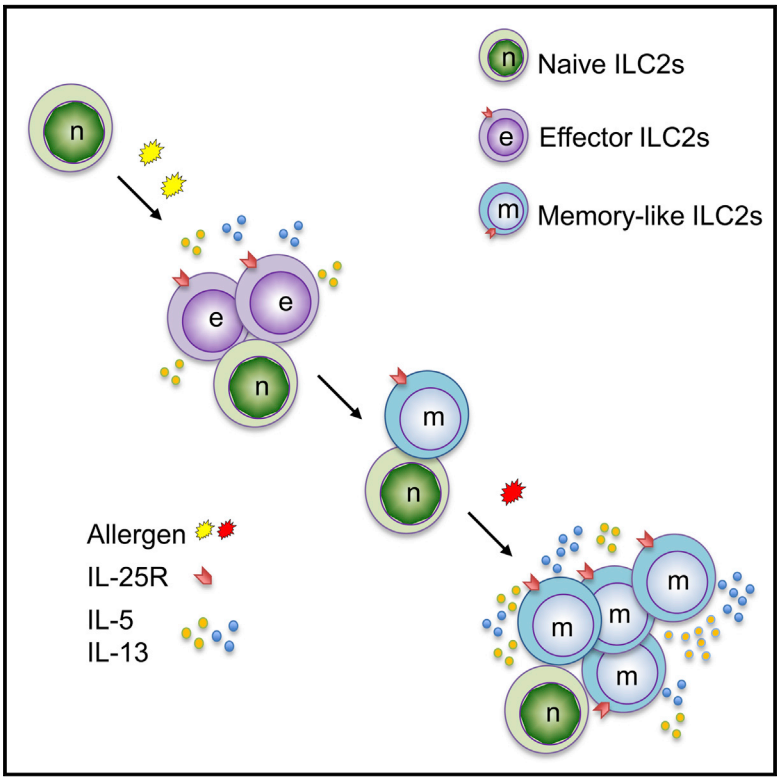
Article

Immunity

Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

Graphical Abstract



Highlights

- Allergen-experienced ILC2s acquire antigen non-specific memory-like properties
- Memory-like ILC2s enhance type 2 lung inflammation upon an unrelated allergen challenge
- Allergen-experienced ILC2s show gene signatures of memory T cells

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In Brief

Group 2 innate lymphoid cells (ILC2s) in the lung are stimulated by inhaled allergens, producing cytokines that contribute to allergic lung inflammation. Takei and colleagues find that allergen-experienced ILC2s respond to unrelated allergens more potently than naive ILC2s and exhibit memory-like properties that may explain why asthma patients are often sensitized to multiple allergens.

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Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

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SUMMARY

Group 2 innate lymphoid cells (ILC2s) in the lung are stimulated by inhaled allergens. ILC2s do not directly recognize allergens but they are stimulated by cytokines including interleukin (IL)-33 released by damaged epithelium. In response to allergens, lung ILC2s produce T helper 2 cell type cytokines inducing T cell-independent allergic lung inflammation. Here we examined the fate of lung ILC2s upon allergen challenges. ILC2s proliferated and secreted cytokines upon initial stimulation with allergen or IL-33, and this phase was followed by a contraction phase as cytokine production ceased. Some ILC2s persisted long after the resolution of the inflammation as allergen-experienced ILC2s and responded to unrelated allergens more potently than naive ILC2s, mediating severe allergic inflammation. The allergen-experienced ILC2s exhibited a gene expression profile similar to that of memory T cells. The memory-like properties of allergen-experienced ILC2s may explain why asthma patients are often sensitized to multiple allergens.

INTRODUCTION

Allergic inflammation is driven by the cytokines interleukin (IL)-4, IL-5, and IL-13. IL-4 promotes the production of IgE by B cells. IgE induces degranulation of mast cells and basophils, resulting in the release of inflammatory mediators including histamine (Steinke and Borish, 2001). IL-5 triggers eosinophil differentiation, recruitment, and activation (Hamelmann and Gelfand, 2001). IL-13 acts on many cell types, including epithelial cells, and promotes mucus hyper-production and tissue remodeling (Wills-Karp, 2004). These cytokines are predominantly produced by T helper 2 (Th2) cells. Upon initial exposure to allergens, some individuals may become sensitized as rare allergen-specific $CD4^+$ T cells proliferate and differentiate into Th2 cells, some of which become long-lived memory T cells. Upon re-exposure to the same allergen, memory Th2 cells rapidly produce large amounts of Th2 cell type (type 2) cytokines, resulting in allergic inflammation.

Whereas the crucial role of Th2 cells in allergic inflammation is well established, group 2 innate lymphoid cells (ILC2s) are also important sources of IL-5 and IL-13 in allergic inflammation (Halim et al., 2012a; Moro et al., 2010). Intranasal injections of the protease allergen papain into naive wild-type (WT) and recombination activating gene 2 (RAG2)-deficient mice induce IL-5 and IL-13 production in bronchoalveolar lavage fluid (BALF), eosinophil infiltration, and mucus hyper-production in the lung (Halim et al., 2012a; Oboki et al., 2010). In contrast, papain injections into ILC2-deficient mice fail to induce strong lung inflammation. Furthermore, transplantation of ILC2s into the ILC2-deficient mice restore the development of type 2 lung inflammation upon papain treatment (Halim et al., 2012b). Stimulation by other allergens including house dust mite extract also results in lung ILC2 stimulation and concomitant production of IL-5 and IL-13 (Gold et al., 2014; Halim et al., 2012b). Epithelial cell-derived cytokines including IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 (Liew et al., 2010) mediate allergen-induced ILC2 activation (Halim et al., 2014; Salmond et al., 2012). Allergic lung inflammation triggered by chitin inhalation is impaired in mice deficient for the receptors for these cytokines (Van Dyken et al., 2014). Papain-induced ILC2 activation and lung inflammation are also severely impaired in IL-33-deficient mice (Halim et al., 2014; Oboki et al., 2010). In addition, purified lung ILC2s are stimulated in vitro by a combination of IL-33 and IL-7 or TSLP. Also, intranasal injections of IL-33 alone strongly stimulate lung ILC2s and induce severe type 2 lung inflammation in vivo (Halim et al., 2014). These findings have led to a model wherein allergen exposure and associated tissue damage result in IL-33 production in the lung, which activates ILC2s and results in allergic lung inflammation that is independent of Th2 cells (Martinez-Gonzalez et al., 2015).

Lung ILC2s also play a role in promoting Th2 cell responses to allergens. Intranasal injections of papain into naive mice stimulate naive $CD4^+$ T cells to differentiate into the Th2 cell pathway. When the papain-primed mice receive challenge injections of papain, large numbers of IL-5- and IL-13-producing Th2 cells are generated in the lung, resulting in severe allergic lung inflammation and high titers of IgE. ILC2-derived IL-13 is essential for effective Th2 cell priming; Th2 cell differentiation in papain-treated naive mice and Th2 cell-mediated allergic inflammation

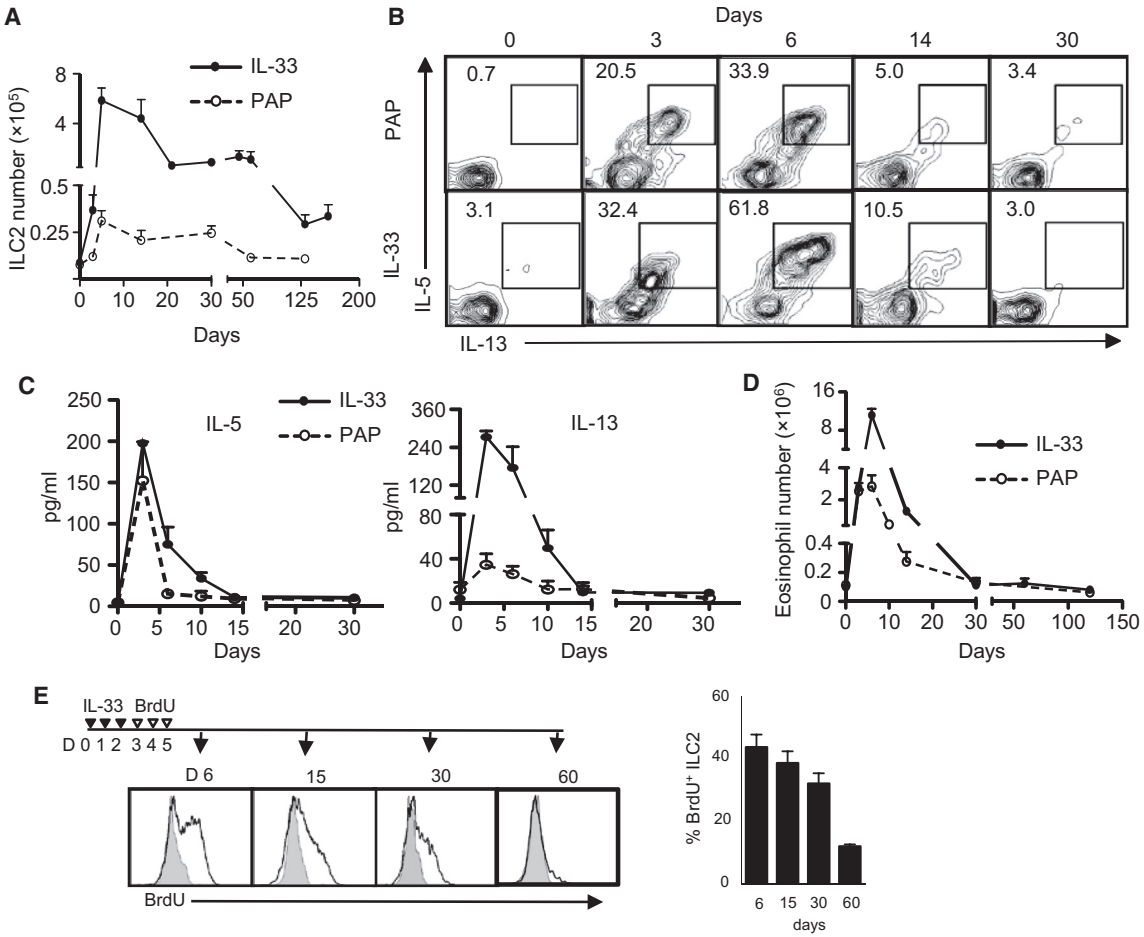


Figure 1. Previously Activated Lung ILC2s Persist after the Resolution of Lung Inflammation

(A and B) Mice received three daily intranasal injections of papain (PAP) or IL-33, and the numbers of lung ILC2s at various time points were determined by flow cytometry and the total numbers of leukocytes in the lung (A). Lung cells at indicated time points were stained for intracellular IL-5 and IL-13 and analyzed by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. ILC2s were gated (as live $CD45^+Lin^-ST2^+Thy1^+$ cells) and intracellular IL-5 and IL-13 fluorescence was plotted on contour plots (B). Numbers in plots indicate the (mean ± SEM) percentages of gated cells among ILC2s.

(C) Amounts of IL-5 and IL-13 in BALF from the mice treated with IL-33 (closed circles, solid lines) and PAP (open circles, dashed lines) were determined by ELISA.

(D) Lung eosinophil numbers at various time points after IL-33 or PAP injections were determined by flow cytometry.

(E) IL-33 and BrdU were intranasally injected into mice as indicated and BrdU-labeled lung ILC2s were analyzed by flow cytometry. Control histogram (shaded) was generated with untreated mice stained with anti-BrdU antibody.

Data represented are the mean ± SEM of three experiments, four to ten mice per group (A, C, D) or representative of two experiments with three mice per group (B, E). *p < 0.05, two-tailed Student's t test. See also Figure S1.

in the challenged mice are impaired in ILC2-deficient mice (Halim et al., 2014).

Thus, although lung ILC2s have been shown to be critically involved in the initiation of allergic inflammation, their role in chronic allergy, which can result in lung diseases including asthma, remains to be elucidated. Here we examine the fate of allergen-stimulated lung ILC2s in mice. We find that after the initial activation and expansion, lung ILC2s undergo a contraction phase and stop producing cytokines, resulting in the resolution of type 2 lung inflammation. A population of ILC2s that persists in the lung and the draining mediastinal lymph node (mLN) display memory-like characteristics and respond more vigorously to unrelated allergen challenge, exacerbating allergic lung inflammation. Our findings suggest that memory-like ILC2s are probably responsible for the sensitization of individuals to multiple allergens.

RESULTS

Papain and IL-33 Elicit Long-Term Changes in the ILC2 Population in the Lung

To study the dynamics of the ILC2 population after stimulation, we gave intranasal injections of IL-33 or papain into naive B6 mice and analyzed lung ILC2s at different time points. Three daily intranasal injections of IL-33 or papain resulted in a rapid increase in the number of ILC2s from fewer than 10^4 in the lung of naive mice to more than 5×10^5 in the IL-33-treated and more than 3×10^4 in the papain-treated mouse lungs (Figure 1A).

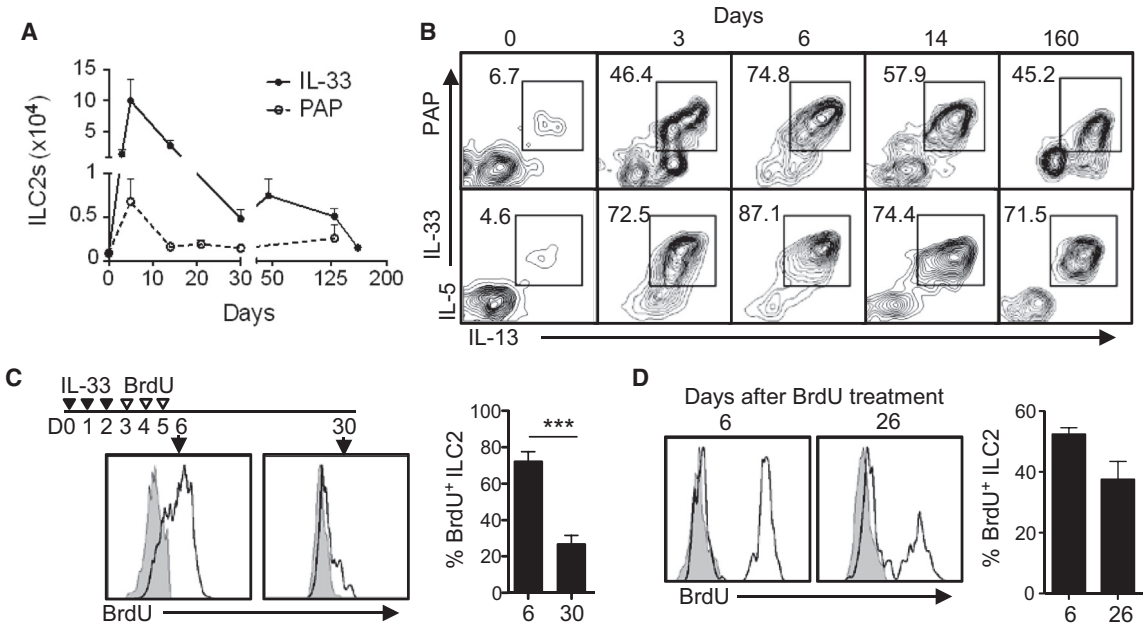


Figure 2. Activated ILC2s Persist in the mLN

(A and B) Mice were treated with IL-33 or papain (PAP) as in Figure 1, and mLN ILC2s at various time points were quantified (A) and analyzed for intracellular IL-5 and IL-13 expression (B) by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. The numbers in the plots show the percentages of cells in the rectangle gates among ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells).

(C and D) Mice received daily intranasal injections of IL-33 and BrdU (C) or PBS and BrdU (D), and BrdU$^+$ mLN ILC2s were analyzed by flow cytometry. Control histogram (shaded) was generated using untreated mice stained with anti-BrdU antibody.

Data represented are the mean ± SEM of three experiments, four to ten mice per group (A) or representative of two experiments with three mice per group (B, C, D). ***$p < 0.001$, two-tailed Student's t test. See also Figure S2.

The stimulated ILC2s produced cytokines as shown by intracellular IL-5 and IL-13 staining (Figure 1B) and the increase in the amounts of those cytokines in BALF (Figure 1C). Accordingly, large numbers of eosinophils infiltrated into the lung (Figure 1D). IL-33 was more potent than papain in stimulating lung ILC2s and induced more cytokine production in the BALF and eosinophil infiltration into the lung. Upon intranasal injections of bromodeoxyuridine (BrdU) into the IL-33-treated mice, most lung ILC2s incorporated BrdU (Figure 1E, histogram). Therefore, the increase in the numbers of lung ILC2s was probably due to proliferation of lung-resident ILC2s rather than recruitment from other tissues.

After the initial expansion, the ILC2 population in the lung started to contract and stopped producing cytokines, and accordingly, cytokine levels in BALF and lung eosinophil numbers declined, indicating the resolution of the inflammation (Figures 1A–1D). Unexpectedly, the numbers of ILC2s in the lung remained much higher in the IL-33-treated (10-fold) and papain-treated (2.5-fold) mice than those in naive mice for more than 4 weeks (Figure 1A). To determine whether the high ILC2 numbers were due to survival of the stimulated ILC2s, we gave intranasal injections of BrdU into the IL-33-treated mice and analyzed ILC2s at various time points (Figure 1E). BrdU-labeled ILC2s were detectable for at least 60 days. However, the intensity of the BrdU staining progressively declined, suggesting that the stimulated ILC2s were slowly dividing. In naive mouse lungs, a lower percentage of ILC2s was labeled by BrdU, which significantly ($p < 0.05$) declined in 20 days whereas the intensity of the BrdU labeling did not decrease much (Figure S1). Altogether, these results suggest that some of the stimulated lung ILC2s live for a long time, resulting in the persistently high ILC2 numbers in the lungs of IL-33-treated mice long after the resolution of the inflammation.

Intracellular Cytokine-Positive ILC2s Persist in the mLN

In naive mice, the mLN was very small and contained fewer than 1,000 ILC2s (873.1 ± 123.8). Upon intranasal injections of papain or IL-33, the mLN enlarged and the numbers of ILC2s in the mLN rapidly increased by more than 100-fold and ~8-fold after the IL-33 and papain treatments, respectively (Figure 2A). The expansion of the mLN ILC2 population was followed by its contraction. The number of ILC2s in the mLN of the papain-treated mice rapidly declined to the level similar to that of a naive mouse whereas ILC2 numbers in the IL-33-treated mice remained higher than those in naive mice for 4 months before eventually returning to the naive mouse level. Most mLN ILC2s in naive mice were negative for intracellular IL-5 and IL-13, whereas the majority of them became IL-5$^+$IL-13$^+$ after the papain or IL-33 injections (Figure 2B). Eosinophils were recruited into the mLN during the expansion phase of ILC2s but the number rapidly declined as the ILC2 population contracted (Figure S2). The percentages of IL-5$^+$IL-13$^+$ ILC2s in the mLN were much higher than those in the lung. Moreover, unlike in the lung, the percentages of IL-5$^+$IL-13$^+$ ILC2s in the mLN remained high for more than 5 months (Figure 2B). However, it seems

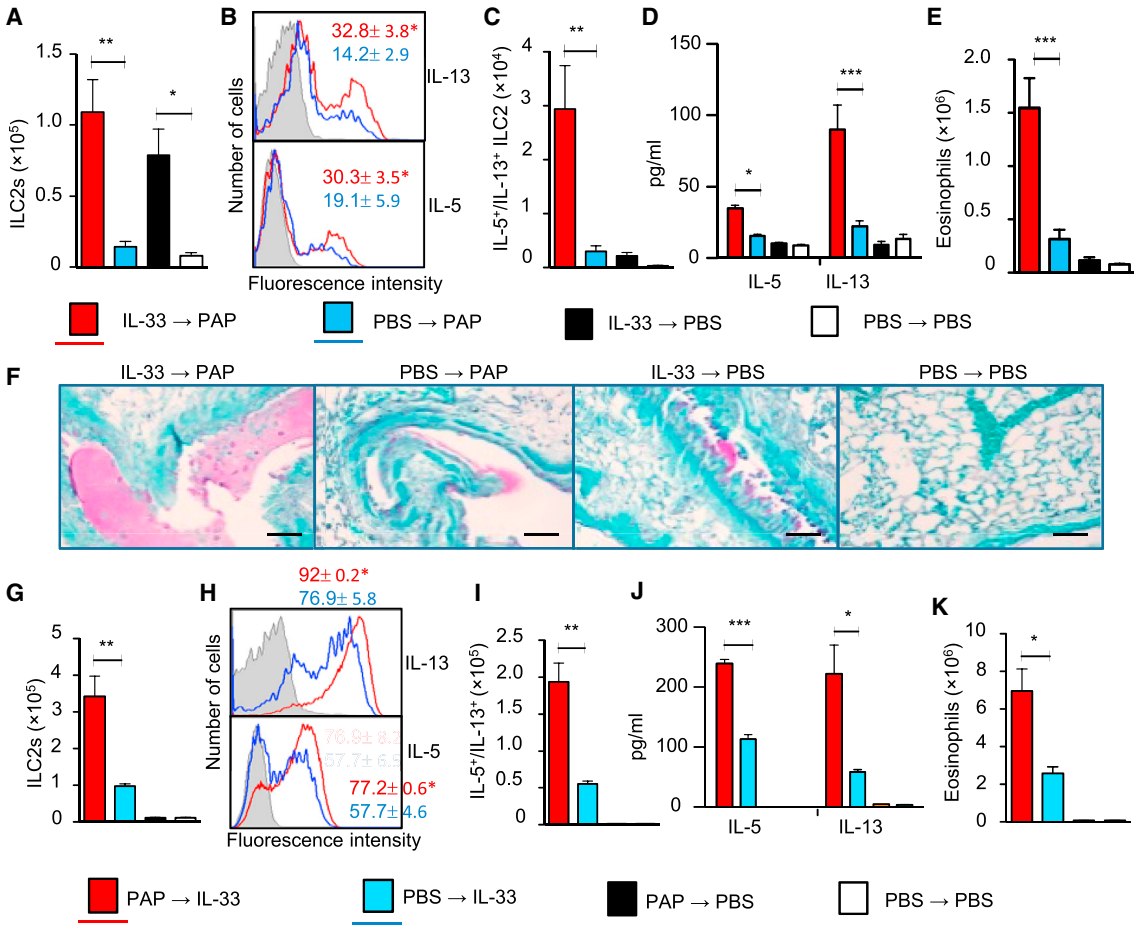


Figure 3. IL-33- or Papain-Experienced Lung ILC2s Respond Vigorously to a Secondary Challenge

(A–F) Mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of papain (PAP) or PBS. Two days later, lung ILC2 numbers (A), intracellular IL-5 and IL-13 staining of lung ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) after 3 hr of re-stimulation with PMA+ionomycin (B), and the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (C) were analyzed by flow cytometry, and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (D). The numbers of eosinophils in the lung were also determined by flow cytometry (E), and lung tissue sections were stained by PAS staining for mucus (F). Scale bar corresponds to 10 μm.

