

Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33

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Background: Asthma in a mouse model spontaneously resolves after cessation of allergen exposure. We developed a mouse model in which asthma features persisted for 6 months after cessation of allergen exposure.

Objective: We sought to elucidate factors contributing to the persistence of asthma.

Methods: We used a combination of immunologic, genetic, microarray, and pharmacologic approaches to dissect the mechanism of asthma persistence.

Results: Elimination of T cells through antibody-mediated depletion or lethal irradiation and transplantation of recombination-activating gene (*Rag1*)^{-/-} bone marrow in mice with chronic asthma resulted in resolution of airway inflammation but not airway hyperreactivity or remodeling. Elimination of T cells and type 2 innate lymphoid cells (ILC2s) through lethal irradiation and transplantation of *Rag2*^{-/-} γ c^{-/-} bone marrow or blockade of IL-33 resulted in resolution of airway inflammation and hyperreactivity. Persistence of asthma required multiple interconnected feedback and feed-forward circuits between ILC2s and epithelial cells. Epithelial IL-33 induced ILC2s, a rich source of IL-13. The latter directly induced epithelial IL-33, establishing a positive feedback circuit. IL-33 autoinduced, generating another feedback circuit. IL-13 upregulated IL-33 receptors and facilitated IL-33 autoinduction, thus establishing a feed-forward circuit.

Elimination of any component of these circuits resulted in resolution of chronic asthma. In agreement with the foregoing, IL-33 and ILC2 levels were increased in the airways of asthmatic patients. IL-33 levels correlated with disease severity. **Conclusions:** We present a critical network of feedback and feed-forward interactions between epithelial cells and ILC2s involved in maintaining chronic asthma. Although T cells contributed to the severity of chronic asthma, they were redundant in maintaining airway hyperreactivity and remodeling. (*J Allergy Clin Immunol* 2015;■■■■:■■■-■■■.)

Key words: Type 2 innate lymphoid cells, IL-33, T cells, feedback circuit, chronic asthma

Asthma is a chronic illness,^{1,2} with airway hyperreactivity and remodeling persisting in asymptomatic patients with normal pulmonary function.^{3,4} Because of the perennial nature of some allergens, it has been difficult to ascertain whether continuous allergen exposure is necessary for the persistence of asthma. Longitudinal observations in patients with occupational asthma indicate that asthma persists in most patients years after removal from occupational exposure.^{5,6} These results suggest that repetitive allergen exposure establishes a biochemical mechanism that sustains asthma in the absence of the inciting allergen. Tremendous progress has been made in uncovering the mechanisms surrounding the inception and development of asthma through animal model studies.^{7,8} However, very little is known about the mechanisms regulating the persistence of chronic asthma.

In the majority of mouse models, asthma resolves spontaneously in 1 to 2 weeks.⁹⁻¹¹ Repetitive allergen exposure in alternative models induces tolerance.^{9,12,13} In others cessation of repetitive allergen exposure results in resolution of inflammation.¹⁴⁻¹⁶ With the longest study period to date, Johnson et al¹⁶ have demonstrated attenuated airway remodeling and airway hyperreactivity persisting for 9 weeks after cessation of repetitive dust mite antigen exposure. The objective of the current study was to investigate the mechanisms of this persistence of asthma in the absence of the inciting allergens. To accomplish this, we used our previously generated mouse model in which asthma persisted for 3 weeks after cessation of repetitive dust mite, ragweed, and *Aspergillus* species allergen exposure.¹⁷

Recent studies have identified a novel IL-5/IL-13-producing type 2 innate lymphoid cell (ILC2) population in mice¹⁸⁻²⁰ and human subjects.^{21,22} Although numerous studies have established the importance of ILC2s in the initiation of airway eosinophilic inflammation,²³⁻²⁸ the role of ILC2s in maintenance of existing airway hyperreactivity has not been established, and their involvement in the airways of asthmatic patients has not been examined.

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Abbreviations used

BAL:	Bronchoalveolar lavage
γ c:	Common γ c chain of the IL-2 receptor
CRTH2:	Chemoattractant receptor-homologous molecule expressed on T _H 2 lymphocytes
DRA:	Dust mite, ragweed and <i>Aspergillus</i> species
ERK:	Extracellular signal-regulated kinase
H&E:	Hematoxylin and eosin
ILC:	Innate lymphoid cell
ILC2:	Type 2 innate lymphoid cell
IL-33R:	IL-33 receptor
Rag:	Recombination-activating gene

METHODS

The materials and methods used in this study are described in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS**Repetitive allergen exposure induces asthma that persists longer than 6 months after cessation of allergen exposure**

To establish experimental asthma in mice, we administered intranasally 3 representative allergens (ie, dust mite, ragweed, and *Aspergillus* species) without adjuvant twice a week for 6 weeks, as described previously.¹⁷ In this model airway hyperreactivity persisted for longer than 6 months ([Fig 1, A](#)) after cessation of allergen exposure. Airway inflammation ([Fig 1, B and C](#)), and epithelial and peribronchial smooth muscle ([Fig 1, D](#)) hypertrophy peaked 1 to 2 months after allergen cessation but remained significantly increased after 6 months. The previously reported increase in mucin and mast cell levels also persisted in patients with chronic asthma (see [Fig E1, A-D](#), in this article's Online Repository at www.jacionline.org).

T cells contribute to the severity but not persistence of asthma

To examine the mechanism of asthma persistence, we designed the following timeline for various interventions ([Fig 2, A](#)). Previous studies have demonstrated resolution of acute asthma within 10 to 14 days after allergen exposure.⁹⁻¹¹ To demonstrate the persistence of asthma, we studied outcomes 3 weeks after the last allergen exposure. Nearly all interventions were carried out in week 10. Lethal irradiation of mice was done at the end of week 9. Lung inflammation and airway hyperreactivity were measured 6 weeks later at week 15 to provide sufficient time for marrow engraftment and restoration of the immune response. We examined the contribution of T cells in asthma maintenance using 3 different approaches.

First, we treated mice with chronic asthma with an anti-CD3 ϵ neutralizing antibody or an isotype control antibody at week 10. This treatment reduced T-cell counts by 91% in the spleen (see [Fig E1, A](#)) and inhibited airway inflammation, as determined by means of quantification of lung histology, by 70% (see [Fig E1, B](#)). However, the anti-CD3 ϵ antibody did not alter airway hyperreactivity ([Fig 2, B](#)). To some extent, our results are similar to those reported by Doherty et al,²⁹ who demonstrated that anti-CD4 T-cell depletion inhibited airway inflammation but not

smooth muscle mass, mucus metaplasia, or airway fibrosis in a mouse model of chronic allergen exposure.

Second, we adoptively transferred splenic CD4 T cells from chronic saline-treated mice or from the chronic asthma model (isolated in week 10) to naive mice and subsequently challenged the recipient mice intranasally with the sensitizing allergens on 3 consecutive days. Adoptively transferred CD4 T cells from mice with chronic asthma potentially induced airway hyperreactivity in naive mice 6 days after the transfer ([Fig 2, C](#)), thus confirming the ability of CD4 T cells from mice with chronic asthma to induce acute asthma in naive mice. However, airway hyperreactivity did not persist when examined on day 21.

Third, we subjected mice with chronic asthma to lethal irradiation at the end of week 9 and transplanted bone marrow from naive mice to sustain mouse viability. Splenic and mediastinal lymph node-derived T cells collected 6 weeks after irradiation proliferated in response to anti-CD3/CD28 antibodies but did not respond to the recall antigens dust mite (see [Fig E2, C](#), in this article's Online Repository at www.jacionline.org), ragweed, and *Aspergillus* species (not shown). This indicates elimination of sensitized T cells in irradiated mice. In addition, antigen stimulation did not increase the number of cytokine-producing cells over baseline (medium stimulation) after immune ablation. In contrast, an increased number of T cells from the control mice with chronic asthma (not subjected to lethal irradiation) produced IL-2 (see [Fig E2, D](#)) and IL-4 (see [Fig E2, E](#)) after dust mite stimulation. The results indicate that lethal irradiation has eliminated antigen-specific T cells. This effect of lethal irradiation will henceforth be referred to as immune ablation.

We monitored airway inflammation at weeks 11, 13, and 15 (2, 4, and 6 weeks after immune ablation). Consistent with the absence of allergen-specific T cells, airway inflammation was nearly absent at week 11 but gradually increased, reaching statistical significance at week 15 ([Fig 2, D](#), and see [Fig E2, F](#)). Epithelial and smooth muscle hypertrophy ([Fig 2, E](#)) and airway hyperreactivity ([Fig 2, F](#)) persisted after immune ablation, although the degree of airway hyperreactivity was reduced. These results suggest that persistence of airway hyperreactivity and remodeling did not require the presence of sensitized T cells. However, the magnitude of airway hyperreactivity was amplified by sensitized T cells.

