL-33 (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). TSLP was discovered in an expression library screen for factors supporting B cell development *in vitro* (Sims et al., 2000), but the expression pattern of its heterodimeric receptor complex, consisting of TSLPR and IL7RA (Pandey et al., 2000; Park et al., 2000), suggested that it has effects on both myeloid and lymphoid cells (Reche et al., 2001). In particular, human dendritic cells primed with TSLP skewed T cells to a Th2 phenotype *in vitro* (Soumelis et al., 2002), and transgenic expression of TSLP in mouse lungs resulted in asthma-like pathology associated with IL-5 and IL-13 (Zhou et al., 2005). ILC2s were later shown to respond to TSLP and express the receptor complex (Halim et al., 2012; Mjosberg et al., 2012). Consistent with a role for each of these cytokines in ILC2 activation, we demonstrated that IL-33, IL-25, and TSLP signaling all had non-redundant contributions to chitin-induced eosinophil accumulation and alternative activation of macrophages (Van Dyken et al., 2014).

Resident Cells in Lung Surveillance

The ability of ILC2s to activate in the absence of antigen suggests that these cells might act as sentinels at mucosal surfaces, ready to express Th2-associated cytokines acutely following epithelial perturbation. Indeed, we have observed that ILC2s populate the mouse lung approximately 1 week after birth and exhibit little turnover (Nussbaum et al., 2013). Their distribution within the lung, however, appears restricted to collagen-rich regions near medium-sized airways and blood vessels (Ikutani et al., 2012; Nussbaum et al., 2013). Thus, surveillance of distal alveoli likely requires additional cells and signaling molecules. Given the roles ascribed to IL-33, IL-25, and TSLP in inducing IL-5 and IL-13 expression in response to chitin (Van Dyken et al., 2014) and helminth infection (Taylor et al., 2009; Neill et al., 2010), these cytokines are the putative molecules that signal lung epithelial perturbation. Significant questions remain, however, regarding the cellular sources of these cytokines, their regulation, and how they engage ILC2 activation. Chapter 2 of this thesis provides data identifying alveolar type II (ATII) cells as the source of IL-33 and TSLP and a mechanism for how these cytokines cooperatively activate ILC2s in two distinct models of lung perturbation.

Chapter 2:

Group 2 innate lymphoid cells coordinate epithelial maintenance of lung homeostasis

Abstract

Group 2 innate lymphoid cells (ILC2s) play an important role in acute allergic lung inflammation. Given their number, distribution and function, lung ILC2s likely coordinate responses with resident epithelial cells that monitor barrier interactions with the environment. Here, we demonstrate that alveolar type II (ATII) cells are the main source of interleukin (IL)-33 and thymic stromal lymphopoietin (TSLP) generated in response to chitin or migratory helminth-induced challenge. IL-33 and TSLP synergistically activate ILC2s to produce IL-5, IL-13 and IL-9, and autocrine IL-9 promotes an ILC2 transcriptional program associated with rapid and robust cytokine production necessary to optimize epithelial gene responses in the conducting airways. Thus, ILC2s link a specialized, distal, epithelial population involved in alveolar function with more proximal epithelia that regulate airway flow, revealing a key role for ILC2s in tissue homeostasis that might underlie similar organizational hierarchies for innate lymphoid cells in other organs.

Introduction

Chitin is a polymer of *N*-acetylglucosamine that is abundant in the environment as a structural constituent of fungi, helminths and arthropods (Bussink et al., 2007). When administered to the lungs of mice, chitin induces an innate inflammatory response characterized by the rapid accumulation of eosinophils and alternatively activated macrophages around impacted beads (Reese et al., 2007). This cellular influx is dependent on activation of interleukin (IL)-5 and IL-13 from resident lung group 2 innate lymphoid cells (ILC2s; Van Dyken et al., 2014). Like other innate lymphoid cells, ILC2s

are dispersed widely in the body tissues of mice and humans, especially at the mucosal barriers of the lung, skin, nasopharynx and bowel (Moro et al., 2010; Neill et al., 2010; Price et al., 2010; Mjosberg et al., 2011; Halim et al., 2012; Kim et al., 2013). This localization suggests a sentinel function to initiate acute responses at sites of epithelial perturbation prior to T cell help. Given the relatively low numbers of tissue ILC2s and, in lung, their spatially clustered organization (Ikutani et al., 2012; Nussbaum et al., 2013), these cells likely integrate signals from other resident cells to maintain tissue homeostasis, but the nature of such interactions remains unknown.

In an earlier study, we detected acute production of the IL-1 family member IL-33 and the IL-7-like cytokine thymic stromal lymphopoietin (TSLP) in lung tissues after chitin challenge; the IL-17 family member IL-25 was induced transiently in bronchoalveolar lavage (BAL) fluid but not in tissue (Van Dyken et al., 2014). Roles for these cytokines in ILC2-dependent inflammation after chitin were revealed genetically (Van Dyken et al., 2014), but the cellular sources of these signals and how ILC2s integrate them remain unclear. Here, we combine immunohistochemistry and cell fractionation experiments to localize acute IL-33 and TSLP after chitin challenge to alveolar type II (ATII) cells, establishing that these signals are spatially and temporally co-regulated. Lung ILC2s are activated synergistically by IL-33 and TSLP to generate effector cytokines, including IL-5, IL-13 and IL-9. In turn, autocrine IL-9 is required for maximal ILC2 IL-5 and IL-13 production, revealing a cell-intrinsic amplification loop for optimal ILC2 activation and subsequent epithelial production of proteins required for tissue defense and repair. These studies link ILC2 activation directly to signals released by specialized epithelial cells responsible for monitoring alveolar integrity and reveal

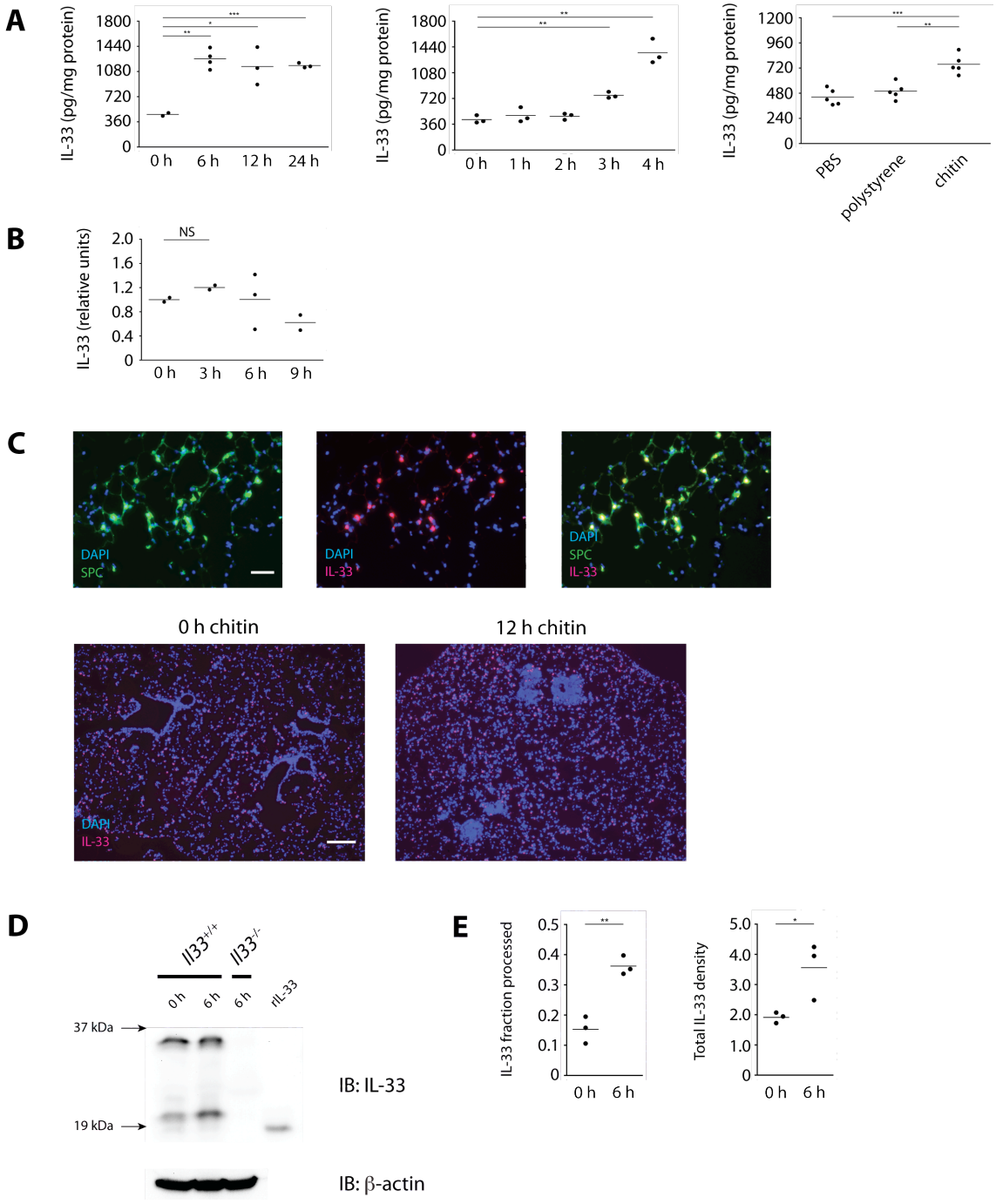
layers of synergy that regulate the rapid response to loss of gas exchange functionality. This model might underpin the role of ILCs in other tissues, where their small numbers and restricted localization may reflect similar organizational hierarchies.

Results

IL-33 is constitutively expressed by ATII cells and processed IL-33 appears in response to chitin

We previously reported increased IL-33 protein in whole lung homogenates from BALB/c mice 6-12 hours after chitin challenge (Van Dyken et al., 2014). C57BL/6 mice developed similar increases in IL-33 after 3-4 hours that remained elevated at 24 hours. IL-33 was not elevated in mice given PBS or polystyrene beads (Figure 2.1A) and was not detected in BAL fluid (data not shown). Compared with the lungs of unchallenged mice, chitin affected neither *Il33* transcription (Figure 2.1B) nor the intensity or localization of IL-33 in the nuclei of lung cells positive for the ATII cell marker surfactant protein C (SPC; Figure 2.1C), suggesting that the increase in IL-33 likely derived post-transcriptionally from a constitutive protein pool. To address this possibility, we assessed IL-33 in lung homogenates from naïve and chitin-challenged mice by western blot. A ~35-kDa band, corresponding to full length IL-33, was present in all homogenates, together with a ~20-kDa band, corresponding to a C-terminal fragment, that was enriched following chitin (Figures 2.1D and 2.1E). Neither band was detected in homogenates from *Il33*$^{-/-}$ mice (Figure 2.1D). Thus, ATII cells comprise the major source of IL-33 in the resting mouse lung and increasing amounts of processed IL-33 appear after chitin challenge.

Figure 2.1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IL-33 (pg/mg protein)
1800
1440
1080
720
360
0
0 h
6 h
12 h
24 h
IL-33 (pg/mg protein)
1800
1440
1080
720
360
0
0 h
1 h
2 h
3 h
4 h
IL-33 (pg/mg protein)
1200
960
720
480
240
0
PBS
polystyrene
chitin
B
IL-33 (relative units)
2.0
1.6
1.2
0.8
0.4
0
NS
0 h
3 h
6 h
9 h
C
DAPI
SPC
DAPI
IL-33
DAPI
SPC
IL-33
0 h chitin
12 h chitin
DAPI
IL-33
D
Il33+/+
Il33-/-
0 h
6 h
6 h
rIL-33
37 kDa
19 kDa
IB: IL-33
IB: β-actin
E
IL-33 fraction processed
0.5
0.4
0.3
0.2
0.1
0
0 h
6 h
Total IL-33 density
5.0
4.0
3.0
2.0
1.0
0
0 h
6 h

Figure 2.1: IL-33 from ATII cells is processed in response to chitin.

(A) ELISA of IL-33 from whole lung homogenates of mice at indicated times after chitin administration (left, middle) and 6 h after the indicated challenge (right). (B) Quantitative RT-PCR analysis of *Il33* expression from whole lung RNA of mice at indicated times after chitin. Values normalized to *18s* expression and graphed as fold increase over the mean of the 0 h mice. NS, not significant (two-tailed *t*-test). (C) Immunohistochemistry of naive mouse lungs (top row) and lungs from mice following chitin (bottom row). Original magnification, x200 (top) and x50 (bottom). Scale bar, 50 μm (top) and 200 μm (bottom). (D) Western blot analysis of whole lung homogenates of mice at indicated times after chitin. "rIL-33" is recombinant protein (Ser109-Ile266; R&D Systems). (E) Analysis of 20-kDa anti-IL-33-reactive band pixel density over total IL-33 pixel density (sum of 20-kDa band and 35-kDa band densities; left). Total IL-33 pixel density (right). Where applicable: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test). Data are representative of at least 3 independent experiments with 2 to 5 mice per exposure (**A**) or 2 independent experiments with 2 mice per exposure (**B**-**D**). Densitometry in **E** is pooled from 3 westerns of similarly treated mice.