(G–K) Mice received three daily intranasal injections of papain or PBS. Two months later, they received a single intranasal injection of IL-33 or PBS, and ILC2 numbers (G), intracellular IL-5 and IL-13 staining of lung ILC2s after 3 hr of re-stimulation with PMA+ionomycin (H), and the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (I) were analyzed by flow cytometry, and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (J). The numbers of eosinophils in the lung were also determined by flow cytometry (K). Bars in the graphs are color coded for each treatment group as shown. Histograms showing intracellular staining of indicated cytokines are also color coded in the same way. Shaded histograms show FMO control.

Data are representative of at least three independent experiments. Mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (two-tailed Student's t test). See also Figure S3.

unlikely they were secreting these cytokines because eosinophils were no longer found in the mLN (Figure S2). Most (72.0% ± 5.5%) mLN ILC2s in the IL-33-injected mice incorporated BrdU (Figure 2C), but the percentage of BrdU$^+$ ILC2s rapidly declined in 1 month. In naive mice, BrdU injection labeled almost 50% of mLN ILC2s, which declined to about 35% in 3 weeks, whereas the intensity of BrdU staining remained mostly the same (Figure 2D). These results suggest that the decrease in the BrdU-labeled mLN ILC2s in the IL-33-treated mice was probably due to a combination of their proliferation, death, and emigration from the mLN whereas in naive mice it was probably due to turnover of the population. The intracellular cytokine staining combined with the BrdU labeling of mLN ILC2s also suggest that a subset of activated ILC2s persist in the mLN for several weeks after the initial activation.

ILC2s in IL-33- or Papain-Sensitized Mice Respond More Vigorously than Naive ILC2s to a Secondary Challenge

To test the function of the lung ILC2s that persisted in IL-33-treated mice, we challenged the mice with a single intranasal administration of papain 1 month after the IL-33 injections (Figure S3A). Without the papain challenge, lung ILC2s in the IL-33-pretreated mice (Figure 3A, black bar) were 10 times more abundant than in control PBS-injected mice (white bar). Although the numbers of lung ILC2s did not significantly increase by the single intranasal papain challenge in both control (blue bar

versus white bar) and the IL-33-pretreated (red bar versus black bar) mice, they became positive for intracellular IL-5 and IL-13 (Figure 3B). Due to much higher numbers of lung ILC2s and higher frequency of intracellular cytokine-positive cells in the IL-33-pretreated mice than in PBS control mice, the former mice had much higher numbers of IL-5$^+$IL-13$^+$ ILC2s than the latter after the papain challenge (Figure 3C, red versus blue). It should be noted that IL-33-pretreated ILC2s had increased intensity of intracellular cytokine staining than control (Figure S3B). The papain challenge also induced significantly more ($p < 0.01$) production of IL-5 and IL-13 in BALF (Figure 3D, red versus blue), more eosinophil infiltration into the lung tissue (Figure 3E, red versus blue), and more mucus production (Figures 3F and S3C) in the IL-33-pretreated mice than in PBS control mice. In contrast, PBS control mice developed barely detectable signs of inflammation upon single papain challenge. Of note, IL-5 (Figure S3D) and IL-13 (not shown) were predominantly produced by ILC2s, but not by CD4$^+$Lin$^+$ (T) cells. These results show that IL-33-experienced ILC2s that persist in the lung for a long time vigorously respond to papain and induce severe lung inflammation.

The number of ILC2s in the lungs of the IL-33-pretreated mice declined 5 months later (Figure S3E) to approximately 2.5×10^4 (Figure S3F, black bar), which was still higher than those in the PBS pretreated control mice (white bar). Papain challenge induced stronger ILC2 activation and more severe lung inflammation (Figures S3G–S3I) in the IL-33-pretreated mice (red bars) than in control mice (blue bars). Interestingly, in the IL-33-pretreated mice, the mLN enlarged after the papain challenge and contained higher numbers of IL-5$^+$IL-13$^+$ ILC2s than in the PBS-pretreated control mice (Figures S3J–S3L). Therefore, IL-33-pretreated mice develop a more severe lung inflammation after a single injection of papain up to 5 months later than untreated mice. This appears to be mainly due to the high numbers of lung ILC2s in the IL-33-treated mice. Whether responsiveness of ILC2s in the IL-33-treated mice contributed to the severe lung inflammation was difficult to assess due to their high numbers.

To further study the responsiveness of allergen-experienced ILC2s, we initially gave three daily intranasal injections of papain and waited for 2 months before giving a single intranasal injection of IL-33 (Figure S3M). The numbers of lung ILC2s in the papain-pretreated mice (Figure 3G, black bar) declined to the levels in control mice pretreated with PBS (white bar). The IL-33 challenge induced much greater increase in the numbers of lung ILC2s in papain-pretreated mice than control mice (Figure 3G, red versus blue). The frequency of intracellular IL-5$^+$ or IL-13$^+$ among ILC2s (Figure 3H), the total numbers of IL-5$^+$IL-13$^+$ ILC2s, and the intensity of intracellular cytokine staining (Figure S3N) were significantly higher in the papain-pretreated mice than control mice (Figure 3I, red versus blue). Accordingly, the amounts of IL-5 and IL-13 in BALF and the eosinophil infiltration into the lung were also much higher in the papain-pretreated than in the control mice (Figures 3J and 3K). The papain-pretreated mice and control naive mice had the same number of ILC2s in the lung at the time of the IL-33 injection, so the severe type 2 lung inflammation in the papain-pretreated mice as compared to control mice can not be explained by higher numbers of ILC2s. Instead, the results indicate that papain-experienced ILC2s are more responsive than naive ILC2s to the IL-33 challenge.

Allergen-Experienced ILC2s Are Highly Responsive to Unrelated Allergens

We gave three daily intranasal injections of fungal allergen *Aspergillus oryzae* serine protease (Kheradmand et al., 2002) and gave a single challenge injection of papain 3.5 months later (Figure S4A). The initial injections of the fungal protease allergen (ASP) activated lung ILC2s (Figures S4B and S4C) and induced eosinophil infiltration into the lung (Figure S4D) as efficiently as papain. At 3.5 months later, the number of lung ILC2s in the treated mice declined to the same level as in the PBS control mice (Figure 4A, black versus white). When the mice received a single intranasal papain challenge injection, the total numbers of ILC2s and IL-5$^+$IL-13$^+$ ILC2s in the lungs were higher in the mice pretreated with ASP than those in control PBS-pretreated mice (Figures 4A and 4B, red versus blue). The intensities of intracellular IL-5 and IL-13 in lung ILC2s in ASP-pretreated mice were also higher than those in the control mice (Figure 4C). Accordingly, the amounts of IL-5 and IL-13 in BALF (Figure 4D), eosinophil infiltration (Figure 4E), and mucus production (Figure 4F) were higher in the mice pretreated with ASP than control PBS-pretreated mice. These results show that allergen-experienced ILC2s are more responsive to an unrelated allergen than naive ILC2s.

Allergen-Experienced ILC2s Are More Efficient than Naive ILC2s in Promoting Th2 Cell Differentiation

We have previously shown that ILC2-derived IL-13 promotes naive CD4$^+$ T cell differentiation into Th2 cells in the mLN (Halim et al., 2014). Because allergen-experienced ILC2s secreted high amounts of IL-13 upon exposure to an unrelated allergen, we tested whether this results in an increase in Th2 cell differentiation in the mLN. We initially treated mice with three daily intranasal injections of ASP (or PBS for control) to generate allergen-experienced ILC2s and gave a single intranasal injection of papain (or PBS for control) 1 month later to induce primary papain-specific Th2 cell response (Figure S5A). Th2 cells in the mLN were defined as CD4$^+$ T cells expressing intracellular IL-4 and IL-13 upon in vitro re-stimulation with PMA plus ionomycin for 3 hr. At 2 days after the papain injection, significantly more Th2 cells were generated in the mLN of the mice that initially received ASP injections (Figure 5A, red bar) than those in control mice that initially received PBS injections (blue bar), those that received PBS injection instead of papain challenge (black bar), or mice that received only PBS injections (white bar). Similar results were obtained with the ASP-treated mice that received the papain challenge 3.5 months later (Figure S5B). More Th2 cells were also generated in the mLN of mice initially receiving IL-33 injections in place of ASP (Figure S5C) and papain injection 1 month later (Figure 5B). Accordingly, 6 days after the papain challenge, Th2 cell numbers in the lung were significantly higher in the papain-challenged mice than those in control mice (Figure S5D, red versus others). We also gave a second papain injection into the mice (Figure 5C). Much higher numbers of Th2 cells were generated in the IL-33-primed mice than control (Figures 5D and 5E, red versus blue bars). These results suggest that

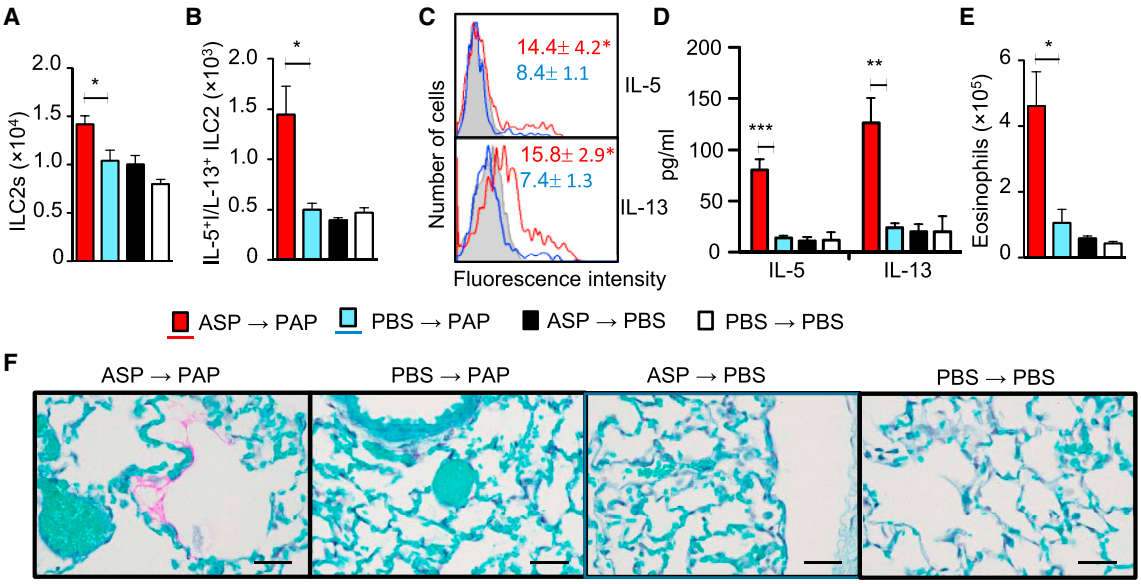


Figure 4. Allergen-Experienced Lung ILC2s Vigorously Respond to an Unrelated Allergen Challenge

Mice received three daily intranasal injections of *Aspergillus* protease (ASP) or PBS and received a single intranasal injection of papain (PAP) or PBS 3.5 months later. Two days later, lung ILC2 numbers (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (A), the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s after 3 hr of re-stimulation with PMA+ionomycin (B), and intracellular IL-5 and IL-13 staining of lung ILC2s (C) were analyzed by flow cytometry. The amounts of IL-5 and IL-13 in BALF were determined by ELISA (D). The numbers of eosinophils in the lung were also determined by flow cytometry (E), and lung tissue sections were stained for mucus (F). Scale bar corresponds to 10 μm. Bars in the graphs are color coded for each treatment group as shown. Histograms showing intracellular staining of indicated cytokines are also color coded in the same way. Shaded histograms show FMO control. Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (two-tailed Student's t test). See also Figure S4.

ILC2s previously activated by ASP or IL-33 enhance the papain-induced CD4$^+$ T cell differentiation into Th2 cells.

The Increased Responsiveness of IL-33-Experienced ILC2s Is Cell Autonomous

To test whether the hyper-responsiveness of allergen-experienced ILC2s was mediated by constitutive release of IL-33 by damaged epithelial cells, we tested IL-33-deficient mice. The mice received intranasal IL-33 injections and were challenged by IL-33 1 month later. The numbers of IL-5$^+$IL-13$^+$ ILC2s and allergic responses were much higher in the IL-33-primed mice than control mice (Figures S6A–S6D). In addition, the generation and maintenance of allergen-experienced ILC2s was T cell independent, as shown by the fact that the hyperresponsive ILC2s were similarly generated in the T cell-deficient *Rag1*$^{-/-}$ mice (Figures S6E–S6I). To test whether allergen-experienced ILC2s undergo intrinsic changes, mice were treated with three daily intranasal injections of IL-33 (or PBS for control) and lung ILC2s were purified 6 months later (Figure S6J). They were cultured with suboptimal doses of IL-33 and TSLP (Zhou et al., 2005), a combination known to stimulate ILC2s (Halim et al., 2012a), for 72 hr. ILC2s from IL-33-treated mice produced more than 4 times more IL-5 and IL-13 than naive ILC2s (Figure 6A). Thus, IL-33-experienced ILC2s are inherently more responsive than naive ILC2s to suboptimal concentrations of cytokines. To further validate this in vivo, we purified ILC2s from the lung of B6 (CD45.2) mice 1 month after the IL-33 treatment and intravenously injected into non-irradiated Pep3b (CD45.1) mice (Figure S6J). We then gave a single intranasal papain injection into the Pep3b mice 24 hr after the ILC2 transfer and analyzed the donor and the recipient ILC2s in the lung 2 days later. In the papain-challenged mice, the donor IL-33-experienced ILC2s (CD45.2$^+$) were readily detected in the lung (Figure 6B, left contour plot) and they were mostly positive for intracellular IL-5 and IL-13 staining (top, shaded histograms). Furthermore, the intensities of intracellular IL-5 and IL-13 staining of the donor ILC2s were higher than those of the recipient (CD45.1$^+$) naive ILC2s (dashed histograms). Without the papain injection, only small numbers of the donor ILC2s were detected in the recipient lungs (Figure 6B, right contour plot), and they were mostly negative for intracellular IL-5 and IL-13 (bottom histograms). Of note, allergen-experienced ILC2s also show higher production of cytokines than naive ILC2s when transplanted into NOD/SCID/*Il2rg*$^{-/-}$ (NSG) mice and challenged with a single intranasal administration of IL-33 (Figure 6C). Therefore, the IL-33-experienced ILC2s respond more vigorously than naive ILC2s to the papain challenge in the naive mouse lung environment. These data suggest that the high responsiveness of the allergen-experienced ILC2s is due to intrinsic changes in ILC2s.

IL-33-Experienced ILC2s Have a Gene Expression Signature Similar to that of Memory T Cells and Are Responsive to IL-25

To further characterize allergen-experienced ILC2s, we purified ILC2s from the lungs of naive and IL-33-treated mice 4 days (d4), 2 weeks (w2), and 4 months (m4) after the intranasal IL-33 injections, extracted RNA, and analyzed global gene expression profiles by Affymetrix microarray. The genes differentially (more

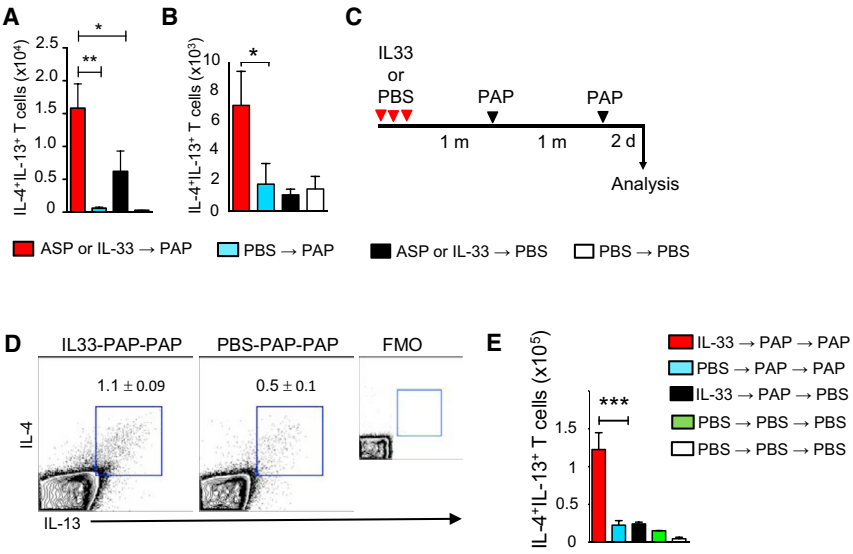


Figure 5. Allergen-Experienced ILC2s Promote Th2 Cell Differentiation upon Unrelated Allergen Challenge

(A) Mice treated with ASP or PBS received a secondary challenge with papain or PBS 1 month later, and $IL4^+IL13^+$ T cell numbers (gated as live $CD45^+CD4^+Thy1^+TCR\text{-}\beta^+$ cells) in the mLN were analyzed by flow cytometry 2 days after the secondary challenge.

(B) Mice treated with IL-33 or PBS received a secondary challenge with papain or PBS 1 month later. $IL\text{-}4^+IL\text{-}13^+$ T cell numbers in the mLN 2 days after the secondary challenge were analyzed by flow cytometry.

(C) Scheme for the treatment of mice with IL-33 and papain in (D) and (E).

(D and E) Lung cells were stained for intracellular IL-4 and IL-13 and analyzed by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. T cells were gated (as live $CD45^+CD4^+Thy1^+TCR\text{-}\beta^+$ cells) and intracellular IL-4 and IL-13 fluorescence was plotted on contour plots (D). Numbers in plots indicate the (mean ± SEM) percentages of gated cells among T cells. Numbers of intracellular $IL\text{-}4^+IL\text{-}13^+$ T cells (E) were analyzed by flow cytometry.

Bars in the graphs are color coded for each treatment group as shown. Data are representative of at least three independent experiments. Mean ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (two-tailed Student's t test). See also Figure S5.

than 2-fold) expressed in the ILC2 populations included *Il1r2*, *Il5*, *Tnfsf18*, *Bcl2a1b*, *Bcl2a1d*, *Ler3*, and *Syne1*, which were more highly expressed in activated (d4) ILC2s than naive ILC2s and remained significantly higher in IL-33-experienced (w2 and m4) than naive ILC2s (Figure 7A). In contrast, *S1pr1*, *Il6st*, *Cd2*, *Cd7*, and *Sell* were lower in d4, w2, and m4 than naive ILC2s. The proliferation/cell cycle-related genes *Mki67* and *Ccnb2* and the chemokine genes *Ccl17*, *Ccl24*, *Cxcl3*, and *Ccl6* were highly expressed in d4 but not naive, w2, or m4 ILC2s, suggesting that IL-33-experienced ILC2s were resting cells. Therefore, IL-33-experienced w2 and m4 ILC2s differed from naive and activated (d4) lung ILC2s in the expression of small subsets of genes. The expression of *Il5* and *Il13* was high in all the ILC2 populations and did not correlate with the intracellular staining of the protein products in ILC2s, suggesting that they are regulated at the translational level.

The differential gene expression profiles of naive, d4, w2, and m4 ILC2s were compared with annotated gene set database (Immunologic Gene Collection: http://software.broadinstitute.org/gsea/msigdb/index.jsp) by gene set enrichment analysis (GSEA). The analysis with the gene expression data of naive and m4 ILC2s resulted in the highest score of enrichment with the differentially expressed gene set of human naive $CD8^+$ T cells compared to memory $CD8^+$ T cells (Figure 7B, left; Abbas et al., 2009; Kaech et al., 2002; Luckey et al., 2006; Wherry et al., 2007). Normalized enrichment score (NES) of 2.27 and false discovery rate (FDR) of 0 indicated that the similarity in the differential gene expression profile of naive versus m4 ILC2s to that of naive versus memory $CD8^+$ T cells was highly significant. Similarly, analysis with the differential gene expression profile in d4 versus m4 lung ILC2s resulted in the highest score of enrichment with the gene set of human effector versus memory $CD8^+$ T cells (Figure 7B, right). GSEA with the data of w2 versus d4 and w2 versus naive also showed high scores of enrichment with effector versus memory $CD8^+$ T cells and naive versus memory $CD4^+$ T cells, respectively (Figure S7A). These results showed that the expression profiles of IL-33-experienced w2 and m4 ILC2s are similar to that of memory T cells.