Gene expression profile shows increased levels of ILC2-promoting molecules in chronic asthma

We compared gene expression in lung tissue from the chronic asthma model using a microarray with 2 different controls, acute asthma and saline controls, as described previously (see the [Methods](#) section in this article's Online Repository).¹⁷ We observed approximately 1000 genes upregulated and downregulated in the acute model by using a cutoff of a 3-fold difference with the saline control and nearly 3 times more genes upregulated than downregulated in mice with chronic asthma compared with saline control animals ([Fig 3, A](#)). The principal component analysis ([Fig 3, B](#))—derived positions of acute and chronic asthma do not overlap, indicating a qualitative difference in gene expression. Because sensitized T cells were not essential for sustaining asthma, we analyzed ILC2-inducing cytokines.^{20,27,30} We observed increased expression of IL-7, IL-33, and their receptors in the setting of chronic asthma ([Fig 3, C](#)), which was confirmed by using real-time PCR ([Fig 3, D-F](#)). Levels of IL-4, but not

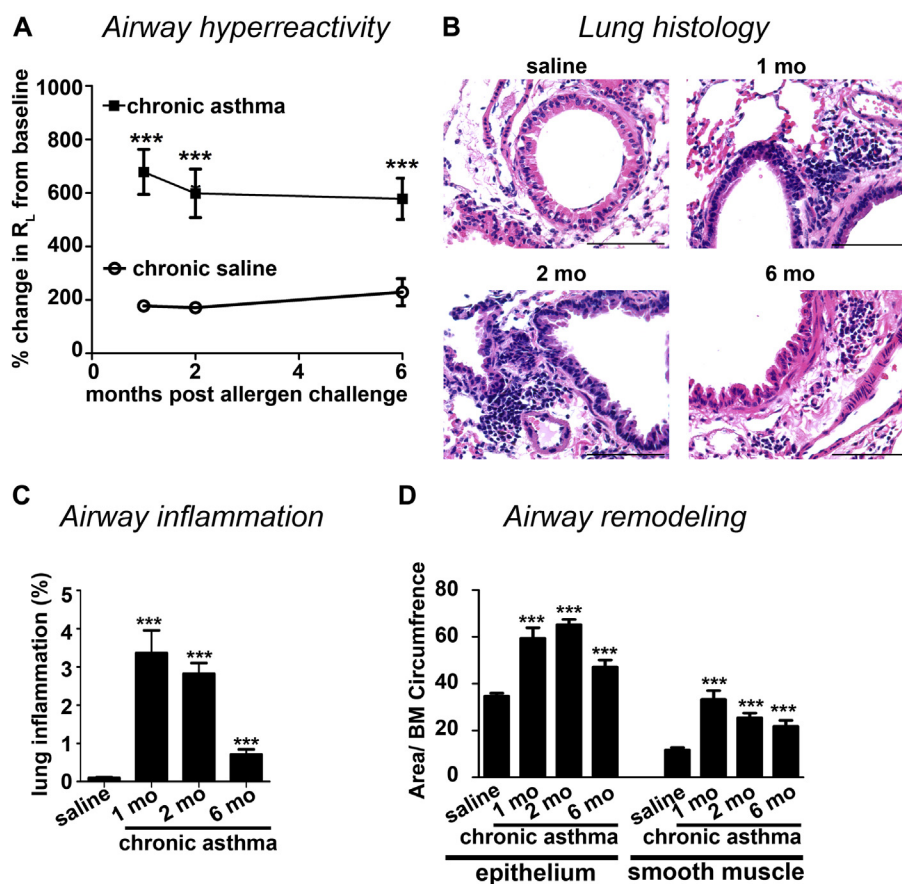


FIG 1. Persistence of an asthma phenotype in a mouse model. **A**, Airway hyperreactivity (total lung resistance [R_L]) measured 1, 2, and 6 months after cessation of allergen exposure. **B**, Corresponding histologic images of the lung from mice of the chronic asthma and saline control groups. Saline control was from the 6-month time point. Scale bar = 100 μ m. **C**, Quantification of peribronchial and perivascular inflammation as a percentage total area in the field. **D**, Quantification of epithelial and peribronchial smooth muscle. BM, Basement membrane. There were 5 to 8 mice per group. *** $P < .001$.

IFN- γ , were increased in both the acute and chronic asthma groups relative to those seen in the saline control group (Fig 3, C). IL-4 expression was confirmed by using real-time PCR (Fig 3, G). In agreement with a recent study that demonstrated a central role for IL-33 compared with IL-25 and thymic stromal lymphopoietin in dust mite and peanut allergic sensitization,³¹ IL-25 and thymic stromal lymphopoietin expression, determined by using real-time PCR, was 100- to 1000-fold lower than that of IL-33 (Fig 3, H and I) in the acute and chronic asthma groups. We confirmed IL-33 expression in the airways after immune ablation using Western blotting (Fig 3, J). The larger 31-kDa form of IL-33 undergoes proteolytic cleavage to produce a more active 20-kDa form.³² The 20-kDa form was present in the chronic asthma group but not in the saline control group and persisted after immune ablation.

Chronic asthma is characterized by increased ILC2 numbers

Previous studies have demonstrated a crucial role for ILC2s in the development of lung inflammation from *Alternaria* species²⁵ and papain,²⁶ airway hyperreactivity caused by influenza,³³ and allergic sensitization from dust mite and peanut.³¹ Our microarray data suggest a role for ILC2s in the maintenance of chronic

asthma. We studied ILC2s in the lung digest, as described previously.^{19,20,26} First, we gated for live CD45⁺ cells (see Fig E3, A-H, in this article's Online Repository at www.jacionline.org). Next, we identified lineage (CD3, Ly-6G/Ly-6C, CD11b, B220, TER-119)-negative but CD25⁺ cells. This lin⁻CD25⁺ population was positive for the ILC2 markers c-Kit, Sca-1, chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTH2), IL-33 receptor (IL-33R), and killer cell lectin-like receptor G superfamily, member 1 (KLRG1) and negative for NK1.1 and Fc ϵ RI. About one fourth of these lin⁻CD25⁺CRTH2⁺IL-33R⁺ ILC2s were positive for IL-5 and IL-13 in nonimmunized mice. This is in agreement with previous reports that showed low basal expression of IL-5 and IL-13 and increased expression on stimulation.¹⁸⁻²⁰ These cells were negative for IL-17 (see Fig E3, I). The frequency of IL-5⁺ innate lymphoid cells (ILCs) was consistently higher than that of IL-5⁺ T cells in all study groups (Fig 3, K).

The total lin⁻CD25⁺ ILC population was increased in the chronic asthma versus saline control groups (Fig 4, A, and see Fig E3, B, J, and K). After immune ablation and naive bone marrow transplantation, the ILC population decreased but remained increased compared with that in the saline control group. IL-5⁺ ILC2s constituted 61% of all IL-5⁺ lung cells in the chronic asthma group (Fig 4, B). Irradiation caused a

