Frontline

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Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma

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Allergic asthma is characterized by chronic airway inflammation and hyperreactivity and is thought to be mediated by an adaptive T helper-2 (Th2) cell-type immune response. Here, we demonstrate that type 2 pulmonary innate lymphoid cells (ILC2s) significantly contribute to production of the key cytokines IL-5 and IL-13 in experimental asthma. In naive mice, lineage-marker negative ILC2s expressing IL-7Rα, CD25, Sca-1, and T1/ST2(IL-33R) were present in lungs and mediastinal lymph nodes (MedLNs), but not in broncho-alveolar lavage (BAL) fluid. Upon intranasal administration of IL-25 or IL-33, an asthma phenotype was induced, whereby ILC2s accumulated in lungs, MedLNs, and BAL fluid. After IL-25 and IL-33 administration, ILC2s constituted ∼50 and ∼80% of IL-5$^+$/IL-13$^+$ cells in lung and BAL, respectively. Also in house dust mite-induced or ovalbumin-induced allergic asthma, the ILC2 population in lung and BAL fluid increased significantly in size and ILC2s were a major source of IL-5 or IL-13. Particularly in OVA-induced asthma, the contribution of ILC2s to the total population of intracellular IL-5$^+$ and IL-13$^+$ cells in the lung was in the same range as found for Th2 cells. We conclude that both ILC2s and Th2 cells produce large amounts of IL-5 and IL-13 that contribute to allergic airway inflammation.

Keywords: Asthma • Cytokines • House dust mite • Innate immunity • Innate lymphoid cells

See accompanying Commentary by Spits

Supporting Information available online

Introduction

Allergic asthma is characterized by a predominant eosinophilic airway inflammation, airway hyperreactivity, and a chronic T helper-2 (Th2) cell-type of immune response to various allergens, such as house dust mites (HDMs), molds, or animal dander [1, 2]. Available experimental models for asthma indicate that allergen-specific Th2 cells are key players in induction and maintenance of allergic asthma [3]. These cells produce vast amounts of cytokines that induce IgE synthesis (IL-4), recruit eosinophils and mast cells (via IL-5 and IL-9, respectively), and cause smooth muscle hyperreactivity via IL-13 (reviewed in [4]).

Although adaptive Th2 cells have been identified as important sources of IL-4, IL-5, and IL-13 [5], many recent studies emphasize the importance of innate cells in cytokine production, including but not limited to mast cells, eosinophils, and basophils. Next to the canonical NK and lymphoid-tissue inducer (LTi) cells, a growing family of cytokine-producing "helper" innate lymphoid cells (ILCs) has been identified [6]. Interestingly, a lineage-negative ILC population that produces high amounts of IL-5 and IL-13 was identified in fat-associated lymphoid clusters (FALCs) and

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mesenteric lymph nodes [7–9]. These cells were named natural helper cells, nuocytes, or multipotent progenitors and, because of their Th2 cytokine production, they have been dubbed type 2 ILCs [6]. ILC2s play a role in defense mechanisms and (re)shaping immune and nonimmune tissues and can be stimulated with IL-25 and IL-33. In a *Nocardia brasiliensis* infection model, ILC2s were essential and sufficient for clearance of this helminth from the mucosa, even in the absence of the adaptive immune system [7, 9].

Recently, various groups demonstrated the presence of ILC2s in the respiratory system of mice and humans [10–12]. ILC2 were shown to accumulate in the lungs of mice after infection with influenza virus, via an IL-33 dependent mechanism. These ILC2 induced airway hyperreactivity through IL-13 secretion [10] and also restored airway epithelial integrity and lung function and contributed to airway remodeling by the production of amphiregulin [12]. In addition, ILC2s were found in nasal polyps of patients with chronic rhinitis, a classical Th2 disease [11].

IL-25 is a member of the IL-17 cytokine family that is expressed in human and mouse in response to allergens, particles, and helminth infection [13–15]. Administration of IL-25 was shown to induce IL-4, IL-5, and IL-13 production, even in the absence of B and T lymphocytes in recombination activating gene (Rag)-deficient mice [15]. The main sources of IL-25 in asthma are the lung epithelium, eosinophils, mast cells, and basophils [16]. Moreover, IL-25 amplifies Th2 responses and causes increased eosinophilic infiltration in mice [17]. IL-33 belongs to the IL-1 family and binds a receptor complex consisting of the IL-1R accessory protein and ST2 [18]. The membrane-bound form of ST2 is highly expressed on mast cells and Th2 cells [19, 20], associating the IL-33 pathway with Th2 conditions. IL-33 is produced by epithelial cells in the lung [18] and epithelial expression of IL-33 increases in asthmatics [21]. The importance of IL-25 and IL-33 for asthma is underlined by the finding that disruption of the IL-25 or IL-33 pathway prevents induction of allergic airway inflammation in mice [22, 23]. Several studies have identified lineage-negative cells in the lung that manifest IL-5 and IL-13 production in response to IL-25 [14, 24] or IL-33 [25, 26] administration in mice.

In this report, we investigated the presence of ILC2s in the respiratory tract and hypothesized that pulmonary ILC2s are involved in allergic asthma by production of Th2 cytokines. We found that ILC2s produced substantial amounts of IL-5 and IL-13 in various murine models of allergic lung inflammation, both upon intranasal administration of IL-25 or IL-33 and in house dustmite (HDM) or ovalbumin (OVA)-induced asthma. We conclude that both ILC2s and Th2 cells produce large amounts of IL-5 and IL-13 that contribute to allergic inflammation in asthma.

Results

Identification and characterization of pulmonary ILC2s

To explore the presence of ILC2s in the respiratory tract, we used flow cytometry and analyzed lung, mediastinal lymph node (MedLN), broncho-alveolar lavage (BAL), and thoracic fat of naive wild-type mice. We identified a lineage-negative, low side scatter population expressing IL-7Rα (CD127), T1/ST2 (IL-1RL1, a subunit of IL-33R), partially positive for c-Kit (CD117), stem cell antigen Sca-1 (Ly6A/E), and IL-2Rα (CD25) in the lung (Fig. 1A). This population resembled the recently identified population of ILC2s in gut-associated tissue and influenza infected lungs [6, 7, 9, 10, 12]. Sca-1 expression levels were higher in B6 mice than in BALB/c mice (data not shown). As ILC2s were initially found in fat-associated lymphoid tissue, we analyzed the thoracic fat attached to the thorax wall and around the aorta, and found varying but steadily limited numbers (<200) of Lin$^-$T1/ST2$^+$CD127$^+$c-Kit$^+$ cells. These ILC2s were present in the lung and MedLNs of naive mice, but virtually absent in BAL (Fig. 1B).

Pulmonary ILC2s depend on common gamma chain cytokine signals

Pulmonary ILC2s were present in the lungs of C57BL/6 and BALB/c mice (Supporting Information Fig. 1). Intestinal ILC2s are found in *Rag2*$^{-/-}$ mice, but their development or survival depends on the common gamma (γ_c) chain, as *Rag2/Il2rg* double-deficient mice lack ILC2 [7]. Accordingly, we found pulmonary ILC2s in comparable numbers in the lungs and MedLNs of *Rag1*$^{-/-}$ mice, but not in *Rag2/Il2rg* double-deficient mice (Supporting Information Fig. 1). We noticed that the absence of *Myd88*, an adapter molecule that functions downstream of TLRs and IL-33R [27], had no significant effect on ILC2 numbers in the lung. IL-22 is an important cytokine in the lung and in the intestine for protection of epithelial surfaces [28] and is rapidly produced in conjunction with IL-17 by LTi cells. We detected no significant alterations in lung ILC2 numbers in *Il22*$^{-/-}$ mice (Supporting Information Fig. 1). Closely related ILC populations, including ILC17 [29] and ILC22 [30–33], depend on the RORγt transcription factor for their development, while intestinal ILC2s do not express RORγt, but RORα and RORβ instead [7]. In agreement with this finding, pulmonary ILC2s were present in mice deficient for the *Rorc* gene, encoding RORγt (Supporting Information Fig. 1).

In summary, we found that development of pulmonary ILC2s requires signaling via a γ_c-dependent cytokine pathway. Because of the high levels of IL-7Rα (CD127), it is conceivable that IL-7 signals are important for ILC2 development.