Among the genes differentially expressed in naive and IL-33-experienced (w2 and m4) ILC2s, those encoding the IL-25 receptor (IL-25R), which consists of IL-17RA and IL-17RB (Figure 7E), were of particular interest because IL-25 is thought to an important ILC2-activating cytokine (Hammad and Lambrecht, 2015). The expression of *Il17rb* mRNA in w2 and m4 ILC2s was 2- to 4-fold higher than that in naive ILC2s whereas lower amounts of *Il17ra* mRNA were expressed in all the ILC2 populations tested (Figure 7C). Flow cytometric analysis confirmed the expression of the IL-25R protein on w2 and m4 ILC2s whereas it was very low on naive ILC2s (Figure 7D). To test the functional significance of the expression of IL-25R, we gave a single intranasal injection of IL-25 in mice pretreated with IL-33 6 months earlier and analyzed the lung and the mLN (Figure S7B). ILC2s in the IL-33-pretreated, but not naive (PBS control), mice were activated by IL-25 as indicated by the high numbers of total ILC2s and cytokine-producing ILC2s in the lung (Figures 7E–7G) and in the mLN (Figures S7C and S7D) as well as high numbers of eosinophils in the lung and increased levels of type 2 cytokines in BALF (Figures 7H and 7I). Of note, naive ILC2s did not respond to three consecutive daily intranasal injections of IL-25 (Figures S7E–S7H). These results showed that IL-33-experienced ILC2s significantly differ from naive ILC2s in their responsiveness to IL-25.

DISCUSSION

In this study, we have shown that allergen-experienced ILC2s generated by intranasal injections of allergen or IL-33 are more readily stimulated by allergen challenge, proliferating more vigorously and producing greater amounts of type 2 cytokines than naive ILC2s stimulated by the same allergen. Although changes

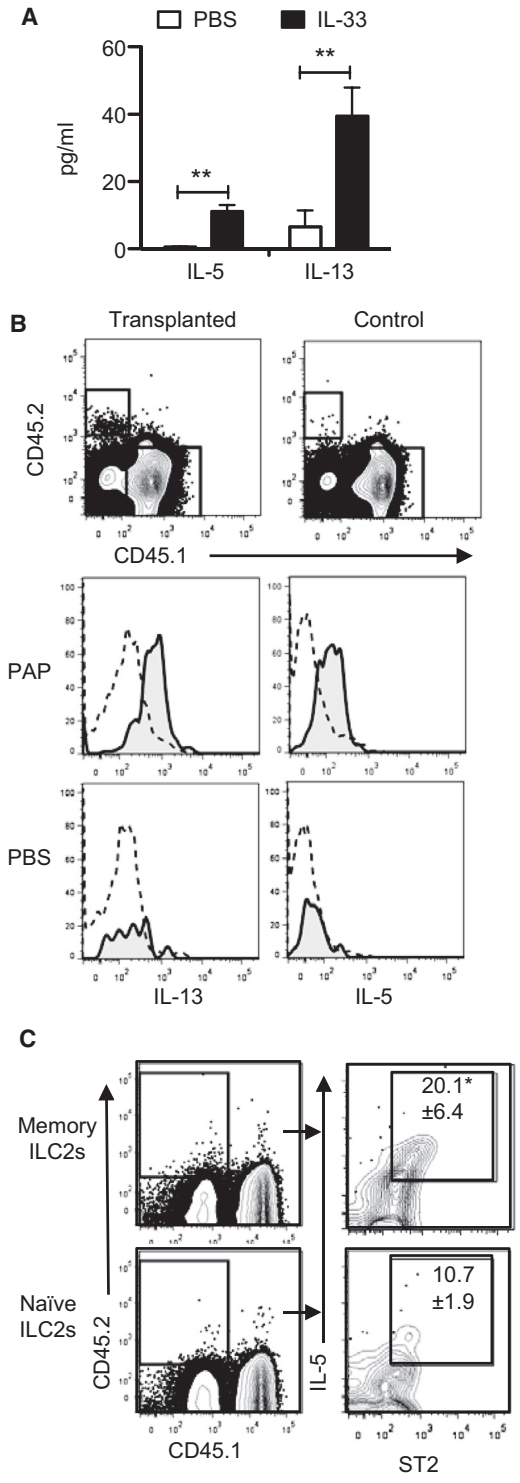


Figure 6. The Increased Responsiveness of IL-33-Experienced ILC2s Is Cell Autonomous

(A) Mice were treated with IL-33 or PBS and lung ILC2s (gated as live CD45^{+-} Lin^{-}ST2^{+}Thy1^{+} cells) were purified 6 months later. The purified ILC2s were cultured for 72 hr with IL-33 (2.5 ng/mL) and TSLP (2.5 ng/mL), and the amounts of IL-5 and IL-13 in the culture supernatants were determined by ELISA.

(B) ILC2s were purified from IL-33-treated B6 mice 1 month after the treatments and transferred into Pep3b mice. One day after the cell transfer, the mice received a single intranasal injection of papain (PAP) or PBS. Donor (CD45.2) and host (CD45.1) lung ILC2s were gated (top contour plots) and analyzed for intracellular IL-5 and IL-13 (histograms) after 3 hr of re-stimulation with PMA+ionomycin. Shaded histograms show donor ILC2s and dashed histograms show the host ILC2s.

(C) ILC2s were purified from naive or IL-33-treated B6 mice 1 month after the treatments and transferred into NSG mice. One day after the cell transfer, the mice received a single intranasal injection of IL-33. Donor (CD45.2) lung ILC2s were gated (left contour plots) and analyzed for intracellular IL-5 (right contour plots) after 3 hr of re-stimulation with PMA+ionomycin.

Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01 (two-tailed Student's t test). See also Figure S6.

in the lung environment induced by initial allergen/IL-33 injections may contribute to the high responsiveness of allergen-experienced ILC2s, purified allergen-experienced ILC2s produce more cytokines than naive ILC2s upon in vitro stimulation by suboptimal amounts of IL-33 and TSLP as well as upon transplantation into naive mice and stimulation by intranasal IL-33 injection. Therefore, the high responsiveness of allergen-experienced ILC2s is probably mediated by cell-intrinsic changes. IL-33-experienced ILC2s differ from naive and effector ILC2s in gene expression profiles, further supporting the notion of cell-intrinsic changes in allergen-experienced ILC2s. Although the molecular basis of the high responsiveness of allergen-experienced ILC2s is still unclear, it seems likely that the increased expression of the IL-25R may play a role. Naive mouse lung ILC2s express very low amounts of IL-25R mRNA and the protein products, and they are not activated by intranasal injections of IL-25, consistent with the results reported by Huang et al. (2015). In contrast, IL-33-experienced ILC2s express higher amounts of IL-25R mRNA and protein, and they are readily activated in vivo by intranasal IL-25 injections. However, it should be noted that purified IL-33-experienced ILC2s are not activated by IL-25 alone in vitro (data not shown), suggesting that other factors are also involved.

The persistence of allergen-experienced ILC2s and their increased responsiveness to a secondary challenge are characteristics of memory lymphocytes (Harrington et al., 2008; Löhning et al., 2008). However, ILC2s are antigen non-specific whereas antigen specificity has been considered to be a key feature of immunological memory (Remakus and Sigal, 2013; Wherry and Ahmed, 2004). Although immunological memory of NK cells, which belong to the innate lymphocyte population, has been reported, memory NK cells are antigen specific (Sun et al., 2009; Paust et al., 2010).

In addition to antigen-specific memory NK cells, cytokine-activated NK cells have been shown to have antigen non-specific memory-like properties (Cooper et al., 2009). Furthermore, monocytes and macrophages have recently been shown to acquire antigen non-specific memory-like characteristics (Cheng et al., 2014; Saeed et al., 2014). Upon recognition of a microbial ligand, macrophages are capable of adapting and reshaping their response to a subsequent microbial assault. Quintin et al. (2014) have proposed the term "trained immunity" to describe the enhanced innate host defense against a second infection. However, trained immunity significantly differs from the memory-like properties of allergen-experienced ILC2s in our current study. In trained immunity, monocytes/macrophages are "trained" to acquire new functions. It involves changes in

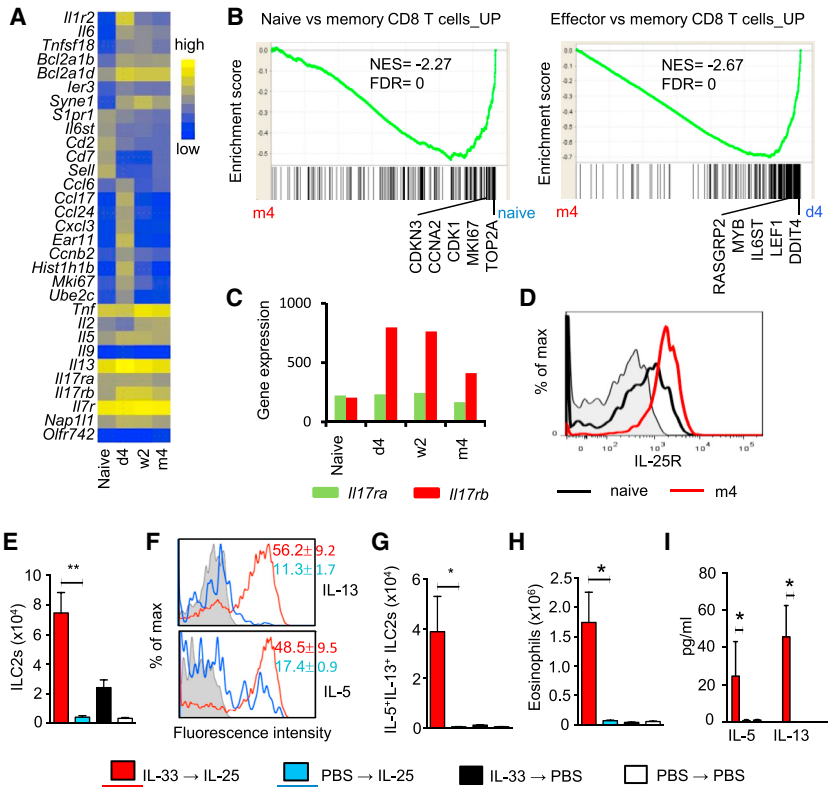


Figure 7. Gene Expression Profiles of Memory-like ILC2s in Lung Show Gene Signatures of Memory T Cells

(A) Heatmap shows the relative expression levels of genes that are differentially expressed in ILC2s in the lung of PBS-injected (naive) mice and 4 days (d4), 2 weeks (w2), and 4 months (m4) after intranasal IL-33 injection.

(B) Gene set enrichment analysis (GSEA) of naive, d4, and m4 ILC2s in the lung. Log2-fold expression values for each comparison were ranked in descending order. The x axis shows individual genes and the y axis shows enrichment score. The five most enriched genes are listed on the x axis of each panel. Left panel shows that genes upregulated in naive CD8$^+$ T cells compared to memory CD8$^+$ T cells were significantly enriched in naive lung ILC2s when compared to m4 ILC2s. Right panel shows that genes upregulated in effector CD8$^+$ T cells compared to memory CD8$^+$ T cells were significantly enriched in d4 lung ILC2s when compared to m4 ILC2s. Abbreviations are as follows: NES, normalized enrichment score; FDR, false discovery rate.

(C) The expression levels of *Il17ra* and *Il17rb* mRNA in naive, d4, w2, and m4 ILC2s as determined by the microarray analyses above were compared. The logarithmic values of the expression levels in the microarray assays were converted into the linear scale. Green bars and red bars show the expression levels of *Il17ra* and *Il17rb*, respectively.

(D) IL-25R expression was analyzed in naive and memory-like ILC2s (red and blue histograms, respectively) by flow cytometry.

(E–I) Mice received three daily intranasal injections of IL-33 or PBS and received a single intranasal injection of IL-25 or PBS 6 months later. Two days after the challenge, ILC2 numbers (E), intracellular IL-5 and IL-13 staining of lung ILC2s (F), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (G), and lung eosinophil numbers (H) after 3 hr of re-stimulation with PMA+ionomycin were analyzed by flow cytometry. The amounts of IL-5 and IL-13 in BALF were determined by ELISA (I). Each treatment group is color coded as indicated in the figure.

Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01 (two-tailed Student's t test).

global gene expression, in part mediated by epigenetic programming (Kleinnijenhuis et al., 2012; Quintin et al., 2012). In contrast, allergen-experienced ILC2s do not acquire a new function. Naive and allergen-experienced ILC2s have the same function, namely producing IL-5 and IL-13 upon stimulation. The main difference is that the latter are more responsive and produce higher amounts of the same cytokines than the former. They differ in the expression of only a small set of genes, not a broad range of genes seen with trained immunity.

Both ILC2s and T/B cells remember a prior activation by changing the expression of selected genes that make them more responsive to secondary stimulation than primary stimulation. The main difference between ILC2s and T/B cells is how the cells are activated. ILC2s are activated by cytokines whereas T/B cells are activated by specific antigens, thus making the latter antigen specific. The recent report of antigen non-specific memory Th2 cells (Guo et al., 2015) is consistent with this view. Therefore, allergen-experienced ILC2s are more similar to memory lymphocytes than trained innate cells.

Activation of lung ILC2s by intranasal injections of IL-33 or papain results in increases in ILC2 numbers not only in the lung but also in the draining mLN. Because most ILC2s in the lung and mLN in the IL-33-injected mice incorporate BrdU, the increases in ILC2 numbers are most probably due to the proliferation of the stimulated ILC2s rather than recruitment from other tissues. It is still unknown whether ILC2s in the mLN proliferate in the mLN or whether proliferating ILC2s in the lung migrate to the mLN. Almost all mLN ILC2s become positive for intracellular IL-5 and IL-13 during the expansion phase, and they seem to secrete the cytokines in vivo as suggested by eosinophil infiltration into the mLN. Unlike lung ILC2s, mLN ILC2s remain positive for intracellular IL-5 and IL-13 after the resolution of inflammation, suggesting that they remain activated. However, the role of memory-like ILC2s in the mLN in type 2 lung inflammation induced by allergen challenge is still unknown.

We have previously shown that intranasal injections of papain into naive mice not only stimulate lung ILC2s but that ILC2-derived IL-13 also promotes naive CD4$^+$ T cell differentiation into the Th2 pathway. Thus, papain injections into papain-primed mice stimulate both ILC2s and Th2 cells, resulting in severe allergic lung inflammation. In the current study, we used different stimuli for priming and challenge to avoid the stimulation of memory Th2 cells generated in the initial allergen priming. Nevertheless, the primary Th2 cell responses to papain in mice primed by fungal protease or IL-33 were higher than those of naive mice. It is possible that IL-33 produced by papain treatment stimulates ASP-specific memory T cells in an antigen non-specific manner as reported by Guo et al. (2015). However, the papain treatment also enhanced Th2 cell differentiation in IL-33-pretreated mice, which unlikely had memory Th2 cells. Therefore, memory-like

ILC2s enhance not only ILC2-mediated innate but also Th2 cell-mediated adaptive type 2 lung inflammation, and they provide a new clue to the question of how individuals become sensitized to a broad range of allergens. Currently, the diagnosis and treatment of allergic diseases rely on identifying specific allergens by skin test and IgE detection. However, many patients react against multiple allergens (Calderón et al., 2012). Furthermore, specific allergens are not readily identified in some asthma patients, who are considered to have non-allergic asthma (DeKruyff et al., 2014). It is conceivable that antigen non-specific memory-like ILC2s play a critical role in such diseases.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6), NSG, and *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratories. *Il33*$^{-/-}$ mice were generated from the breeders obtained from the Knockout Mouse Project Repository. All mice were maintained in the British Columbia Cancer Research Centre animal facility under specific-pathogen-free conditions. 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, FACS Sorting, and Analysis

FITC-conjugated anti-CD3ε, CD19, NK1.1, Mac-1, CD11c, Gr-1, Ter119, TCR-αβ, TCR-γ, PE-conjugated anti-CD127, CD3, PerCP-Cy5.5-conjugated anti-ST2, CD3, PE.Cy7-conjugated anti-DX5, CD4, APC-conjugated anti-CD3, FcεR1α, CD25, APC-eFluor780-conjugated anti-B220, Alexa Fluor 700-conjugated anti-CD11c, eFluor 450-conjugated anti-CD3ε, CD19, NK1.1, Gr-1, CD11b, CD11c, Ter119, TCRαβ, TCRγ, and eFluor 605-conjugated anti-Thy1.2 were purchased from eBioscience. FITC-conjugated anti-7/4 was purchased from Abcam. PE-conjugated anti-IL-13, Siglec-F, APC-conjugated anti-IL-5, and BD Horizon V500-conjugated anti-CD45 was purchased from BD Bioscience. eFluor 780 (eBioscience) was used to exclude non-viable cells. Unconjugated anti-IL-33 and TSLP were purchased from eBioscience. Papain, PMA, and ionomycin were purchased from Sigma Aldrich. *Aspergillus oryzae* protease allergen was purchased from Sigma. BD Fortessa was used for phenotypic analysis; BD FACS Aria II was used for cell sorting and phenotypic analysis. Flowjo v.8.6 was used for data analysis.

Primary Leukocyte Preparation

Cell suspensions were prepared from lung as described (Halim et al., 2014).

Isolation and Detection of ILC2s

Single 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, CD11c, Gr-1, Ter119, TCRαβ, TCRγ), PE-conjugated CD127, PerCP-Cy5.5-conjugated ST2, PE.Cy7-conjugated CD4, APC-conjugated CD25, V500-conjugated CD45, and eFluor 780 fixable viability dye and purified by FACS. EasySep Mouse ILC2 Enrichment Kit (STEMCELL Technologies, cat# 19842) was used prior to cell sorting of naive ILC2s.

ILC2 In Vitro Culture

ILC2s were purified by flow cytometry, 500 cells per well were cultured in 200 μL RPMI-1640 media containing IL-33 (2.5 ng/mL), TSLP (2.5 ng/mL), 10% FBS, penicillin and streptomycin, and 2-Mercaptoethanol at 37°C for 36 hr. The amounts of IL-5 and IL-13 in the culture supernatants were analyzed by ELISA.

ILC2 Transplantation

B6 mice were treated with three consecutive daily intranasal injections of IL-33 (0.25 μg/mouse) and lung ILC2s were purified 1 month later. Five thousand purified ILC2s were injected i.v. into each Pep3b or RAG1-deficient mouse. One day after the cell transfer, the mice received a single intranasal injection of papain (PAP), IL-33, or PBS and lung ILC2s were analyzed by flow cytometry.

In Vivo Stimulation

Mice were anesthetized by isofluorane inhalation, followed by intranasal injection of rIL-33 (0.25 μg), rIL-25 (0.25 μg), *Aspergillus oryzae* protease allergen (50 μg), and papain (15 μg) in 40 μL of PBS on days 0, 1, and 2, and at 1, 2, 3.5, or 5 months later depending on the experiment. Mice were sacrificed at indicated times and spleen, lungs, and BALF (1 mL PBS) were collected or airways were instilled with 1 mL of paraphormadehyde (Sigma) and fixed in formalin. Lung tissue was processed as described previously; lung cells were then counted and identified by flow cytometry. Fixed tissues were embedded in paraffin and processed for PAS staining. For histology, lung sections of three different depths per animal were analyzed (30 fields of view per animal).

Intracellular staining

Intracellular staining for IL-5 and IL-13 was performed using the Cytofix/Cytoperm kit (BD Biosciences) after 3 hr re-stimulation of 2×10^6 total live nucleated cells in 500 μL RPMI-1640 media containing 10% FBS, P+S, 2 ME, Monensin (Golgi Stop, BD Biosciences), PMA (30 ng/mL), and ionomycin (500 ng/mL) at 37°C. Dead cells were stained with eFluor 780 (eBioscience) fixable viability dye before fixation and permeabilization and excluded during analysis.

Quantification of Cytokine

BAL and cell-culture samples were analyzed for IL-5 and IL-13 by ELISA (eBioscience) according to the manufacturer's protocol.

BrdU

Mice received 3 daily intranasal injections of 0.8 mg of BrdU in 80 μL starting 1 day after the last administration of IL-33. Single-cell suspensions were prepared from lung tissues 24 hr after the last injection as above, and cells were stained with antibodies for surface markers and the viability dye as above to identify ILC2s. Cells were then fixed, followed by staining for BrdU using a FITC BrdU Flow Kit (BD Biosciences).