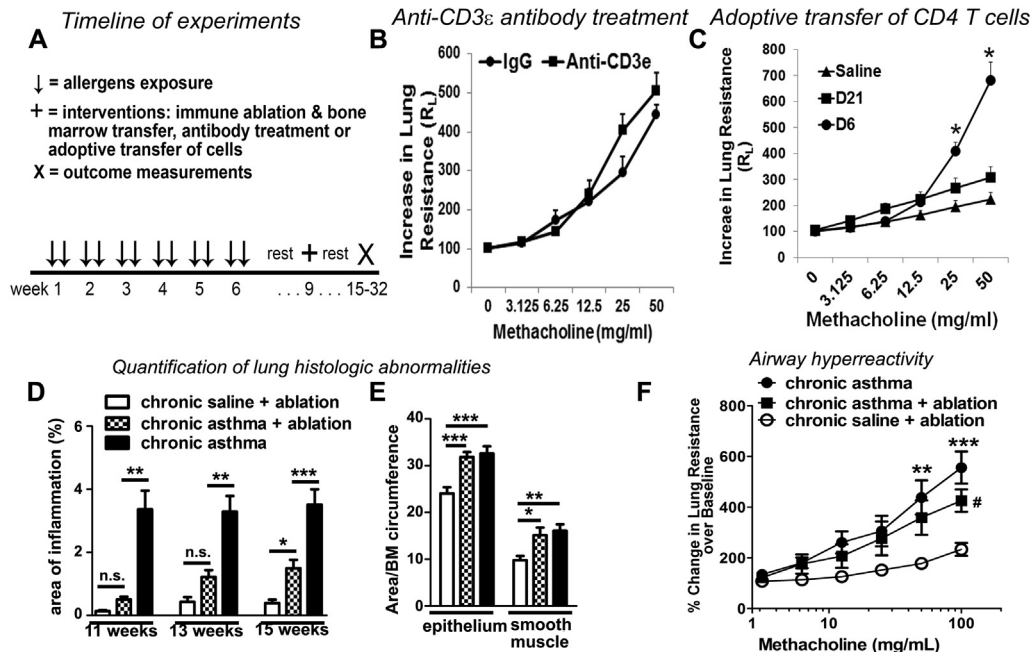


FIG 2. Chronic asthma persists after T-cell depletion. **A**, Timeline of intranasal allergen exposure for 6 weeks, the rest period, and then irradiation, bone marrow transfer, and antibody and inhibitor treatment in weeks 9 and 10 in the chronic asthma model. Outcomes were measured 3 days later, unless otherwise stated. **B**, Effect of an anti-CD3ε or isotype control antibody on airway resistance ($n = 5$). **C**, Effect of adoptive transfer of spleen CD4 T cells from the chronic asthma and saline control groups obtained in week 10 to naive mice. Airway hyperreactivity was measured in chronic asthma CD4 recipient mice on days 6 and 21 and in saline control CD4 recipient mice on day 6 ($n = 4$). * $P < .05$. R_L , Total lung resistance. **D**, Quantification of lung inflammation at weeks 11, 13, and 15 in mice after lethal irradiation and bone marrow transplantation. **E**, Quantification of epithelial hypertrophy and peribronchial smooth muscle hypertrophy at week 15. All morphometric quantifications were performed in at least 5 independent fields per mouse. *BM*, Basement membrane. **F**, Measurement of lung resistance in week 15. * $P < .05$, ** $P < .01$, *** $P < .001$, and # $P < .05$. *n.s.*, Not significant. There were 5 to 6 mice per group for Fig 2, D through F.

near-complete elimination of host-originated IL-5⁺ and IL-13⁺ ILC2s in both the chronic asthma (Fig 4, B and C) and saline control (see Fig E4, D and E, in this article's Online Repository at www.jacionline.org) groups. This was replaced by donor-derived ILCs. Interestingly, the number of donor-derived ILCs increased 3-fold in recipients of marrow from the chronic asthma group compared with that in the saline control group, suggesting a posttransfer expansion likely caused by established increased IL-33 production present in the chronic asthma group.

ILC2s but not antigen-specific T cells are essential for the persistence of asthma

To determine the role of ILC2s in the persistence of asthma, we transferred bone marrow from recombination-activating gene (*Rag1*)^{-/-}, *Rag2*^{-/-} and common γ chain of the IL-2 receptor (γ c)^{-/-}, and wild-type mice to irradiated mice with chronic asthma. *Rag1*^{-/-} mice are deficient in T and B cells but have normal ILC numbers, whereas *Rag2*^{-/-} γ c^{-/-} mice are additionally deficient in ILCs.²⁶ Mice that received marrow from *Rag1*^{-/-} and wild-type mice maintained airway hyperreactivity (Fig 4, D). In contrast, mice that received marrow from *Rag2*^{-/-} γ c^{-/-} mice lost airway hyperreactivity. Airway inflammation and increased inflammatory cells in bronchoalveolar lavage (BAL) fluid persisted in recipients of *Rag1*^{-/-} but not *Rag2*^{-/-} γ c^{-/-} marrow (see Fig E5, A-C, in this article's Online Repository at www.jacionline.org).

The total number of ILCs (lin⁻ CD25⁺) was comparable between recipients of *Rag1*^{-/-} marrow (see Fig E5, D) and naive marrow (Fig 4, A). The ILC population was nearly absent in recipients of *Rag2*^{-/-} γ c^{-/-} marrow (see Fig E5, D). Despite the absence of ILCs and T cells, the recipients of *Rag2*^{-/-} γ c^{-/-} marrow had a significant number of IL-13⁺ cells in the lungs (see Fig E5, E), although this was not sufficient to sustain asthma. This could be due to the difference in the quantity of IL-13 or other cytokines/factors made by ILC2s. The lack of asthma in *Rag2*^{-/-} γ c^{-/-} marrow recipient mice eliminates the possibility that radioresistant antigen-specific T cells or ILC2s that might have persisted after irradiation were responsible for the sustenance of asthma in mice with chronic asthma that received naive marrow (Fig 2, D-F).

IL-33 blockade abolishes airway hyperreactivity, inflammation, and IL-5/IL-13-producing cells

Persistent production of IL-33 after immune ablation (Fig 3, J) suggests that IL-33-driven ILC2s might be critical for the persistence of asthma. To test this hypothesis, we administered 3 doses of an anti-IL-33 antibody or goat IgG to immune-ablated mice with chronic asthma 1 week before the outcome measures in week 15. Anti-IL-33 treatment reduced the number of total lung ILCs (CD45⁺ lin⁻ CD25⁺), IL-5⁺ ILC2s, total IL-5⁺ lung cells (see Fig E6, B, in this article's Online Repository at www.jacionline.org).

