

Lung Natural Helper Cells Are a Critical Source of Th2 Cell-Type Cytokines in Protease Allergen-Induced Airway Inflammation

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SUMMARY

Overproduction of cytokines by T helper 2 (Th2) cells in the lung is thought to be a cause of asthma. Here we report that innate lymphocytes termed lung natural helper (LNH) cells are a T cell-independent source of Th2 cell-type cytokines in protease allergen-treated lungs. LNH ($\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{+/lo} \text{CD25}^+ \text{CD127}^+$) cells, when stimulated by IL-33 plus IL-2, IL-7, or thymic stroma lymphopoietin (TSLP), produced large amounts of IL-5 and IL-13. Intranasal administration of protease allergen papain induced eosinophil infiltration and mucus hyperproduction in the lung of wild-type and *Rag1*^{-/-} mice, but not in *Rag2*^{-/-}*Il2rg*^{-/-} mice that lack LNH cells. LNH cell depletion inhibited papain-induced airway inflammation in *Rag1*^{-/-} mice whereas adoptive transfer of LNH cells enabled *Rag2*^{-/-}*Il2rg*^{-/-} mice to respond to papain. Treatment of lung explants with papain induced IL-33 and TSLP production by stroma cells and IL-5 and IL-13 production by LNH cells. Thus, LNH cells are critical for protease allergen-induced airway inflammation.

INTRODUCTION

Asthma is a chronic inflammatory disease of the lower airways characterized by recurrent airway obstruction and wheezing. It is induced by allergen such as pollen, molds, and dust mites as well as air-borne irritants and infections. Asthma is a heterogeneous disease and includes allergic, nonallergic, and intrinsic forms, of which allergic asthma is the most common form (Kim et al., 2010). Allergic asthma is thought to be driven by T helper 2 (Th2) cell-type cytokines including IL-4, IL-5, and IL-13. IL-4 promotes the production of IgE by B cells and the expression of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells (Steinke and Borish, 2001). IL-5 induces eosinophil development, recruitment to the lung, and activation (Hamelmann and Gelfand, 2001). Activated eosinophils produce a broad range of cytokines and chemokines and are a potent source of

the lipid mediator leukotriene C4 and platelet-activating factor, which induce mucus secretion and smooth muscle contraction. IL-13 is thought to mediate the effector phase of asthma by inducing airway remodeling and hyperresponsiveness as well as mucus hyperproduction (Wills-Karp, 2004). Whereas basophils, mast cells, and eosinophils, which can produce some Th2 cell-type cytokines, are found in the lungs of asthma patients (Voehringer et al., 2006), Th2 cells are generally thought to be the critical source of those cytokines (Cohn et al., 2004).

In most mouse models that simulate asthma, T cells are first sensitized by allergens such as ovalbumin (OVA) to induce a Th2 cell-polarized response; sensitized mice are then challenged by intranasal administration of the same allergen, resulting in cytokine production by Th2 cells recruited to the lung and eosinophilic inflammation (Kim et al., 2010). Although these mouse models have clearly indicated the importance of Th2 cells in lung inflammation, recent studies have revealed that innate cells also play a substantial role in asthma. Lung epithelial cells can produce multiple cytokines in response to stress, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), which induce Th2 cell-type cytokine production and eosinophilic lung inflammation. Intranasal administration of IL-25 reproduces many asthma symptoms (Hurst et al., 2002), and OVA-induced allergic airway inflammation is inhibited by IL-25 antibody (Baltantyne et al., 2007). It is thought that IL-25 stimulates natural killer (NK) T cells, which express the IL-25 receptor (IL-17RB) (Terashima et al., 2008). IL-33 acts on mast cells and basophils (Schmitz et al., 2005; Schneider et al., 2009). Activated basophils produce IL-4 and TSLP, which promote Th2 cell-type responses. IL-33 antibody inhibits eosinophilic lung inflammation, indicating the importance of IL-33. TSLP activates dendritic cells (DCs) and enhances the Th2 cell-type response (Ziegler and Artis, 2010). These epithelial cell-derived cytokines are thought to promote Th2 cell-type responses and induce lung inflammation. However, intranasal administration of IL-25 or IL-33 induces lung eosinophilia in recombination activating gene (RAG)-deficient mice, which lack all T and B cells, indicating that an innate cell population is capable of inducing lung inflammation (Hurst et al., 2002; Kondo et al., 2008).

Recently, novel innate lymphocytes termed multipotent progenitor (MPP) type 2, natural helper cells, and nuocytes have been discovered in the gut-associated mucosa tissues (Moro et al., 2010; Neill et al., 2010; Saenz et al., 2010b).

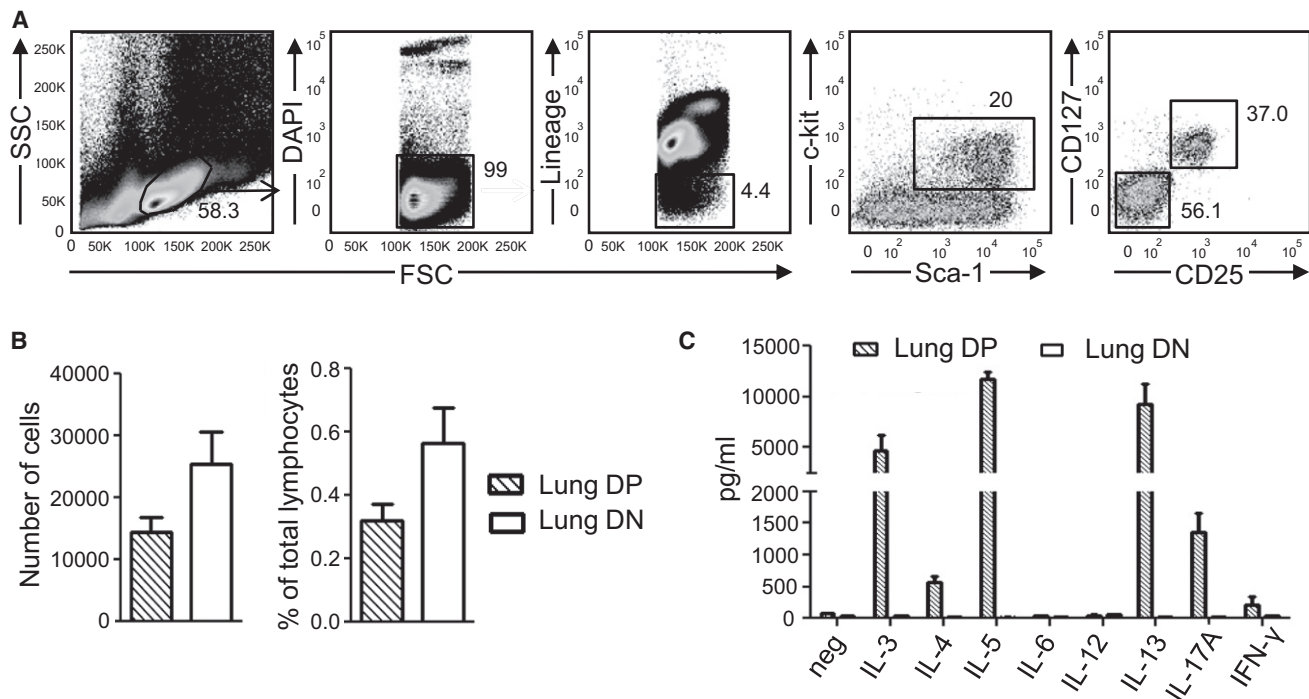


Figure 1. Lung Contains Lin[−]Sca-1⁺c-kit^{+/lo}CD127⁺CD25⁺ Cytokine-Secreting Cells

(A) Gating strategy for identifying LNH (Lin[−]Sca-1⁺c-kit^{+/lo}CD127⁺CD25⁺) cells in naive B6 mouse lungs by flow cytometer is illustrated. The numbers in the plots indicate the percentages of cells in the indicated gates.

(B) Total numbers per whole naive B6 mouse lung and the percentages of Lin[−]Sca-1⁺c-kit⁺CD127⁺CD25⁺ (DP) or Lin[−]Sca-1⁺c-kit⁺CD127[−]CD25[−] (DN) cells among lung lymphocytes are shown.

(C) Flow cytometry-purified lung DP and lung DN cells (3.5×10^3 cells in 200 μ l media per well) were stimulated with PMA plus ionomycin (P/I) for 3 days, and cytokines in culture supernatants were measured by ELISA. Unstimulated negative control (neg) for IL-3 is also shown.

Data are representative of three independent experiments (mean and SEM in B, C). See also Figure S1.

Phenotypically, these cells do not express mature hematopoietic lineage markers (Lin[−]) and produce Th2 cell-type cytokines when stimulated by IL-25 or IL-33. They have been implicated in immune responses in the gut against helminth infections and allergens. Similar Lin[−] cells that produce IL-4 and IL-13 have also been found in other tissues, including the liver, spleen, and lung via IL-4 reporter and IL-13 reporter mice (Price et al., 2010). The detection of those cells in the lung is dependent on the expression of reporter fluorescent proteins, which are induced by IL-25 stimulation. The finding of Th2 cell-type cytokine-producing innate lymphocytes has led to speculations that they may play a role in lung eosinophilic inflammation and asthma (Kaiko and Foster, 2011; Kim et al., 2010; Lloyd and Hessel, 2010). However, IL-4-eGFP⁺ cells are barely detectable in naive mouse lung, and even after stimulation with IL-25 or *Nippostrongylus brasiliensis* infection, very few have been detected. Recently, Chang et al. (2011) reported the presence of Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells in virus-infected BALB/c mouse lung and indicated their importance in viral-induced airway inflammation and macrophage infiltration. Unlike mesenteric natural helper cells, Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells do not induce eosinophil infiltration. Furthermore, their cytokine production capacity is markedly lower than those of nuocytes and mesenteric natural helper cells. Thus, Th2 cell-type cytokine-producing

innate lymphocytes in the lung have not been characterized beyond the initial detection, and their functional significance remains speculative. Here, we have characterized in detail innate lymphocytes in normal mouse lungs that rapidly produce multiple Th2 cell-type cytokines and their regulation in the lung environment. Further, we demonstrate that these innate lymphocytes play a critical role in T cell-independent lung eosinophilia and mucus hypersecretion induced by protease allergen.

RESULTS

Identification of Lung Natural Helper Cells in Normal Mice

Flow cytometry analysis of normal B6 mouse lung leukocytes revealed the presence of cells that did not express leukocyte lineage cell-surface markers (Lin: CD3 ϵ , CD19, CD11b, Gr-1, NK1.1, and Ter119) (Figure 1A). The Lin[−] leukocyte population contained a subpopulation that coexpressed stem cell antigen-1 (Sca-1) and stem cell factor (SCF) receptor CD117 (c-kit). These Lin[−]Sca-1⁺c-kit⁺ cells were further divided into two subsets based on coexpression of IL-2R α (CD25) and IL-7R α (CD127) (Figure 1A). The CD25⁺CD127⁺ and CD25[−]CD127[−] subsets made up $\sim 0.35\%$ and $\sim 0.55\%$ of lung lymphocytes (approximately 1.5×10^4 cells and 2.5×10^4 cells), respectively,

in each 4- to 8-week-old naive B6 mouse lung (Figure 1B). Back-gating analysis of the lung $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+ \text{CD25}^+ \text{CD127}^+$ cells indicated that they formed a distinct population, although a small fraction is c-kit^{lo} (Figure S1A available online). When the two subsets of $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^+$ cells were purified and stimulated by a combination of phorbol 12-myristate 13-acetate (PMA) and ionomycin, the $\text{CD25}^+ \text{CD127}^+$ subset produced large amounts of IL-3, IL-5, and IL-13, more moderate amounts of IL-17A, and low amounts of IL-4 and IFN- γ . In contrast, the $\text{CD25}^- \text{CD127}^-$ subset did not produce any detectable amount of the tested cytokines in response to PMA plus ionomycin (Figure 1C). Purified $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$ cells were also capable of producing Th2 cell-type cytokines when stimulated with PMA plus ionomycin (Figure S1B). Thus, the phenotype and the function of lung $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$ cells are similar to those of nuocytes, innate type 2 helper cells, natural helper cells, and MPP type 2 cells in the gut (Saenz et al., 2010a). We termed lung $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$ cells lung natural helper (LNH) cells.