Pulmonary ILC2s produce IL-5 and IL-13 Th2 cytokines

ILC2s produce large amounts of the Th2 cytokines IL-5 and IL-13 upon phorbol myristate acetate (PMA) and ionomycin stimulation [7, 9]. When we stimulated lung cell suspensions with PMA/ionomycin for 4 h and probed for production of the Th2 cytokines by intracellular flow cytometry, we detected fractions of ILC2s positive for IL-5 and IL-13, while IFN-γ$^+$ and IL-4$^+$ ILC2s were hardly detectable (Fig. 1C). Quantitative RT-PCR analysis of sorted lin$^-$CD127$^+$ T1/ST2$^+$ cells from naive mice stimulated in

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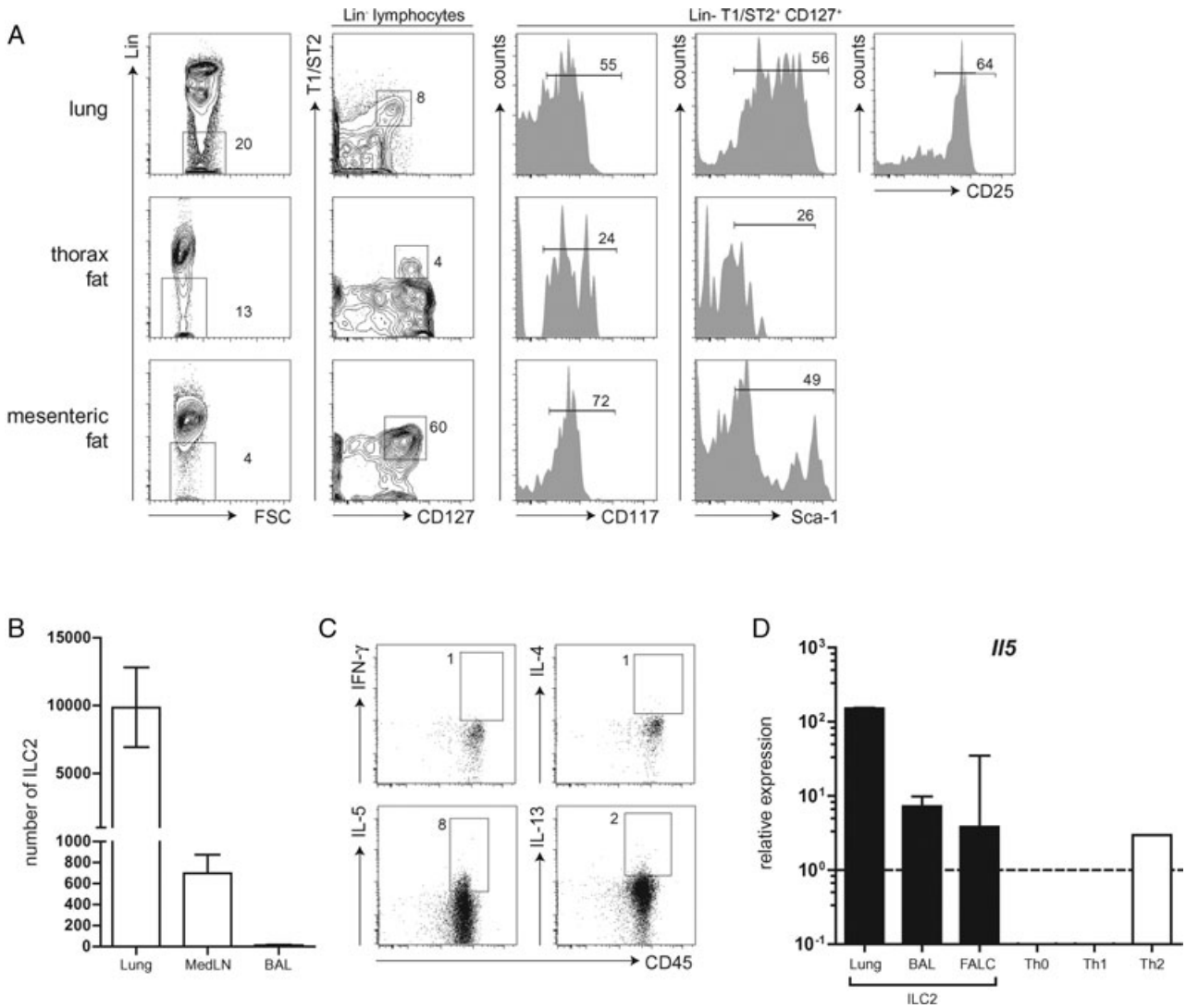

Figure 1. Pulmonary ILC2s are present in the respiratory tract of naive mice. (A) Flow cytometric identification of pulmonary ILC2s is shown. Single-cell suspensions of collagen-treated lungs, thorax fat, and mesenteric fat of naive BALB/c mice were analyzed by flow cytometry. Cells negative for lineage markers were analyzed for expression of IL-33R (T1/ST2) and CD127 (IL-7Rα). T1/ST2$^+$IL-7Rα$^+$ cells were gated and analyzed for c-Kit (CD117), Sca-1, and IL-2Rα (CD25), shown as histograms. Proportions of cells within the gates are given. FSC: Forward Scatter. (B) The quantification of ILC2s (Lin$^-$CD45$^+$CD127$^+$T1/ST2$^+$CD25$^+$ cells) in the pulmonary tract is shown. MedLN: mediastinal lymph node; BAL: bronchoalveolar lavage. Data are shown as mean + SEM of $n \geq 2$ animals per group and all data are representative of four independent experiments. (C) Intracellular cytokine content of ILC2s is shown. Lin$^-$CD127$^+$ T1/ST2$^+$ lymphocytes were gated and analyzed for CD45 versus the indicated cytokines. Results are shown as dot plots. Single-cell suspensions were stimulated with PMA/ionomycin in the presence of monensin for 4 h. (D) RT-PCR quantification of *Il5* expression of the indicated FACS-sorted ILC2 cell suspensions, as well as polarized T-cell cultured under Th0, Th1, and Th2 conditions is shown. *Il5* levels were normalized to *Actb* levels. Data are shown as mean + SEM of $n \geq 3$ samples per group for ILC2 populations and one sample per group for Th populations and data are representative of two independent experiments.

vitro with IL-2, IL-25, and IL-33 showed that lung ILC2 expressed higher levels of *Il5* than BAL ILC2, FALC ILC2, or in vitro cultured polarized Th2 cells (Fig. 1D).

Pulmonary ILC2s are induced in IL-25- and IL-33-induced asthma

Although classic animal models of experimental asthma depend on sensitized Th2 cells, intranasal administration of IL-25 or IL-33 in mice have also been reported to induce an asthma phenotype with airway hyperresponsiveness, eosinophilic inflammation, mucus hypersecretion, and Th2 cytokine production in the lung [14, 25, 34]. Since ILC2s are reported to release cytokines in response to IL-25 or IL-33 stimulation [7, 9], we hypothesized that pulmonary ILC2s contribute to airway inflammation in cytokine-induced asthma. Mice were intranasally treated with five doses of IL-25 or three doses of IL-33 and analyzed 24 h after the last instillation (Fig. 2A). While we observed no significant increase in BAL cellularity in IL-25 treated mice, IL-33 induced a strong increase in total cellularity, eosinophils, and T cells in BAL fluid (Fig. 2B), indicating the onset of airway inflammation. Likewise, lung cellularity also increased upon IL-33 administration (Fig. 2C).