Asthma model microarray analysis

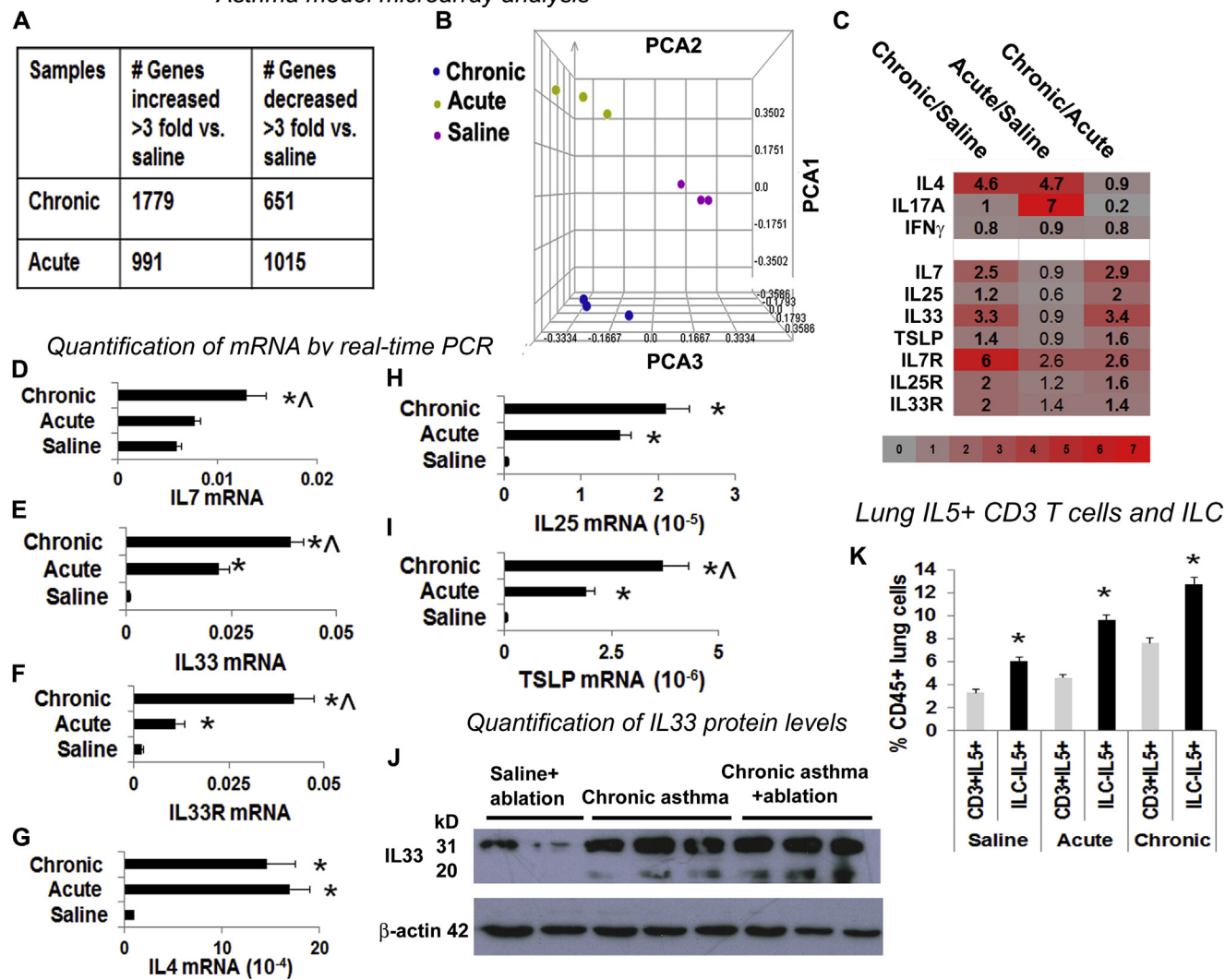


FIG 3. Comparison of the gene expression profile between chronic and acute asthma by using a microarray. **A**, Microarray analysis of lung tissue obtained from mice with chronic and acute asthma ($n = 3$ mice per group). The table shows differentially expressed genes in the asthmatic groups in comparison with the saline control group. **B**, Principal component analysis (PCA) of the expressed genes demonstrating differences among the asthma models, as reflected by their spatial positioning in the 3-dimensional space. **C**, Heat map of select gene expression changes in the chronic and acute asthma compared with saline control groups. **D-I**, Measurement of mRNA for select genes by using real-time PCR. The mRNA level was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression ($n = 5$ mice per group). * $P < .05$ compared to saline control; ^ $P < .05$ compared to acute asthma. **J**, IL-33 levels were confirmed by means of Western blotting to determine the presence of both full-length IL-33 and the mature cleaved product ($n = 3$). **K**, Comparison of lung IL-5⁺ CD3⁺ T cells and IL-5⁺ ILC2s among the study groups ($n = 5$). * $P < .05$ compared with CD3⁺IL-5⁺ cells in each study group.

www.jacionline.org), IL-13⁺ ILC2s, and total IL-13⁺ lung cells to normal nonasthmatic levels (Fig 4, E). This was associated with a complete resolution of airway hyperreactivity (Fig 4, F) and a significant reduction in airway inflammation (see Fig E6, A). IL-33 blockade also reduced total cell, lymphocyte, and eosinophil numbers in BAL fluid (see Fig E6, C).

Adoptive transfer of ILCs induces sustained airway hyperreactivity

The foregoing experiments demonstrated that airway hyperreactivity could not be sustained in the absence of IL-33

or ILC2s. Lung CD45⁺lin⁻CD25⁺ cells were sorted from the chronic asthma and saline control groups (both CD45.1⁺) and adoptively transferred (2×10^5 cells) to naive congenic CD45.2⁺ mice to demonstrate whether activated ILC2s from asthmatic mice were sufficient to sustain airway hyperreactivity. We detected donor-derived ILCs in the recipient lung 21 days after transfer (see Fig E7 in this article's Online Repository at www.jacionline.org). In contrast to adoptive transfer of CD4 T cells from mice with chronic asthma, we observed significant airway hyperreactivity 21 days after adoptive transfer in recipients of ILCs from mice with chronic asthma (Fig 4, G).

Quantification of ILC in chronic asthma with and without ablation/transplantation

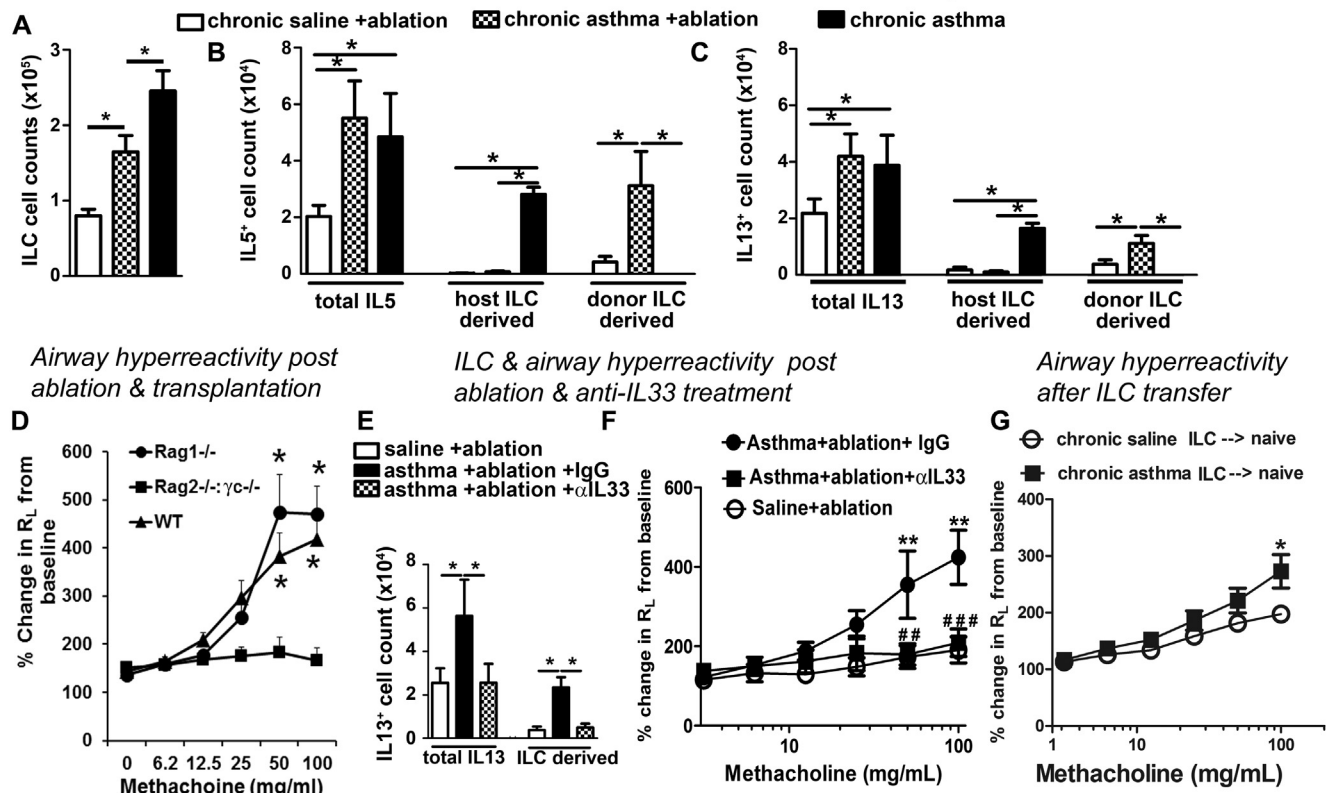


FIG 4. Role of ILCs in the persistence of asthma. **A**, Quantification of the lung ILC population in the chronic asthma group and in the chronic asthma and saline control groups after immune ablation and bone marrow transplantation. **B** and **C**, Total lung cytokine-producing cell populations and cytokine-positive host CD45.2⁺ and transplanted donor CD45.1⁺ ILC populations (mean ± SEM) were quantified for IL-5 (Fig 4, B) and IL-13 (Fig 4, C; n = 5 mice per group). *P < .05. **D**, Bone marrow from either Rag1^{-/-}, Rag2^{-/-}γc^{-/-}, or wild-type mice was transplanted into mice with chronic asthma after irradiation. Airway hyperreactivity was measured 6 weeks after irradiation. **E** and **F**, Effect of IL-33 blockade after immune ablation and bone marrow transplantation: total IL-13⁺ lung cells and IL-13⁺ ILC2s (Fig 4, E) and airway hyperreactivity (Fig 4, F). R_L, Total lung resistance. *P < .05, **P < .01, ***P < .001, ###P < .05, and ###P < .01 in αIL-33 vs IgG. There were 6 to 9 mice per group. Results shown are means ± SEMs. **G**, Effect of adoptive transfer of ILCs from mice with chronic asthma and those in the saline control group to naive mice on airway hyperreactivity measured 21 days later (n = 5). *P < .05.

Airway epithelial cells establish a positive feedback circuit through IL-33 and ILC2s

Self-sustenance of biological processes can be facilitated by the development of a positive feedback circuit or circuits. We tested this putative mechanism by examining the effect of IL-13, a major product of ILC2s, on epithelial production of IL-33 in the human lung epithelial cell line A549. IL-13 was the most potent inducer of IL-33 mRNA compared with IL-33, IL-1β, IL-4, IL-17, TNF, and IFN-γ (Fig 5, A). IL-13 also stimulated secretion of IL-33 (see Fig E8, A, in this article's Online Repository at www.jacionline.org). Similar results were observed with the BEAS2B human bronchial epithelial cell line (not shown). These results suggest that IL-13 can induce epithelial production of IL-33, establishing a positive feedback circuit using ILC2s.