Characterization of LNH Cells

Further flow cytometry analysis showed that LNH ($\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$) cells were negative for CD4, CD11c, NKp46, CD34, and CD122 (not shown), whereas they expressed the pan-leukocyte marker CD45, CD90.2 (Thy1.2), the IL-33 receptor chain T1/ST2, and the T cell costimulatory molecule ICOS (Figure 2A), thus excluding CD4^+ lymphoid tissue-inducing (LTi) cells, CD11c^+ dendritic cells (DCs), NKp46^+ NK cells, CD34^+ mast cells, and immature hematopoietic progenitors. LNH cells were similar in morphology to resting lymphocytes, having a small, round shape with a high nuclear to cytoplasm ratio (Figure 2B). Analysis of other primary and secondary immune organs showed that $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$ cells were barely detectable in the bone marrow, lymph node, spleen, and blood (Figure 2C). Furthermore, no $\text{Lin}^- \text{Sca-1}^+ \text{c-kit}^{\text{lo}} \text{CD25}^+ \text{CD127}^+$ cells were detected in peribronchial lymph nodes in naive B6 mice (data not shown), indicating that LNH cells are resident lung lymphocytes. LNH cells were present in $\text{Rag1}^{-/-}$ and non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice, which are deficient for T and B cells, but they are absent in $\text{Rag2}^{-/-} \text{Il2rg}^{-/-}$ and NOD-SCID $\text{Il2rg}^{-/-}$ mice (Figure 2D). The percentage of LNH cells in wild-type (WT) and $\text{Rag1}^{-/-}$ mice were similar (Figure S2A). Furthermore, CD25 was almost exclusively expressed on LNH cells in $\text{Rag1}^{-/-}$ mice (Figure S2B). Therefore, LNH cells in $\text{Rag1}^{-/-}$ mice could be identified by CD25 staining alone. Purified LNH cells from $\text{Rag1}^{-/-}$ mice were functionally competent, secreted Th2 cell-type cytokines when stimulated in vitro (Figure S2C), and produced similar amounts of cytokines as did WT LNH cells (Figure S2D). Although the phenotype of LNH cells was similar to that of early hematopoietic progenitors and gut MPP type 2 cells, LNH cells did not form myeloid cell colonies in vitro and did not differentiate into T, B, or NK cells in the lymphoid differentiation cultures on OP9/OP9-DL1 stroma (Figures S2E–S2H). Culture of LNH cells on OP9 stroma with IL-2 or IL-7 supported survival and maintenance of cell-surface phenotype, but not expansion, and transplanted LNH cells also failed to engraft or differentiate into T, B, or NK cells in NOD-SCID $\text{Il2rg}^{-/-}$ recipient mice (data not shown).

Gene Expression Profile of LNH Cells

To further characterize LNH cells, the global gene expression patterns of purified LNH cells before and after stimulation with PMA plus ionomycin were analyzed by Affymetrix microarray. The gene expression profile of resting LNH cells differed from those of lung macrophages, lung DCs, CD4 T cells, NK cells, $\gamma\delta$ T, and regulatory T cells (Figure 3A). LNH cells expressed high amounts of *Gata3*, *Cd69*, *Il2ra*, and *Rora* and low amounts of *Notch1* and *Rorc* transcripts. As expected from the flow cytometer analysis, LNH cells expressed high amounts of the *c-kit* (*kit*), *CD127* (*Il7r*), and *CD25* (*Il2ra*) transcripts. They also expressed the genes encoding the receptors for IL-2 (*Il2ra* and *Il2rg*), IL-4 (*Il4ra*), IL-25 (*Il17rb*), and IL-33 (*Il1rl1*). However, the *Il1rap* gene encoding the other chain of the IL-33 receptor was not highly expressed in LNH cells. Stimulation of LNH cells with PMA plus ionomycin induced upregulation of IL-3 (*Il3*), IL-4 (*Il4*), IL-17A (*Il17a*), and IL-17F (*Il17f*) transcripts (Figure 3B). Unstimulated LNH cells expressed the IL-5 gene (*Il5*), and it was only slightly upregulated by the stimulation with PMA plus ionomycin, whereas the IL-33R (*Il1rl1*) and IL-25R (*Il17rb*) transcripts were downregulated by the stimulation.

Cytokine-Mediated Stimulation of LNH Cells

LNH cells were purified and cultured in the presence of various combinations of cytokines, including IL-2, IL-3, IL-4, IL-7, IL-15, IL-25, IL-33, TSLP, stem cell factor (SCF), and Fms-like tyrosinekinase ligand (Flt3L). The flow cytometer and microarray analyses mentioned above showed that LNH cells expressed the receptors for IL-2, IL-4, IL-7, and IL-25, whereas IL-33 has been reported to be important in stimulating other innate helper cells. Unlike gut natural helper cells, which readily respond to IL-2, IL-7, and IL-33 alone (Moro et al., 2010), purified LNH cells produced only low levels of Th2 cell-type cytokines in response to IL-33 alone (Figure 4A). Although IL-33 is required, costimulation with other cytokines was critical in the activation of purified LNH cells. Combinations of IL-33 and IL-2, IL-7, or TSLP resulted in very large amounts of IL-5 (>0.4 pg/cell) and IL-13 (~1.5 pg/cell) production. IL-25 failed to activate LNH cells by itself or with IL-33 but enhanced IL-13 production by LNH cells stimulated with IL-33 plus IL-7. IL-7 had a dampening effect on IL-33 plus TSLP stimulation. None of the cytokine combinations induced IL-3, IL-4, or IL-17A production from purified LNH cells (data not shown).

Because the activation of LNH cells might require factors derived from the lung microenvironment, we employed lung explant cultures, which mimic the physiological state of the lung in vivo (Henjakovic et al., 2008). Freshly isolated lungs were cut into slices of approximately 0.5 mm thickness and cultured in vitro with cytokines, and the amounts of IL-17A, IL-5, and IL-13 secreted into the media were measured. IL-25 alone or IL-2 plus IL-7 readily induced IL-5 and IL-13 (Figure 4B) secretion by the lung explants prepared from WT and $\text{Rag1}^{-/-}$ mice, which have LNH cells but no B, T, or NK cells. In contrast, $\text{Rag2}^{-/-} \text{Il2rg}^{-/-}$ mouse lung explants, which have no LNH cells or other lymphocytes, did not secrete significant amount of IL-13 or IL-5 upon stimulation with cytokine or PMA plus ionomycin (Figures S3A and S3B). The production of IL-5 was rapid, and substantial amounts of IL-5 were secreted even in unstimulated cultures, although IL-25 alone and IL-2 plus IL-7 significantly

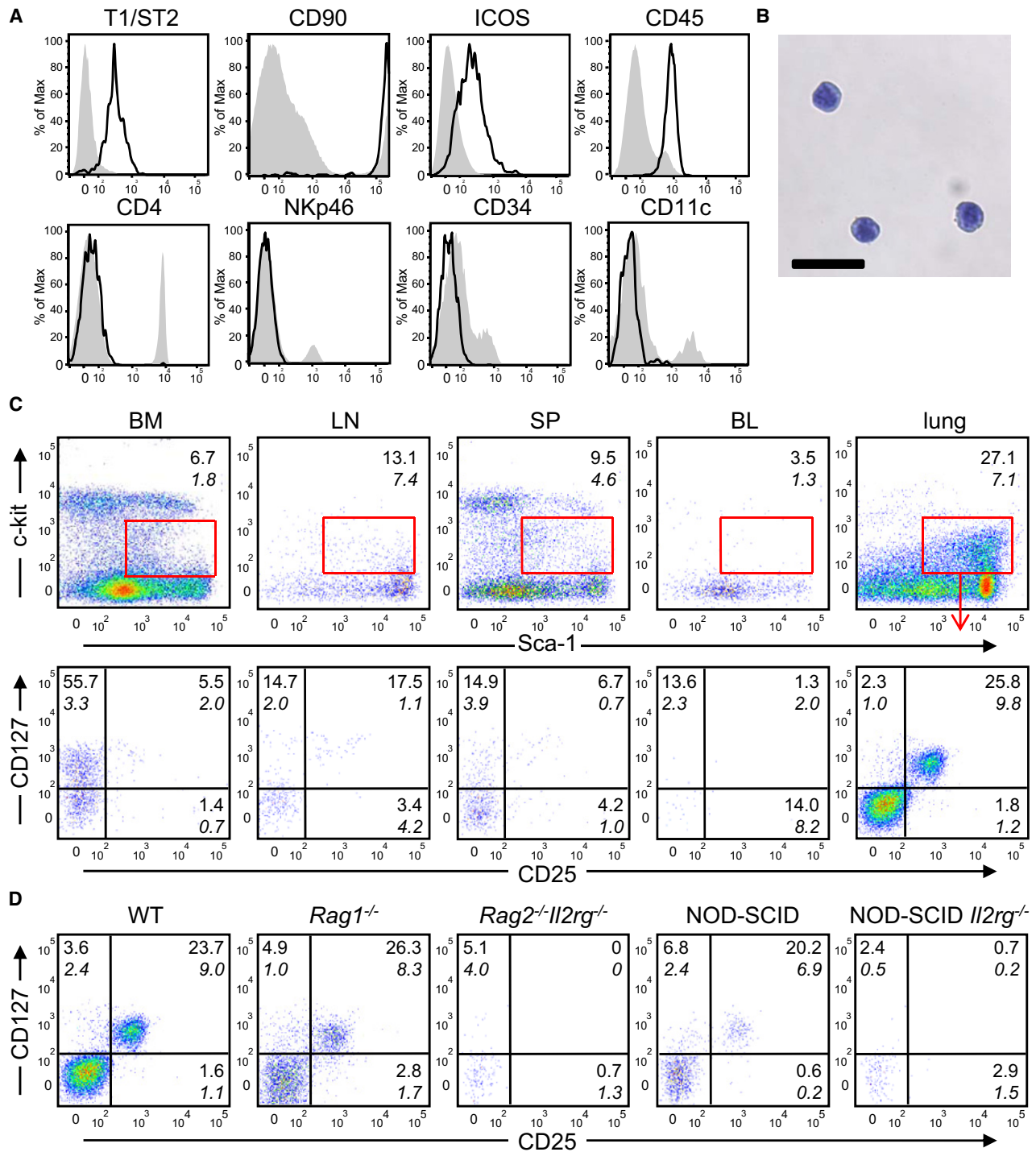


Figure 2. Characterization of LNH Cells

(A) Surface expression of indicated molecules on lung Lin⁻Sca-1⁺c-kit^{+/-}CD127⁺CD25⁺ cells (black line histograms) and control cells (shaded gray histograms) was analyzed by flow cytometry. For CD45 and CD34 staining, BM cells were used as control. For all others, spleen cells were used as control.

(B) The morphology of flow cytometry-purified LNH cells were examined by Diff-Quick staining. Scale bar represents 25 μ m.

(C) Bone marrow (BM), lymph node (LN), spleen (SP), blood (BL), and lung cells were analyzed by flow cytometry for LNH cells. Live (DAPI⁻) lymphocytes (FSC/SSC) were first gated, and Lin⁻ cells were then gated for the analysis of c-kit and Sca-1 expression. Lin⁻Sca-1⁺c-kit⁺ cells were gated as indicated by red boxes (top) and further analyzed for CD25 and CD127 expression (bottom). The numbers in the plots indicate the mean percentages (upper) and SEM (lower in italics) of cells in the gates and quadrants, calculated from three independent analyses.

(D) Lin⁻Sca-1⁺c-kit^{+/-} cells in B6 (WT) and indicated mouse strains (n = 4) were analyzed for the expression of CD25 and CD127.

Data are representative of at least three independent experiments. Numbers in flow cytometry plot are mean (top) and SEM (below in italics). See also Figure S2.

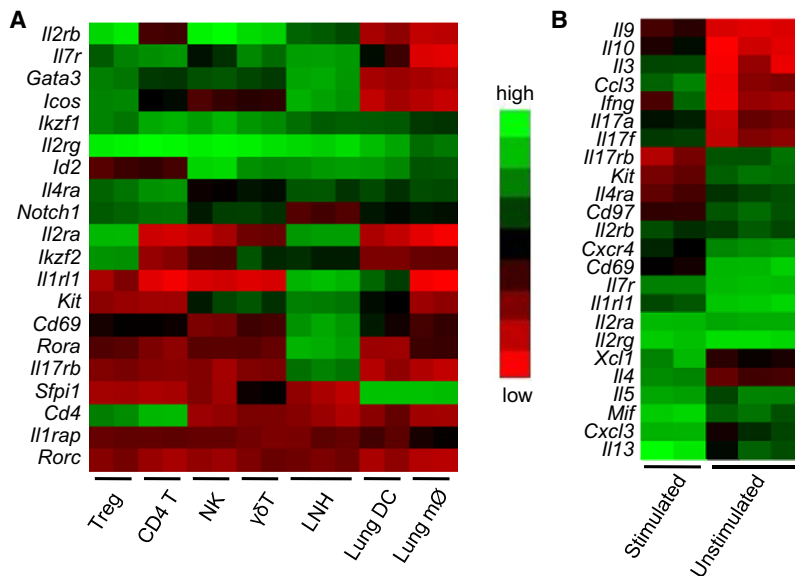


Figure 3. Gene Expression Analysis of LNH Cells

(A) Flow cytometry-purified LNH cells were analyzed for gene expression by Affymetrix microarray. The expression of the indicated genes in LNH cells (triplicate experiment) were compared to available microarray results (duplicates) of other known leukocyte lineages.