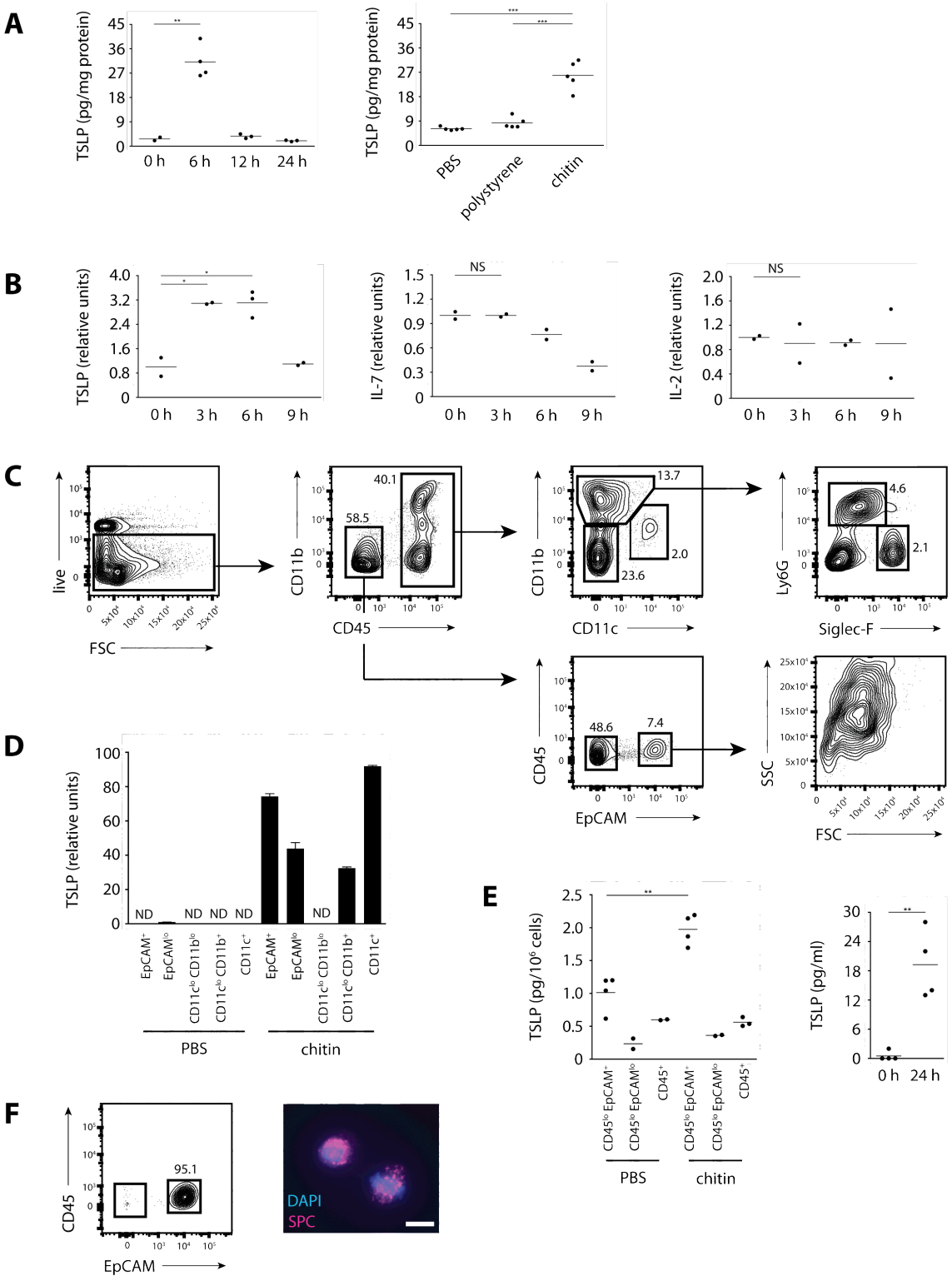
TSLP is produced by ATII cells in response to chitin

We also previously detected increased TSLP in whole lung homogenates of BALB/c mice 6 hours after chitin challenge (Van Dyken et al., 2014). Similarly, in C57BL/6 mice, we observed acute but transient increases in TSLP protein and mRNA 3-6 hours after chitin (Figures 2.2A and 2.2B). TSLP protein did not increase in the lungs of mice given PBS or polystyrene (Figure 2.2A), nor was it detected in BAL (data not shown). In contrast to *Tslp*, transcripts for *Il7* and *Il2* were not increased in response to chitin (Figure 2.2B). We could not detect TSLP in the lung using immunohistochemical methods (data not shown). As an alternative approach, we sorted lung hematopoietic and epithelial populations from resting and chitin-challenged mice and measured *Tslp* transcripts (Figure 2.2C and Figure 2.3). Chitin-induced *Tslp* transcripts were present in $CD45^{lo}$ ($EpCAM^{+}$ or $EpCAM^{lo}$) epithelial cells and in two populations of $CD45^{+}$ cells ($CD11c^{lo}$ $CD11b^{+}$ and $CD11c^{+}$ alveolar macrophages) but absent in $CD45^{+}$ $CD11c^{lo}$ $CD11b^{lo}$ cells (Figure 2.2D). We analyzed the sorted cell populations by ELISA but could detect TSLP protein only in $CD45^{lo}$ $EpCAM^{+}$ cells isolated from chitin-challenged mice (Figure 2.2E). Post-sort analysis and cytospin of these cells revealed highly granular cells that were positive for SPC, consistent with their identification as ATII cells (Figure 2.2F; Fehrenbach, 2001). In support of this, SPC^{+} MLE12 lung epithelial cells (Wikenheiser et al., 1993) also expressed TSLP after stimulation (Figure 2.2E). Thus, ATII cells co-express IL-33 and TSLP rapidly after chitin challenge.

TSLP and IL-33 cooperatively activate ILC2s *in vivo*

The temporal and spatial co-expression of IL-33 and TSLP in response to chitin

Figure 2.2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6 h
12 h
24 h
PBS
polystyrene
chitin
B
TSLP (relative units)
IL-7 (relative units)
IL-2 (relative units)
NS
0 h
3 h
6 h
9 h
C
live
FSC
CD11b
CD45
40.1
58.5
CD11c
13.7
2.0
23.6
Ly6G
Siglec-F
4.6
2.1
EpCAM
48.6
7.4
SSC
D
TSLP (relative units)
ND
EpCAM+
EpCAMlo
CD11clo CD11blo
CD11clo CD11b+
CD11c+
CD11clo CD11b+
PBS
chitin
E
TSLP (pg/10⁶ cells)
CD45lo EpCAM+
CD45lo EpCAMlo
CD45+
CD45lo EpCAMlo
TSLP (pg/ml)
F
95.1
DAPI
SPC

Figure 2.2: TSLP is produced by ATII cells in response to chitin.

(A) ELISA of TSLP from whole lung homogenates of mice at indicated times after chitin (left) and 6 h after the indicated challenge (right). (B) Quantitative RT-PCR analysis of whole lung RNA from mice given chitin, normalized to *18s* expression and presented as fold increase over the mean of the 0 h mice. (C) Sorting scheme for isolation of lung hematopoietic and epithelial populations. Numbers near gates represent percentage of all live ($DAPI^{lo}$) cells. (D) Quantitative RT-PCR analysis of *Tslp* expression using TaqMan probes in lung populations sorted from mice 3 h after PBS or chitin. Values normalized to *18s* expression and graphed in arbitrary units. ND, not detected after 40 cycles of amplification. (E) ELISA of TSLP in lung populations sorted from mice 3 h after PBS or chitin (left). ELISA of TSLP in supernatants from MLE12 cells cultured with 40 ng/ml phorbol 12-myristate 13-acetate for the indicated times (right). (F) Flow cytometry (left) and immunocytochemistry (right) of post-sort ATII cells ($DAPI^{lo}$ $CD45^{lo}$ $CD11b^{lo}$ $CD31^{lo}$ $EpCAM^{+}$) isolated from lungs of mice 3 h after chitin. Original magnification, x200. Scale bar, 10 μm. Where applicable: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test). Data are representative of at least 3 independent experiments with 2-5 mice per exposure (**A** and **C**-**E**) or at least 2 independent experiments with 2 mice per exposure (**B**). Data in **F** are compiled from 4 independent experiments. Bars in **E** represent mean + standard deviation.

Figure 2.3

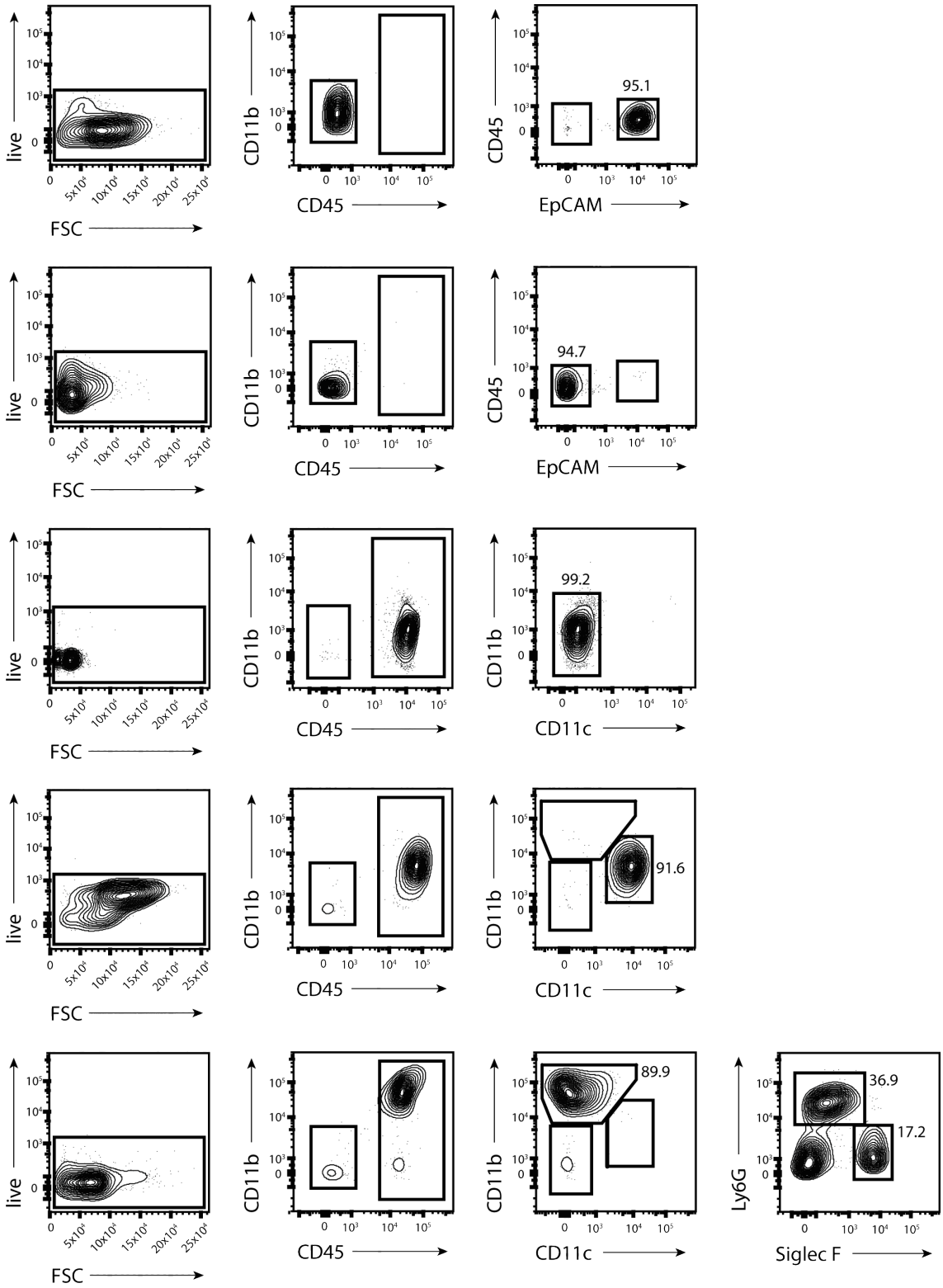

live
FSC
CD11b
CD45
CD45
EpCAM
95.1
94.7
CD11c
99.2
91.6
89.9
Ly6G
Siglec F
36.9
17.2

Figure 2.3: Purity of sorted lung hematopoietic and epithelial populations. Flow cytometry of post-sort samples. Numbers near gates represent percentage of all cells analyzed. Data are representative of at least 3 independent sorts.

suggested these cytokines might function cooperatively. We modeled this by intranasally administering cytokine and analyzing the lung infiltrate after 12 hours. Although TSLP or IL-33 elicited little eosinophil influx when administered alone, a combination of both cytokines resulted in significantly increased eosinophil accumulation (Figures 2.4A and 2.4D). The combination of cytokines also elicited increased surface CD25 expression on ILC2s, consistent with their activation (Figure 2.4B). To establish whether this effect on ILC2s could induce IL-5, we administered IL-33 and TSLP to the lungs of mice homozygous for the *Il5*^{red5} reporter allele, in which a sequence encoding tdTomato is present at the *Il5* start site (Nussbaum et al., 2013). We observed modest increases in IL-5 expression by ILC2s after IL-33 or TSLP alone but a significant increase with the combination (Figure 2.4C). Thus, IL-33 and TSLP function cooperatively *in vivo* to activate and induce cytokine production from ILC2s and to promote lung eosinophil accumulation.