IL-33 autoinduction represents another positive feedback circuit in the setting of chronic asthma

Previous studies have shown that IL-33 induces acute asthma in mice when examined 24 hours after the ultimate

dose.^{24,34-36} To extend these findings to chronic asthma, we examined the persistence of asthma 15 days after intranasal IL-33 administration. IL-33 induced airway hyperreactivity (Fig 5, B) and mild airway inflammation (see Fig E8, B and C) that persisted for 2 weeks. IL-33 also increased the number of lung IL-13⁺ ILC2s (Fig 5, C), the total number of IL-5⁺ and IL-13⁺ lung cells, and IL-5⁺ ILC2 numbers (see Fig E8, D and E). These experiments suggest that IL-33 induces a sustained effect in the airways. To address the mechanism for this sustained effect, we investigated IL-33 autoinduction. Hardman et al³⁶ initially described IL-33 autoinduction 24 hours after intranasal delivery of IL-33. To extend these findings, we observed persistent IL-33 production (Fig 5, D and E) in pro-surfactant protein C-positive epithelial cells (see Fig E9, A, in this article's Online Repository at www.jacionline.org) and CD11b⁺ hematopoietic cells 2 weeks after intranasal IL-33 administration. These results are in agreement with the data from an IL-33 reporter mouse model³⁶ and our previous *in vitro* experiments (Fig 5, A) indicating that IL-33 was capable of autoinduction.

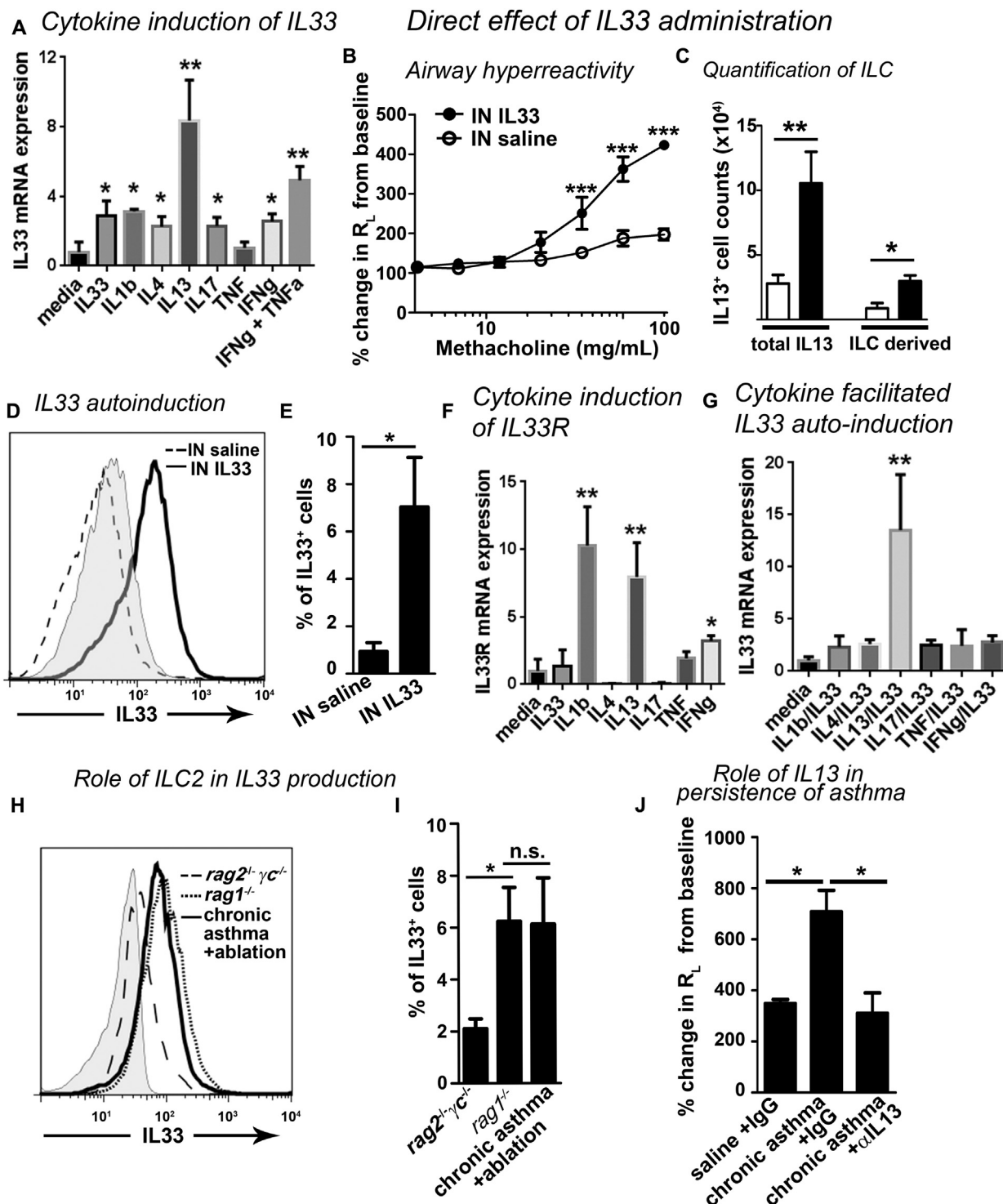


FIG 5. Role of IL-33 in the persistence of asthma. **A**, A549 cells were cultured in medium alone or with IL-33 (10 ng/mL), IL-1 β (2 ng/mL), IL-4 (10 ng/mL), IL-13 (20 ng/mL), IL-17 (10 ng/mL), TNF (2 ng/mL), IFN- γ (10 ng/mL), or IFN- γ plus TNF (same concentrations) for 72 hours, and then mRNA was analyzed for IL-33 normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). * P < .05 and ** P < .01. There were 4 to 8 mice per group. **B-E**, Direct effect of IL-33 on airway hyperreactivity, lung ILCs, and autoinduction. Mice were treated intranasally for 3 consecutive days with 400 ng per dose of IL-33: methacholine-induced airway hyperreactivity (total lung resistance [R_L]; Fig 5, **B**), lung IL-13 $^{+}$ ILCs (Fig 5, **C**), and IL-33 autoinduction (fluorescence intensity [gray, goat IgG control; Fig 5, **D**]; IL-33 $^{+}$ cells [Fig 5, **E**]) were measured after 15 days (n = 5 mice per group). * P < .05, ** P < .01, and *** P < .001. *IN*, Intranasal. **F**, Cytokine induction of IL-33R mRNA in A549 cells (n = 4 per group). * P < .05 and ** P < .01. **G**, A549 cells were pretreated for 24 hours with either medium or the cytokines, as in Fig 5, **A**, and then treated for 72 hours with or without IL-33 (20 ng/mL; n = 4). ** P < .01. **H-I**, Persistence of IL-33 $^{+}$ cells (fluorescence intensity in Fig 5, **H**, and IL-33 $^{+}$ cell numbers in Fig 5, **I**) in mice with chronic asthma after irradiation and transfer of bone marrow from naive, *Rag1* $^{-/-}$, and *Rag2* $^{-/-}$ γ c $^{-/-}$ mice (n = 5 per group). * P < .05. *n.s.*, Not significant. **J**, Effect of 3 doses of an anti-IL-13 antibody on total lung resistance (R_L). The change in R_L with the 25-mg dose of methacholine is shown (n = 5 per group). * P < .05.

IL-13 generates a feed-forward mechanism for IL-33 autoinduction

Airway epithelial cells express low basal levels of mRNA for IL-33R,³⁷ thus permitting IL-33 autoinduction. IL-13 and IL-1 β , but not IL-4 or IL-17, strongly induced IL-33R (Fig 5, F). Next, we investigated whether preincubation with the IL-33–inducing cytokines facilitated IL-33 autoinduction. Only IL-13, but not IL-1 β , IL-4, or IL-17, synergistically enhanced IL-33 autoinduction (Fig 5, G). Although this synergy was modest, the result suggests that IL-13 was mechanistically connected with IL-33 autoinduction. Given that IL-33 stimulates ILC2s, which subsequently produce IL-13 capable of stimulating additional IL-33 release, the result suggests an IL-33–driven feed-forward mechanism. Although IL-1 β was a strong inducer of IL-33R, it was unable to facilitate IL-33 autoinduction. We speculate that this is likely due to competition for the shared IL-1 receptor accessory protein (IL1RAcP) between IL-33R and IL-1 β . To confirm a role for our triple-allergen cocktail used in the induction of the chronic asthma model, we tested their effect on IL-33 production *in vitro*. Dust mite, ragweed, and *Aspergillus* species directly stimulated IL-33 mRNA transcription 24 hours after exposure in A549 epithelial cells (see Fig E9, B). This is in agreement with direct induction of IL-33 by other allergens in previous studies.^{24,25,36} IL-33 production persisted equally in recipients of ILC-sufficient naive and *Rag1*^{−/−} bone marrow but not ILC-deficient *Rag2*^{−/−} γ c^{−/−} bone marrow (Fig 5, H and I). This suggests that ILC2s were essential for sustained IL-33 production *in vivo* during chronic asthma.