(B) Flow cytometry-purified LNH cells were stimulated with PMA plus ionomycin for 12 hr and analyzed for gene expression by microarray. The expression of the indicated genes in stimulated LNH cells (duplicates) were compared to those of nonstimulated samples (triplicates).

increased the amount of IL-5 production. This was consistent with the microarray analysis above, which showed high baseline expression of the IL-5 transcript in LNH cells (Figure 3A). IL-33 did not enhance IL-5 or IL-13 production above the PBS control. None of the tested conditions except PMA and ionomycin resulted in secreted IL-17A from LNH cells in explant cultures (data not shown). Intracellular cytokine staining of lung explants confirmed that CD25⁺ cells in *Rag1*^{-/-} mice were the main producers of IL-5 (Figure 4C). Because LNH cells were the only CD25⁺ cells in the lungs of *Rag1*^{-/-} mice, those results showed that IL-25 or IL-2 plus IL-7 stimulate LNH cells and induce the production of IL-5 and IL-13 in lung explant cultures. Costaining for intracellular IL-17A and IL-5 in *Rag1*^{-/-} mouse explants stimulated with PMA plus ionomycin showed that some LNH cells produced both IL-5 and IL-17A whereas other LNH cells produced one or the other cytokine but not both (Figure 4D). Thus, LNH cells are the major source of IL-5 and IL-13 in cytokine-stimulated lungs. Although a small subset of LNH cells is also capable of producing IL-17A, physiological stimuli that induce IL-17A production by LNH cells are currently unknown.

LNH Cells Are Responsible for Papain-Induced Eosinophilia and Mucus Secretion in RAG1-Deficient Mice

Papain is a protease known to cause occupational asthma (Novey et al., 1979). It also causes asthma-like symptoms in RAG-deficient mice (Oboki et al., 2010). Therefore, we tested whether LNH cells are involved in papain-induced lung inflammation. Intranasal administration of papain (Figure S5A) into WT and *Rag1*^{-/-} mice, which have LNH cells, and into *Rag2*^{-/-}/*Il2rg*^{-/-} mice, which have no LNH cells, showed striking differences among those mice. Eosinophil counts in bronchioalveolar lavage (BAL) were significantly elevated in papain-stimulated WT and *Rag1*^{-/-} mice, compared to *Rag2*^{-/-}/*Il2rg*^{-/-} mice (Figure 5A). Tissue-infiltrating eosinophils were also increased in papain-stimulated WT and *Rag1*^{-/-} mice but not *Rag2*^{-/-}/*Il2rg*^{-/-} mice (Figure 5B). Histological analysis of lung sections

mirrored our flow cytometry findings, showing eosinophilia detected only in lungs of WT and *Rag1*^{-/-} mice treated with papain (Figure S4B). Furthermore, BAL levels of IL-5 and IL-13 were significantly increased in papain-treated WT and *Rag1*^{-/-} mice, but not *Rag2*^{-/-}/*Il2rg*^{-/-} mice (Figure 5C). Periodic acid-Schiff (PAS) staining of mucus in papain-treated lungs also showed mucus production from airway goblet cells in WT and *Rag1*^{-/-} mouse lungs, whereas *Rag2*^{-/-}/*Il2rg*^{-/-} mouse lungs showed no mucus production (Figure 5D). Similar results were obtained with NOD-SCID and NOD-SCID *Il2rg*^{-/-} mice, mirrored by increased serum concentration of IL-5 in WT and NOD-SCID but not NOD-SCID *Il2rg*^{-/-} mice (Figure S4C).

To further investigate the role of LNH cells in T cell-independent lung inflammation, we depleted LNH cells from *Rag1*^{-/-} mice by CD25 antibody injection (Figure S5A). LNH cells identified by CD127 and CD25 (stained by different mAb) expression were barely detectable in the treated mice (Figure 6A). LNH cell-depleted mice showed a significant decrease in allergen-induced eosinophilia (Figure 6B). Histological analysis also showed that LNH cell depletion significantly inhibited lung eosinophilia in papain-treated *Rag1*^{-/-} mice (Figure 6C). PAS staining showed decreased mucus production in LNH cell-depleted *Rag1*^{-/-} mouse lungs (Figure 6D). Similar results were observed in mice stimulated with house dust mite (HDM) extract (Figures S5C and S5D) or IL-25 (Figures S5E–S5G). In addition to depletion, we also transplanted LNH cells into *Rag2*^{-/-}/*Il2rg*^{-/-} mice, followed by papain stimulation (Figure S5B). Transplanted LNH cells were detectable in *Rag2*^{-/-}/*Il2rg*^{-/-} mouse lungs (Figure 6E) and promoted allergen-induced eosinophilia (Figure 6F). Enhanced mucus production was also observed in papain-stimulated *Rag2*^{-/-}/*Il2rg*^{-/-} mouse lungs after LNH cell transplant (Figure 6G). Thus, LNH cells are critical for papain-induced lung eosinophilia and mucus hyperproduction.

Papain Stimulation Enhances Th2 Cell-type Cytokine Production from LNH Cells

IL-5 and IL-13 are known to drive eosinophil maturation and infiltration, goblet cell hyperplasia, and mucus secretion. To investigate the role and regulation of LNH cells in response to papain, we stimulated lung explants from WT, *Rag1*^{-/-}, and *Rag2*^{-/-}/*Il2rg*^{-/-} mice and assessed IL-25, IL-33, TSLP, IL-5, and IL-13 production by ELISA. Papain induced IL-5 and IL-13 secretion in WT and *Rag1*^{-/-} but not in *Rag2*^{-/-}/*Il2rg*^{-/-} mouse lung explant cultures (Figures 7A and 7B), consistent with the

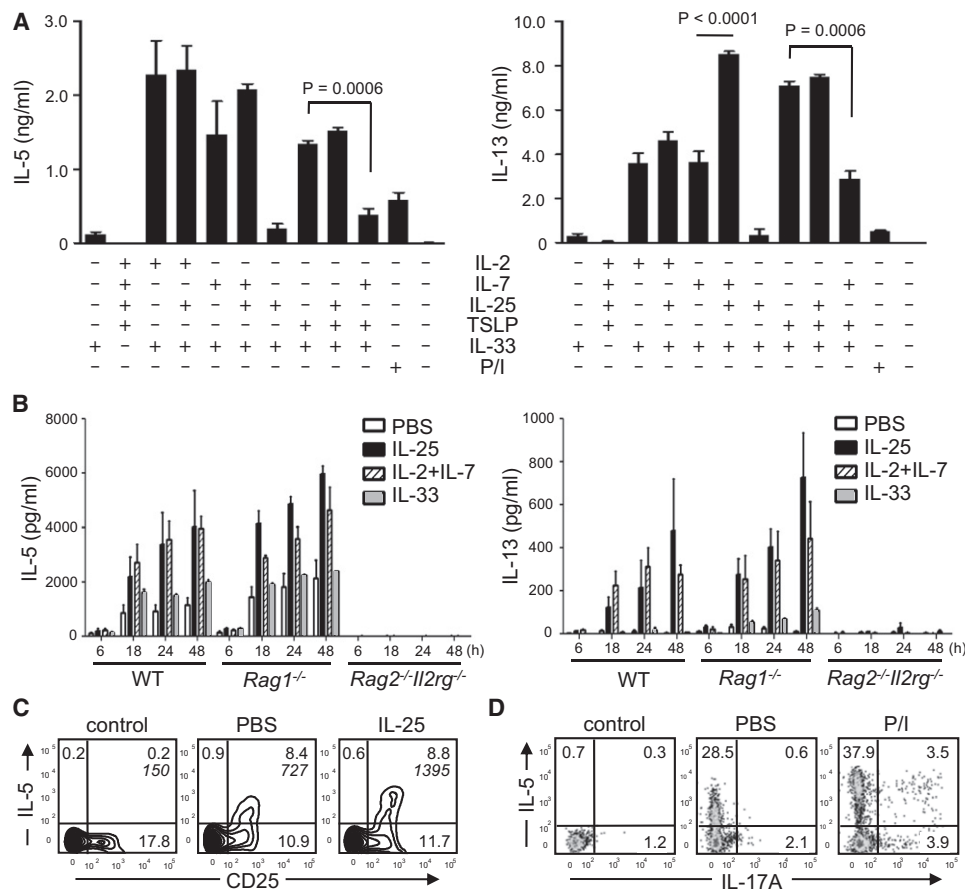


Figure 4. LNH Cells Rapidly Produce Th2 Cell-type Cytokines in Response to IL-33 plus Costimulation

(A) Purified LNH cells (1.0 × 10³ cells in 200 μl media per well) were stimulated with cytokines or PMA plus ionomycin (P/I) as indicated for 3 days, followed by ELISA analysis for IL-5 and IL-13 in culture supernatants.

(B) Lung explants from indicated mouse strains were stimulated with cytokines or PBS. The amounts of IL-5 (left) and IL-13 (right) in the culture supernatants were assayed by ELISA at the times indicated on the x axis.

(C) *Rag1*^{-/-} lung explants were stimulated with IL-25 or PBS for 6 hr, followed by analysis for intracellular IL-5 production. Cells were first electronically gated to exclude blood cells, debris (FSC/SSC), dead cells, and NK cells (NK1.1). Fluorescence minus one (FMO) staining was used as control. The numbers indicate the percentages of cells in the quadrant (top) with mean fluorescence intensity of IL-5-positive cells (italicized below).

(D) *Rag1*^{-/-} mouse lung explants were stimulated with P/I or PBS for 12 hr. Explants were processed and surface-stained as in (C) and CD25⁺ LNH cells were analyzed for intracellular IL-5 and IL-17A by flow cytometry. Fluorescence minus intracellular stain was used for control. The numbers in the plots show the percentage of cell in each quadrant.

Data are representative of at least three independent experiments (mean and SEM in A and B). See also Figure S3.

above in vivo results. These results also indicate that papain induces IL-5 and IL-13 production by lung-resident lymphocytes rather than by recruiting cytokine-producing cells from circulation to the lung. IL-33, IL-25, and TSLP were produced in lung explant cultures from all mouse strains (Figures 7C–7E). Stimulation with papain caused increased IL-25 and TSLP production. IL-33 was constitutively produced in lung explant cultures, and papain stimulation induced a minor but significant increase in concentration. Neutralization of TSLP and/or IL-33 in papain-stimulated explants resulted in a significant reduction of IL-5 and IL-13 production (Figures S6A–S6D). Heat-inactivated papain did not induce cytokine production above PBS levels (Figure S6E).

Intracellular IL-13 staining of cells in papain-treated *Rag1*^{-/-} mouse lung explant cultures showed that the main producers of IL-13 in *Rag1*^{-/-} mouse lung explants were CD25⁺ LNH cells

(Figure 7F). Intracellular IL-5 staining of WT and *Rag1*^{-/-} mouse explants indicated that papain stimulation causes rapid IL-5 production almost exclusively from CD25⁺ LNH cells and not from NK, NKT, or T cells (Figure 7G). The majority of papain-stimulated LNH cells from WT and *Rag1*^{-/-} mice produced IL-5 but not IL-13 whereas small fractions produced IL-13 alone or both IL-5 and IL-13 (Figure 7H). Thus, papain treatment induces production of cytokines by stroma cells, which in turn stimulate LNH cells to produce IL-5 and IL-13. The stimulated LNH cell population is heterogeneous and consists of subsets producing IL-5, IL-13, and both.