TSLP synergizes with IL-33 to stimulate ILC2s

To investigate the interaction of IL-33 and TSLP in a cell-intrinsic manner, we purified lung ILC2s from resting mice and measured cytokine release into the supernatant after 3 days of culture (Figure 2.5). Although IL-33 (10 ng/ml) induced barely detectable amounts of IL-5, IL-13 and IL-9, the addition of 0.1 ng/ml TSLP significantly increased production; no cytokines were detected when ILC2s were cultured with TSLP alone, even at 100-fold higher concentrations (Figure 2.6A). TSLP, IL-2 and IL-7 each synergized comparably with IL-33 to induce ILC2 cytokines (Figure 2.6B). Responses to IL-33 and TSLP were detected at 16 hours by mRNA and protein, but were absent in

Figure 2.4

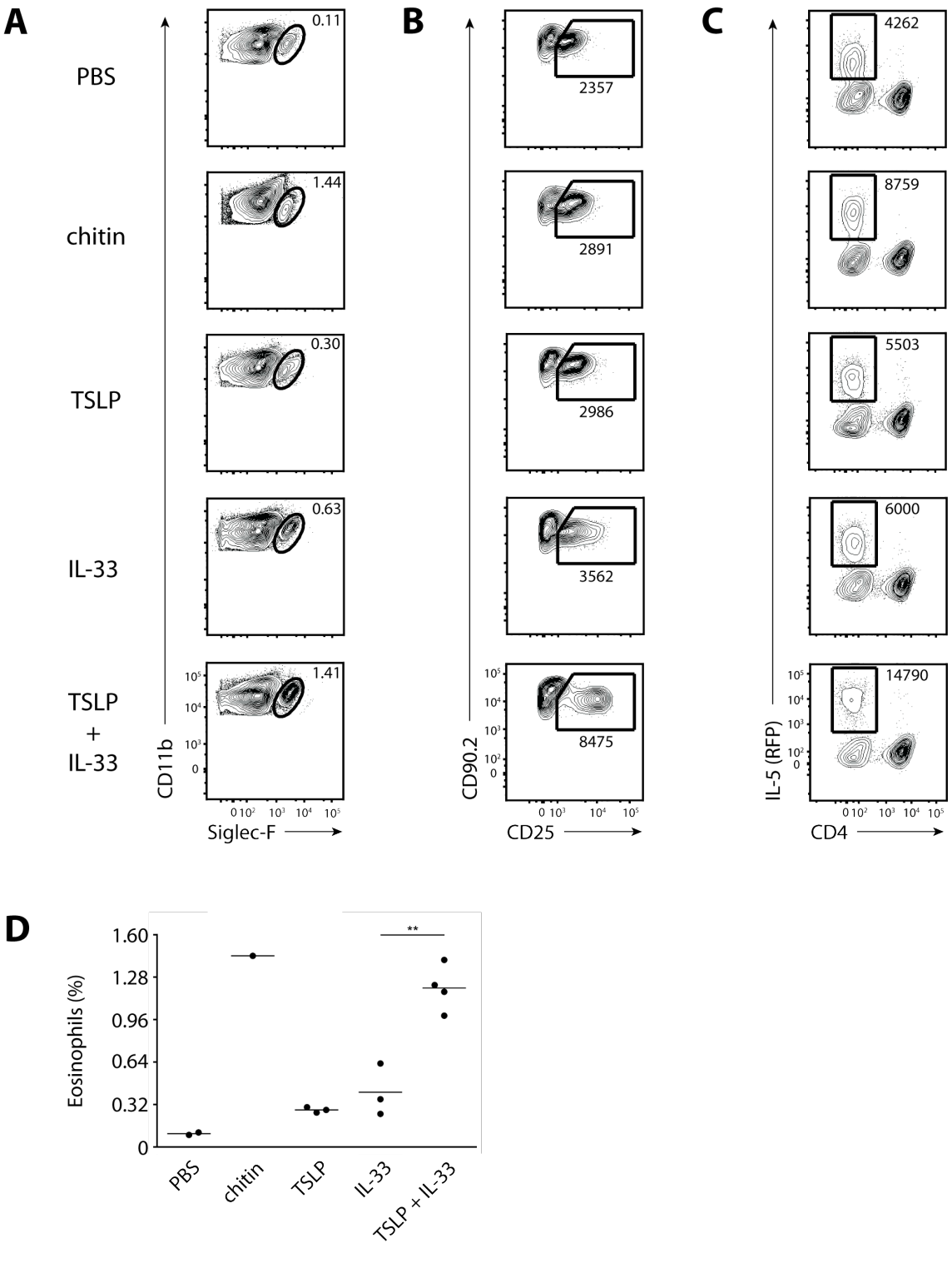

A
B
C
PBS
chitin
TSLP
IL-33
TSLP + IL-33
0.11
1.44
0.30
0.63
1.41
2357
2891
2986
3562
8475
4262
8759
5503
6000
14790
CD11b
Siglec-F
CD90.2
CD25
IL-5 (RFP)
CD4
D
Eosinophils (%)
1.60
1.28
0.96
0.64
0.32
0
**
PBS
chitin
TSLP
IL-33
TSLP + IL-33

Figure 2.4: TSLP and IL-33 cooperatively activate ILC2s *in vivo*.

Mice were analyzed 12 h after administration of PBS, chitin, 1 mg rTSLP, 50 ng rIL-33, or rTSLP + rIL-33. (**A**-**B**) Flow cytometry of eosinophils (**A**; pre-gate: $DAPI^{lo}$ $CD11c^{lo}$; left panels) and ILC2s (**B**; pre-gate: $DAPI^{lo}$ $CD11c^{lo}$ $CD11b^{lo}$ $thy1.2^{+}$ $CD8^{lo}$ $B220^{lo}$ $NK1.1^{lo}$ $CD4^{lo}$) from the lungs of wildtype mice. (C) Flow cytometry of ILC2s, gated as in **B**, from $Il5^{red5/red5}$ mice. Numbers near gates represent percentage of live cells ($DAPI^{lo}$ $CD11c^{lo}$ $CD11b^{+}$ SSC^{hi} Siglec-F^{+} $a4b7^{lo}$; **A**) or mean fluorescence intensity (**B** and **C**). (D) Eosinophils, gated as in **A**, graphed as a percentage of live cells. ** $P < 0.005$ (two-tailed *t*-test). Data are representative of 2 independent experiments with 2-4 mice per exposure.

ILC2s lacking receptors for IL-33 (IL1RL1) and TSLP (TSLPR; data not shown). Production of IL-5 and IL-13 in ILC2s requires GATA3 (Liang et al., 2011), and IL-9 production in helper T cells requires IRF4 (Staudt et al., 2010). Both of these proteins accumulated in ILC2s cultured with IL-33 and TSLP in an IL1RL1- or TSLPR-dependent manner (Figure 2.6C). Thus, IL-33 and TSLP synergistically induce transcriptional reprogramming in lung ILC2s associated with effector cytokine production.

ILC2-derived IL-9 amplifies cell-intrinsic cytokine production *in vitro*

ILC2s constitutively express high levels of the IL-9 receptor, IL9R (Price et al., 2010; Wilhelm et al., 2011), suggesting that autocrine signaling might explain the marked synergy of IL-33 and TSLP in ILC2 activation. To investigate this, we generated IL-9-enhanced yellow fluorescent protein reporter (9er) mice, in which the endogenous *Il9* translation initiation site is replaced with yellow fluorescent protein (YFP) sequence linked by an internal ribosome entry site to a sequence encoding optimized Cre recombinase (Figure 2.7A). When activated under conditions that promote IL-9 expression, naïve $CD4^+$ T cells from $Il9^{9er/9er}$ mice express YFP instead of IL-9, confirming that $Il9^{9er/9er}$ mice are IL-9-deficient (Figure 2.8). To test whether IL-9 deficiency has cell-intrinsic effects, we purified lung ILC2s from $Il9^{+/+}$ and $Il9^{9er/9er}$ mice and cultured them with IL-33 and TSLP for 3 days. $Il9^{9er/9er}$ ILC2s produced no IL-9 and substantially less IL-5 and IL-13 than did wildtype cells, and this deficit was restored by addition of exogenous IL-9 (Figure 2.7B). We observed no differences in viability between wildtype and IL-9-deficient ILC2s under these conditions (Figure 2.7C). Reduced effector cytokine production correlated with decreased expression of

Figure 2.5

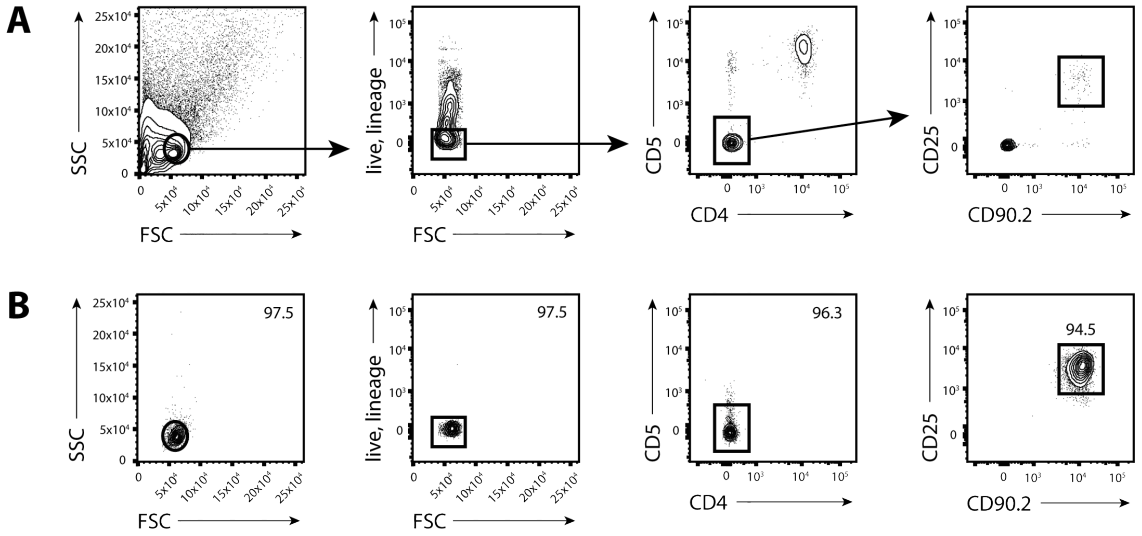
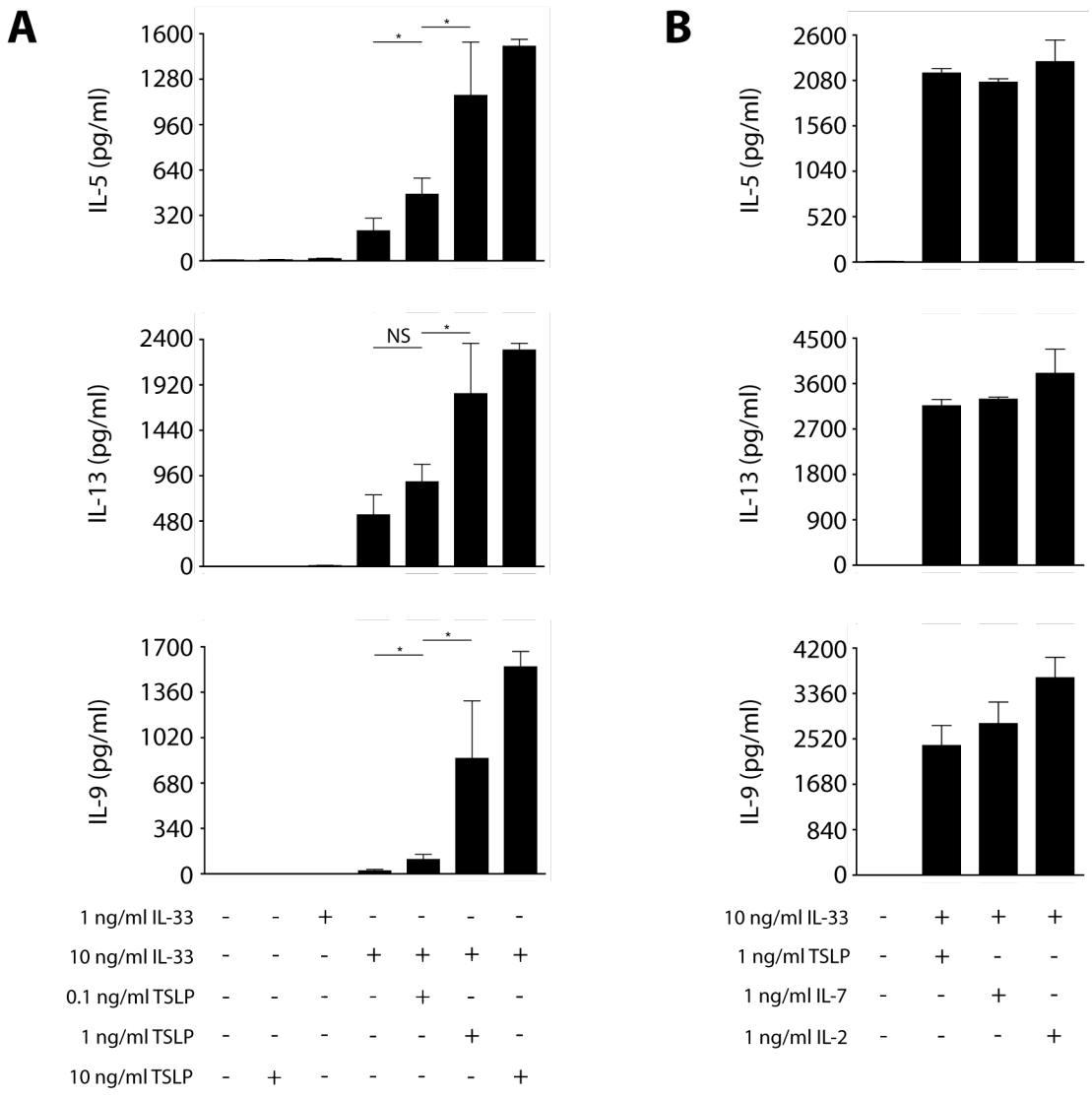


Figure 2.5: Sorting scheme and purity of sorted lung ILC2s.