IL-13 is important for the persistence of asthma

We previously reported that a single administration of an anti-IL-13 antibody inhibited acute but not chronic asthma.¹⁷ Because this study uncovered that IL-13 is likely to be involved in establishing a positive feedback mechanism, we reasoned that repetitive IL-13 blockade would be required to override the feedback mechanism. To test this hypothesis, we administered 3 doses of an anti-IL-13 antibody intraperitoneally to mice with chronic asthma in week 10. The airway hyperreactivity resolved 3 days after the anti-IL-13 antibody treatment (Fig 5, J).

IL-33 and ILC2 levels are increased in human asthma

Next, we examined the clinical relevance of IL-33 and ILC2s. IL-33 levels were significantly increased in BAL fluid from asthmatic patients compared with disease control subjects (median, 560 pg/mL in asthmatic patients vs 295 pg/mL in disease control subjects; Fig 6, A). The demographic and clinical characteristics of the study subjects are provided in Table E1 in this article's Online Repository at www.jacionline.org. BAL fluid IL-33 levels negatively correlated ($r = -0.60$, $P = .0007$) with airway flow volume (Fig 6, B) and Asthma Control Test scores, which reflect symptoms ($r = -0.56$, $P = .04$). In agreement with previous reports,^{38,39} IL-33 was localized to the nucleus of the basal cell layer of airway epithelium (Fig 6, C). Increased IL-33 levels predicted increased ILC2 numbers in the airways. We identified human ILC2s as lin[−]FcεRI[−]CD127⁺IL-33R⁺ cells (see Fig E10 in this article's Online Repository at www.jacionline.org), as reported previously.^{21,40} The majority of these cells expressed IL-5 and IL-13. The frequency of ILC2s in BAL fluid was

significantly increased in asthmatic patients compared with that seen in disease control subjects (median, 1.2% in asthmatic patients vs 0.24% in disease control subjects; Fig 6, D). The number of IL-5⁺ ILC2s was also increased in asthmatic patients (Fig 6, E). Human asthma is heterogeneous. Expectedly, there were high and low expressors of IL-33 and ILC2s. The results suggest that IL-33 and ILC2s are involved in a subgroup of asthmatic patients.

DISCUSSION

Persistence of asthma is generally considered to be mediated by allergen-specific memory T cells.⁴¹ The depletion of allergen-specific memory T cells in our model did not eliminate airway hyperreactivity and remodeling. However, their absence reduced airway hyperreactivity and especially inflammation. On the other hand, the depletion of ILC2s led to resolution of all features of asthma. Although many previous studies demonstrated the ability of ILC2s to induce airway eosinophilic inflammation independent of T cells, this is the first demonstration of ILC2s inducing sustained airway hyperreactivity. We substantiated the role of ILC2s through gain-in-function (adoptive transfer) and loss-of-function (lethal irradiation followed by *Rag*^{−/−} γ c^{−/−} marrow transplantation and anti-IL-33 treatment) approaches.

Epithelial cells and ILC2s established 3 distinct positive feedback and feed-forward mechanisms to sustain asthma, as summarized in Fig 6, F. The outcomes of these interconnected positive feedback and feed-forward circuits were persistent IL-33 production and development of chronic asthma. The importance of the ILC2-driven feedback and feed-forward circuits was illustrated by the loss of IL-33 production in irradiated mice that received *Rag2*^{−/−} γ c^{−/−}, but not *Rag1*^{−/−}, marrow. The interdependence of IL-33 and IL-13 was recently reported in a mouse model of virus-induced chronic obstructive pulmonary disease.⁴²

An important feature of these positive feedback and feed-forward circuits is that they are interconnected. There is strong mathematic and experimental evidence that interconnected positive feedback circuits induce ultrasensitivity and bistability.^{43,44} Ultrasensitivity is manifested when a linear input generates a sigmoidal output. Ultrasensitivity induces system bistability. A system is considered bistable when it is “on” (active) in the absence of any input.^{45–47} The effect of interconnected feed-forward circuits is illustrated by the differential effect of IL-13 versus IL-4 or IL-17 on IL-33 autoinduction. Although all 3 cytokines directly induced IL-33, only IL-13 produced synergy through its feed-forward effect on IL-33R expression. Previously, we demonstrated that repetitive stimulation of epithelial cells with IL-13 led to extracellular signal-regulated kinase (ERK) 1/2 bistability through the establishment of a signaling feedback circuit.⁴⁸ ERK1/2 bistability primed epithelial cells for heightened cytokine production. ERK1/2 signaling is likely relevant because its phosphorylation is increased in airway epithelial cells from asthmatic patients.⁴⁹ Thus IL-13 could establish multiple feedback and feed-forward circuits for sustained IL-33 production.

We demonstrated the biological relevance of our findings in human asthma. In agreement with previous reports,^{50,51} we showed increased IL-33 levels in BAL fluid from asthmatic

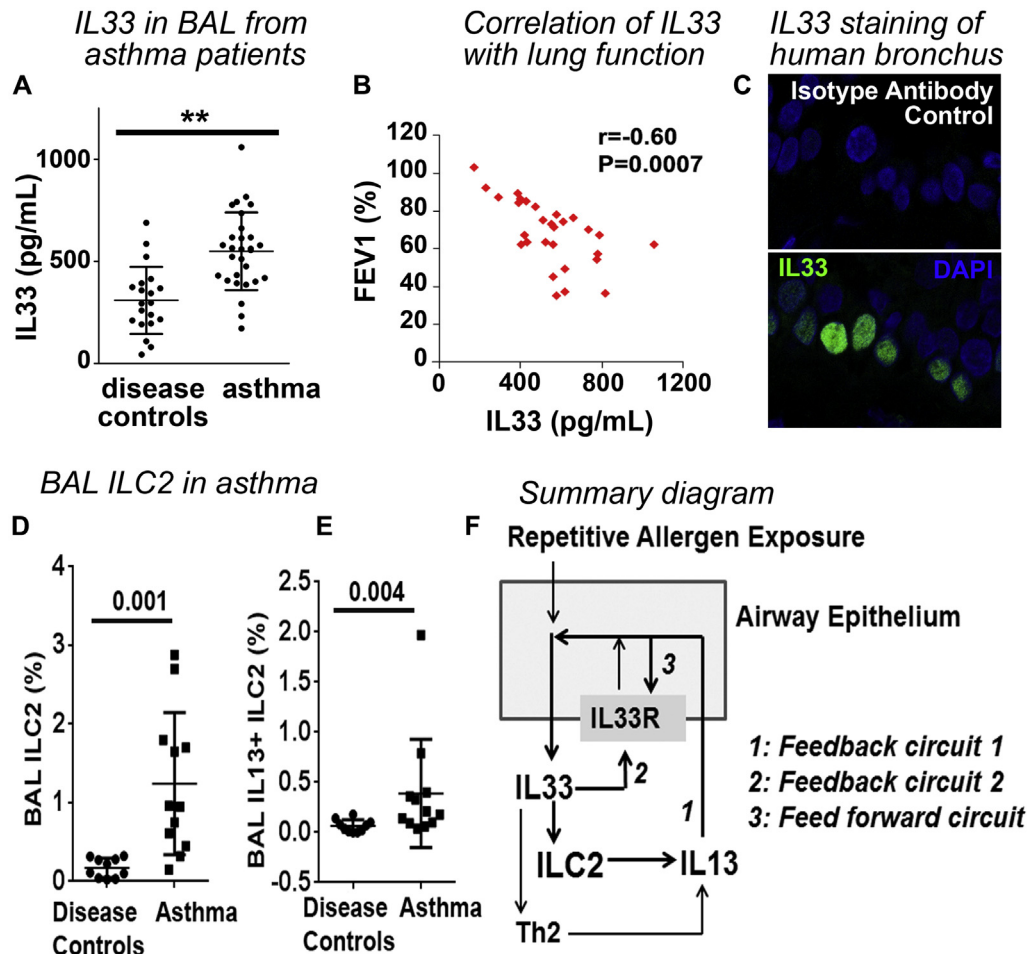


FIG 6. Role of IL-33 and ILC2s in human asthma. **A**, IL-33 levels (ELISA) in BAL fluid from asthmatic patients and disease control subjects. $**P < .005$. **B**, Correlation between BAL fluid IL-33 levels and FEV₁, a measure of airway obstruction. **C**, IL-33 immunostaining of an endobronchial biopsy sample from an asthmatic patient ($n = 9$). Green, IL-33; blue, 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) for nuclear staining. **D** and **E**, Frequency of ILC2s (lin[−]IL-7R α ⁺Fc ϵ RI[−]IL-33R⁺) and IL-13⁺ ILC2s in BAL fluid from asthmatic patients and disease control subjects. **F**, Summary diagram depicting the mechanism of sustained IL-33 production and persistence of asthma. Repetitive allergen exposure induces IL-33 production by epithelial cells. IL-33 induces ILC2s and Th2 cells and stimulates IL-13 production. IL-13 directly induces IL-33 production, which establishes feedback circuit 1. IL-33 autoinduces, which establishes feedback circuit 2. IL-13 augments IL-33R expression and enhances IL-33 autoinduction, thus generating a feed-forward circuit.