RNA Extraction, Microarray Analysis, and GSEA

Total RNA was extracted from FACS-purified ILC2s using TRIzol reagent (Life Technologies) according to manufacturer's protocol. RNA quality check, cDNA amplification, and microarray hybridization were performed by The Centre for Applied Genomics. In brief, the quality of the extracted RNA was tested by Agilent 2100 Bioanalyzer. The samples with RNA integrity number (RIN) above 6 were used to generate cDNA, which was amplified using Ovation Pico WTA kit (Nugen) and hybridized to Affymetrix GeneChip Mouse Gene 2.0ST Array. Two to three samples per group were analyzed. The expression profile was normalized using robust multi-array (RMA) algorithm and all data analyses were performed by Flex Array 1.6.3 (Genome Quebec). Gene set enrichment analysis was performed using GSEA software and the gene set collection C7 (immunological signatures) (http://www.broadinstitute.org/gsea/msigdb/index.jsp).

ACCESSION NUMBERS

GEO accession number for the microarray data is GEO: GSE81700.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.06.017.

AUTHOR CONTRIBUTIONS

I.M.-G. and L.M. designed and performed the experiments and wrote the paper. M.G., G.F.T.P., and C.A.S. performed experiments. F.T. supervised the project, designed the experiments, and wrote the paper.

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Immunity, Volume *45*

Supplemental Information

Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

Itziar Martinez-Gonzalez, Laura Mathä, Catherine A. Steer, Maryam Ghaedi, Grace F.T. Poon, and Fumio Takei

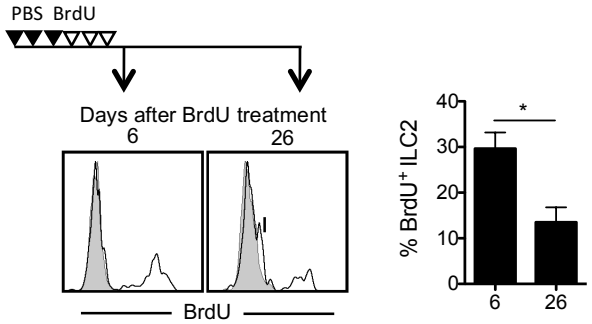


Figure S1. Turnover of naive ILC2s in the lung, related to Figure 1. BrdU was incorporated by lung ILC2s (gated as live $CD45^{+}Lin^{-}ST2^{+}Thy1^{+}$ cells) in control mice injected with PBS and BrdU intranasally and the decrease in BrdU-labeled lung ILC2s over time was analyzed by flow cytometry. Control histogram (shaded) was generated using untreated mice stained with anti-BrdU antibody. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=$p<0.05$) (two-tailed Student's t-test).

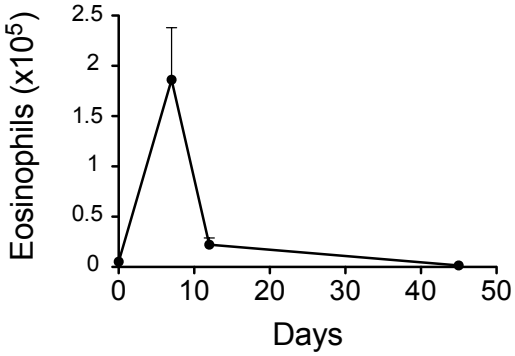


Figure S2. **Eosinophils infiltrate into the mLN after intranasal injection of IL-33, related to Figure 2.** Mice were treated with three daily intranasal injections of IL-33 and eosinophil numbers in the mLN along time were analyzed by flow cytometry.

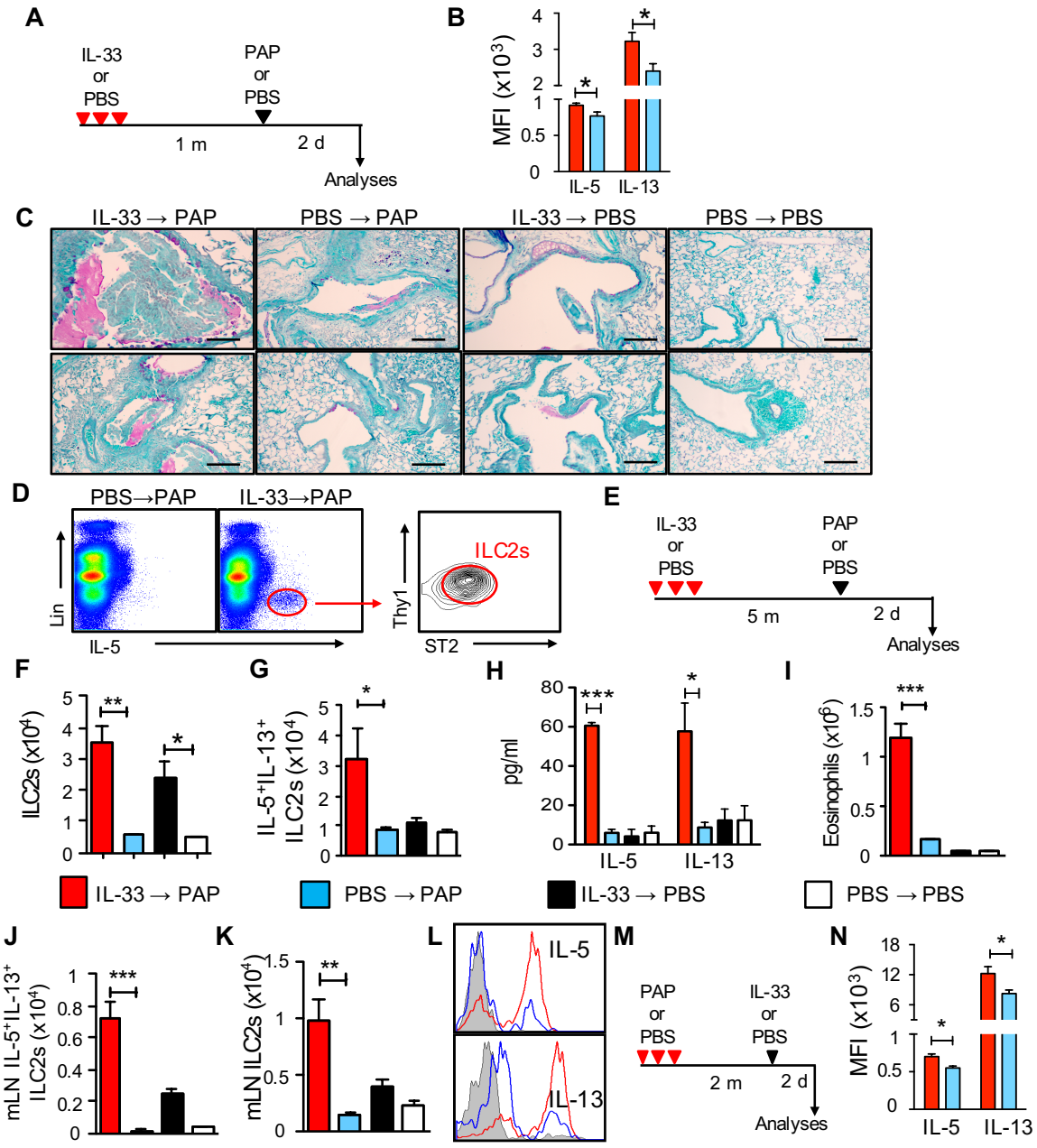


Figure S3. IL-33-experienced ILC2s respond stronger upon challenge 5 months later, related to Figure 3. (A) Scheme for the treatment of mice with IL-33 and papain in Figure 3. (B) Mean Fluorescence Intensity (MFI) for IL-5 and IL-13 expression by lung ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) analyzed by flow cytometry as shown in (A). (C) Lung tissue sections were stained by PAS staining for mucus. Scale bar corresponds to 20 μm. (D) FACS analysis showing that the cytokine-producing cells after papain challenge are ILC2s. (E) Scheme for the treatment of mice with IL-33 and papain. (F-I) Analysis of lung ILC2s and allergic inflammation upon challenge. Lung ILC2 number (F), lung IL-5/IL-13$^+$ producing ILC2s (G), cytokines in BALF (H), Eosinophils (I). (J-L) Analysis of mLN ILC2s upon challenge. mLN IL-5/IL-13$^+$ producing ILC2s (J), mLN ILC2 number (K), intracellular staining for IL-5 and IL-13 in mLN ILC2s (L). (M) Scheme for the treatment of mice with papain and IL-33 in Figure 3. (N) Mean Fluorescence Intensity (MFI) for IL-5 and IL-13 expression by lung ILC2s analyzed by flow cytometry as shown in (M). Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01, ***=p<0.001) (two-tailed Student's t-test).

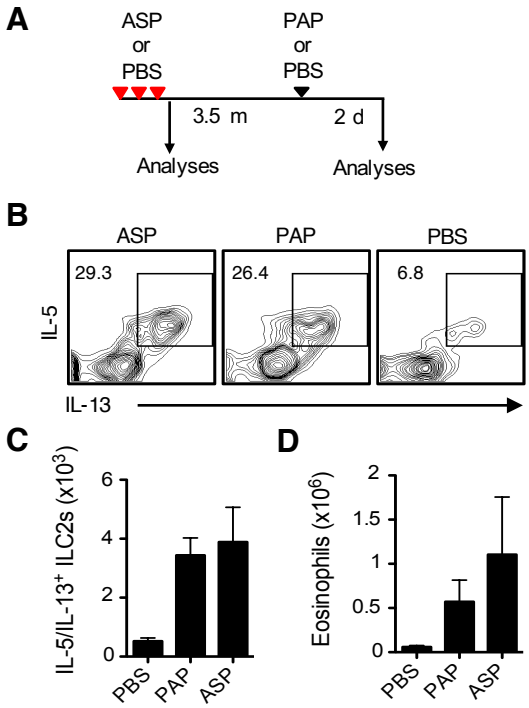


Figure S4. **Intranasal administration of Aspergillus stimulates ILC2s as papain does, related to Figure 4.** (A) Scheme of the treatment of mice treated with Aspergillus protease (ASP) and papain (PAP) in Figure 4. (B-D) Intracellular staining for IL-5/IL-13 of ILC2s (gated as live $CD45^{+}Lin^{-}ST2^{+}Thy1^{+}$ cells) in ASP, PAP and PBS treated mice (B), lung IL-5/IL-13^{+} producing ILC2s (C), eosinophil number (D). Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01, ***=p<0.001) (two-tailed Student's t-test).

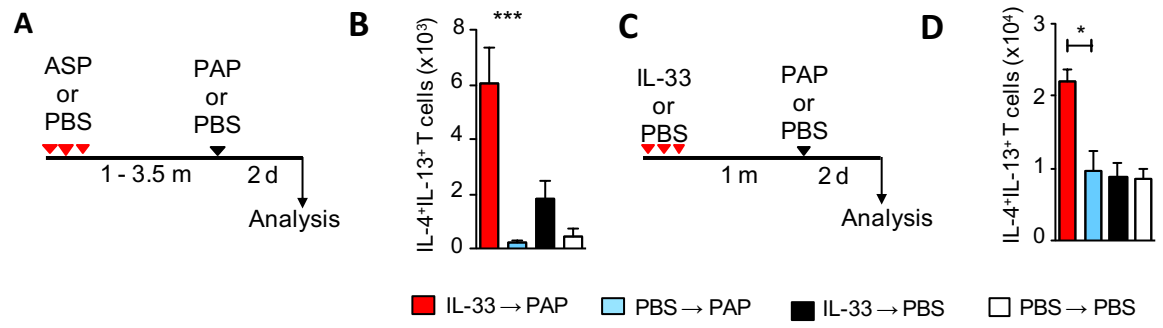


Figure S5. **Scheme of treatments and Th2 cells in lung, related to Figure 5.** (A) Scheme of the treatment of mice with Aspergillus protease (ASP) and papain (PAP) in Figure 5. (B) Th2 cells (gated as live $CD45^+CD4^+Thy1^+TCRb^+$ cells) in the mLN on d2 of mice challenged with papain 3.5 months after the ASP treatment. (C) Scheme of the treatment of mice with IL-33 and papain (PAP) in Figure 5. (D) Th2 cells in the lungs on d6 of the mice pretreated with IL-33 and challenged by PAP. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=$p<0.05$, ***=$p<0.001$) (two-tailed Student's t-test).

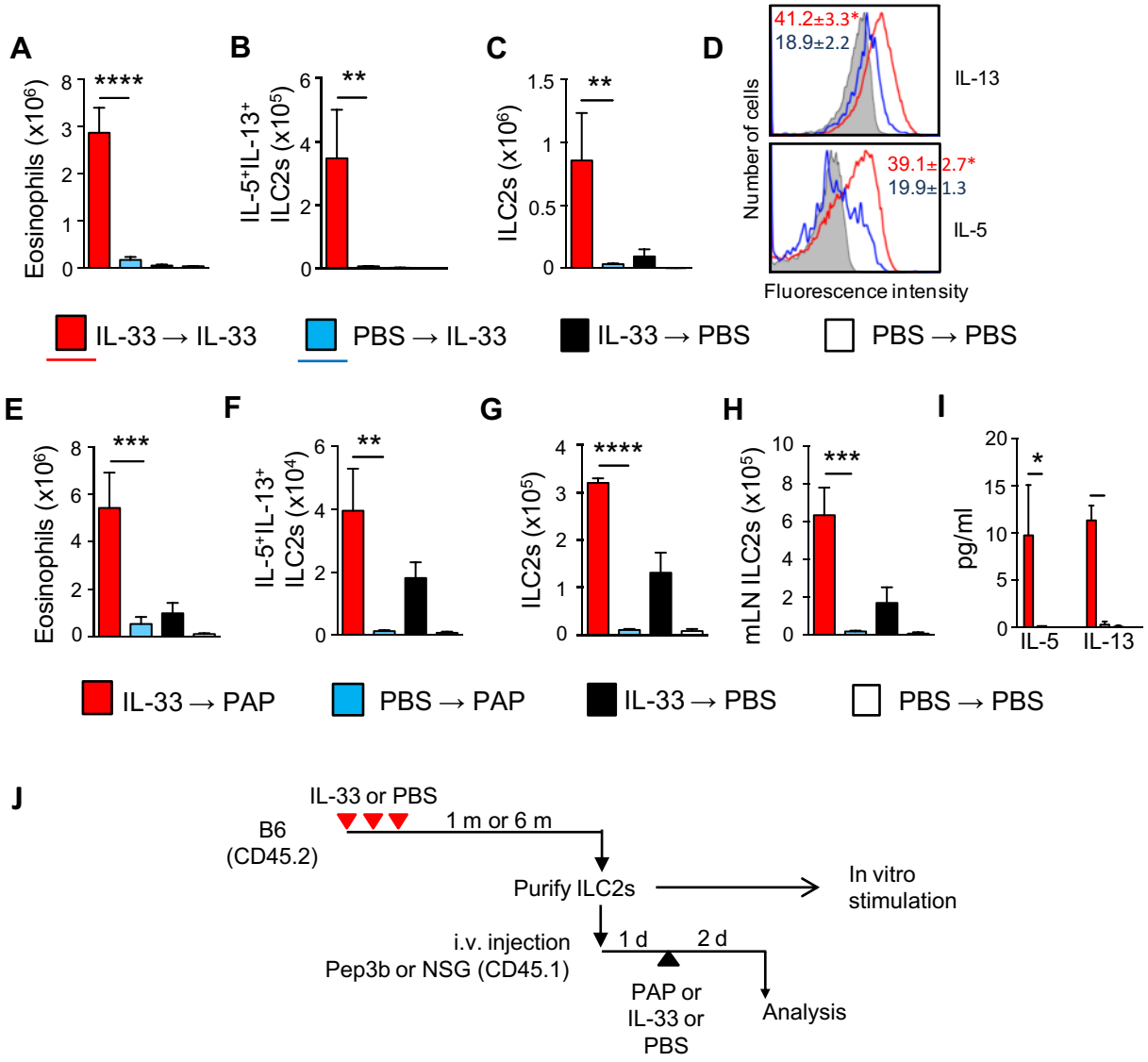


Figure S6. IL-33 and RAG1-deficient mice experiments and scheme of the experiments in Figure 6. (A-D) IL-33-deficient mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of IL-33 or PBS. Two days later, lung eosinophil numbers (A), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (B), lung ILC2 numbers (C) and intracellular IL-5 and IL-13 staining of lung ILC2s (D) after 3 h of re-stimulation with PMA+Ionomycin were analyzed by flow-cytometry. (E-I) RAG1-deficient mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of IL-33 or PBS. Two days later, lung eosinophil numbers (E), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (F), ILC2 numbers in the lung (G) and in the mLN (H) were analyzed by flow-cytometry and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (I). (J) Scheme of the experiments in Figure 6 to generate and purify memory-like ILC2s and to test their responsiveness in vitro and in vivo. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01 ***=p<0.001) (two-tailed Student's t-test).

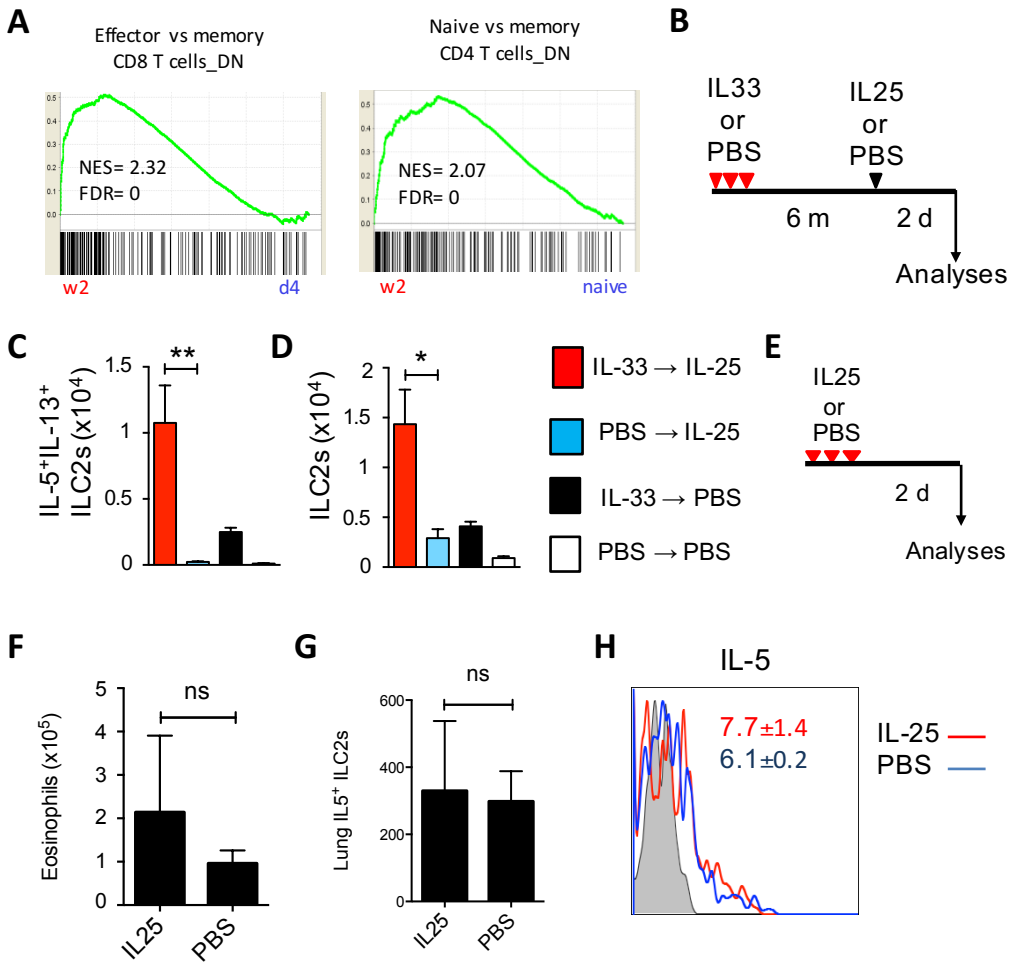


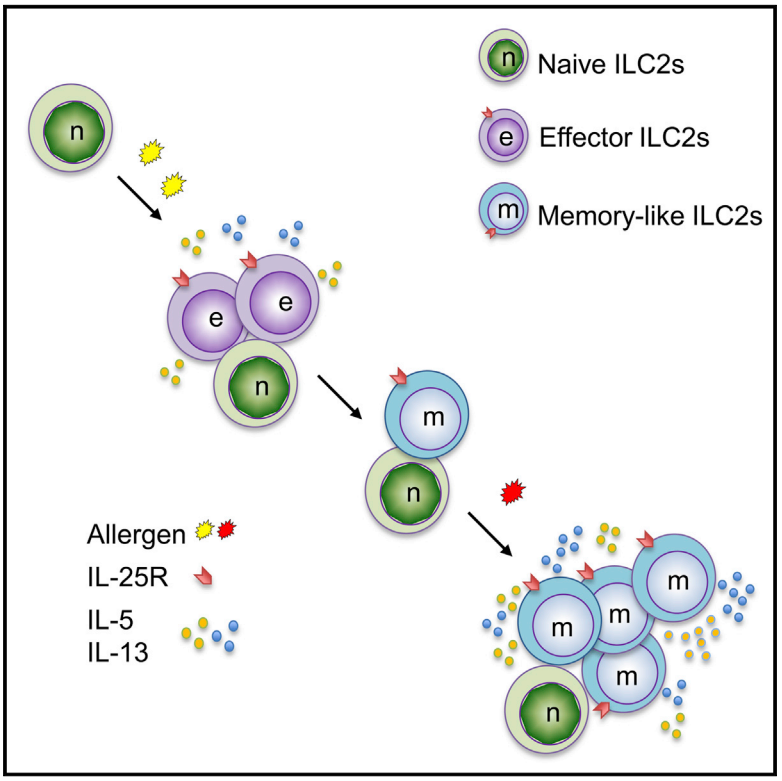
Figure S7. **Gene expression profiles of different populations of ILC2s in lung and mLN and IL-25 related experiments, related to Figure 7.** (A) Gene Set Enrichment Analysis (GSEA) of naïve, d4 and w2 ILC2s in the lung. Log2-fold expression values for each comparison were ranked in descending order. The x-axis shows individual genes and the y-axis shows enrichment score. Left panel shows that genes down regulated in effector CD8 T cells compared to memory CD8 T cells were significantly enriched in w2 lung ILC2s when compared to d4 ILC2s. Right panel shows that genes down regulated in naïve CD8 T cells compared to memory CD8 T cells were significantly enriched in w2 lung ILC2s when compared to naive ILC2s. NES: normalized enrichment score, FDR: false discovery rate. (B) Scheme of the experiments in Figure 7 for IL-25 experiments. (C and D) Numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) in the lung (C) and total lung ILC2 numbers (D) after 3 h of re-stimulation with PMA + Ionomycin were analyzed by flow-cytometry. (E) Scheme for Figure S7 F-H. (F-H) Numbers of eosinophils in the lung (F), intracellular IL-5$^+$ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) in the lung (G) and intracellular IL-5 staining of lung ILC2s (H) after 3 h of re-stimulation with PMA + Ionomycin were analyzed by flow-cytometry. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01) (two-tailed Student's t-test).