(A) Sorting scheme. "Lineage" dump included CD8, CD11b, CD11c, CD19, CD49b, F4/80, Gr-1, and NK1.1. (B) Flow cytometry of post-sort ILC2s. Numbers near gates represent percentage of all cells analyzed. Data are representative of at least 3 independent sorts.

Figure 2.6


A
IL-5 (pg/ml)
1600
1280
960
640
320
0
*
*
IL-13 (pg/ml)
2400
1920
1440
960
480
0
NS
*
IL-9 (pg/ml)
1700
1360
1020
680
340
0
*
*
1 ng/ml IL-33 - - + - - - -
10 ng/ml IL-33 - - - + + + +
0.1 ng/ml TSLP - - - - + - -
1 ng/ml TSLP - - - - - + -
10 ng/ml TSLP - + - - - - +
B
IL-5 (pg/ml)
2600
2080
1560
1040
520
0
IL-13 (pg/ml)
4500
3600
2700
1800
900
0
IL-9 (pg/ml)
4200
3360
2520
1680
840
0
10 ng/ml IL-33 - + + +
1 ng/ml TSLP - + - -
1 ng/ml IL-7 - - + -
1 ng/ml IL-2 - - - +

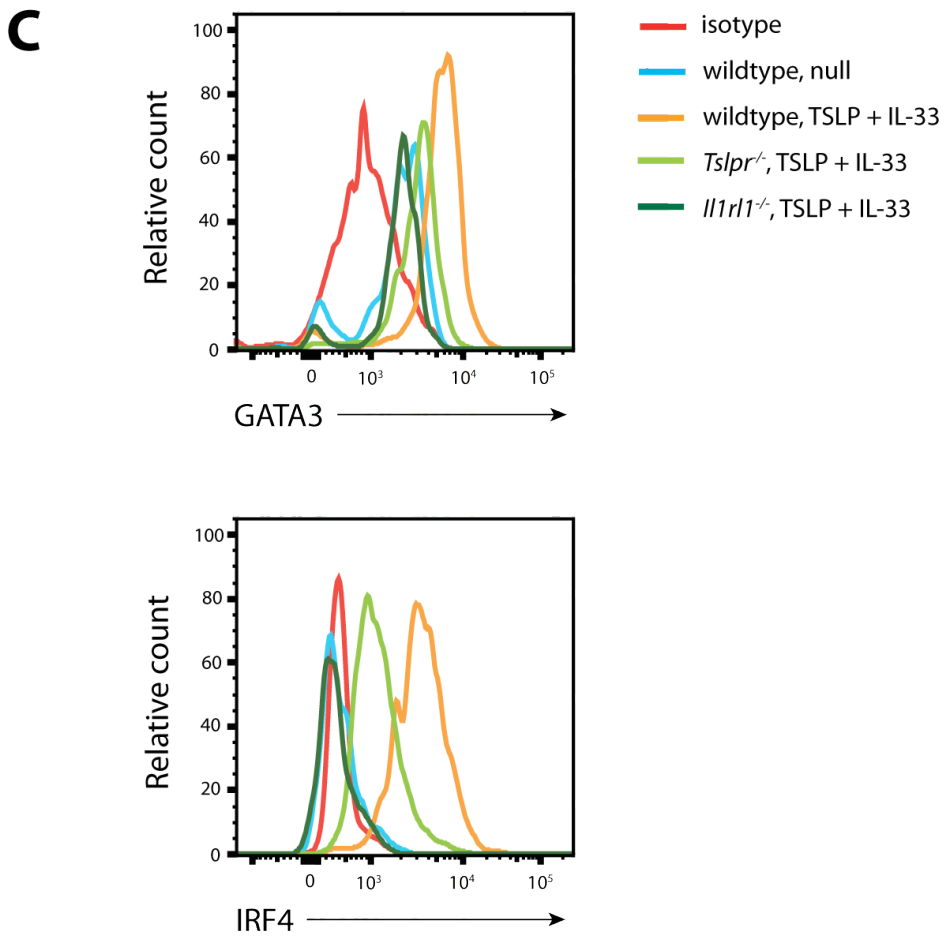


Figure 2.6: TSLP synergizes with IL-33 to stimulate ILC2s.

Lung ILC2s were sorted from wildtype or mutant mice and cultured. (**A-B**) Cytometric bead array (CBA) analysis of IL-5, IL-13 and IL-9 in supernatants after stimulation with IL-33 and/or TSLP (**A**) or with IL-33 and either TSLP, IL-7, or IL-2 (**B**) for 3 days. (C) Flow cytometry of ILC2s stimulated with 10 ng/ml cytokine, as indicated, for expression of GATA3 and IRF4. Where applicable: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test). Data are representative of at least 3 independent sorts, with cells cultured in duplicate or triplicate wells (**A-C**). Bars in **A** and **B** represent mean + standard deviation.

intracellular GATA3 and IRF4 by IL-9-deficient ILC2s, and this was also rescued by the addition of exogenous IL-9 (Figure 2.7D). Thus, ILC2-derived IL-9 signals in an autocrine manner to promote expression of transcription factors necessary to optimize IL-5 and IL-13 production induced by IL-33 and TSLP.

Early IL-9 augments rapid ILC2-directed epithelial responses *in vivo*

Our results demonstrating that ATII cells rapidly produce TSLP and IL-33 after chitin, together with our observation that these cytokines synergize to induce IL-9 in ILC2s, raised the possibility that chitin induces early IL-9 important for rapid, maximal effector cytokine production. Indeed, we observed expression of *Il9* transcripts in the lungs of mice 1 day after chitin that was not explained by any change in the number of lung ILC2s (Figure 2.7E). Moreover, IL-9-deficient mice had reduced induction of *Il13* transcripts as compared to wildtype mice, although induction of *Il5* was not affected under these conditions (Figure 2.7F).

We turned to a second model of lung ILC2 activation to explore the generalized nature of these findings. Hours after subcutaneous inoculation, the helminth *N. brasiliensis* reaches the mouse lung and molts before rupturing into the alveolar sacs and ascending the trachea to complete its migration to the small intestine. Epithelial injury induced during this period is associated with chitin turnover during synthesis of the pharynx (Gause et al., 2003; Zhang et al., 2005). *N. brasiliensis* infection caused the appearance of TSLP and IL-33 in BAL fluid 1 day after infection; both cytokines returned to near baseline by 4 days (Figure 2.9A). Western blot for IL-33 in lung homogenates from infected mice revealed accumulation of a ~20-kDa band, consistent

Figure 2.7

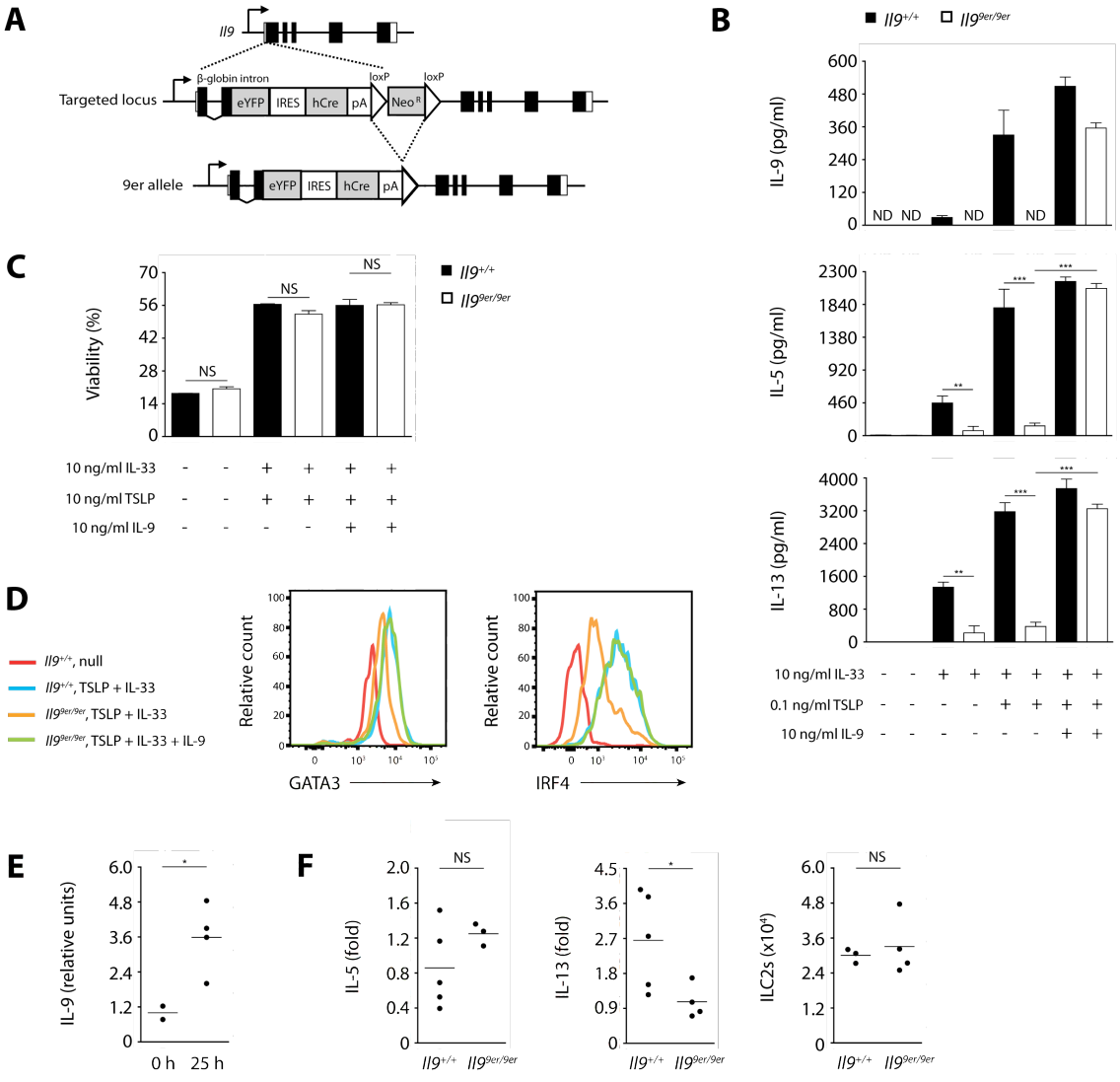

A
Il9
β-globin intron
loxP
loxP
Targeted locus
eYFP
IRES
hCre
pA
Neo^R
9er allele
B
Il9+/+
Il9 9er/9er
IL-9 (pg/ml)
ND ND ND ND
IL-5 (pg/ml)
IL-13 (pg/ml)
10 ng/ml IL-33
0.1 ng/ml TSLP
10 ng/ml IL-9
C
Viability (%)
NS
10 ng/ml IL-33
10 ng/ml TSLP
10 ng/ml IL-9
D
Il9+/+, null
Il9+/+, TSLP + IL-33
Il9 9er/9er, TSLP + IL-33
Il9 9er/9er, TSLP + IL-33 + IL-9
Relative count
GATA3
IRF4
E
IL-9 (relative units)
0 h
25 h
F
IL-5 (fold)
IL-13 (fold)
ILC2s (x10⁴)
NS

Figure 2.7: ILC2-derived IL-9 amplifies cell-intrinsic cytokine production *in vitro* and after chitin.