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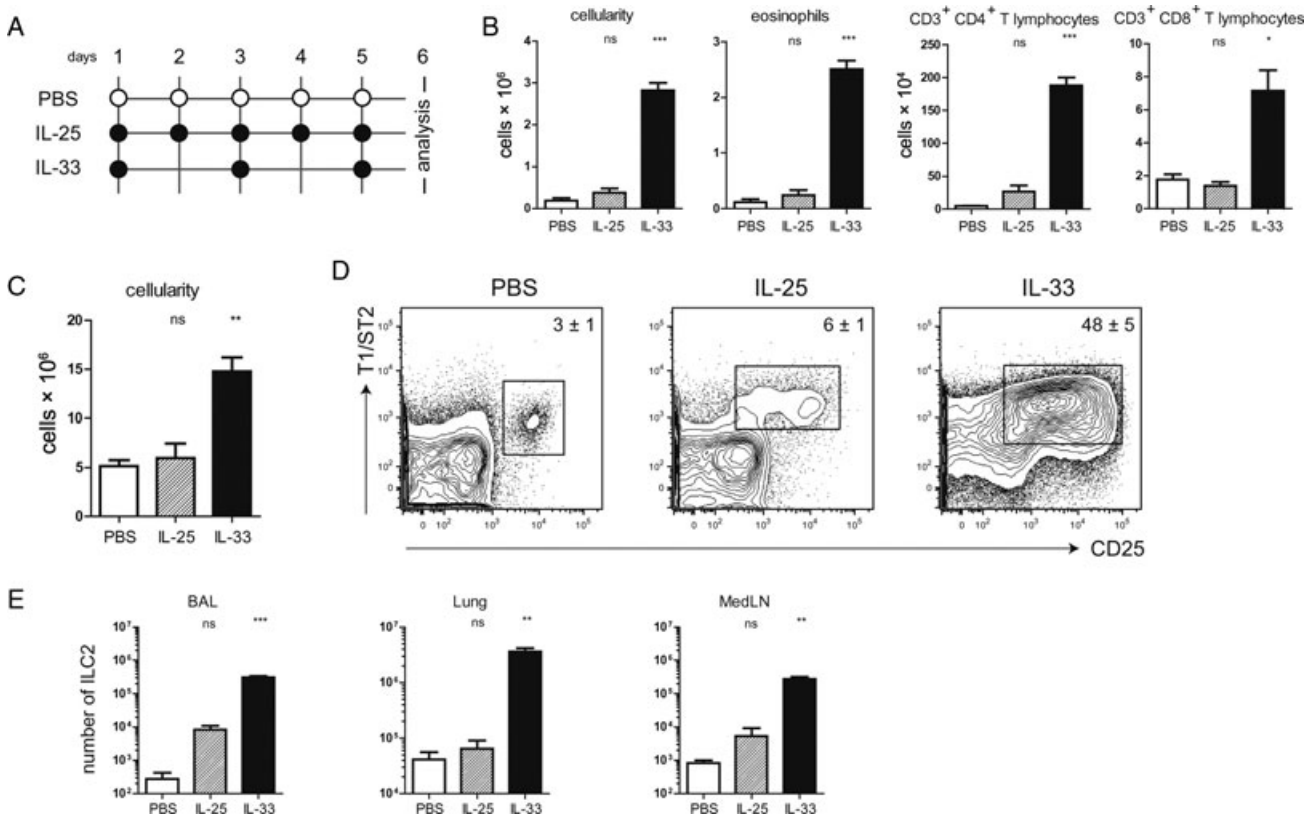

Figure 2. Administration of IL-25 and IL-33 induces eosinophilic airway inflammation and increases ILC2 numbers. (A) Scheme for IL-25 and IL-33 treatment of BALB/c mice. Isoflurane-anesthesized animals received 1 μg intranasal administrations of the indicated cytokines (or PBS as a control) on the days marked with a circle and were analyzed 24 h later. (B) The quantification of indicated cell populations in the BAL fluid after cytokine treatment is shown. (C) The total number of cells in the lungs of treated animals is shown. (D) Flow cytometric identification of pulmonary ILC2s (gated) in single-cell suspensions of the lungs of treated mice. Plots show expression of the indicated markers on cells negative for lineage markers. Numbers indicate the frequency of ILC2s in the total lymphocyte population ± SEM using the gates indicated. (E) Quantification of ILC2s in different compartments of the pulmonary tract is shown. (B, C, E) Data are shown as mean + SEM of $n = 3$–4 mice per group and all panels are representative of two independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA analysis followed by Turkey post-tests to analyze statistical significance between PBS control versus treated animals. ns: not significant.

Administration of IL-25 and IL-33 induced upregulation of T1/ST2 on ILC2s (Fig. 2D). The number of ILC2s strongly increased in mice treated with IL-33 (Fig. 2E).

Pulmonary ILC2s are the major cytokine producing lymphocytes in IL-25- and IL-33-induced asthma

Next, we determined the contribution of ILC2s to production of IL-4, IL-5, and IL-13 in cytokine-induced airway inflammation by intracellular flow cytometry. We observed that intranasal treatment with PBS alone induced increased production of IL-5 and IL-13 (Fig. 3A, compared with naive mice, Fig. 1C). Treatment with IL-25 or IL-33 further increased the fraction of IL-5 and IL-13^{+} ILC2s to more than ~80 and ~60%, respectively, while IL-4 production remained low (Fig. 3A). Comparisons with cytokine content of T1/ST2^{+} CD4^{+} T-cell fractions, which are enriched for Th2 polarized cells [20], and T1/ST2^{-} CD4^{+} T-cell fractions, revealed that the ILC2 population comprised most of the IL-5^{+} and IL-13^{+} lymphocytes upon treatment with IL-25 and IL-33 (up to 3.4×10^{6} IL-5^{+} and 3.1×10^{6} IL-13^{+} cells/lung after IL-33 treatment, respectively) (Fig. 3B). The contribution of ILC2s to IL-4 production strongly increased upon IL-33 but not upon IL-25 administration. Likewise, IL-25 and IL-33 activated IL-5 and IL-13 production in ILC2s in BAL fluid (Supporting Information Fig. 2A). After treatment with IL-25 and IL-33, ILC2 became the most important producers of Th2 cytokines in the BAL (Supporting Information Fig. 2B).

Taken together, these results show that treatment with IL-25 induced a limited increase in BAL cellularity, whereby pulmonary ILC2s were potent producers of IL-5 and IL-13. In contrast, treatment with IL-33 induced a strong increase in total cellularity, and an increase of the numbers of eosinophils and ILC2s in BAL. These ILC2 manifested a high cytokine content for IL-5, IL-13, and to a lesser extent IL-4, all of which contribute to allergic lung inflammation.

ILC2 population is an important target of IL-25 and IL-33, independent of the adaptive immune system

Previous studies have shown that IL-25 and IL-33 can induce an asthma phenotype, even in the absence of B and T lymphocytes

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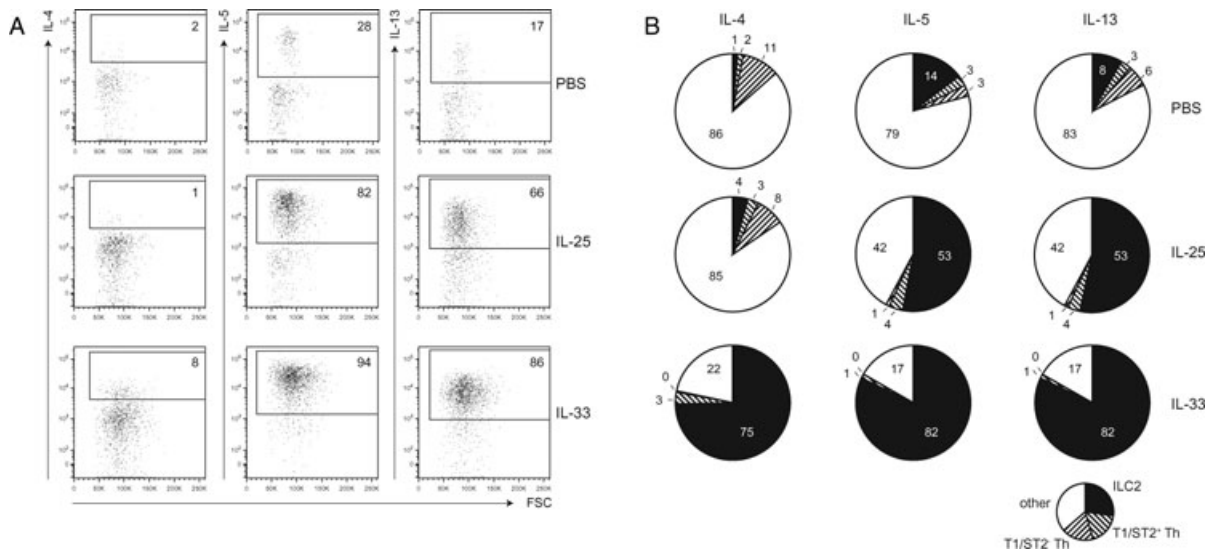

Figure 3. ILC2s produce important amounts of IL-5 and IL-13 in cytokine-induced airway inflammation. (A) Intracellular cytokine content of pulmonary Lin$^-$CD25$^+$CD127$^+$T1/ST2$^+$ ILC2s in mice, treated as specified. Plots show forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4 h. Numbers indicate the proportions of cytokine-positive ILC2s. (B) Contribution of various cell populations to Th2 cytokine production in the lungs after administration of PBS, IL-25, or IL-33. Proportions of cytokine expressing cells were determined by flow cytometry (as in panel A) in ILC2, T1/ST2$^+$, and T1/ST2$^-$ CD3$^+$CD4$^+$ Th cells. Pie charts and percentages represent the contribution of various populations to the total number of cytokine-producing cells. Data are representative of two independent experiments with three to four mice per group.