patients. The IL-33 level negatively correlated with the airway flow volume. For the first time, we demonstrated a significant increase in ILC2 numbers in BAL fluid from asthmatic patients. Previously, increased ILC2 numbers were reported in nasal polyps from patients with chronic rhinosinusitis^{21,40} and in peripheral blood from asthmatic patients.⁵² The frequency in polyps ranged from 0.1% to 3.6% of CD45⁺ cells and that in blood ranged from 0.01% to 0.03% of mononuclear cells. The frequency of ILC2s in BAL fluid from asthmatic patients was similar to that in polyps but was higher than that in peripheral blood.

The findings of this article have implications for chronic illnesses in general. Our results imply that recurrent episodes of any acute illness can establish feedback and feed-forward circuits. Once established, these feedback circuits sustain the disease process during intervals between acute episodes, which facilitates the transition of an acute illness to a chronic one.

Key messages

- A network of interaction between the epithelium and ILC2s generates a mechanism for sustained IL-33 production and persistence of asthma.
- ILC2s are essential for the persistence of asthma.
- T cells, although redundant, contribute to the magnitude of inflammation and airway hyperreactivity.

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METHODS

Animals

C57Bl/6 CD45.1, C57Bl/6 CD45.2, BALB/c, and *Rag1*^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, Me). *Rag2*^{-/-} γ c^{-/-} mice were kindly provided by Dr Roberta Pelanda (National Jewish Health, Denver, Colo). *Erk1*^{-/-} mice were a generous gift from Drs Gilles Pagés and Jacques Pouyssegur (University of Nice, Nice, France), as described previously.^{E1}

Mucosal sensitization of mice

Allergens used include extracts of dust mite (*Dermatophagoides farinae*), ragweed (*Ambrosia artemisiifolia*), and *Aspergillus fumigatus* (Greer Laboratories, Lenoir, NC). Intranasal allergens (dust mite, 5 μ g; ragweed, 15 μ g; *Aspergillus* species, 5 μ g [DRA]) or *Aspergillus* species (5 μ g) were delivered in 15- μ L quantities in saline.

Acute asthma was produced by means of immunization of 8- to 12-week-old BALB/c mice twice 1 week apart with *Aspergillus* species (5 μ g) in adjuvant (1:1 vol/vol). Adjuvant was aluminum and magnesium hydroxide (Pierce, Rockford, Ill). Asthma was initiated by using 3 consecutive intranasal exposures to *Aspergillus* species (5 μ g in 15 μ L of saline), and asthma was evaluated 72 hours after the final exposure.

Chronic asthma was produced by intranasal delivery of the DRA mixture twice a week for 6 consecutive weeks in female mice (C57Bl/6 CD45.2 or BALB/c mice, as appropriate) 8 to 12 weeks of age. For characterization of asthma chronicity experiments, mice were analyzed at the indicated time points (1, 2, or 6 months after cessation of allergen exposure). A timeline of manipulations and interventions for the chronic asthma protocol with irradiation is shown in Fig 2, A. For irradiation experiments, mice were rested for 3 weeks after completion of allergen delivery, and 2 doses of 600 rad were delivered 3 hours apart. Flow cytometric analysis indicates that 6 weeks after irradiation, less than 4% of CD45.2⁺ cells from recipient mice were still present. Bone marrow cells (5×10^6) from C57Bl/6 CD45.1, BALB/c *Rag1*^{-/-}, or BALB/c *Rag2*^{-/-} γ c^{-/-} mice, as indicated in the experimental design, were transferred intravenously, and mice were rested an additional 6 weeks before analyses.

Antibody, pharmacologic, and genetic interventions

For IL-33 blockade experiments, intraperitoneal anti-IL-33 (15 μ g/200 μ L injection in saline; R&D Systems, Minneapolis, Minn) was delivered 3, 5, and 7 days before analysis in week 15 (6 weeks after irradiation). All nonirradiated control mice with asthma were rested an equivalent amount of time before analyses. For CD3 and IL-13 blockade, hamster anti-mouse CD3 ϵ (200 μ g per dose, clone 145 2C11; eBioscience, San Diego, Calif)^{E2} and anti-IL-13 (50 μ g per dose; Calbiochem, San Diego, Calif) antibodies and hamster IgG or rat IgG were administered intraperitoneally on 3 consecutive days in week 10, and outcomes were examined 3 days later.

Adoptive transfer of CD4 T cells and ILC2s

Spleen CD4 T cells were negatively selected by using antibody-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and transferred intravenously (4×10^6 cells) to naive mice. ILC2s (lin⁻CD45⁺CD25⁺) were sorted on MoFlow XDP (Beckman Coulter, Fullerton, Calif) and delivered intravenously (2×10^5 cells) to naive mice 24 hours after sorting. Intranasal DRA (5 μ g, 15 μ g, and 5 μ g, respectively, per 15- μ L dose in saline) was performed on 3 consecutive days after intravenous transfer of CD4 T cells. CD4 T cell–recipient mice were studied on days 6 and 21. ILC recipient mice were analyzed on day 21.

Airway hyperreactivity measurement

Measurement methodologies have been explained in depth elsewhere.^{E3} Briefly, mice were anesthetized with ketamine (180 mg/kg), xylazine (9 mg/kg), and acepromazine (4 mg/kg). After loss of footpad pinch reflex, a

tracheotomy was performed, and the mouse was attached with an 18-gauge cannula to a small-animal ventilator with a computer-controlled piston (flex-iVent; SCIREQ, Montreal, Quebec, Canada). Mice were ventilated at a frequency of 90 breaths/min with a tidal volume of 20 mL/kg during nebulization and otherwise with a frequency of 150 breaths/min with a tidal volume of 20 mL/kg while breathing against an artificial positive end-expiratory pressure of 2.5 to 3 cm H₂O. Lungs were inflated to total lung capacity twice to standardize volume history. Resistance measurements were then taken to establish baselines for total lung resistance and at each methacholine dose. Group averages were expressed as fold increases in baseline resistance (means $\pm$ SEMs).

Histology and immunofluorescence staining

Paraffin-embedded lungs were sectioned and stained with hematoxylin and eosin (H&E) for morphometric analysis or mucin staining or toluidine blue for mast cell staining. Sections for immunofluorescence staining were permeabilized with 0.01% saponin in PBS, blocked with 10% goat serum, and stained with a primary antibody against human IL-33 and visualized with Alexa Fluor 488–conjugated secondary antibody, as described previously,^{E4} and counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride for nuclear staining.

Morphometric measurements

Inflammation was quantified by using Metamorph image acquisition and analysis software on H&E-stained lung sections at $\times 20$ magnification, as described previously.^{E3} Airway epithelial hypertrophy and peribronchial smooth muscle hypertrophy were measured as the area of epithelial or smooth muscle per circumference of airway basement membrane. Airway inflammation was measured as the area of inflammatory infiltrates as a percentage of the total field. A minimum of 5 airways per mouse and 5 to 9 mice per group were quantified.

Images were acquired on a Nikon Eclipse TE2000-U microscope by using $\times 20$ dry lenses at room temperature through a Diagnostics Instruments camera model #4.2 with Spot software 5.0 (Diagnostic Instruments, Inc, Sterling Heights, Mich). H&E sections were mounted with Permount medium (Thermo Fisher Scientific, Inc, Waltham, Mass). Images were adjusted for brightness and contrast to improve viewing.