(A) Gene targeting schematic for generation of the $Il9^{9er}$ allele. (**B-D**) Lung ILC2s were sorted from $Il9^{+/+}$ or $Il9^{9er/9er}$ mice and cultured for 3 days. (B) CBA analysis of IL-9, IL-5 and IL-13 in culture supernatants from cells stimulated as indicated. (C) Viability of cells stimulated as indicated, as assessed by LiveDead exclusion at the end of culture. (D) Flow cytometry of ILC2s stimulated as in **C** for expression of GATA3 and IRF4. (E) Quantitative RT-PCR analysis of whole lung RNA from mice at the indicated times after chitin, normalized to *18s* expression and presented as fold increase over the mean of the 0 h mice. (F) Quantitative RT-PCR analysis of *Il5* (left) and *Il13* (middle) expression and flow cytometric analysis of ILC2s ($DAPI^{lo}$ $CD11c^{lo}$ $CD11b^{lo}$ $CD8^{lo}$ $B220^{lo}$ $thy1.2^{+}$ $CD5^{lo}$ $CD4^{lo}$ $CD45^{+}$ $CD25^{+}$; right) in the lungs of $Il9^{+/+}$ or $Il9^{9er/9er}$ mice 25 h after chitin. RT-PCR values are normalized to *18s* expression and presented as fold increase over unexposed mice (not shown). Where applicable: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test). Data are representative of 2 independent sorts, with cells cultured in duplicate or triplicate wells (**B-D**). Data in **E** are compiled from 2 independent experiments; in **F**, representative of 2 experiments with 3-5 mice per exposure. Bars in **B** and **C** represent mean + standard deviation.

Figure 2.8

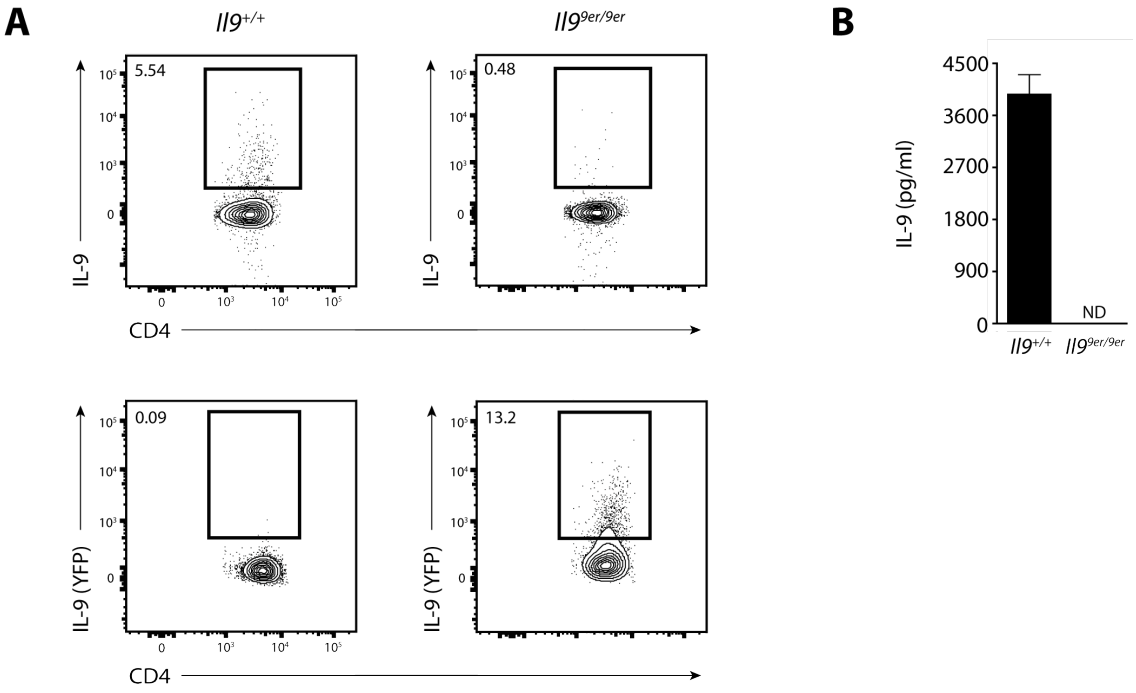


Figure 2.8: Validation of *Il9^{9er}* allele.

Naïve CD4+ T cells from *Il9$^{+/+}$* and *Il9$^{9er/9er}$* mice were sorted and cultured with anti-CD3 and anti-CD28 antibodies and IL-2, IL-4, and TGFβ ("Th9" polarizing conditions) for 4 days. (A) Flow cytometric analysis of IL-9 expression by intracellular cytokine staining (top panels) and by reporter fluorescence (bottom). (B) CBA analysis of culture supernatants. Data are representative of 2 independent sorts. Bars represent mean + standard deviation.

with processing (Figure 2.9B). Concordant with detection of TSLP and IL-33, we quantitated IL-9, IL-5 and IL-13 in BAL fluid at 1 day of infection (Figure 2.9C). Infected Recombination Activating Gene 1 (RAG1)-deficient mice had readily detectable IL-9 production at 24 hours, whereas mice deficient in either TSLPR or IL1RL1 had a significant decrease in recovered IL-9 (Figure 2.9D). These data are consistent with ILC2-derived cytokine production independent of and prior to T cell infiltration of the lungs at day 4-5 after *N. brasiliensis* infection (Licona-Limon et al., 2013). To determine whether early IL-5 and IL-13 expression was dependent on IL-9, we assessed lung RNA expression in $Il9^{+/+}$ and $Il9^{9er/9er}$ mice on day 3 after infection. *Il5* transcripts were significantly reduced, and *Il13* transcripts trended down in mice lacking IL-9 (Figure 2.9E). Further, transcripts for IL-13 target genes in lung epithelial cells, including *Muc5ac*, *Clca3*, *Tff2*, *Retnla* and *Chia1* (Kuperman et al., 2002; Reese et al., 2007), were significantly decreased in the absence of IL-9 (Figure 2.9E). Thus, IL-9 from lung ILC2s is generated acutely by chitin and during helminth infection and is required for optimal ILC2 elaboration of IL-5 and IL-13, which direct subsequent eosinophilia and epithelial responses.

Discussion

We used two models of localized lung injury – chitin bead impaction and migratory helminth infection – to define the cell and cytokine pathways that link activation of ILC2s with tissue homeostasis. We identify IL-33 and TSLP as requisite early cytokines whose simultaneous expression was localized to epithelial ATII cells; the highly active processed form of IL-33 was also recovered. Co-expression of IL-33 and

Figure 2.9

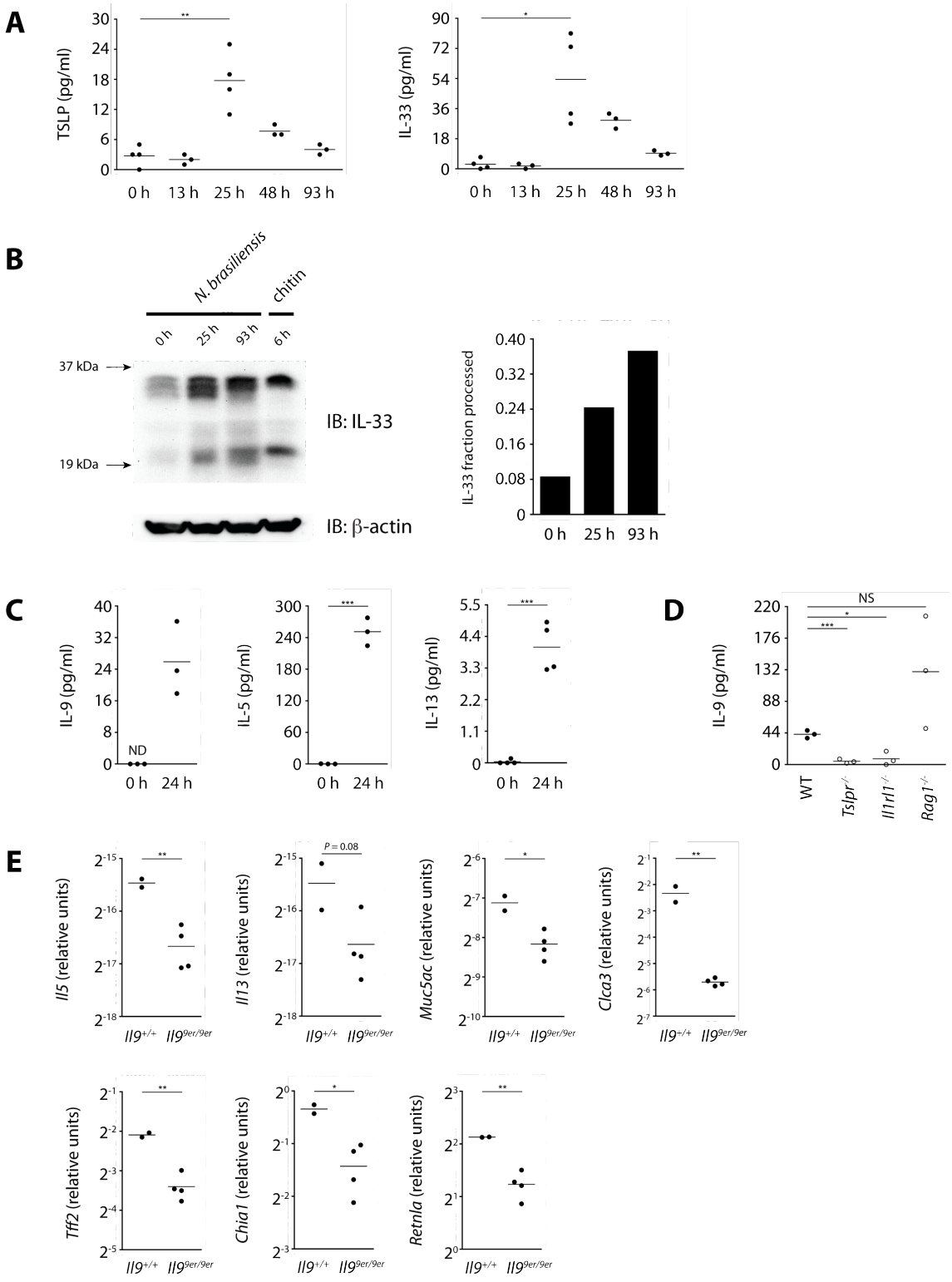

A
TSLP (pg/ml)
IL-33 (pg/ml)
0 h
13 h
25 h
48 h
93 h
B
N. brasiliensis
chitin
0 h
25 h
93 h
6 h
37 kDa
19 kDa
IB: IL-33
IB: β-actin
IL-33 fraction processed
C
IL-9 (pg/ml)
IL-5 (pg/ml)
IL-13 (pg/ml)
ND
0 h
24 h
D
IL-9 (pg/ml)
NS
WT
Tslpr-/-
Il1rl1-/-
Rag1-/-
E
Il5 (relative units)
Il13 (relative units)
Muc5ac (relative units)
Clca3 (relative units)
Tff2 (relative units)
Chia1 (relative units)
Retnla (relative units)
P = 0.08
Il9+/+
Il99er/9er

Figure 2.9: Early IL-9 augments rapid ILC2-directed epithelial responses during *N. brasiliensis* infection.

(A) ELISA of TSLP and IL-33 from BALs of mice at the indicated times after *N. brasiliensis* infection. (B) Western blot analysis (left) of whole lung homogenates from mice at the indicated times after challenge with *N. brasiliensis* or chitin and pixel density analysis (right) of the 20-kDa band in *N. brasiliensis* lanes, as in **Figure 1D**. (C) CBA analysis of IL-9, IL-5 and IL-13 in BALs from mice at the indicated times after *N. brasiliensis* infection. (D) CBA analysis of BALs from mice of the indicated genotypes 24 h after *N. brasiliensis* infection. (E) Quantitative RT-PCR analysis of whole lung RNA for expression of *Il5*, *Il13* and IL-13 target genes from mice 67 h after *N. brasiliensis* infection, normalized to *18s* expression and graphed in arbitrary units. Where applicable: * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ (two-tailed *t*-test). Data are representative of at least 2 experiments with 2-4 mice per exposure (**A**, **B**, and **E**). Data in **C** and **D** are compiled from 3 independent experiments, with each data point representing BALs pooled from 3-5 mice per exposure.

TSLP resulted in synergistic activation of ILC2s to express IL-9, which sustained autocrine amplification of transcription factors, including GATA3 and IRF4, associated with optimal expression of IL-5, IL-13 and IL-9 itself. In the absence of IL-9, IL-13-dependent expression of epithelial genes involved in barrier responses was abrogated. Thus, ILC2s integrate signals from specialized, distal epithelial cells that monitor alveolar function and utilize synergistic and auto-enhancing activation loops to mediate barrier alterations in more proximal conducting epithelia. These alterations, including increases in mucus and enzymatic chitinase, together with IL-13-induced smooth muscle contraction (Lee et al., 2001; Kuperman et al., 2002; Eum et al., 2005), facilitate redirection of blood flow to ventilated areas and eventual airway clearance that work to restore homeostasis.