[14, 25, 34]. Consistent with reported findings [7, 8], we found that ILC2s develop in the absence of Rag1 or Rag2 but are virtually absent from lungs in mice that lack the γc chain (Supporting Information Fig. 1 and 4A). To investigate whether ILC2s are activated in the absence of an adaptive immune system, we employed the cytokine-induced asthma models in *Rag2*-deficient (ILC2$^+$) and *Rag2/Il2rg* double deficient (ILC2$^-$) mice. Administration of IL-25 and IL-33 resulted in an increase in cellularity in lung and BAL and eosinophilic inflammation in *Rag2*-deficient mice, but not in *Rag2/Il2rg* double-deficient mice (Fig. 4B). These findings indicate that a γc chain-dependent population is responsible for inducing airway inflammation upon IL-25/IL-33 administration. Administration of IL-33 induced increased ILC2 numbers in BAL and lung in *Rag2*-deficient mice, while we observed no increase in ILC2 numbers in *Rag/Il2rg*-deficient mice (Fig. 4C). Treatment of mice with PBS, IL-25, and IL-33 induced production of IL-5 and IL-13 but not IL-4 in *Rag2*-deficient lung ILC2 (Supporting Information Fig. 3), demonstrating that ILC2 are activated in the absence of adaptive immunity.

In summary, we found that airway inflammation induced by IL-25 and IL-33 is mediated by a *Rag2*-independent, γc chain-dependent population. Moreover, a strong increase in the number of ILC2s, more than ~80% of which were intracellular IL-5 and IL-13$^+$, was induced. Therefore, we conclude that ILC2s are the most important inducers of eosinophilic inflammation upon intranasal IL-25 or IL-33 administration.

Pulmonary ILC2s are important cytokine producers in HDM-induced allergic asthma

Next, we induced allergic asthma in mice with HDM to determine to which extent ILC2s synergize with an adaptive inflammatory Th2 response in the respiratory tract. Mice were sensitized with HDM and challenged either once, three, or ten times with HDM (Fig. 5A). Mice that were sensitized and challenged with PBS served as controls. As expected, HDM-challenged mice exhibited airway inflammation marked by increased cellularity, eosinophilia and T-cell influx, and BAL fluid, when compared with PBS controls (Fig. 5B). HDM-induced asthma was also accompanied by increased numbers of ILC2s in lung and BAL, but not in MedLNs (Fig. 5C), indicating that during the Th2 inflammation ILC2s undergo proliferation or are attracted, possibly by cytokines.

As observed in cytokine-induced airway inflammation, in HDM-induced allergic asthma pulmonary ILC2s did not only increase in number, but concomitantly also the proportions of cytokine-containing cells increased. Intracellular flow cytometry demonstrated that in HDM-induced allergic asthma ILC2s in the lung produced IL-5, IL-13, and also IL-4 (Fig. 5D). To determine to what extent pulmonary ILC2s contribute to cytokine production in relation to conventional Th2 cells and other Th cells, we calculated absolute numbers of cytokine-expressing cells in lungs and BAL. The contribution of ILC2s to IL-4 was limited, compared with T1/ST2$^+$ or T1/ST2$^-$ Th cells, and only detectable in the lung, where up to ~1400 ILC2 produced IL-4. In the lung up to ~1900 of total IL-5$^+$ cells were ILC2s, which is substantial, as compared to values between 2000 and 4500 for T1/ST2$^+$ or between 1500 and 4800 for T1/ST2$^-$ CD4$^+$ T-cell fractions (Fig. 5E). Likewise, up to 2800 ILC2s produced IL-13, while 3200–5800 T1/ST2$^+$ Th2 cells produced IL-13. Despite the modest numbers of ILC2s detected in BAL fluid, the number of IL-5 and IL-13 producing ILC2 and T1/ST2$^+$ Th2 cells were comparable. Collectively, these results illustrate that pulmonary ILC2s comprised a significant proportion of IL-5$^+$ and IL-13$^+$ cells in lung and BAL in HDM-induced allergic asthma, whereby the contribution of ILC2 cells to the total

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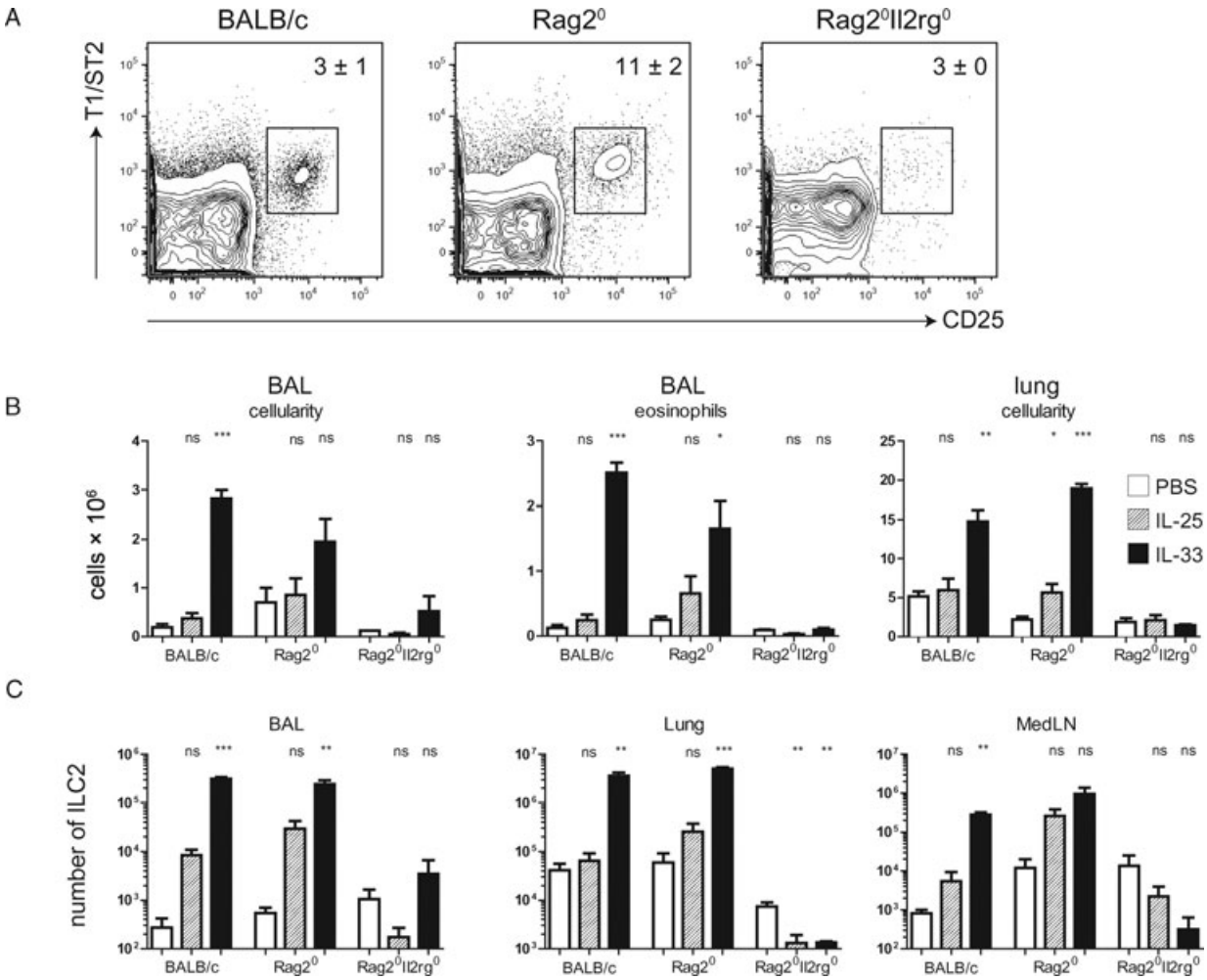

Figure 4. Induction of airway inflammation by IL-25 and IL-33 depends on a γc chain-dependent cell population. (A) Identification of ILC2s in the lungs of mice of the indicated strains is shown. Plots show expression of CD25 and T1/ST2 by Lin$^-$ CD127$^+$ lymphocytes. Values indicate the percentage of ILC2 of Lin-negative lymphocytes ± SEM. (B) Quantification of indicated cell population in the BAL fluid of the indicated mice treated with PBS, IL-25, or IL-33 in BAL and lung is shown. (C) Quantification of Lin$^-$CD25$^+$CD127$^+$T1/ST2$^+$ ILC2s in the pulmonary tract of the indicated mice after treatment is shown. (B, C) Data are shown as mean + SEM of $n = 2$–4 mice per group and are representative of two independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, one-way ANOVA analysis followed by Turkey post-tests to analyze statistical significance between PBS control versus treated animals. ns: not significant.

population of IL-5$^+$ and IL-13$^+$ cells in the BAL was in the same range as found for Th2 cells.