DISCUSSION

We have identified Lin⁻Sca-1⁺c-kit^{+/lo}CD25⁺CD127⁺ cells in naive B6 mouse lung and termed them LNH cells. They have

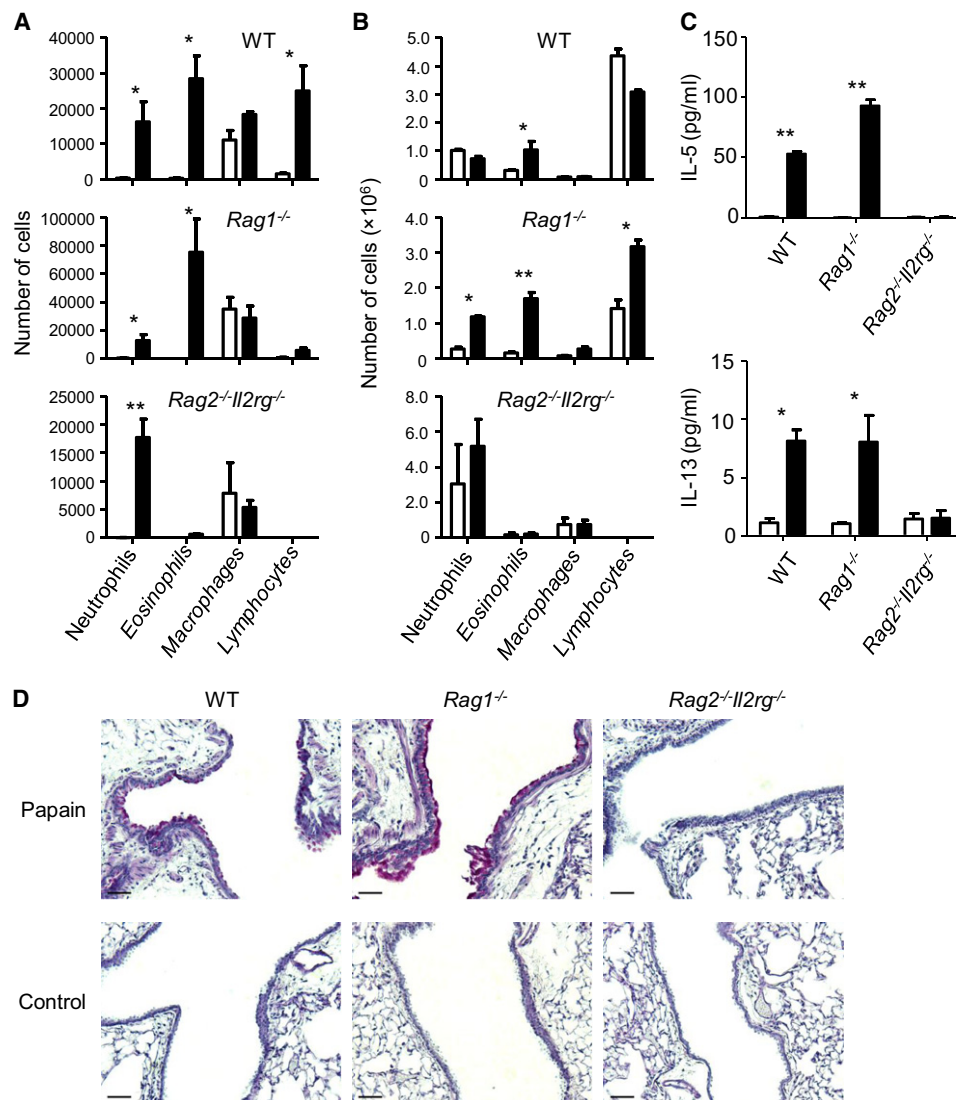


Figure 5. LNH Cells Are Required for T Cell-Independent Eosinophil Infiltration and Mucus Secretion in Papain-Induced Lung Inflammation

(A) Bronchioalveolar lavage (BAL) cells from mice treated with heat-inactivated papain (white) or papain (black) were identified by flow cytometry and quantified. (B) Leukocytes from whole lung tissue from mice treated with heat-inactivated papain (white) or papain (black) were analyzed by flow cytometry and quantified. (C) BAL fluid from heat-inactivated papain- (white) or papain- (black) treated mice was analyzed for IL-5 (top) and IL-13 (bottom) concentration by ELISA. (D) Mucus secretion from heat-inactivated papain- (control) or papain-treated mice was analyzed by PAS staining of formalin-fixed paraffin-embedded lung sections.

Scale bars represent 50 μm. * $p < 0.05$, ** $p < 0.001$ (two-tailed Student's t test). Data are representative of three independent experiments (mean and SEM in A, B, and C). See also Figure S4.

the capacity to rapidly produce large amounts of Th2 cell-type cytokines, and they are critical for induction of eosinophilic lung inflammation by protease allergens, which are known to cause asthma in humans. Whereas T cells are thought to be important for asthma in general, papain has been shown to induce asthma-like symptoms in RAG-deficient mice, indicating the presence of a T cell-independent mechanism. We have demonstrated that intranasal administration of papain into *Rag1*^{-/-} mice, but not *Rag2*^{-/-}*Il2rg*^{-/-} mice, rapidly causes lung eosinophilia, mucus hypersecretion, and elevation in BAL IL-5 and IL-13 levels. LNH cells are present in the former mice but absent in the latter. Furthermore, depletion of LNH cells in

Rag1^{-/-} mice by CD25 mAb injection significantly reduces lung eosinophilia and mucus secretion upon papain administration. Conversely, adoptive transplant of LNH cells into *Rag2*^{-/-}*Il2rg*^{-/-} mice reconstitutes these symptoms, thus providing convincing evidence that LNH cells are critical in T cell-independent allergic lung inflammation. Indeed, LNH cells are the main source of IL-5 and IL-13 in both *Rag1*^{-/-} and WT lung explant cultures treated with papain. Taken together, those results suggest that LNH cells are a critical early source of IL-5 and IL-13 in protease allergen-induced lung inflammation. HDM is another allergen known to depend largely on protease activity (Gregory and Lloyd, 2011). Our results show that LNH cells can

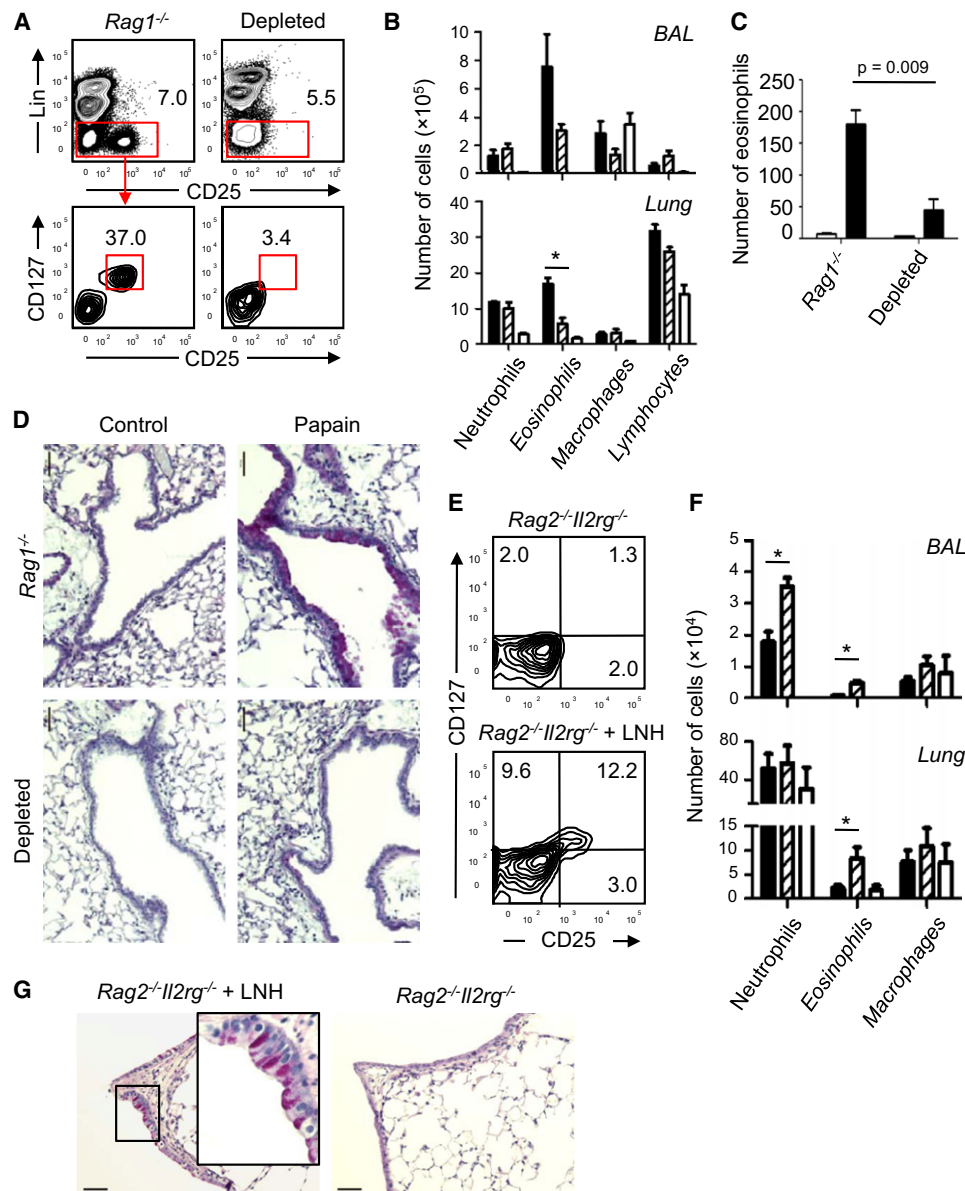


Figure 6. LNH Cell Depletion and Adoptive Transplant Show that LNH Cells Are Required for Papain-Induced Mucus Production and Eosinophil Infiltration

(A) LNH cells were depleted by intraperitoneal injection of CD25 mAb (clone PC61 5.3) into *Rag1*^{-/-} mice. Two days later, lung lymphocytes were stained with Lin-cocktail mAb, CD25 (clone 7D4), and CD127 mAb and analyzed by flow cytometer to confirm LNH cell depletion. The numbers in the plots show the percentages of cells in the indicated gates.

(B) *Rag1*^{-/-} mice were nondepleted and treated with papain (black), LNH depleted and treated with papain (striped), or nondepleted and treated with heat-inactivated papain (white), and leukocytes in BAL and lung tissue were analyzed by flow cytometry and quantified.

(C) Eosinophils were quantified in H&E-stained lung sections of papain- (black) or heat-inactivated papain- (white) treated depleted and nondepleted *Rag1*^{-/-} mice.

(D) Mucus secretion was analyzed by PAS staining of formalin-fixed paraffin-embedded lung sections of heat-inactivated papain- (control) or papain-treated, LNH-depleted, and nondepleted *Rag1*^{-/-} mice. Scale bars represent 50 μm.

(E) In vitro expanded purified LNH cells were intravenously injected (5 × 10⁴ cells per mouse) into *Rag2*^{-/-}*Il2rg*^{-/-} mice, and lymphocytes in the recipient mouse lungs were analyzed by flow cytometry 24 hr after adoptive transplant. Live, Lin⁻Sca-1⁺c-kit^{+/lo} cells were first gated and analyzed for cells expressing CD127 and CD25. Top plot shows control without LNH injection, and bottom plot shows lung lymphocytes of mice injected with LNH cells. The numbers in the plots indicate the percentage of cells in each quadrant.

(F) BAL and lung tissue of *Rag2*^{-/-}*Il2rg*^{-/-} + papain- (black), *Rag2*^{-/-}*Il2rg*^{-/-} + LNH cell + papain- (striped), or *Rag2*^{-/-}*Il2rg*^{-/-} + LNH cell + heat-inactivated papain- (white) treated mice were analyzed by flow cytometry and quantified.

(G) Mucus secretion in lungs was analyzed by PAS staining of papain-treated *Rag2*^{-/-}*Il2rg*^{-/-} mice with or without transplanted LNH cells. Scale bars represent 50 μm, magnified image taken with 40× objective.

**p* < 0.05 (two-tailed Student's *t* test). Data are representative of at least three independent experiments (mean and SEM in B, C, and F). See also Figure S5.