The presence of constitutive, nuclear IL-33 in the lung epithelial cells of resting mice has been noted, as has transcriptional induction in a variety of resident and recruited cells during inflammation (Kouzaki et al., 2011; Chang et al., 2011; Kim et al., 2012; Yasuda et al., 2012; Wills-Karp et al., 2012; Halim et al., 2012; Pichery et al., 2012; Juncadella et al., 2013; Hardman et al., 2013; Byers et al., 2013). How the active C-terminal, IL-1-like domain of IL-33 (Lefrancais et al., 2012; Luzina et al., 2012; Bessa et al., 2014) is released from chromatin (Carriere et al., 2007) to signal neighboring cells remains unclear. Here, we demonstrate that a ~20-kDa protein, reactive with antibodies raised against Ser109-Ile266 of mouse IL-33 (R&D Systems), accumulates *in vivo* after chitin or helminth exposure. Processed IL-33 also accumulates in oleic acid-induced lung damage (Lefrancais et al., 2012) and in RIPK1-deficient mice that die of uncontrolled necroptosis (Rickard et al., 2014), consistent with injury as an elicitor of

processing. Intriguingly, we also identified ATII cells as the predominant source of TSLP after challenge with chitin or migratory worms. TSLP has been detected by transcript profiling in various cells at mucosal sites (Sims et al., 2000; Soumelis et al., 2002; Ying et al., 2005; Li et al., 2005; Zaph et al., 2007; Kashyap et al., 2011), consistent with our findings. Our observation of discordance between mRNA and protein expression suggests, however, that post-transcriptional regulation of TSLP may be the relevant mechanism of control (Kato et al., 2007).

Synergistic activation of lung ILC2s by combinations of IL-33 and TSLP, IL-2 or IL-7 has been described *in vitro* (Wilhelm et al., 2011; Halim et al., 2012; Mjosberg et al., 2012). Using two distinct *in vivo* challenges, we demonstrate acute induction of IL-33 and TSLP, but not IL-2 or IL-7, and observe direct synergy between them. IL-2 and IL-7 may become more relevant during the adaptive phases of inflammation. Recent studies implicating IL-33 (Moffatt et al., 2010) and TSLP (Gauvreau et al., 2014) in human asthma are consistent with a role in allergic inflammation. ILC2-derived IL-9, robustly induced by the combination of IL-33 and TSLP, was involved in an auto-amplification loop necessary to promote high levels of intracellular GATA3 and IRF4, implicating IL-9 as the mediator of synergistic ILC2 activation and revealing a mechanism for rapid cytokine expression. Indeed, our studies reveal a previously unexplored role for IL-9, aside from those in late lung repair (Turner et al., 2013) or adaptive Th9-mediated worm immunity (Licona-Limon et al., 2013), in tuning acute mucosal homeostasis, since IL-13-mediated epithelial responses were highly attenuated in the absence of IL-9. Identification of IRF4 in association with effector function in ILC2s is consistent with its role in controlling cytokine production by Th9, Th2 and Th17

cells as part of a larger complex regulating chromatin accessibility (Staudt et al., 2010; Glasmacher et al., 2012).

ATII cells have been designated sentinels of the alveoli, reflecting their critical roles in surfactant homeostasis (Fehrenbach, 2001), clearance of apoptotic cells (Juncadella et al., 2013) and alveolar regeneration (Desai et al., 2014). Our finding that ATII cells are the major source of IL-33 and TSLP involved in the acute lung challenges investigated here supports this designation and will serve to focus interest on the upstream signals that induce release of these cytokines. Multiple pathways, including physicochemical (Rangasamy et al., 2005; Jang et al., 2013), metabolic (Kouzaki et al., 2011; Hara et al., 2014) and structural (Demehri et al., 2008) elements, are the focus of further study, with different inputs potentially underlying each cytokine. The physical circuits by which these signals are transmitted also remain unclear. Our data position lung ILC2s in a pathway between specialized epithelial cells that sense and regulate alveolar function and more proximal conducting epithelial cells that control airway access to the alveoli. Interactions between resident innate lymphoid cells and structural cells, likely established during mucosal tissue development (Nussbaum et al., 2013), may define a generalized framework for homeostasis.

Experimental Procedures

Mice

Wildtype C57BL/6J, *ROSA26*$^{fl\text{-}STOP\text{-}eYFP}$ (Srinivas et al., 2001), and *Rag1*$^{-/-}$ (Mombaerts et al., 1992) mice were obtained from The Jackson Laboratory. *Il33*$^{Gt/Gt}$ (IL-33-deficient; Pichery et al., 2012), *Tslpr*$^{-/-}$ (Carpino et al., 2004), and *Il1rl1*$^{-/-}$ (Hoshino et

al., 1999) mice were generous gifts from J.-P. Girard (Univ. de Toulouse), S. Ziegler (Benaroya Research Inst.), and M. Steinhoff (UCSF), respectively. Other strains used include *Il4*$^{4get/4get}$ (Mohrs et al., 2001) and *Il5*$^{red5/red5}$. Mice were backcrossed to C57BL/6J for at least 10 generations and maintained in the UCSF specific pathogen-free animal facility in accordance with guidelines established by IACUC and LARC. All experiments were performed on 8- to 12-week old mice.

IL-9 reporter (9er) mice were generated by homologous gene targeting in C57BL/6 embryonic stem cells (PRXB6T). We used a reporter cassette derived from the pKO915-DT plasmid (Lexicon; Sullivan et al., 2011), containing (in order from 5' to 3') genomic sequence for rabbit β-globin gene partial exon 2-3, enhanced YFP (Clontech), encephalomyocarditis virus IRES, humanized Cre recombinase, bovine growth hormone poly(A) and *loxP*-flanked neomycin resistance. Homologous arms straddling the *Il9* translation initiation site (3.4 kb 5', containing the promoter and 5' UTR, and 3.0 kb 3', containing the start ATG through 3' UTR) were amplified from C57BL/6 genomic DNA using Phusion polymerase (Finnzymes) and ligated into the modified pKO915-DT plasmid, flanking the reporter cassette. The construct was linearized with NotI and transfected by electroporation into C57BL/6 embryonic stem cells. Cells were grown on irradiated feeders with media containing G418, and neomycin-resistant clones were screened for 5' and 3' homologous recombination by PCR. Two of 10 positive clones were injected into albino C57BL/6 blastocysts to generate chimeras, and male pups from a single clone were bred with homozygous CMV-Cre transgenic C57BL/6 females (The Jackson Laboratory) to excise the neomycin resistance cassette. Males from this cross were bred to wild-type C57BL/6 females to remove the CMV-Cre allele.

In vivo challenges

Purified chitin (New England Biolabs) and 60-mm polystyrene beads (Polysciences) were prepared and administered as described (Van Dyken et al., 2014). Carrier-free, recombinant TSLP and IL-33 (R&D Systems) were reconstituted in sterile PBS and administered intranasally in 50-ml doses. Third-stage larvae of *N. brasiliensis* were prepared and administered under isoflurane anesthesia as described (Voehringer et al., 2004).

Lung preparation

For BAL collection, lungs were instilled intratracheally with 1 ml PBS. The lavage fluid was centrifuged, and the supernatant was concentrated using Amicon Ultra filters (MWCO 10 kDa; EMD Millipore).

For flow cytometry or sorting of ILC2s, lungs were perfused with 10 ml PBS via the right ventricle. Lung lobes were excised and processed with a gentleMACS automated tissue dissociator in C tubes (Miltenyi Biotec), running program "m_lung_01", followed by shaking incubation for 30 min at 37^{o} C, 200 RPM, in HBSS (with Ca^{2+}, Mg^{2+}) containing 0.26 Wunsch units/ml Liberase TM and 25 μg/ml DNase I (Roche). Following incubation, a second dissociation was performed with program "m_lung_02".

For flow cytometry or sorting of ATII cells, lungs were instilled with 2 ml protease solution [300 U/ml collagenase Type I (Life Technologies), 4 U/ml elastase (Worthington Biochemical Corp.), 5 U/ml dispase (Life Technologies) and 200 U/ml DNase I in HBSS], minced by razor, and incubated in a total volume of ~2.5 ml (with protease solution) in a shaker for 25 min at 37^{o} C, 200 RPM. Suspension was washed in

PBS with 3% fetal bovine serum (FBS; Atlanta Biologicals), washed in HBSS, and resuspended in 2 ml HBSS with 0.1% Trypsin-EDTA (Life Technologies) + 200 U/ml DNase I for 20 min at 37^{o} C, 200 RPM. The protocol was adapted from Rock et al. (2011).

Following tissue dissociation, cell suspensions were filtered through a 70-mm nylon mesh, washed and treated with ACK solution to lyse red blood cells before final suspension in PBS with 3% FBS. Cells were stained for flow cytometry or sorting as described in Supplemental Experimental Procedures.

Cell culture

Sorted ILC2s were cultured (37^{o} C, 5% CO_2) at 5000 cells per well in 100 ml RPMI 1640 (Caisson Laboratories) containing 10% heat-inactivated FBS, penicillin-streptomycin-L-glutamine, 2-mercaptoethanol (Bio-Rad Laboratories), and recombinant mouse cytokines (R&D Systems), as indicated. At harvest, cell-free culture supernatants were collected and cells were analyzed by flow cytometry. To assess intracellular protein expression, cells were fixed and permeabilized using the eBioscience Transcription Factor Staining Buffer Set.

MLE12 cells (American Type Culture Collection) were cultured (37^{o} C, 5% CO_2) in 1:1 DMEM:Ham's F-12 (Life Technologies) containing 10% heat-inactivated FBS, penicillin-streptomycin-L-glutamine and 2-mercaptoethanol. Cells were passaged with 0.05% Trypsin-EDTA and adhered for 24 h at 70% confluency, prior to exposure to phorbol 12-myristate 13-acetate (Sigma) for 24 h. Culture supernatants were concentrated using Amicon Ultra filters.

Sorted naïve $CD4^+$ T cells were cultured (37^o C, 5% CO_2) at $2x10^6$ cells per well in 2 mL RPMI 1640, prepared as described for ILC2 cultures. Wells were pre-coated with 5 mg/ml anti-CD3 and anti-CD28 antibodies 1 day prior to culture. For Th9 polarization, 20 ng/ml recombinant mouse IL-2, 10 ng/ml IL-4, and 10 ng/ml TGFβ were added to wells for 4 days (R&D Systems; Veldhoen et al., 2008). At harvest, culture supernatants were collected and cells were analyzed by flow cytometry as described above.

Immunohistochemistry

Lungs were inflated with 1:1 Tissue-Tek OCT (Sakura Finetek):PBS, and lobes were embedded in OCT, prior to frozen sectioning at 7 mm onto glass slides using a CM 3050S cryomicrotome (Leica Microsystems). Alternatively, sorted cells were spun onto glass slides using a Cytospin 2 cytocentrifuge (Shandon). After tissue adherence, slides were dehydrated in cold acetone. Goat anti-GFP (Abcam), goat anti-mouse IL-33 (R&D Systems) and rabbit anti-mouse SPC (Abcam) antibodies were applied to sections and detected with fluorophore-conjugated, anti-goat IgG and anti-rabbit IgG secondary antibodies (Life Technologies). Nuclei were counterstained with DAPI (4′, 6-diamidine-2′-phenylindole dihydrochloride; Roche). Images were acquired with an AxioCam HRm camera and an AxioImager M2 upright microscope (Carl Zeiss).

Protein extraction and analysis

Lung lobes were snap-frozen in liquid nitrogen and later homogenized in TNT buffer with cOmplete Protease Inhibitor Cocktail (Roche) and Antifoam Y-30 (Sigma) in

M tubes using gentleMACS, running program "Protein_01". The suspension was centrifuged for 5 min at 1500 RPM, and the supernatant was incubated on ice for at least 30 min. Protein homogenate from sorted or cultured cells was prepared by triturating cell pellets in TNT buffer with protease inhibitor. Following incubation on ice for 30 minutes, suspensions were centrifuged at 16,100 x *g* for 10 min at 4^{o} C. Total protein concentration was measured by BCA (Thermo Scientific).

Specific protein expression assays were normalized to total protein amounts for homogenates or equal volumes for culture supernatants and BALs. Amounts of IL-5, IL-9 and IL-13 were quantified by CBA using a LSRII flow cytometer and FCAP Array analysis software (BD Biosciences). TSLP and IL-33 protein were measured by ELISA (R&D Systems). To quantify TSLP protein in sorted lung populations, the ELISA protocol was modified to avoid spurious measurements due to endogenous biotin in ATII cells (Kuhn, 1988). Briefly, cell homogenates were incubated with goat anti-mouse TSLP detection antibodies (R&D Systems) and subsequently probed with HRP-conjugated donkey anti-goat IgG antibodies (Southern Biotech), followed by application of Substrate Solution (R&D Systems). Processing of IL-33 was assessed by western blotting of denatured lung homogenates separated by SDS/PAGE with gradient gels (Life Technologies) and transferred to PVDF membranes (EMD Millipore). Membranes were probed with goat anti-mouse IL-33 (R&D Systems) and rabbit anti-mouse β-actin antibodies (Cell Signaling), which were detected with HRP-conjugated mouse anti-goat IgG and goat anti-rabbit IgG secondary antibodies (Southern Biotech). Peroxidase signal was developed using Pierce ECL Substrate (Thermo Scientific), and data were acquired with a Kodak Image Station 440CF and Kodak Molecular Imaging software.