ILC2 are major IL-5 and IL-13 producers in OVA-induced asthma

Finally, we determined the contribution of ILC2 to cytokine production in an OVA-induced asthma model. After i.v. injection of Th2-polarized T cells bearing the DO11.10 OVA-specific T-cell receptor [35], mice were challenged five times with OVA aerosols and analyzed 2 days after the last challenge. In this asthma model, total BAL cellularity increased to $3.0 \pm 0.5 \times 10^6$ cells and flow cytometric analysis revealed that 68 ± 2% of BAL cells were eosinophils (data not shown), confirming induction of eosinophilic airway inflammation. Flow cytometric analyses of the lungs revealed that ILC2s contained substantial proportions IL-5$^+$ and IL-13$^+$ cells, while only small proportions of the cells were IL-4$^+$ (Fig. 6A).

When we determined the contribution of ILC2s to Th2 cytokine production, we noticed that ILC2s were poor producers of IL-4 in both lung and BAL, but were major producers of IL-5. In the lung ~21,000 ILC2s produced IL-5 (compared with ~32,000 T1/ST2$^+$ Th2 cells), while in BAL ILC2 was the main IL-5-producing cell population: ~7500 ILC2s, compared with ~1900 T1/ST2$^+$ Th2 cells (Fig. 6B). In addition, we found ~12,000 IL-13 producing ILC2 in the lung, compared to ~16, 000 IL-13 producing T1/ST2$^+$ Th2 cells. In contrast, the contribution of ILC2s to IL-13 production in the BAL was limited.

In summary, these results indicate an important role for pulmonary ILC2s in the production of IL-5 and IL-13 in OVA-induced asthma. In particular, pulmonary ILC2s are potent producers of IL-5 and make up the largest IL-5-producing population in the BAL fluid in OVA-induced asthma.

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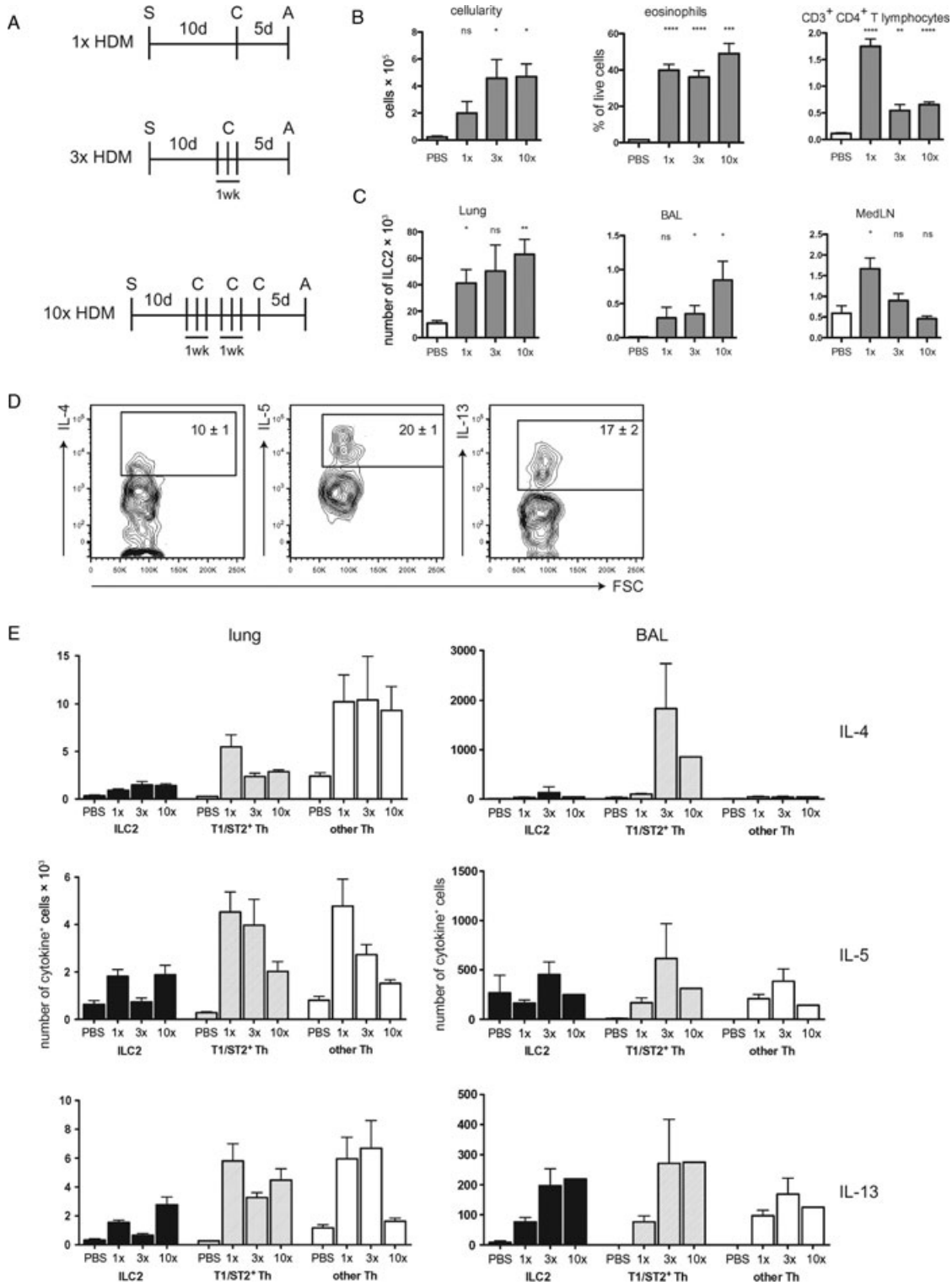

Figure 5. Pulmonary ILC2s are major Th2 cytokine producers in HDM asthma. (A) Asthma protocols showing sensitization (S) and challenges (C) with house dust mite (HDM) in BALB/c mice, followed by analysis (A). Control mice were treated with PBS instead of HDM. (B) Flow cytometric quantification of the indicated cell populations in the BAL fluid after various HDM-asthma treatments is shown. (C) Quantification of $Lin^{-}CD127^{+}T1/ST2^{+}$ ILC2 numbers in lung, BAL, and mediastinal lymph nodes (MedLNs) of HDM- or PBS-treated animals is shown. (D) The intracellular cytokine content in HDM-challenged mice is shown. Total lung cell suspensions were stimulated with PMA/ionomycin for 4 h. $CD3^{-}CD4^{-}T1ST2^{+}CD127^{+}$ cells were gated and analyzed for the indicated cytokines. Values indicate the proportions of cytokine positive cells ± SEM. (E) Cytokine production by lymphocyte populations in lung and BAL fluid is shown. Bars show the absolute numbers of intracellular cytokine-positive cells within the indicated lymphocyte populations. (B, C, E) Data are shown as mean + SEM of $n > 4$ mice per group and are representative of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, unpaired *t*-test. ns: not significant.