Article

Immunity

Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

Graphical Abstract



Highlights

- Allergen-experienced ILC2s acquire antigen non-specific memory-like properties
- Memory-like ILC2s enhance type 2 lung inflammation upon an unrelated allergen challenge
- Allergen-experienced ILC2s show gene signatures of memory T cells

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In Brief

Group 2 innate lymphoid cells (ILC2s) in the lung are stimulated by inhaled allergens, producing cytokines that contribute to allergic lung inflammation. Takei and colleagues find that allergen-experienced ILC2s respond to unrelated allergens more potently than naive ILC2s and exhibit memory-like properties that may explain why asthma patients are often sensitized to multiple allergens.

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Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

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SUMMARY

Group 2 innate lymphoid cells (ILC2s) in the lung are stimulated by inhaled allergens. ILC2s do not directly recognize allergens but they are stimulated by cytokines including interleukin (IL)-33 released by damaged epithelium. In response to allergens, lung ILC2s produce T helper 2 cell type cytokines inducing T cell-independent allergic lung inflammation. Here we examined the fate of lung ILC2s upon allergen challenges. ILC2s proliferated and secreted cytokines upon initial stimulation with allergen or IL-33, and this phase was followed by a contraction phase as cytokine production ceased. Some ILC2s persisted long after the resolution of the inflammation as allergen-experienced ILC2s and responded to unrelated allergens more potently than naive ILC2s, mediating severe allergic inflammation. The allergen-experienced ILC2s exhibited a gene expression profile similar to that of memory T cells. The memory-like properties of allergen-experienced ILC2s may explain why asthma patients are often sensitized to multiple allergens.

INTRODUCTION

Allergic inflammation is driven by the cytokines interleukin (IL)-4, IL-5, and IL-13. IL-4 promotes the production of IgE by B cells. IgE induces degranulation of mast cells and basophils, resulting in the release of inflammatory mediators including histamine (Steinke and Borish, 2001). IL-5 triggers eosinophil differentiation, recruitment, and activation (Hamelmann and Gelfand, 2001). IL-13 acts on many cell types, including epithelial cells, and promotes mucus hyper-production and tissue remodeling (Wills-Karp, 2004). These cytokines are predominantly produced by T helper 2 (Th2) cells. Upon initial exposure to allergens, some individuals may become sensitized as rare allergen-specific $CD4^+$ T cells proliferate and differentiate into Th2 cells, some of which become long-lived memory T cells. Upon re-exposure to the same allergen, memory Th2 cells rapidly produce large amounts of Th2 cell type (type 2) cytokines, resulting in allergic inflammation.

Whereas the crucial role of Th2 cells in allergic inflammation is well established, group 2 innate lymphoid cells (ILC2s) are also important sources of IL-5 and IL-13 in allergic inflammation (Halim et al., 2012a; Moro et al., 2010). Intranasal injections of the protease allergen papain into naive wild-type (WT) and recombination activating gene 2 (RAG2)-deficient mice induce IL-5 and IL-13 production in bronchoalveolar lavage fluid (BALF), eosinophil infiltration, and mucus hyper-production in the lung (Halim et al., 2012a; Oboki et al., 2010). In contrast, papain injections into ILC2-deficient mice fail to induce strong lung inflammation. Furthermore, transplantation of ILC2s into the ILC2-deficient mice restore the development of type 2 lung inflammation upon papain treatment (Halim et al., 2012b). Stimulation by other allergens including house dust mite extract also results in lung ILC2 stimulation and concomitant production of IL-5 and IL-13 (Gold et al., 2014; Halim et al., 2012b). Epithelial cell-derived cytokines including IL-25, thymic stromal lymphopoietin (TSLP), and IL-33 (Liew et al., 2010) mediate allergen-induced ILC2 activation (Halim et al., 2014; Salmond et al., 2012). Allergic lung inflammation triggered by chitin inhalation is impaired in mice deficient for the receptors for these cytokines (Van Dyken et al., 2014). Papain-induced ILC2 activation and lung inflammation are also severely impaired in IL-33-deficient mice (Halim et al., 2014; Oboki et al., 2010). In addition, purified lung ILC2s are stimulated in vitro by a combination of IL-33 and IL-7 or TSLP. Also, intranasal injections of IL-33 alone strongly stimulate lung ILC2s and induce severe type 2 lung inflammation in vivo (Halim et al., 2014). These findings have led to a model wherein allergen exposure and associated tissue damage result in IL-33 production in the lung, which activates ILC2s and results in allergic lung inflammation that is independent of Th2 cells (Martinez-Gonzalez et al., 2015).

Lung ILC2s also play a role in promoting Th2 cell responses to allergens. Intranasal injections of papain into naive mice stimulate naive $CD4^+$ T cells to differentiate into the Th2 cell pathway. When the papain-primed mice receive challenge injections of papain, large numbers of IL-5- and IL-13-producing Th2 cells are generated in the lung, resulting in severe allergic lung inflammation and high titers of IgE. ILC2-derived IL-13 is essential for effective Th2 cell priming; Th2 cell differentiation in papain-treated naive mice and Th2 cell-mediated allergic inflammation

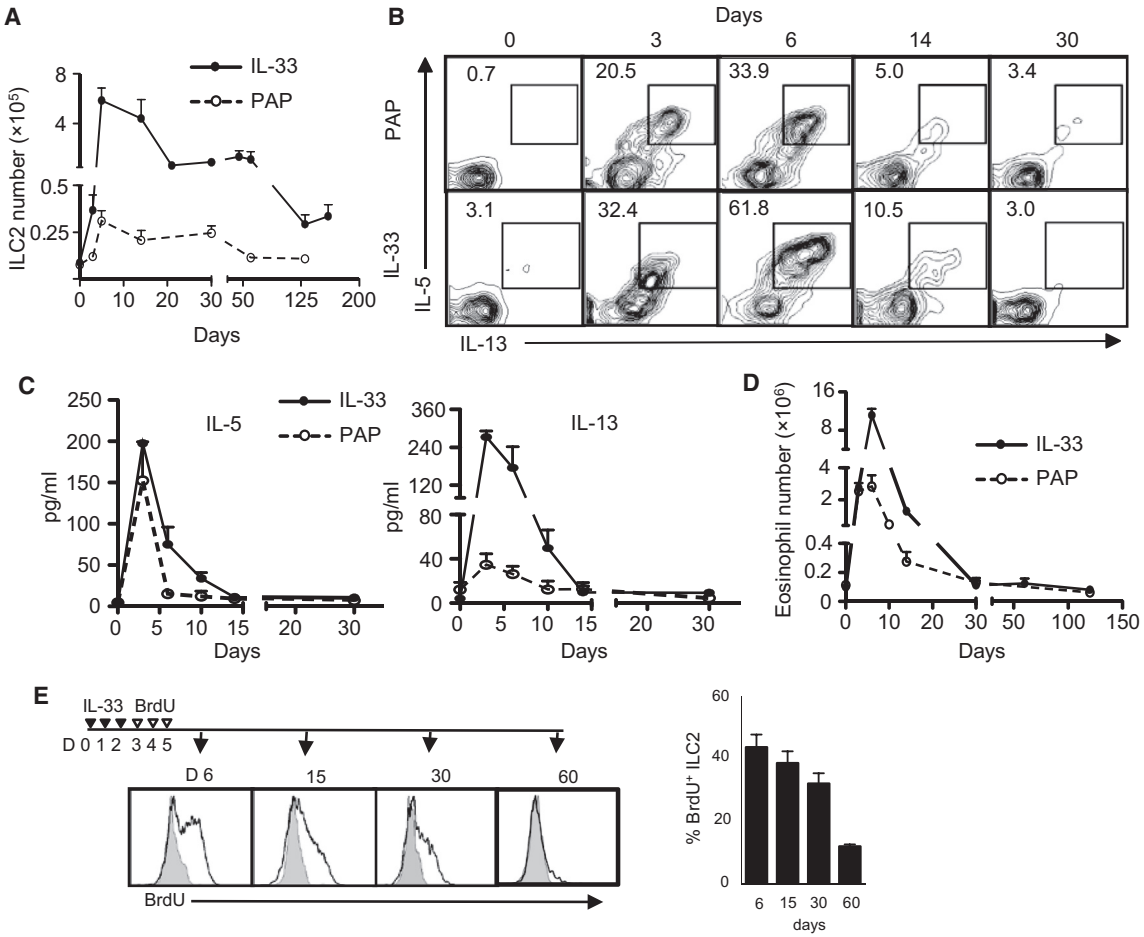


Figure 1. Previously Activated Lung ILC2s Persist after the Resolution of Lung Inflammation

(A and B) Mice received three daily intranasal injections of papain (PAP) or IL-33, and the numbers of lung ILC2s at various time points were determined by flow cytometry and the total numbers of leukocytes in the lung (A). Lung cells at indicated time points were stained for intracellular IL-5 and IL-13 and analyzed by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. ILC2s were gated (as live $CD45^+Lin^-ST2^+Thy1^+$ cells) and intracellular IL-5 and IL-13 fluorescence was plotted on contour plots (B). Numbers in plots indicate the (mean ± SEM) percentages of gated cells among ILC2s.

(C) Amounts of IL-5 and IL-13 in BALF from the mice treated with IL-33 (closed circles, solid lines) and PAP (open circles, dashed lines) were determined by ELISA.

(D) Lung eosinophil numbers at various time points after IL-33 or PAP injections were determined by flow cytometry.

(E) IL-33 and BrdU were intranasally injected into mice as indicated and BrdU-labeled lung ILC2s were analyzed by flow cytometry. Control histogram (shaded) was generated with untreated mice stained with anti-BrdU antibody.

Data represented are the mean ± SEM of three experiments, four to ten mice per group (A, C, D) or representative of two experiments with three mice per group (B, E). *p < 0.05, two-tailed Student's t test. See also Figure S1.

in the challenged mice are impaired in ILC2-deficient mice (Halim et al., 2014).

Thus, although lung ILC2s have been shown to be critically involved in the initiation of allergic inflammation, their role in chronic allergy, which can result in lung diseases including asthma, remains to be elucidated. Here we examine the fate of allergen-stimulated lung ILC2s in mice. We find that after the initial activation and expansion, lung ILC2s undergo a contraction phase and stop producing cytokines, resulting in the resolution of type 2 lung inflammation. A population of ILC2s that persists in the lung and the draining mediastinal lymph node (mLN) display memory-like characteristics and respond more vigorously to unrelated allergen challenge, exacerbating allergic lung inflammation. Our findings suggest that memory-like ILC2s are probably responsible for the sensitization of individuals to multiple allergens.

RESULTS

Papain and IL-33 Elicit Long-Term Changes in the ILC2 Population in the Lung

To study the dynamics of the ILC2 population after stimulation, we gave intranasal injections of IL-33 or papain into naive B6 mice and analyzed lung ILC2s at different time points. Three daily intranasal injections of IL-33 or papain resulted in a rapid increase in the number of ILC2s from fewer than 10^4 in the lung of naive mice to more than 5×10^5 in the IL-33-treated and more than 3×10^4 in the papain-treated mouse lungs (Figure 1A).

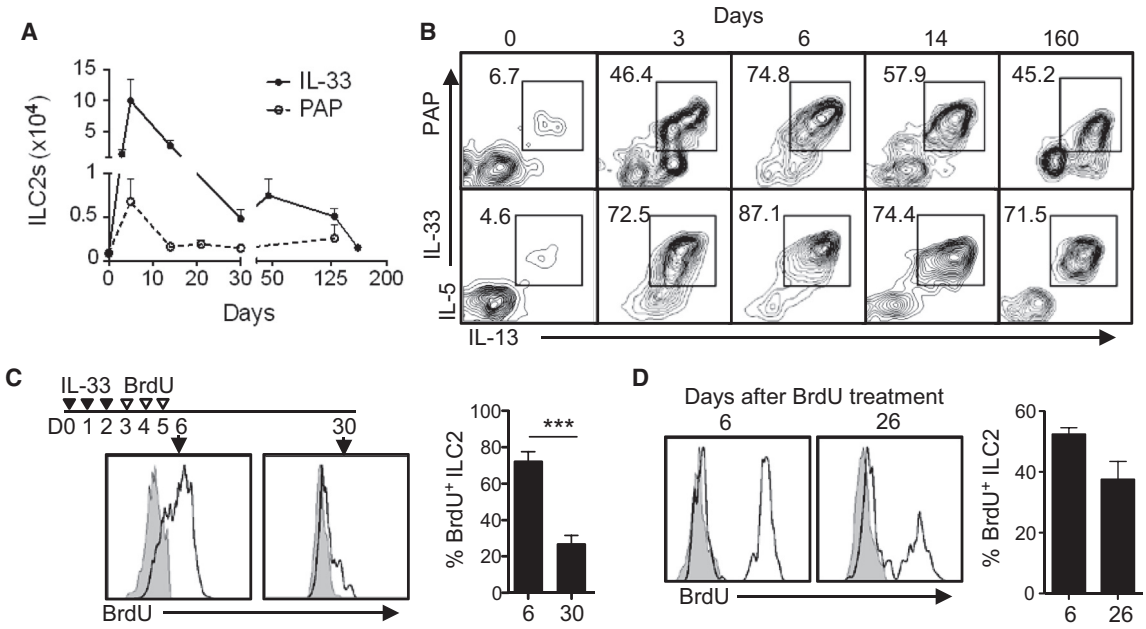


Figure 2. Activated ILC2s Persist in the mLN

(A and B) Mice were treated with IL-33 or papain (PAP) as in Figure 1, and mLN ILC2s at various time points were quantified (A) and analyzed for intracellular IL-5 and IL-13 expression (B) by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. The numbers in the plots show the percentages of cells in the rectangle gates among ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells).

(C and D) Mice received daily intranasal injections of IL-33 and BrdU (C) or PBS and BrdU (D), and BrdU$^+$ mLN ILC2s were analyzed by flow cytometry. Control histogram (shaded) was generated using untreated mice stained with anti-BrdU antibody.

Data represented are the mean ± SEM of three experiments, four to ten mice per group (A) or representative of two experiments with three mice per group (B, C, D). ***$p < 0.001$, two-tailed Student's t test. See also Figure S2.

The stimulated ILC2s produced cytokines as shown by intracellular IL-5 and IL-13 staining (Figure 1B) and the increase in the amounts of those cytokines in BALF (Figure 1C). Accordingly, large numbers of eosinophils infiltrated into the lung (Figure 1D). IL-33 was more potent than papain in stimulating lung ILC2s and induced more cytokine production in the BALF and eosinophil infiltration into the lung. Upon intranasal injections of bromodeoxyuridine (BrdU) into the IL-33-treated mice, most lung ILC2s incorporated BrdU (Figure 1E, histogram). Therefore, the increase in the numbers of lung ILC2s was probably due to proliferation of lung-resident ILC2s rather than recruitment from other tissues.

After the initial expansion, the ILC2 population in the lung started to contract and stopped producing cytokines, and accordingly, cytokine levels in BALF and lung eosinophil numbers declined, indicating the resolution of the inflammation (Figures 1A–1D). Unexpectedly, the numbers of ILC2s in the lung remained much higher in the IL-33-treated (10-fold) and papain-treated (2.5-fold) mice than those in naive mice for more than 4 weeks (Figure 1A). To determine whether the high ILC2 numbers were due to survival of the stimulated ILC2s, we gave intranasal injections of BrdU into the IL-33-treated mice and analyzed ILC2s at various time points (Figure 1E). BrdU-labeled ILC2s were detectable for at least 60 days. However, the intensity of the BrdU staining progressively declined, suggesting that the stimulated ILC2s were slowly dividing. In naive mouse lungs, a lower percentage of ILC2s was labeled by BrdU, which significantly ($p < 0.05$) declined in 20 days whereas the intensity of the BrdU labeling did not decrease much (Figure S1). Altogether, these results suggest that some of the stimulated lung ILC2s live for a long time, resulting in the persistently high ILC2 numbers in the lungs of IL-33-treated mice long after the resolution of the inflammation.

Intracellular Cytokine-Positive ILC2s Persist in the mLN

In naive mice, the mLN was very small and contained fewer than 1,000 ILC2s (873.1 ± 123.8). Upon intranasal injections of papain or IL-33, the mLN enlarged and the numbers of ILC2s in the mLN rapidly increased by more than 100-fold and ~8-fold after the IL-33 and papain treatments, respectively (Figure 2A). The expansion of the mLN ILC2 population was followed by its contraction. The number of ILC2s in the mLN of the papain-treated mice rapidly declined to the level similar to that of a naive mouse whereas ILC2 numbers in the IL-33-treated mice remained higher than those in naive mice for 4 months before eventually returning to the naive mouse level. Most mLN ILC2s in naive mice were negative for intracellular IL-5 and IL-13, whereas the majority of them became IL-5$^+$IL-13$^+$ after the papain or IL-33 injections (Figure 2B). Eosinophils were recruited into the mLN during the expansion phase of ILC2s but the number rapidly declined as the ILC2 population contracted (Figure S2). The percentages of IL-5$^+$IL-13$^+$ ILC2s in the mLN were much higher than those in the lung. Moreover, unlike in the lung, the percentages of IL-5$^+$IL-13$^+$ ILC2s in the mLN remained high for more than 5 months (Figure 2B). However, it seems

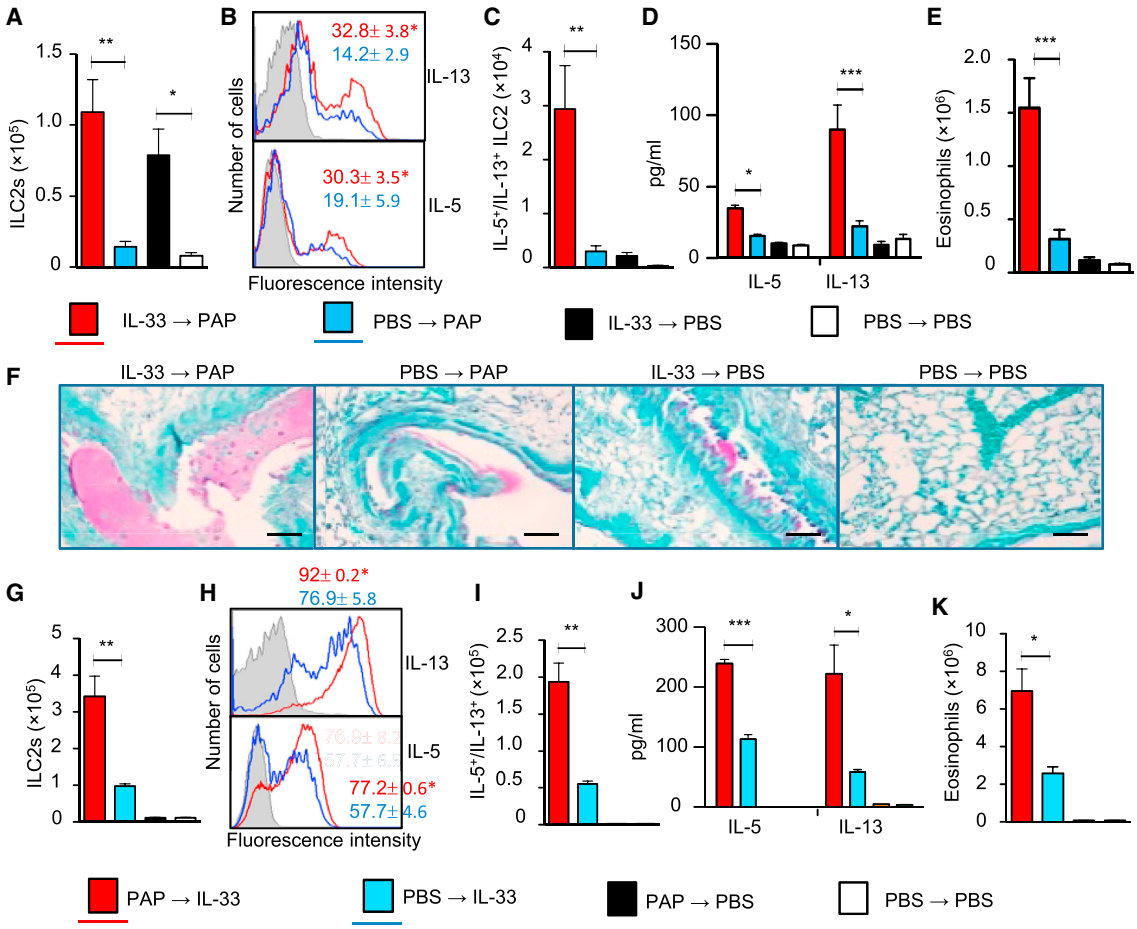


Figure 3. IL-33- or Papain-Experienced Lung ILC2s Respond Vigorously to a Secondary Challenge

(A–F) Mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of papain (PAP) or PBS. Two days later, lung ILC2 numbers (A), intracellular IL-5 and IL-13 staining of lung ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) after 3 hr of re-stimulation with PMA+ionomycin (B), and the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (C) were analyzed by flow cytometry, and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (D). The numbers of eosinophils in the lung were also determined by flow cytometry (E), and lung tissue sections were stained by PAS staining for mucus (F). Scale bar corresponds to 10 μm.