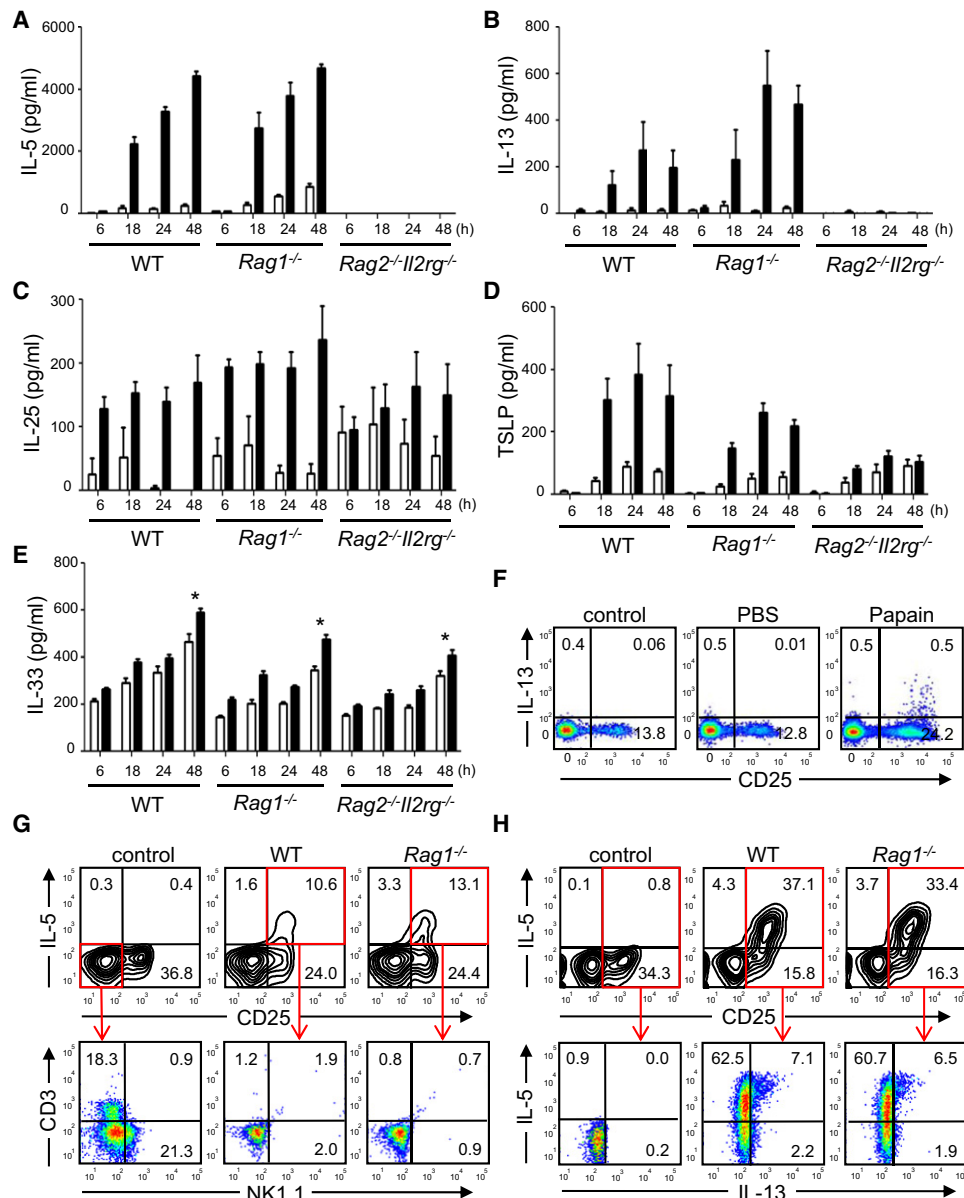


Figure 7. LNH Cells Respond to Lung Stroma-Derived Cytokines and Are the Main Producers of IL-5 and IL-13 after Papain Treatment

(A–E) Lung explants of indicated mouse strains were stimulated with papain (black) or PBS (white). The amount of indicated cytokine in the culture supernatants were assayed by ELISA at the times indicated on the x axis.

(F) Rag1^{-/-} lung explants were cultured for 18 hr with PBS or papain and analyzed for intracellular IL-13 and surface expression of CD25. Cells were first electronically gated to exclude blood cells and debris (FSC/SSC), dead cells, and NK cells (NK1.1). FMO (minus anti-IL-13 staining) of papain-treated explants is also shown (control).

(G) Rag1^{-/-} and WT mouse lung explants were stimulated with papain for 12 hr and analyzed for intracellular IL-5 by flow cytometry. IL-5-producing cells were gated and analyzed for surface expression of CD3 and NK1.1 (below). Cells were first electronically gated to exclude blood cells and debris (FSC/SSC) and dead cells. FMO was used as a negative control.

(H) Costaining for intracellular IL-5 and IL-13 in Rag1^{-/-} and WT mouse lung. Lung explants were stimulated with papain for 18 hr and analyzed by flow cytometry. CD25⁺ cells were gated (top) and analyzed for intracellular IL-5 and IL-13 (bottom). Cells were first electronically gated to exclude blood cells and debris (FSC/SSC), dead cells and NK (NK1.1), and T (CD3) cells. Fluorescence minus intracellular stain was used as a negative control.

*p < 0.05 (two-tailed Student's t test). Data are representative of at least three independent experiments (mean and SEM in A–E). See also Figure S6.

play an important role in mediating a rapid Th2 cell-type response to HDM exposure.

Proteases are important components of many allergens (Reed and Kita, 2004) and thought not only to disrupt mucosa integrity

but also to act on protease-activated receptors and activate airway epithelial cells (Thompson et al., 2001). Activated epithelial cells produce a range of cytokines including TSLP, IL-25, and IL-33 (Strickland et al., 2010). Our current study has shown that

papain-treated lung explants produce IL-25 and TSLP, whereas IL-33 production is enhanced upon papain stimulation. IL-33 is known to be constitutively expressed in endothelial and epithelial cells and is released as an alarmin upon cell damage (Moussion et al., 2008). IL-33 has also been detected in naive mouse lungs (Oboki et al., 2010), which explains the low levels of IL-33 detected in unstimulated lung explant cultures in our study. Neutralization of IL-33 in explant cultures significantly decreases Th2 cell-type cytokine production, and simultaneous neutralization of TSLP and IL-33 effectively blocks IL-13 production. This is consistent with a previous study by Oboki et al. (2010) showing that papain-induced lung eosinophilia is IL-33 dependent. Purified LNH cells are stimulated by a combination of IL-33 and TSLP and secrete very large amounts of IL-5 and IL-13. Therefore, lung stroma-derived IL-33 and TSLP probably stimulate LNH cells and induce IL-5 and IL-13 production in papain-treated lungs, resulting in lung eosinophilia, goblet cell hyperplasia, and mucus production. Interestingly, unstimulated LNH cells express IL-5 transcript and have intracellular IL-5, although purified LNH cells do not secrete IL-5 without stimulation. Thus, LNH cells seem to be poised to respond to cytokines produced by activated lung stromal cells and rapidly release preexisting IL-5.

Intranasal administration of IL-25 or IL-33 is known to induce lung eosinophilia in RAG-deficient mice (Hurst et al., 2002; Kondo et al., 2008). We now have demonstrated that intranasal IL-25 stimulates LNH cells, resulting in Th2 cell-type cytokine production and eosinophil infiltration. IL-33 is also critical for the stimulation of purified LNH cells, although it requires an additional cytokine, IL-2, IL-7, or TSLP, for efficient stimulation of LNH cells in vitro. In contrast, IL-25 on its own or in combination with other cytokines does not stimulate purified LNH cells although it enhances IL-13 production by LNH cells stimulated by IL-33 plus IL-7. On the other hand, RAG-deficient mouse lung explants are stimulated by IL-25 alone, but not IL-33. Baseline production of IL-33 in the explant cultures explains why exogenous IL-33 does not stimulate lung explants and why lung explants produce low levels of IL-5 without additional cytokines, because IL-33 neutralization inhibits IL-5 and IL-13 secretion. However, how IL-25 alone stimulates mouse lung explants or LNH cells in vivo is still unknown. It is possible that IL-25 promotes the production of IL-7 or TSLP by stromal cells, which in combination with IL-33 stimulates LNH cells in the explants cultures. Indeed, neutralization of IL-33 and TSLP inhibits cytokine production by LNH cells in lung explant cultures stimulated by IL-25. Alternatively, the lung microenvironment may provide cell-surface ligands that participate in LNH cell stimulation. LNH cells express the T cell costimulatory receptor ICOS and also express the transcript for 4-1BB (data not shown), but their functional significance is currently unknown.

The phenotype and the function of LNH cells are similar to those of recently discovered innate helper cells (termed MPP type 2), natural helper cells (Moro et al., 2010), nuocytes, and innate type 2 helper cells (Saenz et al., 2010a). MPP type 2 cells were detected by the expression of GFP in IL-4-eGFP reporter mice stimulated with IL-25 (Saenz et al., 2010b). They are Lin⁻Sca-1⁺c-kit^{int}, but unlike LNH cells, they do not express CD127 (Saenz et al., 2010a). Furthermore, *Il4* transcript is absent in unstimulated LNH cells and no IL-4 is secreted from cytokine-

activated LNH cells, although the most striking difference is that MPP type 2 have progenitor capacity and can differentiate into myeloid cells, whereas no progenitor activity is detected with LNH cells. As such, MPP type 2 cells probably constitute an entirely separate lineage. Innate type 2 helper cells were also identified in IL-4-eGFP mice and were found in naive mouse mesenteric LN, spleen, and liver. They are also Lin⁻, but Sca-1⁻, CD122^{lo}, heterogeneous for c-kit expression, and express high baseline *Il4* transcript, unlike LNH cells (Price et al., 2010). It should be noted that LNH cells do not produce detectable level of IL-4 upon stimulation with cytokine combinations or papain-treated lung explant cultures. Nuocytes were identified in the mesenteric LN and spleen of IL-13-eGFP mice upon induction by IL-25 or IL-33 administration or helminth infection (Neill et al., 2010). The phenotype of nuocytes (Lin⁻Sca-1⁺c-kit⁺CD127⁺ICOS⁺) is similar to that of LNH cells. Functionally, nuocytes produce IL-5 and IL-13, but differ from LNH cells in their secretion of substantial amounts of IL-6. Natural helper cells found in fat associated lymphoid cluster (FALC) in the mesentery are Lin⁻Sca-1⁺c-kit⁺CD25⁺CD127⁺ and also produce IL-6 in addition to IL-5 and IL-13 when stimulated (Moro et al., 2010). Thus, LNH cells, nuocytes, and FALC natural helper cells are similar to each other and they probably belong to the same innate lymphocyte lineage, although differences in cytokine production capacity and some cell-surface markers are evident. Most notably, IL-6 is not produced by LNH cells although IL-17A is. It is possible that the differences are partly due to different ways of identifying those cells. MPP type 2, nuocytes, and innate type 2 helper cells were identified by the reporter eGFP expression upon stimulation or helminth infection, and thus probably are activated cells and might have migrated into the affected tissue, whereas FALC natural helper cells and LNH cells were identified in naive mice and probably are unstimulated and tissue resident. It is also possible that they may be in different stages of development in the same cell lineage. Alternatively, their phenotypes and functions may be influenced by the microenvironment, as indicated by the fact that they are located in different anatomical sites.

The recently described Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells, also termed natural helper cells, in BALB/c mouse lungs have been shown to play a role in influenza virus-induced airway hyperreactivity (Chang et al., 2011). Although Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells may partially overlap with LNH cells in our study, significant differences in phenotype and function are also apparent. Most strikingly, Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells are far less efficient in Th2 cell-type cytokine production than LNH cells. Purified Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells (4 × 10⁴ cells per well) stimulated with IL-33 plus IL-2 produce less than 500 pg/ml IL-13 and less than 125 pg/ml IL-5 whereas purified LNH cells (10³ cells per well) stimulated in the same way produce almost 4 ng/ml IL-13 and >2 ng/ml IL-5. In fact, it is unclear whether Lin⁻ST2⁺c-kit⁺Sca-1⁺ cells in BALB/c mouse lungs are the major source of Th2 cell-type cytokines upon influenza virus infection; lung macrophages also produce significant levels of IL-13, with at least 12,500 IL-13⁺ macrophages per lung, compared to 200 Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells. The inability to produce a significant amount of IL-5 probably explains why lung Lin⁻ST2⁺Sca-1⁺c-kit⁺ cells do not induce eosinophilic lung inflammation, even upon adoptive transplantation of in vivo expanded cells at

2,000 times more than naive cell numbers per animal. Eosinophilic inflammation induced by IL-5 is a hallmark Th2 cell response, and the lack of eosinophilia in influenza virus infection suggests that Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells are unlikely to be involved in Th2 cell-mediated lung inflammation. Furthermore, viral infection did not appear to stimulate IL-13 production from Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells because artificial stimulation with PMA plus ionomycin was required. It is possible that viral infection may induce macrophage IL-33 production and prime Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells in BALB/c mice. In our study, LNH cells are the main, if not the only, source of IL-5 and IL-13 in papain-treated *Rag1*^{−/−} mouse lungs as indicated by intracellular cytokine staining as well as depletion of LNH cells. Depletion of Lin[−]ST2⁺Sca-1⁺c-kit⁺ by anti-CD90.2 injection results in significant decrease in virally induced airway hyperreactivity in *Rag2*^{−/−} mice, but the effect is complicated by the presence of other CD90-positive cells in the lung, most notably NK cells (Koo et al., 1980), which respond to viral infections (Mandelboim et al., 2001). Because detailed phenotypic analysis of Lin[−]ST2⁺c-kit⁺Sca-1[−] population is lacking, it is difficult to directly compare the two populations. Nevertheless, the inefficient Th2 cell-type cytokine production by Lin[−]ST2⁺Sca-1⁺c-kit⁺ cells clearly separates them from LNH cells and other innate Th2 cell-type cytokine-producing lymphocytes.