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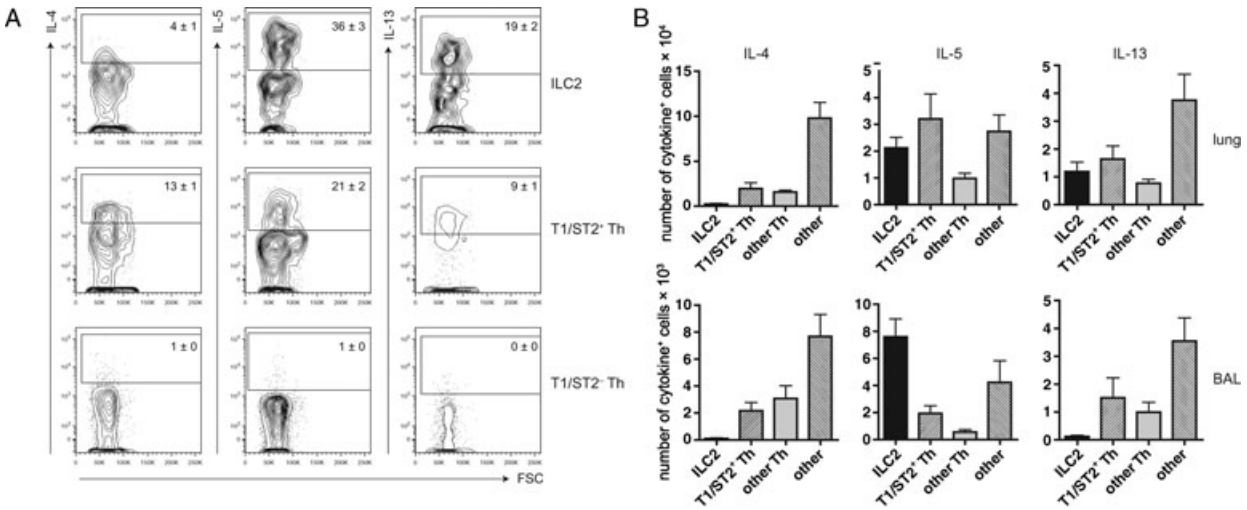

Figure 6. Pulmonary ILC2s are major Th2 cytokine producers in OVA-induced asthma. (A) Flow-cytometric analysis of intracellular cytokine content in OVA-challenged mice is shown. Total lung cell suspensions were stimulated with PMA/ionomycin for 4 h. ILC2s were gated as $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$. Th cells were gated as $T1/ST2^{+}$ or $T1/ST2^{-}$ $CD3^{+}CD4^{+}$ lymphocytes. Values indicate the proportions cells ± SEM positive for the indicated cytokines. Plots are from representative samples of seven animals per group. (B) Contribution of lymphocyte populations indicated in panel A to production of IL-4 and IL-5, IL-13 in lungs, and BAL fluid, upon OVA treatment in vivo. Data are shown as mean + SEM of $n = 7$ animals. Experiment was performed once.

Discussion

ILC2 were shown to mediate airway hyperreactivity, independent of the adaptive immune system, in a nonallergic form of asthma induced by influenza virus infection. However, whether ILC2 have the capacity to synergize with allergen-specific Th2 cells in allergic asthma remained unexplored. Our studies in naive mice as well as in cytokine-, HDM-, and OVA-induced asthma demonstrate the presence of ILC2s in the pulmonary tract and their substantial contribution to allergic inflammation. We conclude that ILC2s, defined as non-B/non-T lymphocytes expressing IL-7Rα, CD25, Sca-1, c-Kit, and T1/ST2/IL-33R are major producers of IL-5 and IL-13, even in allergen-induced asthma, where Th2 cells are thought to be the most important sources of IL-5 and IL-13.

It was previously reported that the lineage-negative cells that produce IL-5 and IL-13 in response to intranasal administration of IL-25 were T-cell independent, because they were also detected in *Rag2*$^{-/-}$ mice [14]. We have now shown that these cells are ILC2s and we additionally found that they were efficiently induced upon IL-25 or IL-33 administration in *Rag2*$^{-/-}$ mice, but not in *Rag2/Il2gc* double-deficient mice. While our studies were in progress, two other studies have demonstrated the activation of ILC2s in response to IL-25 and IL-33 administration [36] or in response to glycolipid antigens, which activate NKT cells and thereby induce IL-33 production by alveolar macrophages [37], although these papers focus on the production of IL-13 by ILC2s. From our findings, we can conclude that ILC2s are an important source of both IL-5 and IL-13, not only in IL-25- or IL-33-induced allergic lung inflammation, but also in models of allergic asthma based on OVA and HDM allergens. In addition, we noticed that upon IL-33 stimulation in vivo (Fig. 3A) or in OVA- or HDM-induced airway inflammation (Fig 5D and 6A) low but detectable proportions of ILC2 had the capacity to produce IL-4. We have compared the expression levels of CD127/IL-7Rα, IL-2Rα, T1/ST2, and Sca-1 by flow cytometry in IL-4, IL-5, and IL-13 expressing ILC2, but did not detect significant differences.

ILC2s express c-Kit, IL-2Rα, and IL-7Rα, suggesting a lymphoid origin. Although the exact nature of ILC2 precursors remains unknown, recent data confirm that bone marrow lymphoid-primed multipotent progenitors and common lymphoid progenitors but not myeloid–erythryoid precursors can give rise to ILC2s [38]. Like other ILC subtypes that have recently been described (reviewed in [6]) and NK cells, ILC2s depend on the transcription factor Id2 [7, 39, 40]. The absence of ILC2s in *Rag2/Il2rg* double-deficient mice demonstrates dependency on a γ_c-chain cytokine. More specifically, ILC2s found in FALCs were found to be dependent on IL-7 for their development [7]. We observed pulmonary ILC2s in mice lacking *Il2rb* (the β-chain of the IL-2R/IL-15R), showing that IL-2 and IL-15 are not crucial for their development and maintenance, or that their function can be compensated for by other cytokines (R.K.W., Vosshenrich et al., unpublished findings). It is possible that the ILC2 populations that have been recently described share a common precursor and that the local environment plays a decisive role in their terminal differentiation.

In summary, we have shown that the novel population of ILC2 surprisingly constitutes an important fraction of IL-5 and IL-13 cytokine producing cells, next to the classical Th2 cells, in HDM or OVA allergen-induced allergic asthma in mice. It is conceivable that ILC2s also play a crucial role in "classical" allergic asthma in human, because recent genome-wide association studies have demonstrated associations with variations in genes coding for IL-33 and its receptor ST2 [41]. ILC2s could therefore be

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an important novel target for development of future asthma therapies.

Materials and methods

Mice and asthma induction

C57BL/6 and BALB/c mice were purchased from Harlan. All other mice were bred and kept at the Erasmus MC Laboratory Animal Center. Mice were kept under specific pathogen-free conditions and provided with food and water ad libitum. Mice were 6–15 weeks at time of analysis.

In the cytokine-induced asthma models, 6-week-old anesthetized BALB/c mice were intranasally treated three (IL-25) or five (IL-33 and PBS) times, with 1 μg recombinant mouse IL-25 or IL-33 (both R&D Systems) in 50 μL PBS or PBS alone. Mice were sacrificed and analyzed 24 h later.

For the HDM asthma protocol, 6-week-old BALB/c mice were anesthetized using isoflurane and sensitized intratracheally using 100 μg HDM (Greer, Lenoir, NC, USA) or PBS (GIBCO, Invitrogen). Ten days later, mice were challenged once, three, or ten times (three times per week) with 10 μg HDM or PBS intranasally. Five days after the last challenge, mice were sacrificed for analysis. OVA asthma was induced by transfer of polarized Th2 cells bearing an OVA-specific TCR as described previously [42]. In brief, DO11.10 cells obtained from lymph nodes of naive mice were stimulated in vitro for 6 days in the presence of IL-4 (10 ng/mL, PeproTech), anti-IFN-γ (10 μg/mL, Bioceros B.V., The Netherlands), anti-IL-12 (1 μg/mL), and OVA peptide (5 μg/mL, Worthington Biochemical Corp). Th2 differentiation was confirmed by flow cytometric analysis of intracellular contents for IFN-γ, IL-4, IL-5, IL-13, and IL-17 production. After washing, 3×10^6 cells were injected into naive BALB/c mice. Mice were challenged with daily OVA aerosols (Sigma-Aldrich) for the subsequent 5 days and sacrificed 48 h after the last challenge.

BAL fluid was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA. All experiments were approved by the Erasmus MC Animal Ethics Committee (DEC).

Preparation of single-cell suspensions

Single-cell suspensions were prepared from the spleen, lungs, MedLNs, and BAL fluid. Spleen and lymph nodes were mechanically disrupted using a 100 μm cell strainer (BD Falcon). Lungs were mechanically disrupted using a cell strainer or digested using collagenase type I or collagenase IV, according to the manufacturer's protocol. Mesenteric and thoracic fat was digested using 2 mg/mL collagenase I in DMEM supplemented with 4% FCS (Lonza) rocking at 37°C for 1 h. Red blood cells from lung and spleen were lysed using osmotic lysis buffer ($NH_4Cl + KHCO_3 + Na_2EDTA$ in H_2O).

Th cell cultures

For in vitro T-cell stimulations and Th1/Th2 polarization cultures, naive $CD62L^+CD4^+$ T cells were purified by cell sorting using a FACSAria equipped with BD FACSDiva software (BD Biosciences) and polarized in vitro as described previously [43]. The purity of obtained fractions was >98%.