(G–K) Mice received three daily intranasal injections of papain or PBS. Two months later, they received a single intranasal injection of IL-33 or PBS, and ILC2 numbers (G), intracellular IL-5 and IL-13 staining of lung ILC2s after 3 hr of re-stimulation with PMA+ionomycin (H), and the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (I) were analyzed by flow cytometry, and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (J). The numbers of eosinophils in the lung were also determined by flow cytometry (K). Bars in the graphs are color coded for each treatment group as shown. Histograms showing intracellular staining of indicated cytokines are also color coded in the same way. Shaded histograms show FMO control.

Data are representative of at least three independent experiments. Mean ± SEM. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ (two-tailed Student's t test). See also Figure S3.

unlikely they were secreting these cytokines because eosinophils were no longer found in the mLN (Figure S2). Most (72.0% ± 5.5%) mLN ILC2s in the IL-33-injected mice incorporated BrdU (Figure 2C), but the percentage of BrdU$^+$ ILC2s rapidly declined in 1 month. In naive mice, BrdU injection labeled almost 50% of mLN ILC2s, which declined to about 35% in 3 weeks, whereas the intensity of BrdU staining remained mostly the same (Figure 2D). These results suggest that the decrease in the BrdU-labeled mLN ILC2s in the IL-33-treated mice was probably due to a combination of their proliferation, death, and emigration from the mLN whereas in naive mice it was probably due to turnover of the population. The intracellular cytokine staining combined with the BrdU labeling of mLN ILC2s also suggest that a subset of activated ILC2s persist in the mLN for several weeks after the initial activation.

ILC2s in IL-33- or Papain-Sensitized Mice Respond More Vigorously than Naive ILC2s to a Secondary Challenge

To test the function of the lung ILC2s that persisted in IL-33-treated mice, we challenged the mice with a single intranasal administration of papain 1 month after the IL-33 injections (Figure S3A). Without the papain challenge, lung ILC2s in the IL-33-pretreated mice (Figure 3A, black bar) were 10 times more abundant than in control PBS-injected mice (white bar). Although the numbers of lung ILC2s did not significantly increase by the single intranasal papain challenge in both control (blue bar

versus white bar) and the IL-33-pretreated (red bar versus black bar) mice, they became positive for intracellular IL-5 and IL-13 (Figure 3B). Due to much higher numbers of lung ILC2s and higher frequency of intracellular cytokine-positive cells in the IL-33-pretreated mice than in PBS control mice, the former mice had much higher numbers of IL-5$^+$IL-13$^+$ ILC2s than the latter after the papain challenge (Figure 3C, red versus blue). It should be noted that IL-33-pretreated ILC2s had increased intensity of intracellular cytokine staining than control (Figure S3B). The papain challenge also induced significantly more ($p < 0.01$) production of IL-5 and IL-13 in BALF (Figure 3D, red versus blue), more eosinophil infiltration into the lung tissue (Figure 3E, red versus blue), and more mucus production (Figures 3F and S3C) in the IL-33-pretreated mice than in PBS control mice. In contrast, PBS control mice developed barely detectable signs of inflammation upon single papain challenge. Of note, IL-5 (Figure S3D) and IL-13 (not shown) were predominantly produced by ILC2s, but not by CD4$^+$Lin$^+$ (T) cells. These results show that IL-33-experienced ILC2s that persist in the lung for a long time vigorously respond to papain and induce severe lung inflammation.

The number of ILC2s in the lungs of the IL-33-pretreated mice declined 5 months later (Figure S3E) to approximately 2.5×10^4 (Figure S3F, black bar), which was still higher than those in the PBS pretreated control mice (white bar). Papain challenge induced stronger ILC2 activation and more severe lung inflammation (Figures S3G–S3I) in the IL-33-pretreated mice (red bars) than in control mice (blue bars). Interestingly, in the IL-33-pretreated mice, the mLN enlarged after the papain challenge and contained higher numbers of IL-5$^+$IL-13$^+$ ILC2s than in the PBS-pretreated control mice (Figures S3J–S3L). Therefore, IL-33-pretreated mice develop a more severe lung inflammation after a single injection of papain up to 5 months later than untreated mice. This appears to be mainly due to the high numbers of lung ILC2s in the IL-33-treated mice. Whether responsiveness of ILC2s in the IL-33-treated mice contributed to the severe lung inflammation was difficult to assess due to their high numbers.

To further study the responsiveness of allergen-experienced ILC2s, we initially gave three daily intranasal injections of papain and waited for 2 months before giving a single intranasal injection of IL-33 (Figure S3M). The numbers of lung ILC2s in the papain-pretreated mice (Figure 3G, black bar) declined to the levels in control mice pretreated with PBS (white bar). The IL-33 challenge induced much greater increase in the numbers of lung ILC2s in papain-pretreated mice than control mice (Figure 3G, red versus blue). The frequency of intracellular IL-5$^+$ or IL-13$^+$ among ILC2s (Figure 3H), the total numbers of IL-5$^+$IL-13$^+$ ILC2s, and the intensity of intracellular cytokine staining (Figure S3N) were significantly higher in the papain-pretreated mice than control mice (Figure 3I, red versus blue). Accordingly, the amounts of IL-5 and IL-13 in BALF and the eosinophil infiltration into the lung were also much higher in the papain-pretreated than in the control mice (Figures 3J and 3K). The papain-pretreated mice and control naive mice had the same number of ILC2s in the lung at the time of the IL-33 injection, so the severe type 2 lung inflammation in the papain-pretreated mice as compared to control mice can not be explained by higher numbers of ILC2s. Instead, the results indicate that papain-experienced ILC2s are more responsive than naive ILC2s to the IL-33 challenge.

Allergen-Experienced ILC2s Are Highly Responsive to Unrelated Allergens

We gave three daily intranasal injections of fungal allergen *Aspergillus oryzae* serine protease (Kheradmand et al., 2002) and gave a single challenge injection of papain 3.5 months later (Figure S4A). The initial injections of the fungal protease allergen (ASP) activated lung ILC2s (Figures S4B and S4C) and induced eosinophil infiltration into the lung (Figure S4D) as efficiently as papain. At 3.5 months later, the number of lung ILC2s in the treated mice declined to the same level as in the PBS control mice (Figure 4A, black versus white). When the mice received a single intranasal papain challenge injection, the total numbers of ILC2s and IL-5$^+$IL-13$^+$ ILC2s in the lungs were higher in the mice pretreated with ASP than those in control PBS-pretreated mice (Figures 4A and 4B, red versus blue). The intensities of intracellular IL-5 and IL-13 in lung ILC2s in ASP-pretreated mice were also higher than those in the control mice (Figure 4C). Accordingly, the amounts of IL-5 and IL-13 in BALF (Figure 4D), eosinophil infiltration (Figure 4E), and mucus production (Figure 4F) were higher in the mice pretreated with ASP than control PBS-pretreated mice. These results show that allergen-experienced ILC2s are more responsive to an unrelated allergen than naive ILC2s.

Allergen-Experienced ILC2s Are More Efficient than Naive ILC2s in Promoting Th2 Cell Differentiation

We have previously shown that ILC2-derived IL-13 promotes naive CD4$^+$ T cell differentiation into Th2 cells in the mLN (Halim et al., 2014). Because allergen-experienced ILC2s secreted high amounts of IL-13 upon exposure to an unrelated allergen, we tested whether this results in an increase in Th2 cell differentiation in the mLN. We initially treated mice with three daily intranasal injections of ASP (or PBS for control) to generate allergen-experienced ILC2s and gave a single intranasal injection of papain (or PBS for control) 1 month later to induce primary papain-specific Th2 cell response (Figure S5A). Th2 cells in the mLN were defined as CD4$^+$ T cells expressing intracellular IL-4 and IL-13 upon in vitro re-stimulation with PMA plus ionomycin for 3 hr. At 2 days after the papain injection, significantly more Th2 cells were generated in the mLN of the mice that initially received ASP injections (Figure 5A, red bar) than those in control mice that initially received PBS injections (blue bar), those that received PBS injection instead of papain challenge (black bar), or mice that received only PBS injections (white bar). Similar results were obtained with the ASP-treated mice that received the papain challenge 3.5 months later (Figure S5B). More Th2 cells were also generated in the mLN of mice initially receiving IL-33 injections in place of ASP (Figure S5C) and papain injection 1 month later (Figure 5B). Accordingly, 6 days after the papain challenge, Th2 cell numbers in the lung were significantly higher in the papain-challenged mice than those in control mice (Figure S5D, red versus others). We also gave a second papain injection into the mice (Figure 5C). Much higher numbers of Th2 cells were generated in the IL-33-primed mice than control (Figures 5D and 5E, red versus blue bars). These results suggest that

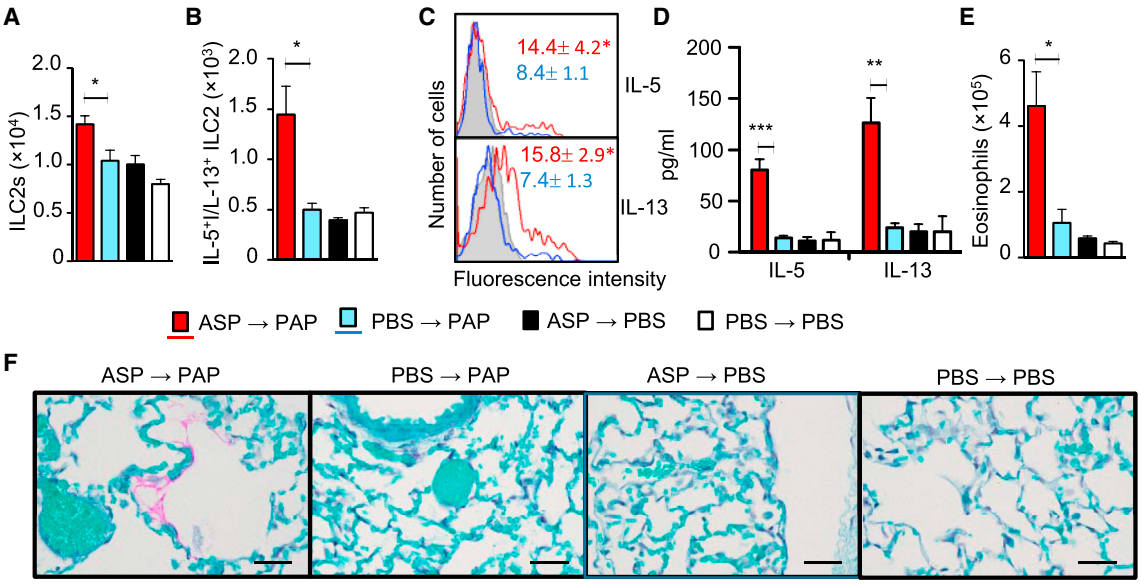


Figure 4. Allergen-Experienced Lung ILC2s Vigorously Respond to an Unrelated Allergen Challenge

Mice received three daily intranasal injections of *Aspergillus* protease (ASP) or PBS and received a single intranasal injection of papain (PAP) or PBS 3.5 months later. Two days later, lung ILC2 numbers (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (A), the numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s after 3 hr of re-stimulation with PMA+ionomycin (B), and intracellular IL-5 and IL-13 staining of lung ILC2s (C) were analyzed by flow cytometry. The amounts of IL-5 and IL-13 in BALF were determined by ELISA (D). The numbers of eosinophils in the lung were also determined by flow cytometry (E), and lung tissue sections were stained for mucus (F). Scale bar corresponds to 10 μm. Bars in the graphs are color coded for each treatment group as shown. Histograms showing intracellular staining of indicated cytokines are also color coded in the same way. Shaded histograms show FMO control. Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 (two-tailed Student's t test). See also Figure S4.

ILC2s previously activated by ASP or IL-33 enhance the papain-induced CD4$^+$ T cell differentiation into Th2 cells.

The Increased Responsiveness of IL-33-Experienced ILC2s Is Cell Autonomous

To test whether the hyper-responsiveness of allergen-experienced ILC2s was mediated by constitutive release of IL-33 by damaged epithelial cells, we tested IL-33-deficient mice. The mice received intranasal IL-33 injections and were challenged by IL-33 1 month later. The numbers of IL-5$^+$IL-13$^+$ ILC2s and allergic responses were much higher in the IL-33-primed mice than control mice (Figures S6A–S6D). In addition, the generation and maintenance of allergen-experienced ILC2s was T cell independent, as shown by the fact that the hyperresponsive ILC2s were similarly generated in the T cell-deficient *Rag1*$^{-/-}$ mice (Figures S6E–S6I). To test whether allergen-experienced ILC2s undergo intrinsic changes, mice were treated with three daily intranasal injections of IL-33 (or PBS for control) and lung ILC2s were purified 6 months later (Figure S6J). They were cultured with suboptimal doses of IL-33 and TSLP (Zhou et al., 2005), a combination known to stimulate ILC2s (Halim et al., 2012a), for 72 hr. ILC2s from IL-33-treated mice produced more than 4 times more IL-5 and IL-13 than naive ILC2s (Figure 6A). Thus, IL-33-experienced ILC2s are inherently more responsive than naive ILC2s to suboptimal concentrations of cytokines. To further validate this in vivo, we purified ILC2s from the lung of B6 (CD45.2) mice 1 month after the IL-33 treatment and intravenously injected into non-irradiated Pep3b (CD45.1) mice (Figure S6J). We then gave a single intranasal papain injection into the Pep3b mice 24 hr after the ILC2 transfer and analyzed the donor and the recipient ILC2s in the lung 2 days later. In the papain-challenged mice, the donor IL-33-experienced ILC2s (CD45.2$^+$) were readily detected in the lung (Figure 6B, left contour plot) and they were mostly positive for intracellular IL-5 and IL-13 staining (top, shaded histograms). Furthermore, the intensities of intracellular IL-5 and IL-13 staining of the donor ILC2s were higher than those of the recipient (CD45.1$^+$) naive ILC2s (dashed histograms). Without the papain injection, only small numbers of the donor ILC2s were detected in the recipient lungs (Figure 6B, right contour plot), and they were mostly negative for intracellular IL-5 and IL-13 (bottom histograms). Of note, allergen-experienced ILC2s also show higher production of cytokines than naive ILC2s when transplanted into NOD/SCID/*Il2rg*$^{-/-}$ (NSG) mice and challenged with a single intranasal administration of IL-33 (Figure 6C). Therefore, the IL-33-experienced ILC2s respond more vigorously than naive ILC2s to the papain challenge in the naive mouse lung environment. These data suggest that the high responsiveness of the allergen-experienced ILC2s is due to intrinsic changes in ILC2s.

IL-33-Experienced ILC2s Have a Gene Expression Signature Similar to that of Memory T Cells and Are Responsive to IL-25

To further characterize allergen-experienced ILC2s, we purified ILC2s from the lungs of naive and IL-33-treated mice 4 days (d4), 2 weeks (w2), and 4 months (m4) after the intranasal IL-33 injections, extracted RNA, and analyzed global gene expression profiles by Affymetrix microarray. The genes differentially (more

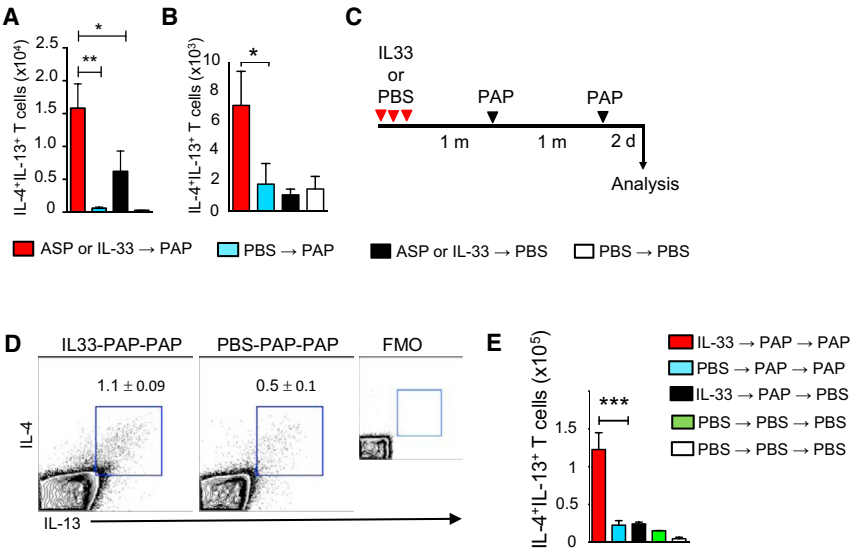


Figure 5. Allergen-Experienced ILC2s Promote Th2 Cell Differentiation upon Unrelated Allergen Challenge

(A) Mice treated with ASP or PBS received a secondary challenge with papain or PBS 1 month later, and $IL4^+IL13^+$ T cell numbers (gated as live $CD45^+CD4^+Thy1^+TCR\text{-}\beta^+$ cells) in the mLN were analyzed by flow cytometry 2 days after the secondary challenge.

(B) Mice treated with IL-33 or PBS received a secondary challenge with papain or PBS 1 month later. $IL\text{-}4^+IL\text{-}13^+$ T cell numbers in the mLN 2 days after the secondary challenge were analyzed by flow cytometry.

(C) Scheme for the treatment of mice with IL-33 and papain in (D) and (E).

(D and E) Lung cells were stained for intracellular IL-4 and IL-13 and analyzed by flow cytometry after 3 hr of re-stimulation with PMA+ionomycin. T cells were gated (as live $CD45^+CD4^+Thy1^+TCR\text{-}\beta^+$ cells) and intracellular IL-4 and IL-13 fluorescence was plotted on contour plots (D). Numbers in plots indicate the (mean ± SEM) percentages of gated cells among T cells. Numbers of intracellular $IL\text{-}4^+IL\text{-}13^+$ T cells (E) were analyzed by flow cytometry.

Bars in the graphs are color coded for each treatment group as shown. Data are representative of at least three independent experiments. Mean ± SEM. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ (two-tailed Student's t test). See also Figure S5.

than 2-fold) expressed in the ILC2 populations included *Il1r2*, *Il5*, *Tnfsf18*, *Bcl2a1b*, *Bcl2a1d*, *Ler3*, and *Syne1*, which were more highly expressed in activated (d4) ILC2s than naive ILC2s and remained significantly higher in IL-33-experienced (w2 and m4) than naive ILC2s (Figure 7A). In contrast, *S1pr1*, *Il6st*, *Cd2*, *Cd7*, and *Sell* were lower in d4, w2, and m4 than naive ILC2s. The proliferation/cell cycle-related genes *Mki67* and *Ccnb2* and the chemokine genes *Ccl17*, *Ccl24*, *Cxcl3*, and *Ccl6* were highly expressed in d4 but not naive, w2, or m4 ILC2s, suggesting that IL-33-experienced ILC2s were resting cells. Therefore, IL-33-experienced w2 and m4 ILC2s differed from naive and activated (d4) lung ILC2s in the expression of small subsets of genes. The expression of *Il5* and *Il13* was high in all the ILC2 populations and did not correlate with the intracellular staining of the protein products in ILC2s, suggesting that they are regulated at the translational level.

The differential gene expression profiles of naive, d4, w2, and m4 ILC2s were compared with annotated gene set database (Immunologic Gene Collection: http://software.broadinstitute.org/gsea/msigdb/index.jsp) by gene set enrichment analysis (GSEA). The analysis with the gene expression data of naive and m4 ILC2s resulted in the highest score of enrichment with the differentially expressed gene set of human naive $CD8^+$ T cells compared to memory $CD8^+$ T cells (Figure 7B, left; Abbas et al., 2009; Kaech et al., 2002; Luckey et al., 2006; Wherry et al., 2007). Normalized enrichment score (NES) of 2.27 and false discovery rate (FDR) of 0 indicated that the similarity in the differential gene expression profile of naive versus m4 ILC2s to that of naive versus memory $CD8^+$ T cells was highly significant. Similarly, analysis with the differential gene expression profile in d4 versus m4 lung ILC2s resulted in the highest score of enrichment with the gene set of human effector versus memory $CD8^+$ T cells (Figure 7B, right). GSEA with the data of w2 versus d4 and w2 versus naive also showed high scores of enrichment with effector versus memory $CD8^+$ T cells and naive versus memory $CD4^+$ T cells, respectively (Figure S7A). These results showed that the expression profiles of IL-33-experienced w2 and m4 ILC2s are similar to that of memory T cells.