The gene expression profile of LNH cells is very different from those of lung macrophages and DCs and indicates their distinct lymphoid origin. Interestingly, LNH cells do not express the *Rorc* gene encoding ROR γ , a member of the ROR family of nuclear hormone receptors, known to be important for Th17 cells (Ivanov et al., 2006). The lack of *Rorc* expression also separates LNH cells from another family of innate lymphocytes that includes LTi-like cells and IL-22-producing NK-like cells (Sawa et al., 2010). Those innate lymphocytes express ROR γ t, an isoform of ROR γ , and produce IL-22.

The lung is a unique immune site because it is constantly exposed to potential pathogens. Innate lymphocytes that are capable of rapidly responding to infections are probably important for the lung immune system. LNH cells comprise a distinct population of innate lymphocytes that rapidly respond to lung epithelium-derived cytokines. Thus, innate lymphocytes in the lung can be divided into two functionally distinct populations, namely cytotoxic NK cells and cytokine-producing NH cells, similar to cytotoxic CD8 T cells and helper CD4⁺ T cells in the adaptive immune system.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (WT), NOD-SCID, and NOD-SCID *Il2rg*^{-/-} mice were maintained in the BCCRC pathogen-free animal facility. B6.*Rag1*^{-/-} mice were purchased from The Jackson Laboratories. B6.*Rag2*^{-/-}*Il2rg*^{-/-} mice were purchased from Taconic Farms. Mice were used at 4–8 weeks of age. All animal use was approved by the animal care committee of the University of British Columbia, and animals were maintained and euthanized under humane conditions in accordance with the guidelines of the Canadian Council on Animal Care.

Antibodies, Reagents, Flow Cytometry Sorting, and Analysis

Phycoerythrobilin (PE)-conjugated anti-CD4, CD11c, CD90, CD122, CD127, FITC-conjugated anti-CD3ε, CD19, NK1.1, Mac-1, Gr-1, Ter119, MHCII, CD45.2, Allophycocyanin (APC)-conjugated anti-CD117, IL-5, CD25, Alexa

647-conjugated anti-CCR3, PerCP-cy5.5 conjugated anti-CD25, NK1.1, CD3, B220, and PE.cy7-conjugated Sca-1 were purchased from BD Biosciences, and PE-conjugated anti-NKP46, ICOS, IL-5, and IL-13 was purchased from eBioscience. FITC-conjugated anti-T1/ST2 was purchased from MD Bioproducts. Propidium iodide (PI), eFluor 780 (eBioscience), or DAPI reagents were used to exclude nonviable cells. IL-2, IL-7, IL-12, IL-25, IL-33, SCF, and TSLP were purchased from eBiosciences, IL-4 was purchased from STEMCELL Technologies, and PMA and ionomycin were purchased from Sigma Aldrich. Polyclonal goat anti-mouse IL-33 IgG was purchased from R&D Systems. BD Caliber (Cytek 6 color upgrade) and Canto II were used for phenotypic analysis, BD FACS Aria II was used for cell sorting and phenotypic analysis, and FlowJo v. 8.6 was used for data analysis.

Primary Leukocyte Preparation

Cell suspensions were prepared from lung, spleen, LN, blood, or BM tissue as described (Veinotte et al., 2008).

Isolation of LNH Cells

Lung cells were incubated with 2.4G2 to block Fc receptors and then stained with FITC-conjugated lineage marker mAbs (CD3, CD19, NK1.1, Mac-1, GR-1, and Ter119), PE-CD127, PE.cy7-Sca-1, PerCP-cy5.5-CD25, and APC-CD117 and purified by flow cytometry.

Cytokine Production Assay

Flow cytometry-purified B6 or *Rag1*^{-/-} mouse LNH cells were cultured in 200 μ l RPMI-1640 media containing 10% FBS, penicillin and streptomycin (P/S), and 50 mM 2-mercaptoethanol (2ME) at 37°C. Cells were stimulated with IL-25 (10 ng/ml), PMA (30 ng/ml) and ionomycin (500 ng/ml), IL-2 (1000 U/ml), IL-7 (10 ng/ml), TSLP (10 ng/ml), and IL-33 (10 ng/ml).

Lung Explant Culture

Mice were sacrificed by CO₂ asphyxiation. Lungs were instilled via 18G catheter with 1.5 ml 1% low melting point agar in RPMI-1640 + 10% FCS, 2-ME P/S at 37°C and cooled on ice. Lungs were sliced by razor into ~0.5 mm thick sections placed in 2 ml RPMI-1640, 10% FCS, 2ME, P/S, and stimulated with IL-25 (10 ng/ml), PMA (30 ng/ml) and ionomycin (500 ng/ml), papain (10 µg/ml), IL-2 (1000 U/ml), and IL-7 (10 ng/ml). Lung explants from one animal were divided into six separate cultures; total cell number per culture was counted to allow normalization of ELISA data. For intracellular cytokine staining, Golgi-Plug (BD Biosciences) was added to the lung explant cultures 6 hr before collection, and lung explants were passed through a 70 µm filter to make single-cell suspension.

Intracellular Staining

Intracellular staining for IL-17A, IL-5, and IL-13 was performed with the Cytofix/Cytoperm Plus kit (BD Biosciences) according to the manufacturer's protocol. Dead cells were stained with eFluor 780 (eBioscience) fixable viability dye before fixation/permeabilization and were gated out during analysis.

ELISA Assay

IFN- γ , IL-5, IL-6, IL-12, IL-13, IL-17A, IL-25, IL-33, TSLP (eBioscience), IL-4 (R&D Systems), and IL-3 (BD Bioscience) ELISAs were performed according to the manufacturer's protocol.

RNA Isolation and Microarray

Total RNA was isolated from flow cytometry-purified unstimulated cells or cells stimulated for 12 hr with PMA plus ionomycin by Trizol (Invitrogen). Agilent Bioanalyzer 2100, RNA amplification, and microarray services were performed by McGill University and Génome Québec Innovation Centre with the Affymetrix Mouse Gene 1.0 arrays. All data analysis was performed with FlexArray 1.5 (Genome Quebec). Microarray data sets for other cells were obtained from data assembled by the ImmGen consortium (Heng and Painter, 2008).

In Vivo Papain Stimulation, LNH Cell Depletion, and Adoptive Transplant

For LNH cell depletion, mice were twice injected intraperitoneal every 24 hr with 200 μ g of anti-CD25 produced from PC61 5.3 hybridoma 2 days before papain stimulation. For LNH cell adoptive transplant, LNH cells were purified

from WT mice by flow cytometry and expanded in IL-25 and IL-33 (10 ng/ml) for 5 days. Expanded LNH cells or PBS control were injected by tail vein on day 0. Mice were anesthetized by isoflurane inhalation, followed by intranasal injection of papain or heat-inactivated papain (10 μ g) in 40 μ l of PBS on days 0–2. Blood was collected from the tail vein on days 1 and 2 and cardiac puncture on day 3. Mice were sacrificed on day 3 and lungs and BAL (1 ml PBS) were collected or airways were instilled with 50:50 Tissue-Tek O.C.T. Compound/PBS (Adwin Scientific) and fixed in formalin. Lung tissue was processed as described previously and lung and BAL cells were then counted and identified by flow cytometry (van Rijt et al., 2004). Fixed lungs were embedded in paraffin and processed for H&E or PAS staining by the Centre for Translational and Applied Genomics (Vancouver, Canada).

Statistics

Data were analyzed with GraphPad Prism 5 (GraphPad Software). A Student's *t* test was used to determine statistical significance between groups, with $p \leq 0.05$ being considered significant. The statistical analysis of microarray results was carried out with FlexArray (Genome Quebec).

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number GSE6057.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at [doi:10.1016/j.immuni.2011.12.020](https://doi.org/10.1016/j.immuni.2011.12.020).

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Supplemental Information

Lung Natural Helper Cells Are a Critical Source of Th2 Cell-type Cytokines in Protease Allergen-Induced Airway Inflammation

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Inventory of supplemental information

Figure S1. Backgating analysis of LNH cells in the lung. This figure compliments Figure 1.

Figure S2. The frequency, phenotype and cytokine producing capacity of LNH cells in *Rag1*^{-/-} mice, and myeloid and lymphoid potential of LNH cells. This figure extends on the finding of LNH cells in *Rag1*^{-/-} mice in Figure 2.

Figure S3. Response of explant cultures to PMA and ionomycin. This figure relates to Figure 4, and shows that PMA and ionomycin induces Th2 cytokine production in explants from WT and *Rag1*^{-/-}, but not from *Rag2*^{-/-}*Il2rg*^{-/-} mice.

Figure S4. The *in vivo* papain stimulation protocol is shown in diagram format. Eosinophil quantification by histology following papain stimulation. Increase in serum IL-5 concentration following papain stimulation. This figure compliments the *in vivo* response to papain results shown in Figure 5.

Figure S5. Intranasal HDM and IL-25 stimulation. Panels A and B illustrates the LNH cell depletion and transplantation protocol, and accompanies Figure 6. Panels C and D show *in vivo* HDM stimulation results. Panels E, F and G show *in vivo* IL-25 stimulation results.

Figure S6. TSLP and/or IL-33 neutralization, and response of explant cultures to heat-inactivated papain and PBS are shown to confirm negative control. This figure compliments the explant culture results shown in Figure 7.

Supplemental Experimental Procedures

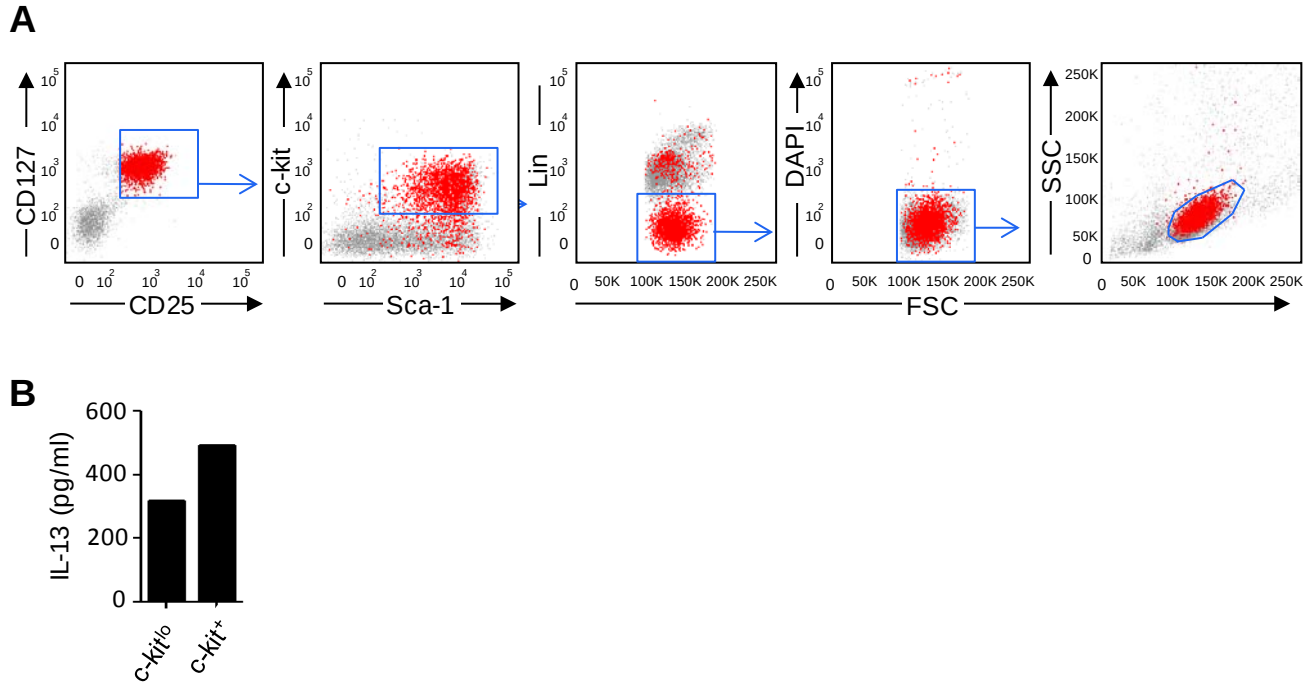


Figure S1. Backgating analysis of LNH cells. **(A)** Backgating analysis of the main Lin⁻Sca-1⁺c-kit⁺CD25⁺CD127⁺ population was conducted in FlowJo. A small number of Lin⁻Sca-1⁺c-kit^{lo}CD25⁺CD127⁺ cells are present in the lung. **(B)** FACS purified Lin⁻Sca-1⁺c-kit^{lo}CD25⁺CD127⁺ and Lin⁻Sca-1⁺c-kit⁺CD25⁺CD127⁺ were stimulated with PMA plus ionomycin (1.0×10^3 cells per well) and culture supernatant was analyzed for IL-13 production after 3 days. Mean of two independent experiments shown.