Cell sorting, RNA extraction, and PCR

FALC cells, BAL, and lung ILC2 were purified by cell sorting using a FACSAria. Fifty cells per well were dispensed directly into 96-well plates and were stimulated for 4 h using IL-2, IL-25, and IL-33 in the presence of GolgiStop. Polarized Th subsets were also dispensed in 96-wells plates using a FACSAria. After stimulation, cells were directly lysed, resuspended, and frozen until analysis in SuperScript III One-Step RT-PCR reaction buffer containing Igepal CA-630 (Sigma) and RNase Out. Multiplex PCR was performed to directly amplify mRNA using primers for *Il4*, *Il5*, *Il6*, *Il13*, and *Actb*. mRNA levels were quantified in a nested RT-PCR reaction using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas) and gene-specific primers. Quantitative real-time PCR was performed using an Applied Biosystems Prism 7700 sequence detection system. The expression of Il5 was calculated by substraction of *Actb* levels from the test mRNA, followed by calculation of the inverse $\log_2$ of the difference. Primers were ordered from Invitrogen and sequences were as follows for external primers: Il5-extF GTGGGGGTACTGTGGAAATG, Il5-extR ACCAAGGAACTCTTGCAGGT, Il4-extF ACGGATGCGACAAAAATCAC, Il4-extR TTGGAAGCCCTACAGACGAG, Il6-extF GATGGATGCTACCAAACTGGA, Il6-extR TGAAGGACTCTGGCTTTGTCT, Il13-extF CCATCTACAGGACCCAGAGG, Il13-extR GCGAAACAGTTGCTTTGTGT, Actb-extF CCAACCGTGAAAAGATGACC, Actb-extR TCTCCGGAGTCCATCACAAT. Internal primers were as follows: ActbF TCAACACCCCAGCCATGTA, ActbR GTGGTACGACCAGAGGCATAC, Il5-forward ACATTGACCGCCAAAAAGAG, Il5-reverse ATCCAGGAACTGCCTCGTC.

Flow cytometric analysis

Cells were stained with a mix of fluorescently tagged antibodies in FACS buffer containing BSA. For measurements of cytokine production by flow cytometry, cells were restimulated for 4 h at 37°C using PMA and ionomycin supplemented with GolgiStop. After extracellular staining, cells were fixed using 4% PFA and permeabilized using 0.5% saponin in FACS buffer. Lineage-negative cells were gated as cells not expressing CD3, CD4, CD8α, CD11b, CD11c, CD19, B220, NK1.1, FcεRIα, Ter-119, and Gr-1. Purchased antibodies are described in Supporting Information Table 1. Biotin-conjugated antibodies were detected using Pacific Blue or allophycocyanin-Cy7-conjugated (BD) streptavidin. Data acquisition was done using a LSR II flow cytometer equipped with three lasers and FACSDiva™ software. Data analysis was done using FlowJo 8.8.7 (Tree Star Inc., Ashland, OR, USA).

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Statistical analysis

For comparisons between two groups either unpaired *t*-tests (normal distribution) or Mann–Whitney U tests (if no normal distribution was expected) were used. Statistical significance of differences between more than two groups was calculated using one-way ANOVA analysis followed by Turkey post-tests. All analyses were performed using Prism (GraphPad software, La Jolla, CA, USA).

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Abbreviations: **BAL**: broncho-alveolar lavage · **FALC**: fat-associated lymphoid cluster · **γc**: common gamma · **HDM**: house dust mite · **ILC**: innate lymphoid cell. **MedLN**: mediastinal lymph node

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Supporting Information to:

Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma

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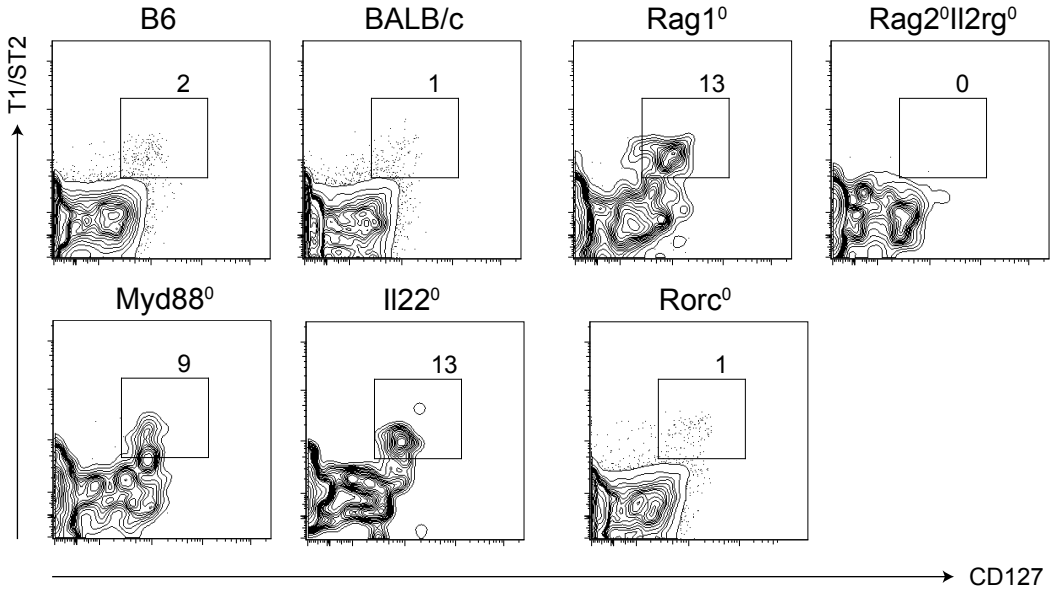
Supporting Information Table 1. Antibodies used for flow cytometric analyses.

Antibody	Conjugate	Clone	Company
B220	biotin	RA3-6B2	eBioscience
CD3	PE	145-2C11	eBioscience
CD3	PECy7	145.2C11	eBioscience
CD4	PE	GK1.5	eBioscience
CD4	PECy7	GK1.5	eBioscience
CD4	biotin	RM4-5	eBioscience
CD8a	PE	53-6.7	eBioscience
CD8a	PECy7	53-6.7	eBioscience
CD8a	biotin	53-6.7	eBioscience
CD11b	PE	M1/70	eBioscience
CD11b	PECy7	M1/70	eBioscience
CD11b	biotin	M1/70	eBioscience
CD11c	PE	N418	eBioscience
CD11c	PECy7	N418	Biolegend
CD11c	biotin	N418	BD Pharmingen
CD19	PE	1D3	BD Pharmingen
CD19	PECy7	1D3	BD Pharmingen
CD19	biotin	1D3	BD Pharmingen
CD25	PerCPCy5.5	PC61	BD Pharmingen
CD25	APCCy7	PC61	BD Pharmingen
CD45	PECy7	30-F11	eBioscience
CD45.2	PerCPCy5.5	104	eBioscience
CD117	APC	2B8	eBioscience
CD127	PE	A7R34	eBioscience
CD127	APC	A7R34	eBioscience
FceRIa	PE	MAR-1	eBioscience
FceRIa	biotin	MAR-1	eBioscience
Gr-1	PE	RB6-8C5	BD Pharmingen
Gr-1	PECy7	RB6-8C5	eBioscience
Gr-1	biotin	RB6-8C5	BD Pharmingen
IFN-g	PECy7	XMG1.2	BD Pharmingen
IL-4	PECy7	11B11	BD Pharmingen
IL-5	biotin	TRFK5	BD Pharmingen
IL-10	PerCPCy5.5	JES5-16E3	eBioscience
IL-13	Alexa Fluor 647	eBio13A	eBioscience
NK1.1	PE	PK136	eBioscience
NK1.1	PECy7	PK136	BD Pharmingen
NK1.1	biotin	PK136	eBioscience
Sca-1	APC	D7	eBioscience
Sca-1	PECy7	D7	eBioscience
SiglecF	PE	E50-2440	BD Pharmingen
Streptavidin	APCCy7		BD Pharmingen