Densitometry analysis was performed using ImageJ software.

RNA isolation and quantitative RT-PCR

Lung lobes were snap-frozen in liquid nitrogen and homogenized in M tubes in RNAzol (Molecular Research Center) by gentleMACS, running program "RNA_02". Total RNA was extracted, dissolved in RNase-free water and purified using RNeasy Mini columns (Qiagen). To isolate RNA from sorted cells, pellets were lysed in RLT Plus, and total RNA was eluted from RNeasy Plus Micro columns (Qiagen).

RNA was reverse transcribed with VILO cDNA Synthesis Kit, and the cDNA was used as a template for quantitative RT-PCR using the Power SYBR Green Kit on a StepOnePlus cycler (Life Technologies). Primers used for SYBR Green PCRs are described in Supplemental Experimental Procedures. Where indicated, TaqMan probes and the TaqMan Gene Expression Kit were used (Life Technologies).

Statistical analysis

Statistical analysis was performed as indicated in figure legends using Prism software (GraphPad, Inc.).

Chapter 3: Conclusion

Model

The findings presented in Chapter 2 suggest a highly regulated program by which the distal airway responds to epithelial perturbation, as assessed by inhalation of chitin and infiltration of helminths (Figure 3.1). Perturbation results in acute transcription of *Tslp* and processing of IL-33 in ATII cells. These signals are simultaneously sensed by ILC2s positioned near medium-sized blood vessels and bronchi and synergize to induce IL-9 production. IL-9 has an autocrine effect on ILC2s that amplifies TSLP- and IL-33-mediated production of IL-5 and IL-13, which is required for expression of epithelial target genes important for restoration of tissue homeostasis.

Our model suggests several questions for future studies. We still have not determined the characteristics of our chitin beads that cause acute lung inflammation following intranasal administration. Moreover, the mechanism by which the beads are sensed in the lung to induce IL-33 and TSLP remains to be addressed. These questions are relevant to our understanding of what makes a given stimulus allergenic. Irrespective of the stimulus, we also do not understand how the IL-33 and TSLP generated in alveoli reach ILC2s situated near bronchi, and how these signals are integrated within ILC2s to initiate an effector program.

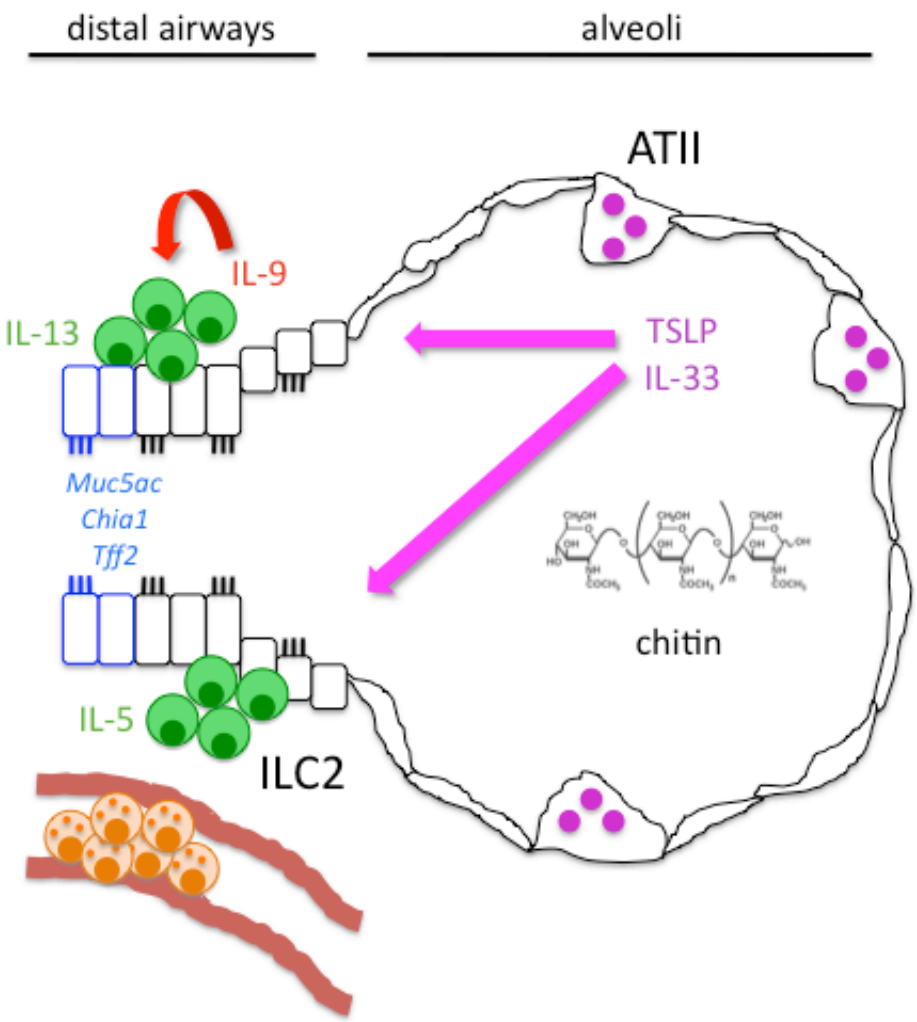


Figure 3.1: ATII cells and ILC2s interact via cytokines. Granular ATII cells in alveoli produce IL-33 and TSLP in response to stimuli such as chitin. ILC2s (green), located near bronchi (black) and blood vessels (bottom left), express IL-9-dependent cytokines IL-5 and IL-13, required for expression of genes (blue) and accumulation of cells (orange) that support tissue defense and repair.

Parameters Defining Chitin-Induced Cytokine Expression

Several groups, using a chitin powder from Sigma, have observed patterns of cytokine expression that differ from that described in our Model. For example, administration of a sonicated chitin powder solution, filtered to contain particles less than 10 μm in diameter, induced interferon gamma production in alveolar macrophages *in vitro* (Shibata et al., 1997). This was shown to involve macrophage phagocytosis (Shibata et al., 1998), and intranasal administration of this chitin preparation abrogated Th2-dependent allergic inflammation (Strong et al., 2002). Further, Da Silva et al. detected IL-10 and tumor necrosis factor alpha (TNFα) from alveolar and peritoneal macrophages exposed to chitin particles less than 10 μm in diameter (2009). A solution of similarly sized chitin particles generated with methanesulfonic acid (Hirano and Nagao, 1988) was observed to limit Th2 polarization *in vivo* and was attributed to macrophage expression of B7-H1 *in vitro* (Wagner et al., 2010). These studies raise the possibility that the size and composition of chitin used and the route of exposure can affect macrophage activation and local cytokine expression, suggesting that the response to chitin is not simply based on recognition of a pathogen-associated molecular pattern. This size-dependent, inhibitory effect, however, may not be chitin-dependent, as nanometer-sized polystyrene beads have been observed to inhibit allergic inflammation *in vivo* (Hardy et al., 2012).

The chitin particles used in our work are too large to be immediately phagocytosed by resident alveolar macrophages, but we do observe gradual disintegration of impacted chitin beads over time, presumably due to the action of AMCase and chitotriosidase (Bussink et al., 2007; Van Dyken et al., 2014). This breakdown of chitin correlates with resolution of inflammation (Van Dyken et al., 2014), which we infer is

due to the loss of chitin oligosaccharides of sufficient size for signaling (Shibuya et al., 1996). It is possible, however, that degradation of large chitin beads in the lung parenchyma results in generation of phagocytosable fragments that subsequently alter macrophage expression, limiting further IL-5- and IL-13-mediated inflammation. In contrast, a sonicated solution of chitin powder composed of particles 40-70 μm in diameter, a range similar to that used in our studies, induced parallel inflammatory responses driven by IL-5 and IL-13 and IL-17A (Da Silva et al., 2010), as we have observed (Van Dyken et al., 2014). Moreover, in this size range, particles made of other polymers, including sephadex (Kogiso et al., 2011), polystyrene, and polymethylmethacrylate (Van Dyken et al., 2014), have not been observed to induce any inflammatory responses when inhaled. Given the proposed link between allergic inflammation and macro-parasite defense, one hypothesis regarding this size and composition dependence is that disruption of lung epithelial structure induces eosinophil recruitment and alternative activation of macrophages. Particles of sufficient size would be necessary for disruption, while chitin multimers exposed to the epithelial surface might prevent bead expulsion. One approach to test this hypothesis is to synthesize beads with a similar chemical composition to that of the chitin beads used in our studies and determine whether they induce similar patterns of lung inflammation. In general, our observations with chitin-induced inflammation must still be reconciled with other well-characterized elicitors of allergic inflammation, including plant- and insect-derived proteases. Although host responses to chitin and proteases, which are both common to mammalian macro-parasites, may have evolved in parallel, it is also possible that

epithelial disruption is the final common signal for the inflammatory module driven by Th2-associated cytokines.

Chitin Sensors

Known Receptor Families in Pathogenesis

To understand the role of chitin as either a pathogen-associated molecular pattern or a direct inducer of epithelial damage, the sensing machinery required for inflammation will need to be defined. Members of the toll-like receptor (TLR) family bind a wide range of conserved molecular patterns in bacteria, fungi, and viruses (Beutler et al., 2006). In previous work, we found no evidence of a role for TLR4 in chitin-induced lung inflammation (Reese et al., 2007). There may be a partial dependence on the TLR adaptor MyD88, but this is confounded by its role in IL-33 signaling (Reese et al., 2007; Arend et al., 2008; Van Dyken et al., 2014). Da Silva et al. found a role for TLR2 in a model of experimental allergic asthma in which intraperitoneal injection of chitin was used to sensitize mice to ovalbumin (2010). While we have not specifically studied the response of TLR2-deficient mice to intranasal chitin, we have previously observed that the peritoneal response to particle injection is somewhat non-specific; whereas polystyrene beads do not induce lung inflammation, we observed eosinophil accumulation following polystyrene injection in the peritoneum to a level similar to that observed with chitin injection (unpublished data). Cytokine responses to fungi mediated by TLR2 can be augmented by signaling through the C-type lectin family member Dectin-1 (Brown et al., 2003; Gantner et al., 2003). These conserved lectins bind a variety of carbohydrates in a calcium-dependent manner (Geijtenbeek and Gringhuis, 2009). Although Dectin-1 has

only been observed to detect β-1,3- and β-1,6-linked glucans (Brown et al., 2002), fungal cell walls contain these β-glucans interwoven with chitin (Van Dyken et al., 2011). Peritoneal macrophage expression of TNFα in response to chitin particles less than 10 μm in diameter *in vitro* was observed to depend on Dectin-1 and another C-type lectin, type 1 mannose receptor (MRC1; Da Silva et al., 2009). We have not assessed whether Dectin-1 is required for inflammation following intranasal chitin, but MRC1 has been observed to bind mannose, fucose, and *N*-acetylglucosamine (Lennartz et al., 1987). MRC1-deficient mice, however, had wildtype induction of IL-33 and TSLP when intranasally challenged with chitin (unpublished data), suggesting that MRC1 is, at best, redundant for chitin sensing in the lung. The conserved response to chitin-containing organisms, especially fungi, may also involve germline-encoded, B1 cell-derived, "natural" IgM (Baumgarth et al., 2005). Although the BALB/c and C57BL/6 mice strains used in all of our chitin studies express significant amounts of natural IgM specific for *N*-acetylglucosamine (Love et al., 2000; Rapaka et al., 2010), we observed no change in chitin-induced lung inflammation in B cell-deficient *Rag2*$^{-/-}$ mice (Reese et al., 2007).

Chilectins

Although chitin sensing may occur via some of these known pathways, our work on chitin began with the observation that several previously described chitin-binding proteins, or "chilectins", were induced in mouse lungs following infection with *N. brasiliensis* (Reese et al., 2007). Based on amino acid sequence similarities, AMCase, CHIL3, and CHIL4, along with others, all belong to glycosyl hydrolase family 18, encoded in two loci on mouse chromosomes 1 and 3 (Bussink et al., 2007). Whereas only

chitotriosidase and AMCase possess true chitinase activity, all other characterized proteins in this family have retained chitin-binding ability but lost hydrolase activity (Bussink et al., 2007). Comparative analysis across species suggests that these loci have undergone multiple rounds of expansion, contraction, and diversification under significant evolutionary pressure (Funkhouser and Aronson, 2007). Thus, the retention of catalytically-inactive chilectins in syntenic regions of the mouse and human genomes suggests that chitin-binding proteins could be important for digestion, pathogen recognition, and host defense, since vertebrates do not employ chitin for structural purposes (Bussink et al., 2007). Indeed, CHIL3 and CHIL4, like AMCase, were induced by IL-13 in a model of experimental allergic asthma (Webb et al., 2001), implying that at least some chilectins are part of the Th2-associated inflammatory program. Chitin-binding proteins in this setting may act as co-receptors. We developed transgenic mice expressing CHIL4 and observed neither spontaneous inflammation nor alterations in the immune response to *N. brasiliensis* infection (unpublished data). Although the abundance of chilectins in the mouse genome suggests the potential for functional redundancy, new technologies may enable deletion of multiple chilectin genes to assess the necessity of these loci in chitin-induced lung inflammation.