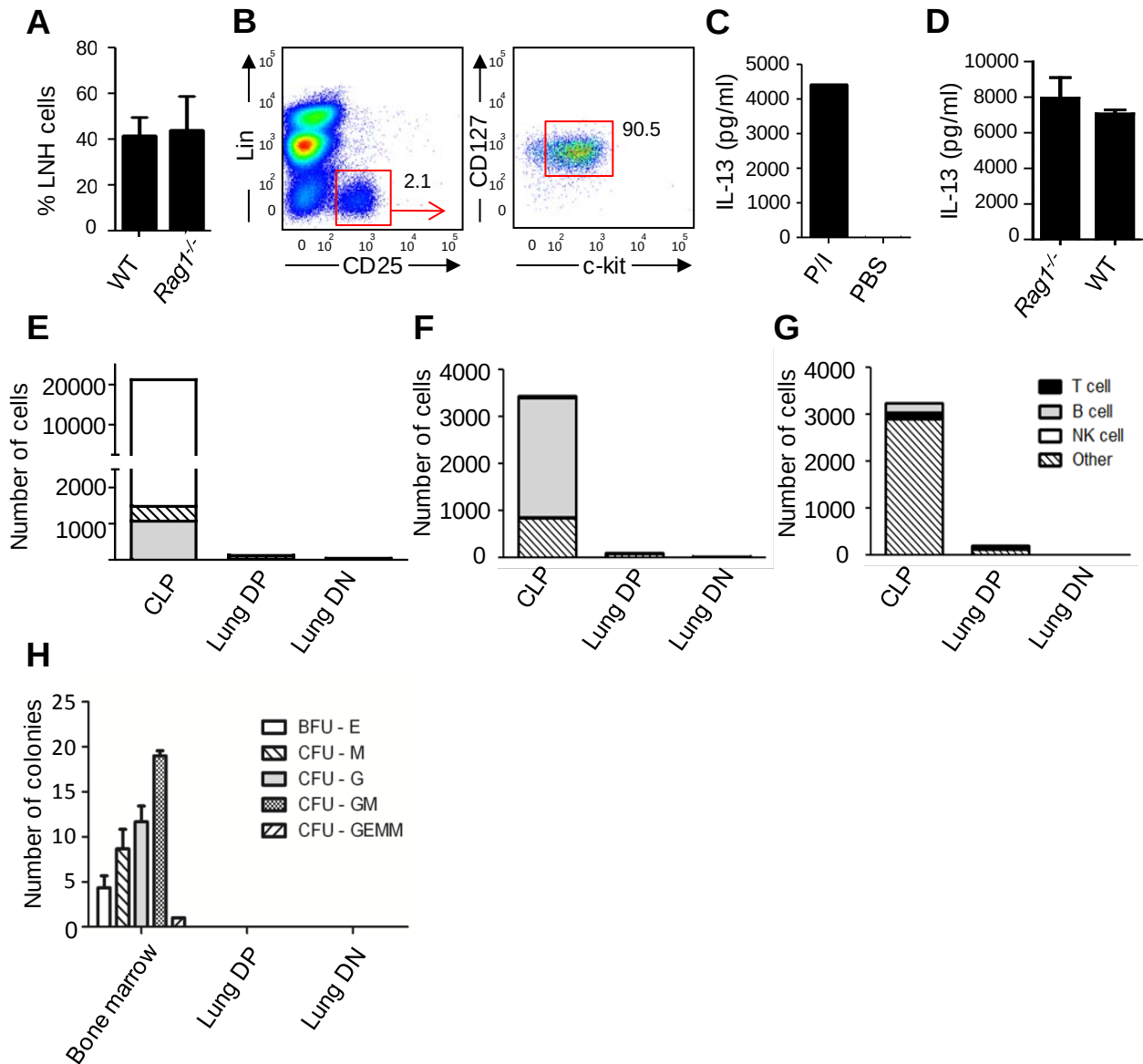


Figure S2. *Rag1*^{-/-} mice have functionally competent LNH cells. **(A)** The percentage of LNH cells amongst Lin⁻Sca-1⁺c-kit^{+/lo} cells in WT and *Rag1*^{-/-} mice was compared. **(B)** CD25 is expressed exclusively on LNH cells in *Rag1*^{-/-} mice. *Rag1*^{-/-} lung cells were analyzed by flow cytometry, plot shown was electronically gated for lymphocytes by FSC/SCC and live cells by DAPI-positive cell exclusion. Result shown is representative of at least 3 independent experiments. **(C)** *Rag1*^{-/-} LNH cells are functionally competent. FACS purified *Rag1*^{-/-} lung Lin⁻Sca-1⁺c-kit^{+/lo}CD25⁺CD127⁺ cells (3.5×10^3 cells per well) were stimulated with PMA plus ionomycin for 3 days, followed by ELISA of cell supernatant. **(D)** FACS purified WT and *Rag1*^{-/-} lung Lin⁻Sca-1⁺c-kit^{+/lo}CD25⁺CD127⁺ cells (1.0×10^3 cells per well) were stimulated with IL-33 (10 ng/ml) and TSLP (10 ng/ml) for 3 days, followed by ELISA of cell supernatant. Lymphoid potential of FACS purified lung Lin⁻Sca-1⁺c-kit⁺CD25⁺CD127⁺ (Lung DP), Lin⁻Sca-1⁺c-kit⁺CD25⁻CD127⁻ (Lung DN), or bone marrow common lymphoid progenitors

(CLP) was assayed using *in vitro* differentiation cultures (1000 cells/culture) for NK cell (E), B cell (F) or T cells (G). Cells were harvested after 2 weeks, counted, and analyzed for percent CD3 ϵ (T-cell), CD19 (B-cell) or NK1.1 (NK cell) expression by flow cytometry. Data in bar charts is the mean of 2 independent experiments. (H) Myeloid potential of FACS purified lung DP, lung DN (1000 cells/culture) or whole bone marrow control (20,000 cells/culture) was examined by methylcellulose colony forming assay. Colonies were counted and categorized based on morphology as erythroid (BFU-E), macrophage (CFU-M), granulocyte (CFU-G), granulocyte/macrophage (CFU-GM), granulocyte/erythroid/monocyte/megakaryocyte (CFU-GEMM). Data are representative of 3 independent experiments (mean and SEM in A, D and H).

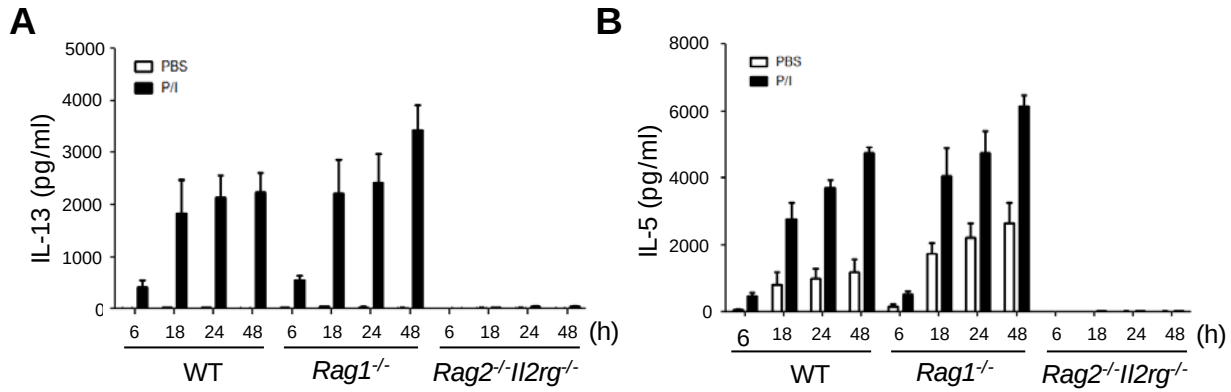


Figure S3. Stimulation by PMA plus ionomycin (P/I) induced IL-5 and IL-13 production from WT and *Rag1*^{-/-} lung explants, but not from *Rag2*^{-/-}*Il2rg*^{-/-} lung explants (1/6 lung in 2 ml media per well). Culture supernatant IL-13 (A) or IL-5 (B) levels were assayed by ELISA at the times indicated on the x-axis. Data are representative of 3 independent experiments (mean and SEM).

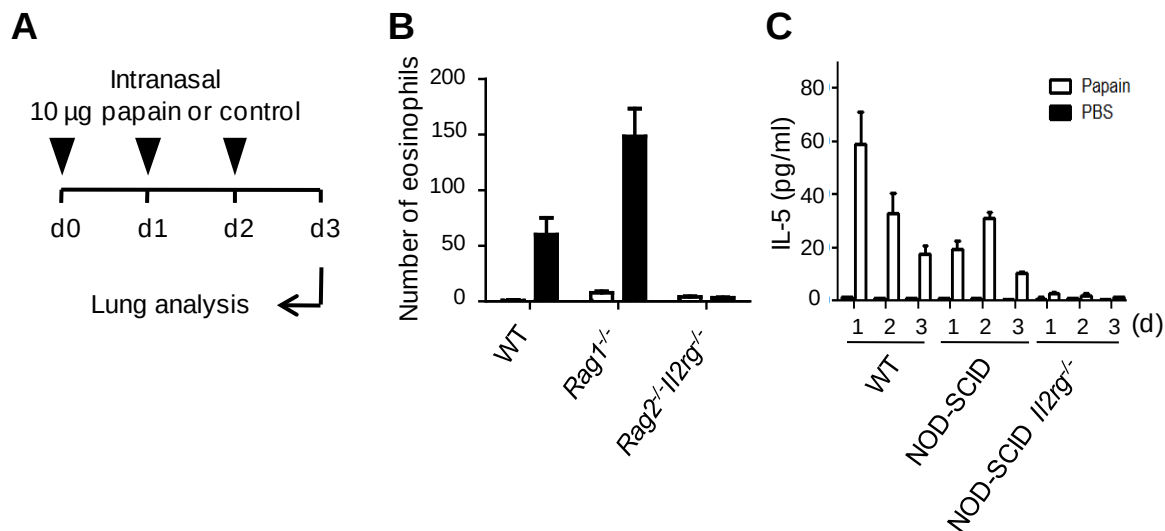


Figure S4. Papain stimulation induces a LNH cell dependent Th2 cell-type response. **(A)** Mice were injected intranasally with papain or control (PBS or heat-inactivated papain) once a day for three days. Mice were sacrificed 24 hours after the last injection and BAL was collected and lungs were dissected for analysis. **(B)** Lung sections prepared from heat-inactivated (white) or papain (black) treated mice were stained with H&E stain. The number of eosinophils in ten 20× fields was counted. **(C)** WT and NOD-SCID but not NOD-SCID *Il2rg*^{-/-} mice respond to intranasal papain treatment by IL-5 production, but not to PBS control. Blood samples were taken on day 1 to 3 after first injection and analyzed for serum IL-5 by ELISA. Data are representative of 3 independent experiments (mean and SEM in B and C).