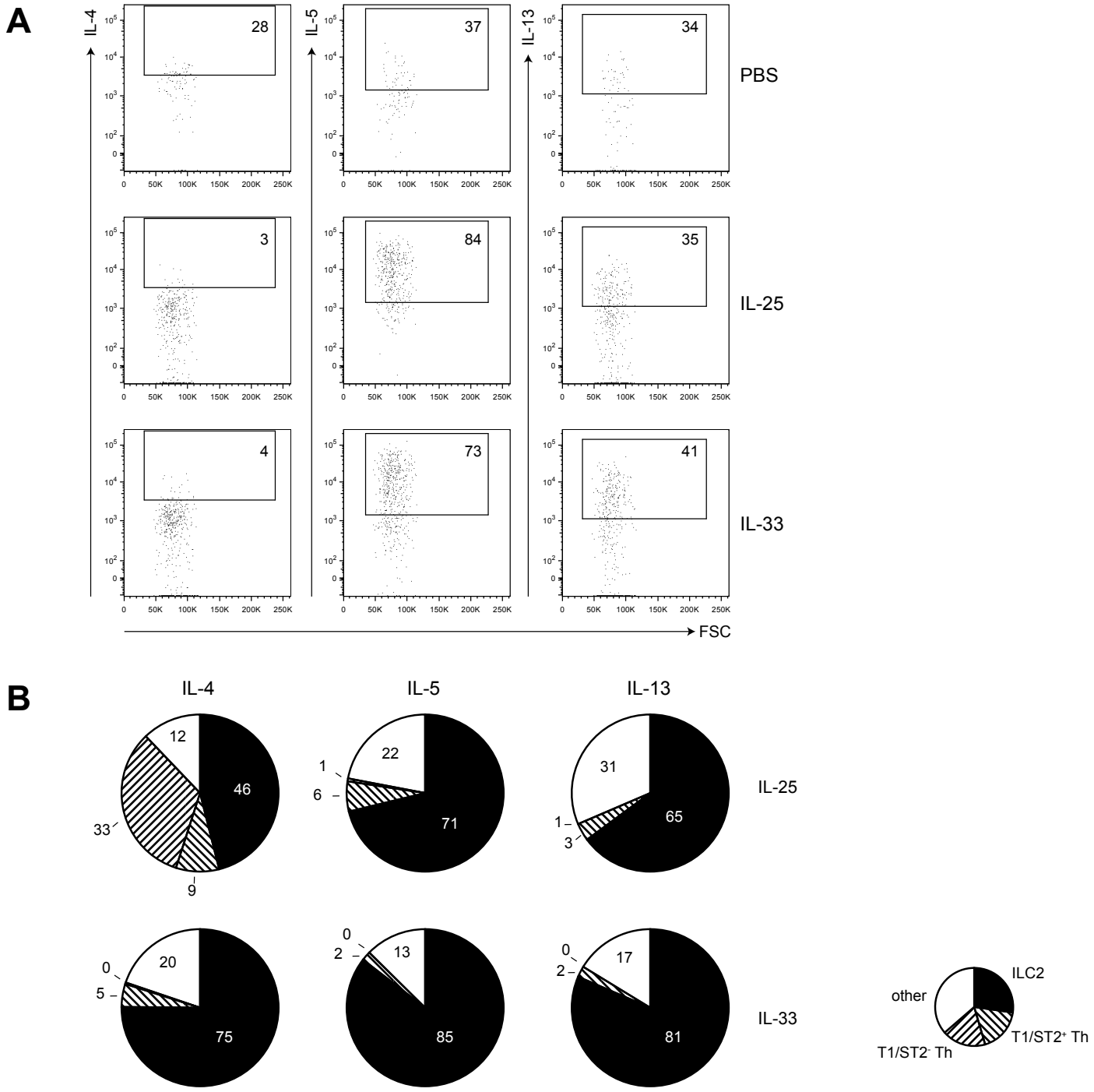
Supporting Information Table 1 (continued). Antibodies used for flow cytometric analyses.

Antibody	Conjugate	Clone	Company
Streptavidin	Pacific Blue		Invitrogen
T1/ST2	FITC	DJ8	MD Bioproducts
T1/ST2	biotin	DJ8	MD Bioproducts
Ter-119	PE	TER119	eBioscience
Ter-119	PECy7	TER119	eBioscience
Ter-119	biotin	TER119	BD Pharmingen
TNF-a	PE	MP6-TX22	eBioscience



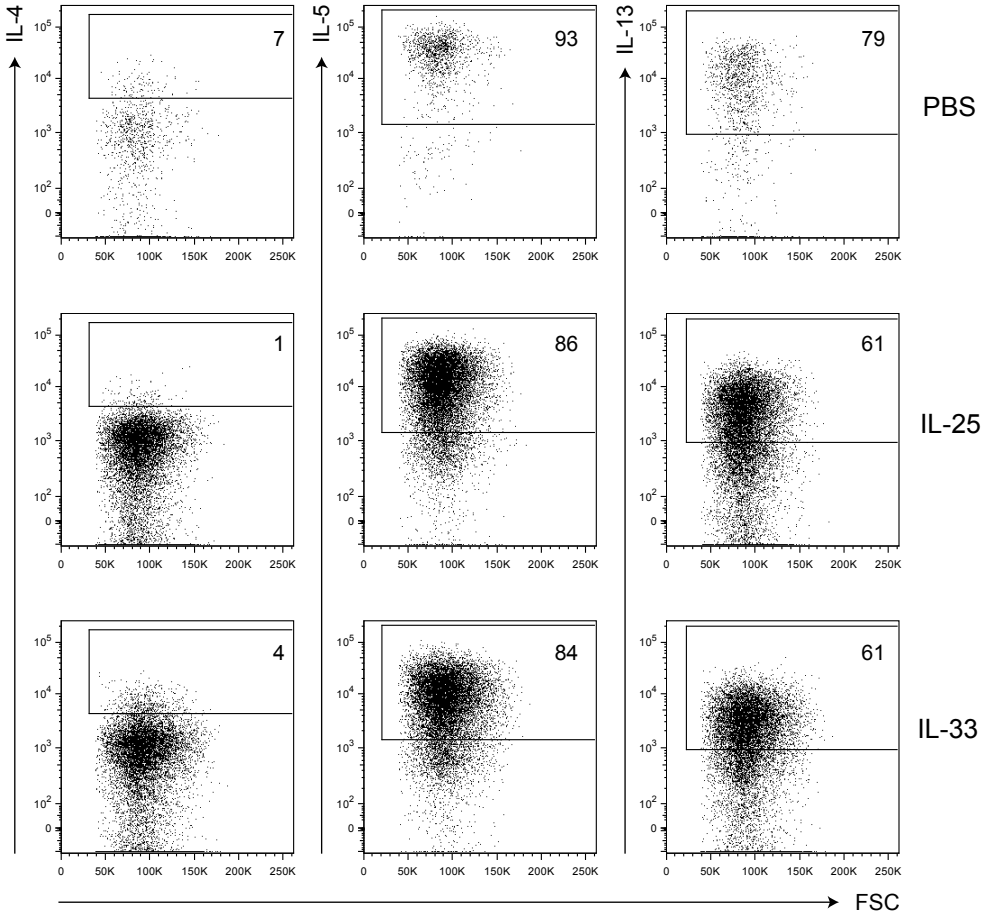
Supporting Information Figure 1. ILC2 development does not depend on *Rag, Myd88*, *Il22* or *Rorc*.

Identification of ILC2s in the lungs of mice of the indicated strains. Plots show expression of CD127 and T1/ST2 by gated Lin$^-$ lymphocytes. Numbers indicate the percentage of ILC2 of Lin-negative lymphocyte population ± s.e.m.



Supporting Information Figure 2. ILC2 are major producers of IL-5 and IL-13 in BAL in cytokine-induced airway inflammation.

A) Intracellular cytokine content of BAL $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$ ILC2s in mice treated as specified. Plots show forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4h. Numbers indicate the proportions of cytokine-positive ILC2s. B) Contribution of various cell populations to production of Th2 cytokines in BAL fluid upon administration of IL-25 or IL-33. Proportions of cytokine expressing cells were determined by flow cytometry (as in panel A) in ILC2, $T1/ST2^{+}$ and $T1/ST2^{-}$ $CD3^{+}CD4^{+}$ T helper cells. Pie charts and percentages represent the contribution of various populations to the total number of cytokine-producing cells. Representative data of two independent experiments with 3-4 mice per group.



Supporting Information Figure 3. The majority of ILC2s produce IL-5 and IL-13 in cytokine-induced airway inflammation.

Intracellular cytokine content of $Lin^{-}CD25^{+}CD127^{+}T1/ST2^{+}$ ILC2s of *Rag2*-deficient mice treated 5 times with IL-25 or 3 times with IL-33, shown as dot plots of forward scatter (FSC) versus the indicated cytokines. Single-cell suspensions of lungs were stimulated with PMA/ionomycin in the presence of monensin for 4h. Plots show one representative sample of two experiments with n ≥ 3 mice per group.