Novel Candidate Receptors

Evidence from other organisms, however, suggests that distinct chitin-binding systems have evolved multiple times aside from glycosyl hydrolase family 18. For example, the human pathogen *Vibrio cholerae* utilizes a chitin-binding protein to survive on zooplankton exoskeletons and bind intestinal epithelial cells (Kirn et al., 2005). A

variant of this protein may be important for *Vibrio fischeri* to chemotax to the light organ of its symbiont, the squid *Euprymna scolopes* (McFall-Ngai, 2014). Another example of chitin-dependent symbiosis is that between nitrogen-fixing bacteria and legumes, in which a modified oligosaccharide of *N*-acetylglucosamine is the signal for initiation of root nodulation (Lerouge et al., 1990). For two such legumes, *Lotus japonicus* (Radutoiu et al., 2003) and *Medicago truncatula* (Limpens et al., 2003), the respective plant receptors for the chitooligosaccharide signal were identified and found to contain a conserved domain, lysin-like motif (LysM), originally identified in peptidoglycan-degrading enzymes but also present in several chitinases (Ponting et al., 1999). Chitin receptors containing the LysM motif have also been identified in *Oryza sativa* (Kaku et al., 2006) and *Arabidopsis thaliana* (Miya et al., 2007; Wan et al., 2008) and mediate host defense. While sequences predicted to encode LysM or other unique chitin-binding domains exist in the mouse and human genomes, we have not yet confirmed their expression in the lung.

While we continue to search for gene orthologs of these and other chitin-binding proteins in mouse and human, a parallel approach is to screen known, expressed receptors for binding to chitin particles or oligosaccharides. Glycan arrays, in which various carbohydrate structures are printed onto glass slides, can be used to screen proteins of interest for binding (Liang and Wu, 2009). For example, a search of the Consortium for Functional Glycomics database for previously screened proteins reveals other C-type lectins, including type 2 mannose receptor and LY75, which bind *N*-acetylglucosamine (www.functionalglycomics.org). An affinity column-based approach has also been used to identify novel proteins from invertebrate tissue samples that bind a *N*-

acetylglucosamine resin (Vitashenkova et al., 2012). With all of these methods, care must be taken to validate potential binding proteins in an *in vitro* assay that closely resembles the *in vivo* setting of chitin-induced lung inflammation. Given the ability of various lectins to bind multiple carbohydrate moieties (Geijtenbeek and Gringhuis, 2009), any screening assay will require the versatility to over-express or repress the expression of multiple targets to study the potential redundancy in chitin detection *in vivo*. We continue to work toward developing such an assay.

Implications of a Dual-Signal Pathway

Our studies suggest that two differentially regulated signals, IL-33 and TSLP, are generated in response to chitin bead impaction in the lung. Even if direct chitin sensing is involved in production of these signals, our data raise the possibility that the chitin bead is sensed in other ways by the lung epithelium and that IL-33 and TSLP are induced independently of each other. Broadly, damage to the lung epithelium due to bead impaction might result in loss of epithelial integrity and/or local hypoxia, and several studies in other systems have suggested how these two events might result in TSLP- and IL-33-mediated inflammation.

Inputs to *Tslp* Transcription

Recent data suggests that ATII cells can regenerate type I cells following injury (Desai et al., 2014) and clear apoptotic alveolar cells homeostatically (Juncadella et al., 2013), implying that they can directly sense epithelial integrity. Chitin beads might damage contacts between neighboring cells by mechanically disrupting the distal airway

following inhalation. Notch signaling has been implicated as a sensor of integrity in intestinal epithelial cells (Obata et al., 2012), and in a skin model, loss of this signaling in epidermal cells was implicated in TSLP production (Demehri et al., 2012; Di Piazza et al., 2012). The role of Notch in the adult lung has been difficult to assess due to its effects on embryonic lung development (Guseh et al., 2009), but a variety of tools exist to determine whether Notch signaling is active in alveolar cells near chitin beads.

Local hypoxia, potentially induced by chitin bead occlusion of or injury to a distal alveolar sac, might also drive acute production of TSLP. Indeed, hypoxia-inducible factors (HIFs), a family of transcriptional regulators implicated in the stress response to low oxygen levels, are expressed in ATII cells (Shimoda and Semenza, 2011). Further, in a skin model of ultraviolet radiation-induced damage, HIF-1α was shown to induce TSLP and directly bind the *Tslp* promoter, although it is unclear what factors drive HIF-1α activity in this case (Jang et al., 2013). Another stress sensor related to oxygenation is the NF-E2-related factor 2 (NRF2)/Kelch-like ECH-associated protein 1 (KEAP1) complex (Cho et al., 2014). NRF2 is constitutively degraded in the cytoplasm by the actions of KEAP1, but reactive oxygen species alter KEAP1 binding to NRF2, enabling it to accumulate and enter the nucleus, where it induces transcription of stress response genes (Huebner et al., 2012). In a model of experimental allergic asthma (Rangasamy et al., 2005) and in lung fibroblasts *in vitro* (Fourtounis et al., 2012), loss of NRF2 correlated with increased IL-13-mediated effects. Given that alveolar cells express NRF2 (Ballinger et al., 2012), it is possible that this transcription factor is constitutively active in the hyperoxic environment of the lung and represses genes such as *Tslp*. Thus, both the HIF

family and NRF2 are candidates we can characterize to determine the potential role of hypoxia in chitin-induced lung inflammation.

Inputs to IL-33 Processing

Although we have never observed this directly, chitin bead impaction, through either the signaling described above or other means, might result in ATII cell necrosis. Necrosis is one means by which nuclear IL-33 could be exposed for extracellular binding to IL1RL1, but it does not itself explain how the protein would be freed from chromatin complexes (Carriere et al., 2007). *In vitro* data suggesting that full-length IL-33 is released from intact cells also fails to address this point (Talabot-Ayer et al., 2009; Kouzaki et al., 2011; Kakkar et al., 2012; Hara et al., 2014). Our observation that IL-33 is processed *in vivo*, likely separating the chromatin-binding N-terminal portion of the protein from the IL-1-like domain, suggests a mechanism by which optimal signaling might occur (Lefrancais et al., 2012; Luzina et al., 2012; Bessa et al., 2014). Thus, we predict that IL-33-inducing stimuli activate or induce a protease that cleaves the full-length form, prior to or following release into the extracellular compartment. To identify potential proteases, we are currently sequencing the 20-kDa product we have isolated *in vivo* to determine the cleavage site. This product may be similar to those identified *in vitro* after exposure of full-length IL-33 to neutrophil elastase or cathepsin G (Lefrancais et al., 2012). Indeed, Voynow et al. reported increased IL-5- and IL-13-mediated effects following intranasal administration of recombinant neutrophil elastase to mice (2004). Although we have not determined whether these proteases are specifically induced following chitin, we observed no difference in IL-33 processing with antibody-mediated

depletion of neutrophils prior to chitin administration (unpublished data). Given the acuity with which IL-33 processing occurs, we predict that either alveolar epithelial cells or alveolar macrophages express the relevant protease. In anticipation of screening candidate proteases, we have generated a stable cell line that expresses full-length IL-33.

Although hypoxia, as sensed by HIF-1α, has been linked to IL-33 regulation (Hu et al., 2013), signaling via extracellular adenosine triphosphate (ATP; Kouzaki et al., 2011; Byers et al., 2013) or uric acid (Hara et al., 2014) has also been proposed to regulate release of the protein and/or induction of protease activity. We have never detected ATP in BAL fluid from chitin-exposed mice, but we have not studied mice lacking ATP receptors. Similarly, we intend to measure uric acid production and assess the potential role of the inflammasome in chitin-induced lung inflammation. Identifying the pathways by which chitin, a simple stimulus, induces *Tslp* transcription and IL-33 processing may yield insight into how more complex allergens and helminths trigger these signals.

ILC2 Integration of Two Signals

Our data regarding the simultaneous expression of IL-33 and TSLP following chitin or *N. brasiliensis* infection also raise questions about the integration of these signals in ILC2s. The TSLP receptor complex is proposed to activate JAK-STAT signaling (Rochman et al., 2010), whereas the IL-33 receptor complex has been linked to NFκB and MAP kinases (Schmitz et al., 2005). Furusawa et al., however, proposed that IL-33-induced production of IL-5 and IL-13 in cultured ILC2s was only dependent on p38 MAP kinase (2013), which was observed to phosphorylate GATA3 within minutes,

resulting in nuclear translocation; IL-33 stimulation also resulted in increased total GATA3 over days. We previously identified GATA3 as a regulator of IL-5 and IL-13 in ILC2s (Liang et al., 2011) and observed increased transcription of *Gata3* in ILC2s cultured with IL-33 and TSLP for 16 h; intracellular IRF4, a transcription factor implicated in *Il9* regulation (Staudt et al., 2010), also increased (unpublished data). We plan to study IRF4-deficient mice to validate the potential role of this factor in ILC2 production of IL-9. Although Furusawa et al. observed no role for NFκB in ILC2s cultured with IL-33 alone, we observed a substantial role for the transcription factor in the cytokine output of ILC2s exposed to both IL-33 and TSLP (unpublished data). This suggests one mechanism by which TSLP might augment IL-33-induced signaling. Further, chromatin immunoprecipitation of STAT5 and NFκB in cultured ILC2s suggests that these factors bind the promoters of *Gata3* and *Irf4* in the presence of IL-33 and TSLP (unpublished data), though the contribution of IL-33 to these processes remains unclear. We have not, however, assessed the effect of inhibiting JAK-STAT signaling on TSLP-mediated cytokine production. In general, the extent to which GATA3 and IRF4 are regulated by transcription versus post-translational modification remains unclear. To clarify these questions and identify other factors regulated by the combination of IL-33 and TSLP, we could profile whole transcriptome changes in ILC2s cultured with these cytokines for various times. Mass spectrometry could provide a similar analysis of intracellular protein modifications and the secretome.

This information might also be useful for establishing how IL-25, which may induce similar signaling modules to that of IL-33 (Wong et al., 2005; Furusawa et al., 2013), activates ILC2s. Although we and others have observed diminished lung ILC2

effector responses in mice unable to signal through IL-25 (Barlow et al., 2013; Hams et al., 2013; Van Dyken et al., 2014), we have not been able to consistently detect IL-25 induction in response to chitin instillation or *N. brasiliensis* infection. Indeed, lung ILC2s generally exhibit decreased responsiveness to IL-25 as compared to IL-33 (Wilhelm et al., 2011; Ikutani et al., 2012; Halim et al., 2012; Barlow et al., 2013). Induction of similar signaling profiles by IL-25 and IL-33 would be consistent with a model in which tissue expression of these cytokines is mutually exclusive, resulting in tissue-specific ILC2 subsets that are primed to respond to either IL-25 or IL-33. To test this hypothesis, we plan to sort ILC2s from various mucosal sites and assess their responsiveness to these cytokines *in vitro*.

Although many of these questions regarding ILC2 integration can be answered through cultures of isolated cells, we cannot study how TSLP and IL-33 reach ILC2s *in vitro*. This is relevant because ILC2s appear to be located in collagen-rich regions near medium-sized airways and blood vessels (Ikutani et al., 2012; Nussbaum et al., 2013), distant from most alveolar sacs. Although IL-33 and TSLP are soluble proteins, it is unclear whether they could traverse these distances. One hypothesis is that perturbation-induced collapse of an alveolar sac might cause it to retract proximally toward the bronchus from which it derives, reducing physical distance and potentially altering lymphatic drainage. Alternatively, dendritic cells, which have been observed to leave alveoli and migrate to nearby larger airways to activate T cells (Thornton et al., 2012), may act as carriers or activated intermediaries. We can begin to address these hypotheses with live imaging of lung slices from mice exposed to chitin. However, we will likely

need to generate mice in which IL-33 or TSLP are tagged or cells receiving a signal through the IL-33 or TSLP receptor complex can be tracked in real time.

Summary

Allergic inflammation is likely an unintended consequence of a conserved program evolved to deal with chronic helminth colonization. Although important questions remain, this work has made important strides in furthering our understanding of how mucosal epithelia, which mediate interactions with the external environment, respond to perturbations to maintain and restore tissue homeostasis. Our data reinforce the notion that multicellular organisms compartmentalize functions within specialized cells. Following perturbation, epithelial cells sense tissue status and communicate via cytokine with resident lymphoid effector cells. These effectors integrate combinatorial cytokine expression and then use feed forward signaling to tightly regulate epithelial programs that begin to restore homeostasis. Recent studies implicating IL-33 (Moffatt et al., 2010) and TSLP (Gauvreau et al., 2014) in human allergic disease demonstrate how these principles can guide therapy by highlighting the factors that initiate inflammation proximal to allergen exposure.

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