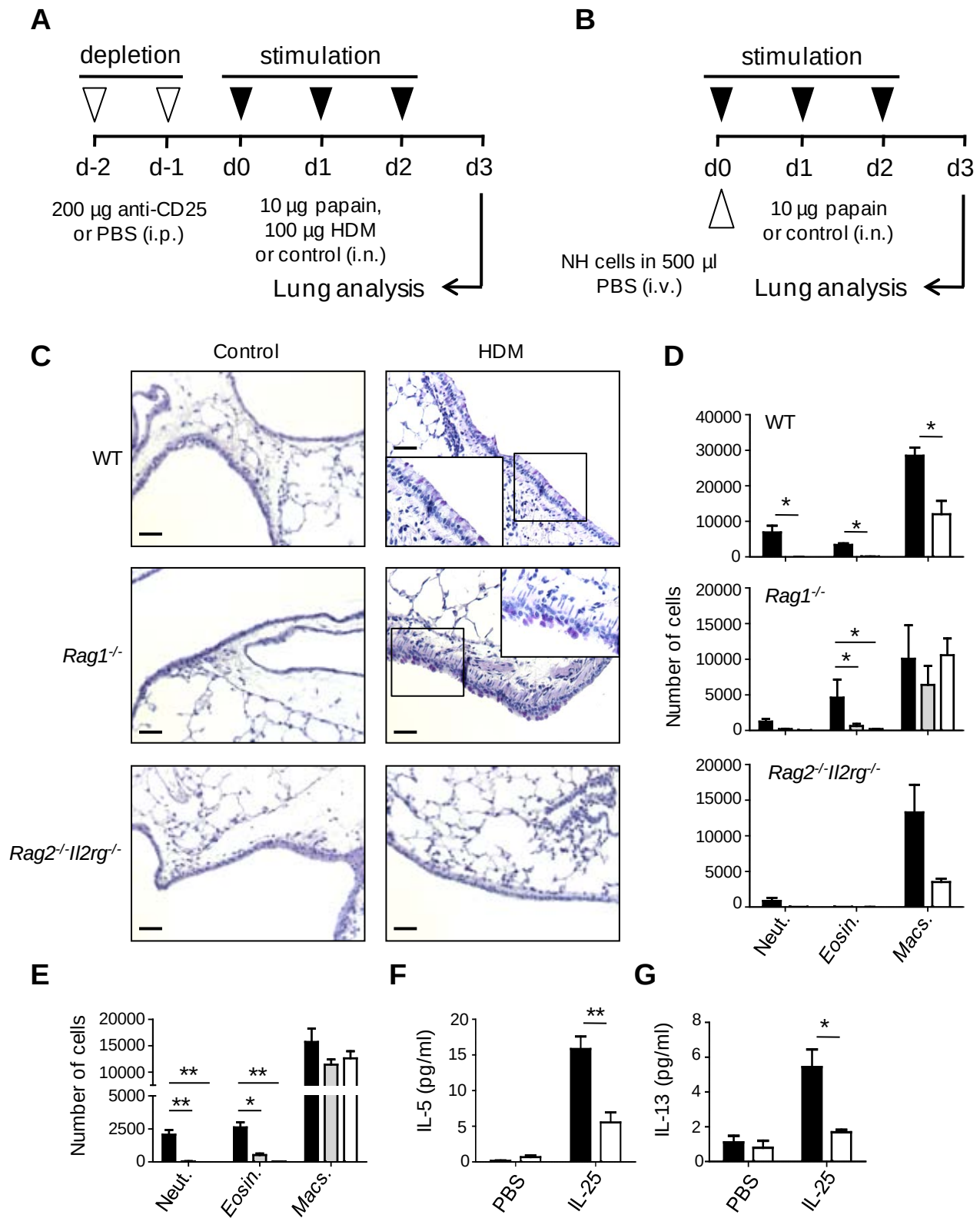


Figure S5. LNH cell depletion in *Rag1*^{-/-} mice followed by intranasal stimulation with protease allergen or house dust mite (HDM). **(A)** LNH cells in *Rag1*^{-/-} mice were depleted by two intra-peritoneal injections of 200 µg anti-CD25 antibody (clone PC61 5.3) or PBS control. LNH cell-depleted and control *Rag1*^{-/-} mice were injected intra-nasally with papain, heat-inactivated papain (control) or HDM once a day for three days. Mice were sacrificed 24 h after the last injection and the lungs and Bronchioalveolar lavage (BAL) were analyzed. **(B)** *Rag2*^{-/-}*Il2rg*^{-/-} mice were injected i.v. with 50,000 LNH cells or PBS, followed by intranasal stimulation with papain or heat-inactivated papain once a day for three days. Mice were sacrificed 24 h after the last injection and the lungs and BAL were analyzed. **(C)** HDM stimulation causes mucus production in WT and *Rag1*^{-/-} mice, but not *Rag2*^{-/-}*Il2rg*^{-/-} mice or PBS (Control) treated mice. Scale bar represents 50 µm, magnified image taken at 63× with immersion oil. **(D)** BAL cells from indicated mice strains treated with PBS (white) or HDM (black) were identified by flow cytometry and quantified. *Rag1*^{-/-} mice were not depleted and treated with HDM (black), LNH-depleted and treated with HDM (grey) or LNH-depleted and treated with PBS (white). **(E)** BAL cells from *Rag1*^{-/-} mice treated with IL-25 (black), LNH-depleted *Rag1*^{-/-} mice treated with IL-25 (grey) or LNH-depleted *Rag1*^{-/-} mice treated with PBS (white) were identified by flow cytometry and quantified. BAL fluid from *Rag1*^{-/-} mice (black) or LNH-depleted *Rag1*^{-/-} mice (white) treated with IL-25 or PBS was analyzed for IL-5 **(F)** or IL-13 **(G)** concentration by ELISA. *p < 0.05 **p < 0.001 (two-tailed Student's t-test). Data are representative of 3 independent experiments (mean and SEM in D, E, F and G).

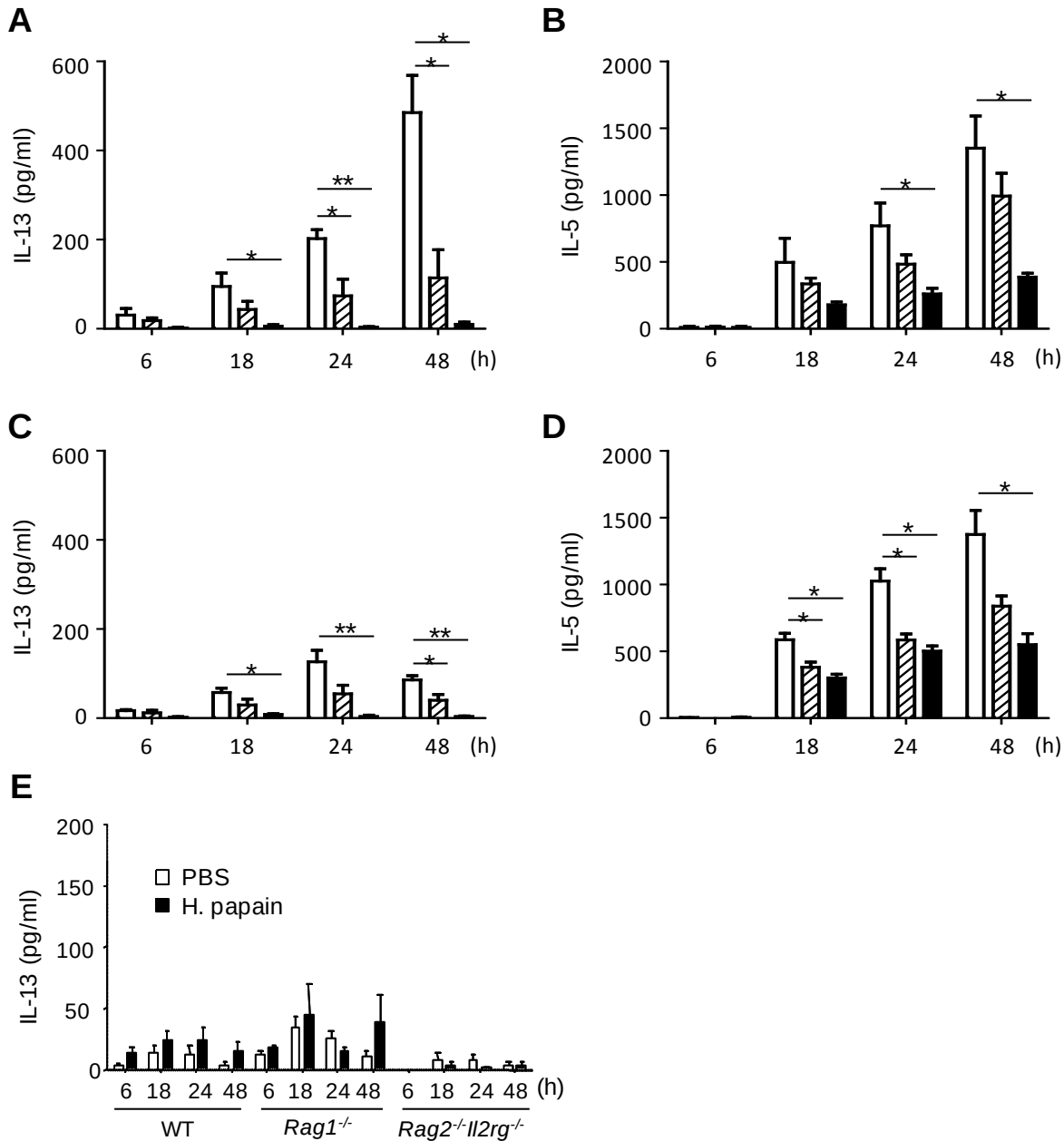


Figure S6. TSLP and/or IL-33 neutralization in explant cultures significantly decreases papain or IL-25 induced Th2 cell-type cytokine production. Explant cultures were prepared from WT mice with anti-IL-33 (striped) or anti-IL-33 plus anti-TSLP neutralizing antibody (black), or IgG control (white). Cultures were stimulated with IL-25 (**A** and **B**) or papain (**C** and **D**). Culture supernatant samples were taken at the indicated time points and analyzed for IL-5 or IL-13 concentration. (**E**) Heat-inactivated papain and PBS were used to stimulate explant cultures (1/6 lung in 2 ml media per well) of the indicated strains, and culture supernatant was collected for IL-13 analysis by ELISA. * $p < 0.05$ ** $p < 0.001$ (two-tailed Student's t-test). Data are representative of 3 independent experiments (mean and SEM).

Supplemental Experimental Methods

***In vitro* differentiation assay.** For lymphoid cell differentiation, FACS sorted LNH cells or BM CLP were seeded onto pre-formed OP9 or OP9-DL1 stroma in 500 μ l alpha-MEM with 10% FBS, P/S, 150 μ M monothioglycerol, 30 ng/ml stem cell factor (SCF), 100 ng/ml recombinant human Flt-3 ligand (Flt3L), 1 ng/ml IL-7, and 25 ng/ml IL-15 for NK cell differentiation. Half of the media was replaced on day 7. Cultures were harvested after 14 days and analyzed by flow cytometer. For myeloid cell differentiation, FACS sorted LNH cells or whole BM was cultured in the Methocult® myeloid differentiation assay (STEMCELL) according to the manufacturer's protocol. Colonies were scored with the aid of an unbiased expert.

Transplantation assays. LNH cells were sorted from B6 mice and injected by tail vein in 500 μ l PBS into NOD-SCID *Il2rg*^{-/-} mice. BM, spleens, and lungs were examined by flow cytometry after 4 weeks. PBS was used as negative control in the transplantation assays.

***In vivo* House Dust Mite stimulation.** Lipolized crude *D.pteronyssinus* (GREER Laboratories, Lenoir NC) was injected i.n. into anaesthetized mice (100 μ g in 40 μ l of PBS) once a day for 3 days. Lungs and BAL were analyzed as previously described.

Intranasal IL-25 stimulation. LNH-cell depleted *Rag1*^{-/-} or non-depleted *Rag1*^{-/-} mice were injected with 5 μ g of carrier-free IL-25 (eBiosciences) in 50 μ l of PBS once a day for 2 days. Mice were sacrificed 24 h after the last injection and BAL was analyzed.

Cytokine neutralization. Lung explants from B6 mice were prepared and cultured as described before. 12 cultures were prepared from a single animal and cultured in 1.5 ml of media with anti-

TSLP (2.5 µg/ml) (R&D Systems) and/or anti-IL-33 (2.5 µg/ml) (R&D Systems). Explants were stimulated with IL-25 (10 ng/ml) (eBiosciences) or papain (10 µg/ml). Supernatant was analyzed for cytokines as previously described.

Eosinophil quantification. Eosinophils were quantified in 20× fields of view of H&E stained lung sections (Axioplan 2 Imaging, Carl Zeiss), captured by camera (Retiga EX, QImaging). Using the digital image to define field boundaries, eosinophils were then positively identified at 63× using immersion oil. Total eosinophil number in ten 20× fields of view was calculated per mouse lung (5 fields of view per section, 2 sections taken at different depths, separated by 500 µm).