Among the genes differentially expressed in naive and IL-33-experienced (w2 and m4) ILC2s, those encoding the IL-25 receptor (IL-25R), which consists of IL-17RA and IL-17RB (Figure 7E), were of particular interest because IL-25 is thought to an important ILC2-activating cytokine (Hammad and Lambrecht, 2015). The expression of *Il17rb* mRNA in w2 and m4 ILC2s was 2- to 4-fold higher than that in naive ILC2s whereas lower amounts of *Il17ra* mRNA were expressed in all the ILC2 populations tested (Figure 7C). Flow cytometric analysis confirmed the expression of the IL-25R protein on w2 and m4 ILC2s whereas it was very low on naive ILC2s (Figure 7D). To test the functional significance of the expression of IL-25R, we gave a single intranasal injection of IL-25 in mice pretreated with IL-33 6 months earlier and analyzed the lung and the mLN (Figure S7B). ILC2s in the IL-33-pretreated, but not naive (PBS control), mice were activated by IL-25 as indicated by the high numbers of total ILC2s and cytokine-producing ILC2s in the lung (Figures 7E–7G) and in the mLN (Figures S7C and S7D) as well as high numbers of eosinophils in the lung and increased levels of type 2 cytokines in BALF (Figures 7H and 7I). Of note, naive ILC2s did not respond to three consecutive daily intranasal injections of IL-25 (Figures S7E–S7H). These results showed that IL-33-experienced ILC2s significantly differ from naive ILC2s in their responsiveness to IL-25.

DISCUSSION

In this study, we have shown that allergen-experienced ILC2s generated by intranasal injections of allergen or IL-33 are more readily stimulated by allergen challenge, proliferating more vigorously and producing greater amounts of type 2 cytokines than naive ILC2s stimulated by the same allergen. Although changes

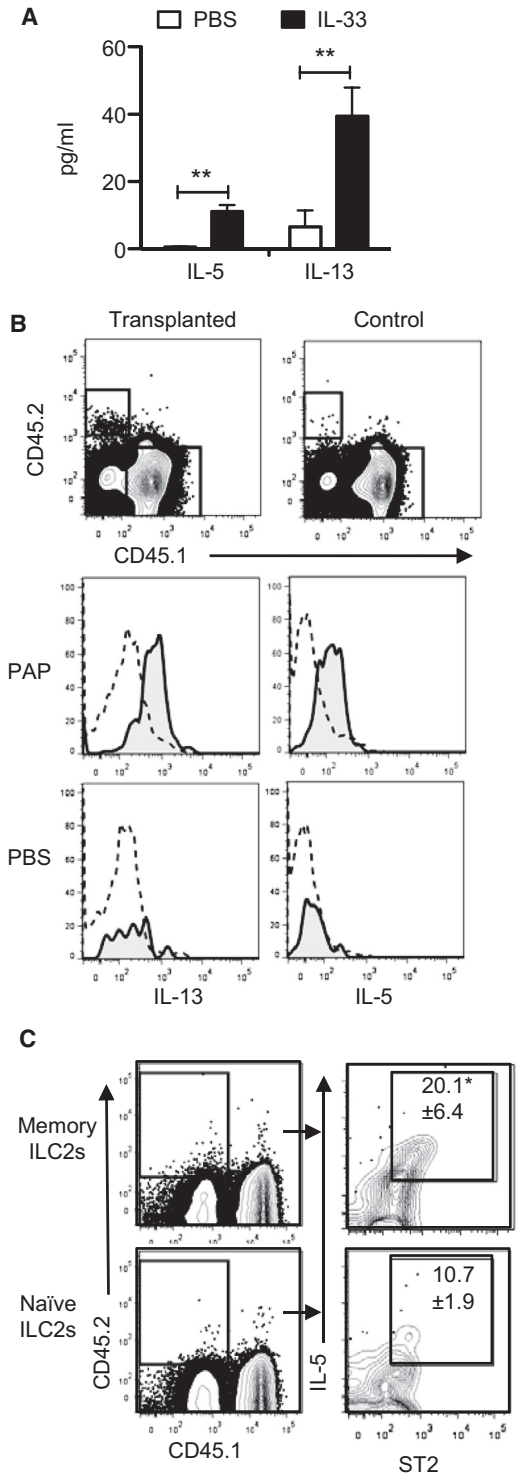


Figure 6. The Increased Responsiveness of IL-33-Experienced ILC2s Is Cell Autonomous

(A) Mice were treated with IL-33 or PBS and lung ILC2s (gated as live CD45^{+-} Lin^{-}ST2^{+}Thy1^{+} cells) were purified 6 months later. The purified ILC2s were cultured for 72 hr with IL-33 (2.5 ng/mL) and TSLP (2.5 ng/mL), and the amounts of IL-5 and IL-13 in the culture supernatants were determined by ELISA.

(B) ILC2s were purified from IL-33-treated B6 mice 1 month after the treatments and transferred into Pep3b mice. One day after the cell transfer, the mice received a single intranasal injection of papain (PAP) or PBS. Donor (CD45.2) and host (CD45.1) lung ILC2s were gated (top contour plots) and analyzed for intracellular IL-5 and IL-13 (histograms) after 3 hr of re-stimulation with PMA+ionomycin. Shaded histograms show donor ILC2s and dashed histograms show the host ILC2s.

(C) ILC2s were purified from naive or IL-33-treated B6 mice 1 month after the treatments and transferred into NSG mice. One day after the cell transfer, the mice received a single intranasal injection of IL-33. Donor (CD45.2) lung ILC2s were gated (left contour plots) and analyzed for intracellular IL-5 (right contour plots) after 3 hr of re-stimulation with PMA+ionomycin.

Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01 (two-tailed Student's t test). See also Figure S6.

in the lung environment induced by initial allergen/IL-33 injections may contribute to the high responsiveness of allergen-experienced ILC2s, purified allergen-experienced ILC2s produce more cytokines than naive ILC2s upon in vitro stimulation by suboptimal amounts of IL-33 and TSLP as well as upon transplantation into naive mice and stimulation by intranasal IL-33 injection. Therefore, the high responsiveness of allergen-experienced ILC2s is probably mediated by cell-intrinsic changes. IL-33-experienced ILC2s differ from naive and effector ILC2s in gene expression profiles, further supporting the notion of cell-intrinsic changes in allergen-experienced ILC2s. Although the molecular basis of the high responsiveness of allergen-experienced ILC2s is still unclear, it seems likely that the increased expression of the IL-25R may play a role. Naive mouse lung ILC2s express very low amounts of IL-25R mRNA and the protein products, and they are not activated by intranasal injections of IL-25, consistent with the results reported by Huang et al. (2015). In contrast, IL-33-experienced ILC2s express higher amounts of IL-25R mRNA and protein, and they are readily activated in vivo by intranasal IL-25 injections. However, it should be noted that purified IL-33-experienced ILC2s are not activated by IL-25 alone in vitro (data not shown), suggesting that other factors are also involved.

The persistence of allergen-experienced ILC2s and their increased responsiveness to a secondary challenge are characteristics of memory lymphocytes (Harrington et al., 2008; Löhning et al., 2008). However, ILC2s are antigen non-specific whereas antigen specificity has been considered to be a key feature of immunological memory (Remakus and Sigal, 2013; Wherry and Ahmed, 2004). Although immunological memory of NK cells, which belong to the innate lymphocyte population, has been reported, memory NK cells are antigen specific (Sun et al., 2009; Paust et al., 2010).

In addition to antigen-specific memory NK cells, cytokine-activated NK cells have been shown to have antigen non-specific memory-like properties (Cooper et al., 2009). Furthermore, monocytes and macrophages have recently been shown to acquire antigen non-specific memory-like characteristics (Cheng et al., 2014; Saeed et al., 2014). Upon recognition of a microbial ligand, macrophages are capable of adapting and reshaping their response to a subsequent microbial assault. Quintin et al. (2014) have proposed the term "trained immunity" to describe the enhanced innate host defense against a second infection. However, trained immunity significantly differs from the memory-like properties of allergen-experienced ILC2s in our current study. In trained immunity, monocytes/macrophages are "trained" to acquire new functions. It involves changes in

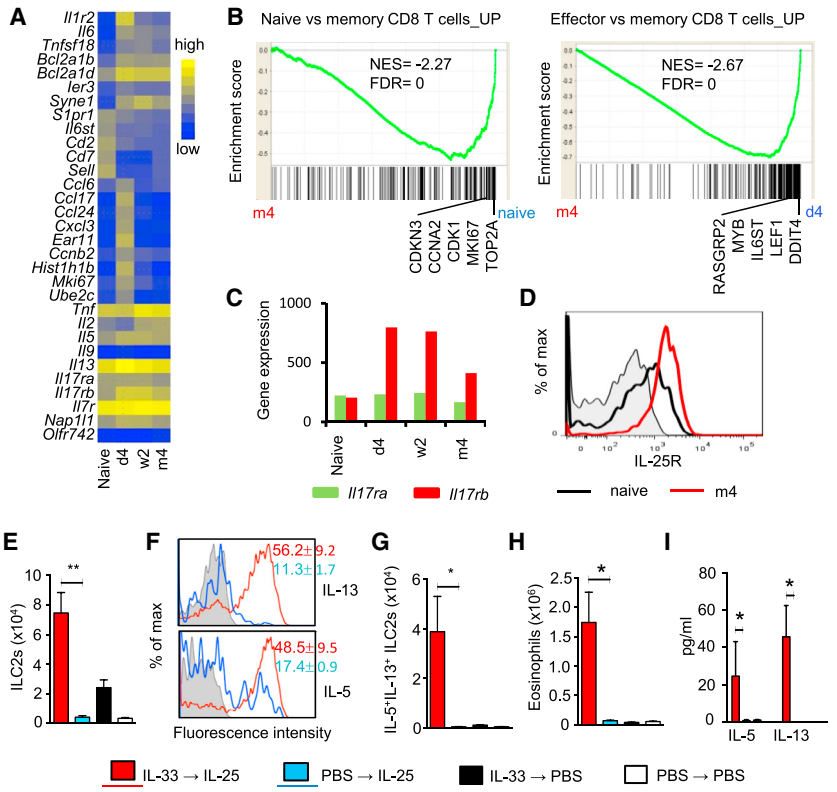


Figure 7. Gene Expression Profiles of Memory-like ILC2s in Lung Show Gene Signatures of Memory T Cells

(A) Heatmap shows the relative expression levels of genes that are differentially expressed in ILC2s in the lung of PBS-injected (naive) mice and 4 days (d4), 2 weeks (w2), and 4 months (m4) after intranasal IL-33 injection.

(B) Gene set enrichment analysis (GSEA) of naive, d4, and m4 ILC2s in the lung. Log2-fold expression values for each comparison were ranked in descending order. The x axis shows individual genes and the y axis shows enrichment score. The five most enriched genes are listed on the x axis of each panel. Left panel shows that genes upregulated in naive CD8$^+$ T cells compared to memory CD8$^+$ T cells were significantly enriched in naive lung ILC2s when compared to m4 ILC2s. Right panel shows that genes upregulated in effector CD8$^+$ T cells compared to memory CD8$^+$ T cells were significantly enriched in d4 lung ILC2s when compared to m4 ILC2s. Abbreviations are as follows: NES, normalized enrichment score; FDR, false discovery rate.

(C) The expression levels of *Il17ra* and *Il17rb* mRNA in naive, d4, w2, and m4 ILC2s as determined by the microarray analyses above were compared. The logarithmic values of the expression levels in the microarray assays were converted into the linear scale. Green bars and red bars show the expression levels of *Il17ra* and *Il17rb*, respectively.

(D) IL-25R expression was analyzed in naive and memory-like ILC2s (red and blue histograms, respectively) by flow cytometry.

(E–I) Mice received three daily intranasal injections of IL-33 or PBS and received a single intranasal injection of IL-25 or PBS 6 months later. Two days after the challenge, ILC2 numbers (E), intracellular IL-5 and IL-13 staining of lung ILC2s (F), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (G), and lung eosinophil numbers (H) after 3 hr of re-stimulation with PMA+ionomycin were analyzed by flow cytometry. The amounts of IL-5 and IL-13 in BALF were determined by ELISA (I). Each treatment group is color coded as indicated in the figure.

Data are representative of at least three independent experiments. Mean ± SEM. *p ≤ 0.05, **p ≤ 0.01 (two-tailed Student's t test).

global gene expression, in part mediated by epigenetic programming (Kleinnijenhuis et al., 2012; Quintin et al., 2012). In contrast, allergen-experienced ILC2s do not acquire a new function. Naive and allergen-experienced ILC2s have the same function, namely producing IL-5 and IL-13 upon stimulation. The main difference is that the latter are more responsive and produce higher amounts of the same cytokines than the former. They differ in the expression of only a small set of genes, not a broad range of genes seen with trained immunity.

Both ILC2s and T/B cells remember a prior activation by changing the expression of selected genes that make them more responsive to secondary stimulation than primary stimulation. The main difference between ILC2s and T/B cells is how the cells are activated. ILC2s are activated by cytokines whereas T/B cells are activated by specific antigens, thus making the latter antigen specific. The recent report of antigen non-specific memory Th2 cells (Guo et al., 2015) is consistent with this view. Therefore, allergen-experienced ILC2s are more similar to memory lymphocytes than trained innate cells.

Activation of lung ILC2s by intranasal injections of IL-33 or papain results in increases in ILC2 numbers not only in the lung but also in the draining mLN. Because most ILC2s in the lung and mLN in the IL-33-injected mice incorporate BrdU, the increases in ILC2 numbers are most probably due to the proliferation of the stimulated ILC2s rather than recruitment from other tissues. It is still unknown whether ILC2s in the mLN proliferate in the mLN or whether proliferating ILC2s in the lung migrate to the mLN. Almost all mLN ILC2s become positive for intracellular IL-5 and IL-13 during the expansion phase, and they seem to secrete the cytokines in vivo as suggested by eosinophil infiltration into the mLN. Unlike lung ILC2s, mLN ILC2s remain positive for intracellular IL-5 and IL-13 after the resolution of inflammation, suggesting that they remain activated. However, the role of memory-like ILC2s in the mLN in type 2 lung inflammation induced by allergen challenge is still unknown.

We have previously shown that intranasal injections of papain into naive mice not only stimulate lung ILC2s but that ILC2-derived IL-13 also promotes naive CD4$^+$ T cell differentiation into the Th2 pathway. Thus, papain injections into papain-primed mice stimulate both ILC2s and Th2 cells, resulting in severe allergic lung inflammation. In the current study, we used different stimuli for priming and challenge to avoid the stimulation of memory Th2 cells generated in the initial allergen priming. Nevertheless, the primary Th2 cell responses to papain in mice primed by fungal protease or IL-33 were higher than those of naive mice. It is possible that IL-33 produced by papain treatment stimulates ASP-specific memory T cells in an antigen non-specific manner as reported by Guo et al. (2015). However, the papain treatment also enhanced Th2 cell differentiation in IL-33-pretreated mice, which unlikely had memory Th2 cells. Therefore, memory-like

ILC2s enhance not only ILC2-mediated innate but also Th2 cell-mediated adaptive type 2 lung inflammation, and they provide a new clue to the question of how individuals become sensitized to a broad range of allergens. Currently, the diagnosis and treatment of allergic diseases rely on identifying specific allergens by skin test and IgE detection. However, many patients react against multiple allergens (Calderón et al., 2012). Furthermore, specific allergens are not readily identified in some asthma patients, who are considered to have non-allergic asthma (DeKruyff et al., 2014). It is conceivable that antigen non-specific memory-like ILC2s play a critical role in such diseases.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 (B6), NSG, and *Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratories. *Il33*$^{-/-}$ mice were generated from the breeders obtained from the Knockout Mouse Project Repository. All mice were maintained in the British Columbia Cancer Research Centre animal facility under specific-pathogen-free conditions. 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, FACS Sorting, and Analysis

FITC-conjugated anti-CD3ε, CD19, NK1.1, Mac-1, CD11c, Gr-1, Ter119, TCR-αβ, TCR-γ, PE-conjugated anti-CD127, CD3, PerCP-Cy5.5-conjugated anti-ST2, CD3, PE.Cy7-conjugated anti-DX5, CD4, APC-conjugated anti-CD3, FcεR1α, CD25, APC-eFluor780-conjugated anti-B220, Alexa Fluor 700-conjugated anti-CD11c, eFluor 450-conjugated anti-CD3ε, CD19, NK1.1, Gr-1, CD11b, CD11c, Ter119, TCRαβ, TCRγ, and eFluor 605-conjugated anti-Thy1.2 were purchased from eBioscience. FITC-conjugated anti-7/4 was purchased from Abcam. PE-conjugated anti-IL-13, Siglec-F, APC-conjugated anti-IL-5, and BD Horizon V500-conjugated anti-CD45 was purchased from BD Bioscience. eFluor 780 (eBioscience) was used to exclude non-viable cells. Unconjugated anti-IL-33 and TSLP were purchased from eBioscience. Papain, PMA, and ionomycin were purchased from Sigma Aldrich. *Aspergillus oryzae* protease allergen was purchased from Sigma. BD Fortessa was used for phenotypic analysis; BD FACS Aria II was used for cell sorting and phenotypic analysis. Flowjo v.8.6 was used for data analysis.

Primary Leukocyte Preparation

Cell suspensions were prepared from lung as described (Halim et al., 2014).

Isolation and Detection of ILC2s

Single 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, CD11c, Gr-1, Ter119, TCRαβ, TCRγ), PE-conjugated CD127, PerCP-Cy5.5-conjugated ST2, PE.Cy7-conjugated CD4, APC-conjugated CD25, V500-conjugated CD45, and eFluor 780 fixable viability dye and purified by FACS. EasySep Mouse ILC2 Enrichment Kit (STEMCELL Technologies, cat# 19842) was used prior to cell sorting of naive ILC2s.

ILC2 In Vitro Culture

ILC2s were purified by flow cytometry, 500 cells per well were cultured in 200 μL RPMI-1640 media containing IL-33 (2.5 ng/mL), TSLP (2.5 ng/mL), 10% FBS, penicillin and streptomycin, and 2-Mercaptoethanol at 37°C for 36 hr. The amounts of IL-5 and IL-13 in the culture supernatants were analyzed by ELISA.

ILC2 Transplantation

B6 mice were treated with three consecutive daily intranasal injections of IL-33 (0.25 μg/mouse) and lung ILC2s were purified 1 month later. Five thousand purified ILC2s were injected i.v. into each Pep3b or RAG1-deficient mouse. One day after the cell transfer, the mice received a single intranasal injection of papain (PAP), IL-33, or PBS and lung ILC2s were analyzed by flow cytometry.

In Vivo Stimulation

Mice were anesthetized by isofluorane inhalation, followed by intranasal injection of rIL-33 (0.25 μg), rIL-25 (0.25 μg), *Aspergillus oryzae* protease allergen (50 μg), and papain (15 μg) in 40 μL of PBS on days 0, 1, and 2, and at 1, 2, 3.5, or 5 months later depending on the experiment. Mice were sacrificed at indicated times and spleen, lungs, and BALF (1 mL PBS) were collected or airways were instilled with 1 mL of paraphormadehyde (Sigma) and fixed in formalin. Lung tissue was processed as described previously; lung cells were then counted and identified by flow cytometry. Fixed tissues were embedded in paraffin and processed for PAS staining. For histology, lung sections of three different depths per animal were analyzed (30 fields of view per animal).

Intracellular staining

Intracellular staining for IL-5 and IL-13 was performed using the Cytofix/Cytoperm kit (BD Biosciences) after 3 hr re-stimulation of 2×10^6 total live nucleated cells in 500 μL RPMI-1640 media containing 10% FBS, P+S, 2 ME, Monensin (Golgi Stop, BD Biosciences), PMA (30 ng/mL), and ionomycin (500 ng/mL) at 37°C. Dead cells were stained with eFluor 780 (eBioscience) fixable viability dye before fixation and permeabilization and excluded during analysis.

Quantification of Cytokine

BAL and cell-culture samples were analyzed for IL-5 and IL-13 by ELISA (eBioscience) according to the manufacturer's protocol.

BrdU

Mice received 3 daily intranasal injections of 0.8 mg of BrdU in 80 μL starting 1 day after the last administration of IL-33. Single-cell suspensions were prepared from lung tissues 24 hr after the last injection as above, and cells were stained with antibodies for surface markers and the viability dye as above to identify ILC2s. Cells were then fixed, followed by staining for BrdU using a FITC BrdU Flow Kit (BD Biosciences).

RNA Extraction, Microarray Analysis, and GSEA

Total RNA was extracted from FACS-purified ILC2s using TRIzol reagent (Life Technologies) according to manufacturer's protocol. RNA quality check, cDNA amplification, and microarray hybridization were performed by The Centre for Applied Genomics. In brief, the quality of the extracted RNA was tested by Agilent 2100 Bioanalyzer. The samples with RNA integrity number (RIN) above 6 were used to generate cDNA, which was amplified using Ovation Pico WTA kit (Nugen) and hybridized to Affymetrix GeneChip Mouse Gene 2.0ST Array. Two to three samples per group were analyzed. The expression profile was normalized using robust multi-array (RMA) algorithm and all data analyses were performed by Flex Array 1.6.3 (Genome Quebec). Gene set enrichment analysis was performed using GSEA software and the gene set collection C7 (immunological signatures) (http://www.broadinstitute.org/gsea/msigdb/index.jsp).

ACCESSION NUMBERS

GEO accession number for the microarray data is GEO: GSE81700.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2016.06.017.

AUTHOR CONTRIBUTIONS

I.M.-G. and L.M. designed and performed the experiments and wrote the paper. M.G., G.F.T.P., and C.A.S. performed experiments. F.T. supervised the project, designed the experiments, and wrote the paper.

ACKNOWLEDGMENTS

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Immunity, Volume *45*

Supplemental Information

Allergen-Experienced Group 2 Innate Lymphoid Cells Acquire Memory-like Properties and Enhance Allergic Lung Inflammation

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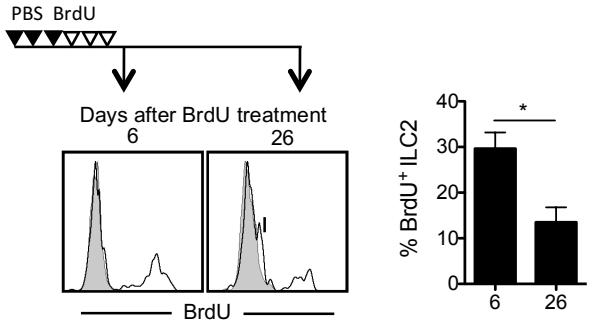


Figure S1. Turnover of naive ILC2s in the lung, related to Figure 1. BrdU was incorporated by lung ILC2s (gated as live $CD45^{+}Lin^{-}ST2^{+}Thy1^{+}$ cells) in control mice injected with PBS and BrdU intranasally and the decrease in BrdU-labeled lung ILC2s over time was analyzed by flow cytometry. Control histogram (shaded) was generated using untreated mice stained with anti-BrdU antibody. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=$p<0.05$) (two-tailed Student's t-test).

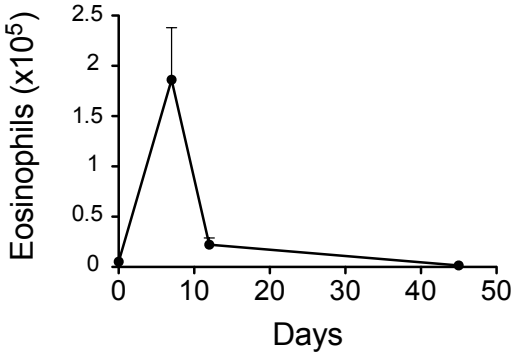


Figure S2. **Eosinophils infiltrate into the mLN after intranasal injection of IL-33, related to Figure 2.** Mice were treated with three daily intranasal injections of IL-33 and eosinophil numbers in the mLN along time were analyzed by flow cytometry.

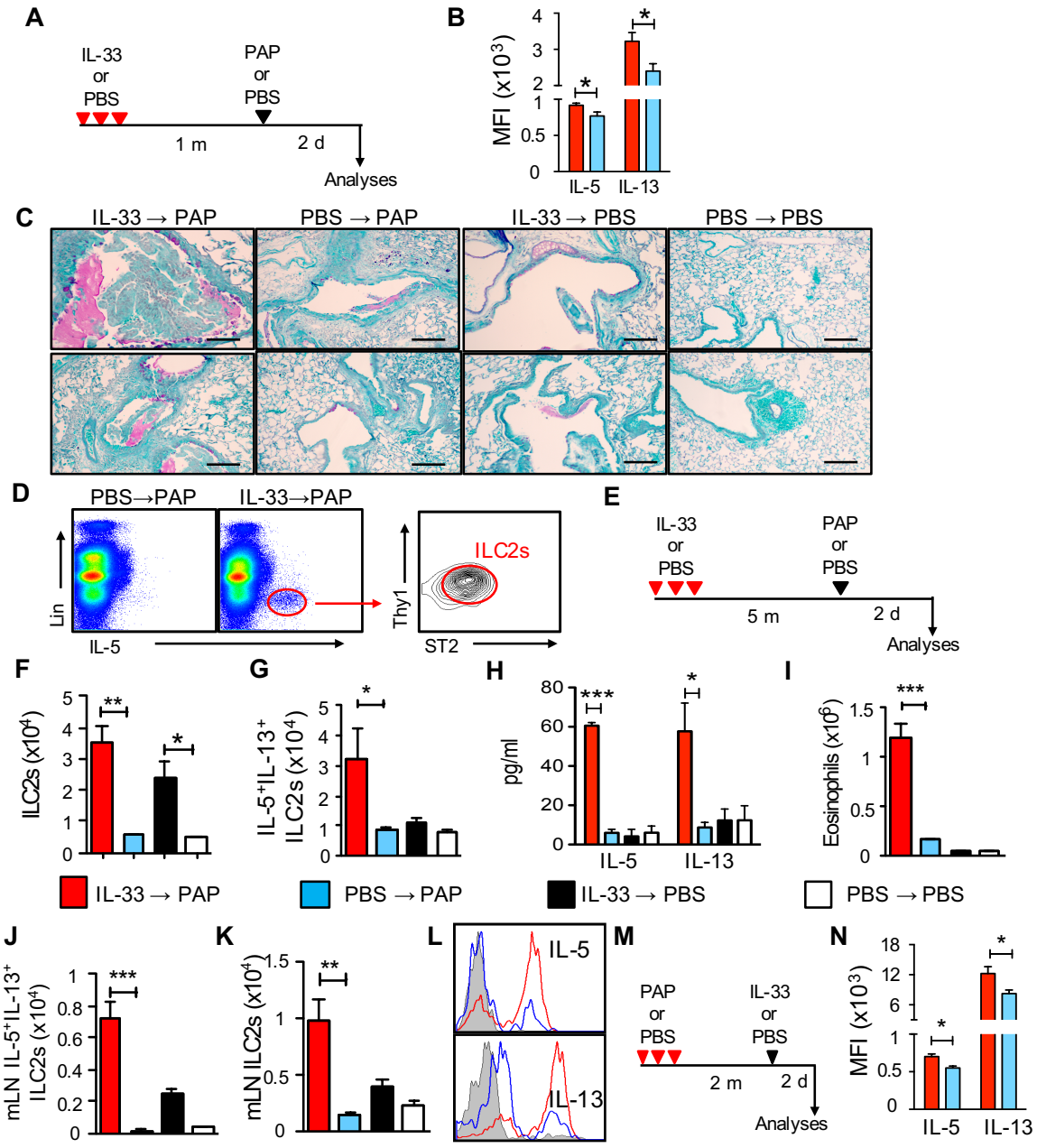


Figure S3. IL-33-experienced ILC2s respond stronger upon challenge 5 months later, related to Figure 3. (A) Scheme for the treatment of mice with IL-33 and papain in Figure 3. (B) Mean Fluorescence Intensity (MFI) for IL-5 and IL-13 expression by lung ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) analyzed by flow cytometry as shown in (A). (C) Lung tissue sections were stained by PAS staining for mucus. Scale bar corresponds to 20 μm. (D) FACS analysis showing that the cytokine-producing cells after papain challenge are ILC2s. (E) Scheme for the treatment of mice with IL-33 and papain. (F-I) Analysis of lung ILC2s and allergic inflammation upon challenge. Lung ILC2 number (F), lung IL-5/IL-13$^+$ producing ILC2s (G), cytokines in BALF (H), Eosinophils (I). (J-L) Analysis of mLN ILC2s upon challenge. mLN IL-5/IL-13$^+$ producing ILC2s (J), mLN ILC2 number (K), intracellular staining for IL-5 and IL-13 in mLN ILC2s (L). (M) Scheme for the treatment of mice with papain and IL-33 in Figure 3. (N) Mean Fluorescence Intensity (MFI) for IL-5 and IL-13 expression by lung ILC2s analyzed by flow cytometry as shown in (M). Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01, ***=p<0.001) (two-tailed Student's t-test).

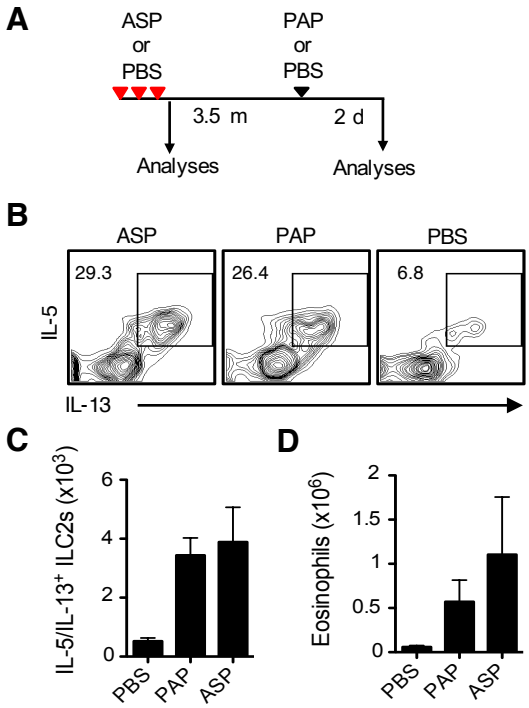


Figure S4. **Intranasal administration of Aspergillus stimulates ILC2s as papain does, related to Figure 4.** (A) Scheme of the treatment of mice treated with Aspergillus protease (ASP) and papain (PAP) in Figure 4. (B-D) Intracellular staining for IL-5/IL-13 of ILC2s (gated as live $CD45^{+}Lin^{-}ST2^{+}Thy1^{+}$ cells) in ASP, PAP and PBS treated mice (B), lung IL-5/IL-13^{+} producing ILC2s (C), eosinophil number (D). Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01, ***=p<0.001) (two-tailed Student's t-test).

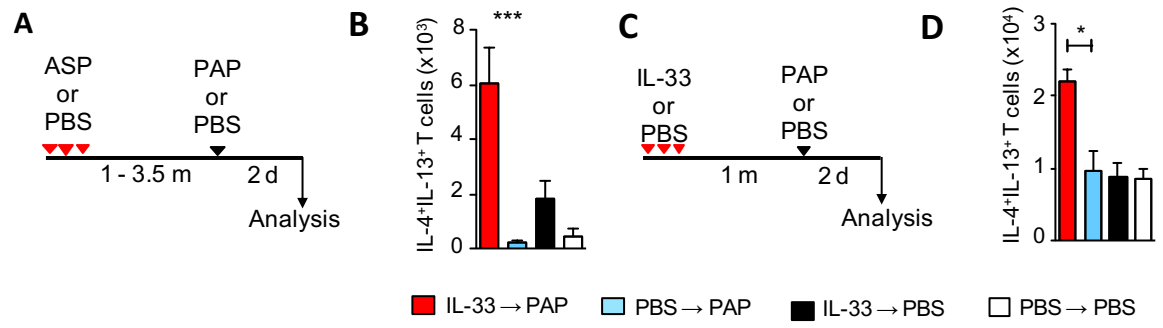


Figure S5. **Scheme of treatments and Th2 cells in lung, related to Figure 5.** (A) Scheme of the treatment of mice with Aspergillus protease (ASP) and papain (PAP) in Figure 5. (B) Th2 cells (gated as live $CD45^+CD4^+Thy1^+TCRb^+$ cells) in the mLN on d2 of mice challenged with papain 3.5 months after the ASP treatment. (C) Scheme of the treatment of mice with IL-33 and papain (PAP) in Figure 5. (D) Th2 cells in the lungs on d6 of the mice pretreated with IL-33 and challenged by PAP. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=$p<0.05$, ***=$p<0.001$) (two-tailed Student's t-test).

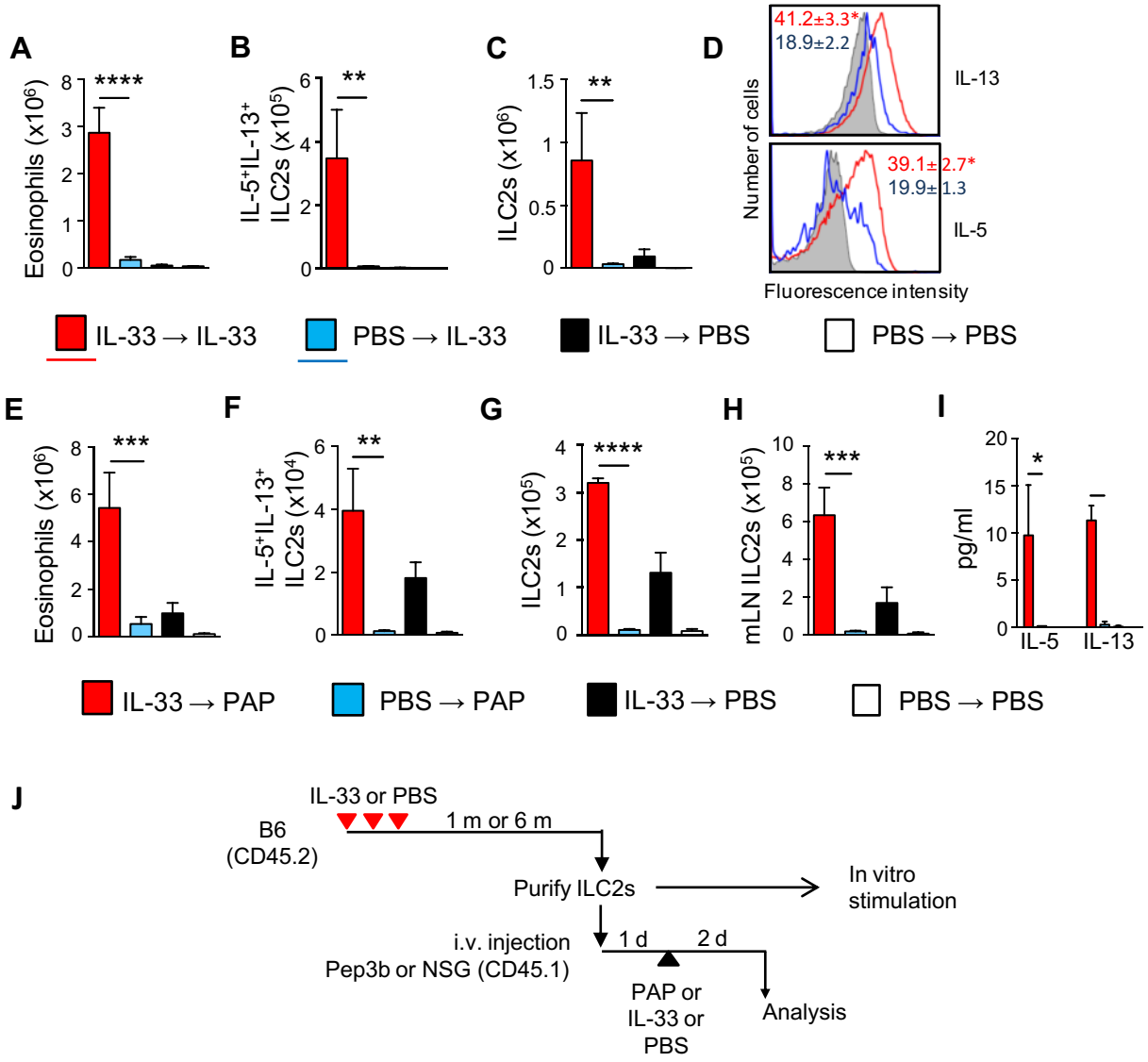


Figure S6. IL-33 and RAG1-deficient mice experiments and scheme of the experiments in Figure 6. (A-D) IL-33-deficient mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of IL-33 or PBS. Two days later, lung eosinophil numbers (A), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (B), lung ILC2 numbers (C) and intracellular IL-5 and IL-13 staining of lung ILC2s (D) after 3 h of re-stimulation with PMA+Ionomycin were analyzed by flow-cytometry. (E-I) RAG1-deficient mice received three daily intranasal injections of IL-33 or PBS. One month later, the mice received a single intranasal injection of IL-33 or PBS. Two days later, lung eosinophil numbers (E), numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) (F), ILC2 numbers in the lung (G) and in the mLN (H) were analyzed by flow-cytometry and the amounts of IL-5 and IL-13 in BALF were determined by ELISA (I). (J) Scheme of the experiments in Figure 6 to generate and purify memory-like ILC2s and to test their responsiveness in vitro and in vivo. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01 ***=p<0.001) (two-tailed Student's t-test).

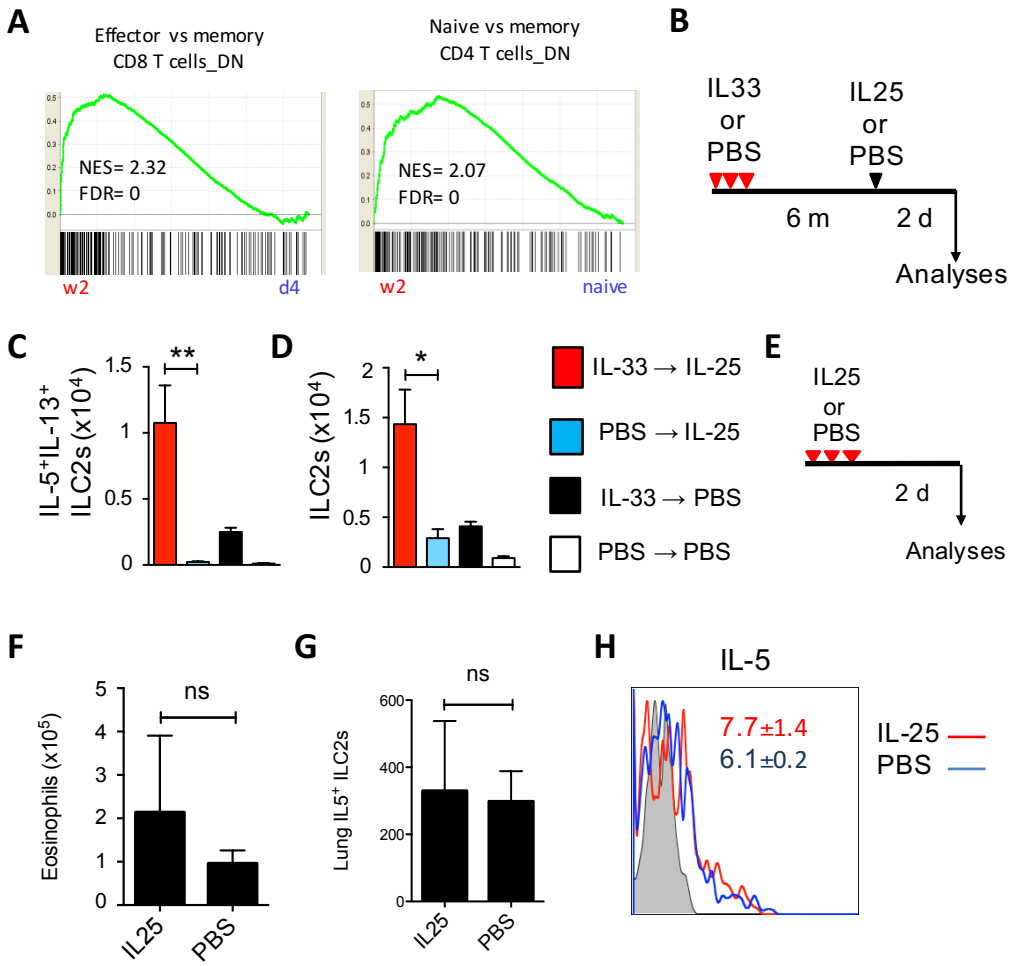


Figure S7. **Gene expression profiles of different populations of ILC2s in lung and mLN and IL-25 related experiments, related to Figure 7.** (A) Gene Set Enrichment Analysis (GSEA) of naïve, d4 and w2 ILC2s in the lung. Log2-fold expression values for each comparison were ranked in descending order. The x-axis shows individual genes and the y-axis shows enrichment score. Left panel shows that genes down regulated in effector CD8 T cells compared to memory CD8 T cells were significantly enriched in w2 lung ILC2s when compared to d4 ILC2s. Right panel shows that genes down regulated in naïve CD8 T cells compared to memory CD8 T cells were significantly enriched in w2 lung ILC2s when compared to naive ILC2s. NES: normalized enrichment score, FDR: false discovery rate. (B) Scheme of the experiments in Figure 7 for IL-25 experiments. (C and D) Numbers of intracellular IL-5$^+$IL-13$^+$ ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) in the lung (C) and total lung ILC2 numbers (D) after 3 h of re-stimulation with PMA + Ionomycin were analyzed by flow-cytometry. (E) Scheme for Figure S7 F-H. (F-H) Numbers of eosinophils in the lung (F), intracellular IL-5$^+$ILC2s (gated as live CD45$^+$Lin$^-$ST2$^+$Thy1$^+$ cells) in the lung (G) and intracellular IL-5 staining of lung ILC2s (H) after 3 h of re-stimulation with PMA + Ionomycin were analyzed by flow-cytometry. Data represented are the mean ± S.E.M. of two experiments with 3 mice per group (*=p<0.05, **=p<0.01) (two-tailed Student's t-test).