Lung digestions

Lungs were perfused with saline, and single-cell suspensions of lung cells were acquired by using mechanical mincing of lungs followed by digestion at 37°C for 45 minutes in RPMI with 10% FBS, 1% penicillin/streptomycin, and collagenase A (1 mg/mL). Cell suspensions were agitated at room temperature for 10 minutes in RPMI with 100 U/mL DNase I before filtration through 40- μ m filters and red blood cell lysis. Single-cell suspensions were subsequently fixed in 4% paraformaldehyde for flow cytometric analysis.

Flow cytometric analyses of ILC2s and other cell-surface markers

Mouse ILC2s. For mouse flow cytometric staining, all conjugate antibodies were purchased from BioLegend (San Diego, Calif), unless otherwise stated. Mouse lung digest cells were fixed with 4% paraformaldehyde and incubated with 10% donkey serum and 1% 2.4G2. ILCs were stained with PerCp/Cy5.5-labeled CD45.2 (clone 104), phycoerythrin/Cy7-labeled CD45.1 (clone A20), or both, as appropriate; Pacific Blue–labeled lineage marker antibodies (CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/erythroid cells); Alexa Fluor 488–conjugated CD25 (clone PC61); allophycocyanin-labeled IL-5 (TRFK5); and phycoerythrin-labeled IL-13 (clone eBio13A, eBioscience). ILC2s were initially characterized with the addition of the primary antibody anti-IL-33R (ST-2; clone AF1004, R&D Systems), followed by allophycocyanin-labeled secondary antibody against the primary antibody and allophycocyanin-eFluor 780–conjugated IL-7R α (clone eBioRDR5, eBioscience), allophycocyanin-labeled CD117 (c-kit; clone ACK2), phycoerythrin-labeled Ly-6A/E (Sca-1; clone D7),

phycoerythrin-Cy7-labeled NK1.1 (clone PK136), and allophycocyanin-labeled FcεRI (clone MAR-1).

Lung IL-33⁺ cells. IL-33 levels and localizations were characterized with the primary antibody against IL-33 (clone Poly5165), followed by fluorescein isothiocyanate-labeled secondary and primary antibodies against pro-surfactant protein C (AB3786; Millipore, Temecula, Calif), followed by allophycocyanin-labeled secondary or phycoerythrin/Cy7-labeled CD11b (clone M1/70) or the primary antibody against E-cadherin (clone H108; Santa Cruz Biotechnology, Dallas, Tex), followed by a Pacific Blue-labeled secondary antibody or allophycocyanin-labeled FcεRI (clone MAR-1).

Human BAL fluid ILC2s. BAL fluid cells were fixed in 4% paraformaldehyde and incubated in 10% goat serum. ILC2s were stained with Pacific Blue-labeled lineage marker antibodies (CD3, CD14, CD16, CD19, CD20, and CD56), fluorescein isothiocyanate-labeled IL-7Rα (clone A019D5), PerCp/Cy5.5-labeled IL-13 (clone JES10-5A2), or phycoerythrin-labeled IL-5 (clone JES1-39D10). IL-33R was identified with the addition of a primary antibody against IL-33R (ST-2; clone HB12; MBL International, Woburn, Mass), followed by allophycocyanin-labeled secondary antibody against the primary antibody. After washing, cells were analyzed by using flow cytometry with the CyAn ADP cytometer (Beckman Coulter). Data were analyzed with FlowJo version 7.6.5 software (TreeStar, Ashland, Ore).

T helper cell proliferation and cytokine production

Splenocytes and mediastinal lymph node cells (2×10^6 /mL) were cultured for 96 hours on anti-CD3 and anti-CD28 (1 μg/mL)-coated 48-well plates or stimulated separately with the following allergens: dust mite (10 μg/mL), ragweed (50 μg/mL), and *Aspergillus* species (10 μg/mL).^{E4} In one set of cultures, cells were labeled with 1 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, Calif). For measurement of cytokines (IL-2 and IL-4), monensin was added to another culture set 6 hours before the conclusion. T cells were detected by addition of allophycocyanin-labeled anti-CD4 (clone GK1.5, BioLegend) before flow cytometric analysis on the CyAn ADP cytometer (Beckman Coulter) for proliferation and intracellular cytokine expression.

Western blotting

Immediately after flexiVent analysis, lung sections were collected in RIPA buffer (50 mmol/L Tris [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 1 mmol/L Na₃VO₄, and 0.1 mmol/L phenylmethylsulfonyl fluoride) containing protease and phosphatase inhibitors and homogenized. Lysed samples were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and then immunoblotted with primary antibodies (IL-33 [R&D Systems], tubulin [H-235], and β-actin [Santa Cruz Biotechnology, Dallas, Tex]). After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody. After additional washing, the membranes were developed with ECL reagent, as previously described.^{E4}

A549 cell culture

A549 cells were cultured in RPMI in 10% FBS (HyClone; Thermo Scientific, Waltham, Mass) and 1% penicillin/streptomycin (Gibco, Carlsbad, Calif) and maintained at 37°C in a humidified 5% CO₂ incubator. All cytokines were purchased from PeproTech (Rocky Hill, NJ).

In one experiment cells were treated with medium, IL-1β (2 ng/mL), IL-4 (10 ng/mL), IL-13 (20 ng/mL), IL-17 (10 ng/mL), IL-33 (20 ng/mL), TNF (2 ng/mL), IFN-γ (10 ng/mL), and TNF (2 ng/mL) plus IFN-γ (10 ng/mL) for 72 hours. Cellular RNA was analyzed for mRNA for IL-33 and IL-33R. In a second experiment cells were pretreated for 24 hours with medium, IL-1β (2 ng/mL), IL-4 (10 ng/mL), IL-13 (20 ng/mL), IL-17 (10 ng/mL), TNF (2 ng/mL), or IFN-γ (10 ng/mL) before stimulation for 72 hours with IL-33 (20 ng/mL). Cellular RNA was analyzed for mRNA for IL-33. In a third experiment cells were treated for 24 hours with DRA (5 μg/mL dust mite, 15 μg/mL ragweed, and 5 μg/mL *Aspergillus* species). Cellular RNA was analyzed for mRNA for IL-33. In a fourth experiment cells were treated for 24 hours with IL-13 (20 ng/mL) and ATP, and the supernatant was collected and

analyzed for IL-33 levels by means of ELISA, according to the manufacturer's instructions (R&D systems).

Real-time PCR

Total RNA was isolated from frozen tissues by using Trizol (Invitrogen) or from cells by using a kit (Purelink Mini RNA kit, Life Technologies), and cDNA was synthesized with an ImProm-II cDNA synthesis kit (Promega, Madison, Wis), according to the manufacturer's instructions, as described previously.^{E4} Gene-specific PCR products were amplified with SYBR Green (Thermo Scientific) and primers outlined in Table E2 by using an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif). The levels of target gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression by using the $2^{-\Delta\Delta C_t}$ method.

Microarray and analysis

We used the OneArray microarray service from Phalanx Biotech (Palo Alto, Calif) in which 29,922 mouse genome probes and 1,880 experimental control probes were used for the array. Each sample was studied in triplicates. The following analyses of the microarray results were performed and provided with the service: (1) Rosetta profile error model calculation; (2) normalized intensities (excluding flagged and control data) with median scaling; (3) basic statistic plot and Pearson correlation coefficient; (4) pairwise ratio calculation; (5) principal component analysis; and (6) gene ontology analysis.

Patient samples

Subjects were recruited from our outpatient clinic at National Jewish Health (Denver, Colo). Human subject protocols were approved by the institutional review board. We studied BAL fluid cells from asthmatic patients and disease control subjects. Asthma was diagnosed based on the presence of reversible airway obstruction, positive methacholine test results (PC₂₀, <8 mg/mL), or both, per the Expert Panel Report 3 criteria. The clinical characteristics of the study subjects are shown in Table E1. Bronchoscopy, BAL, and endobronchial biopsy were performed, as described previously.^{E5} BAL fluid was processed immediately for flow cytometry. IL-33 was analyzed in BAL fluid by means of ELISA, according to the manufacturer's directions (R&D Systems).

Statistical analyses

Data are presented as means ± SEMs. For comparison of airway hyperreactivity, 2-way ANOVA for repeated measures with a Bonferroni *post hoc* test was used. For pairwise comparisons, a Student *t* test was used. Data from human subjects were analyzed by using nonparametric tests (Mann-Whitney *U* test and Kruskal-Wallis test). A *P* value less than .05 was considered significant.

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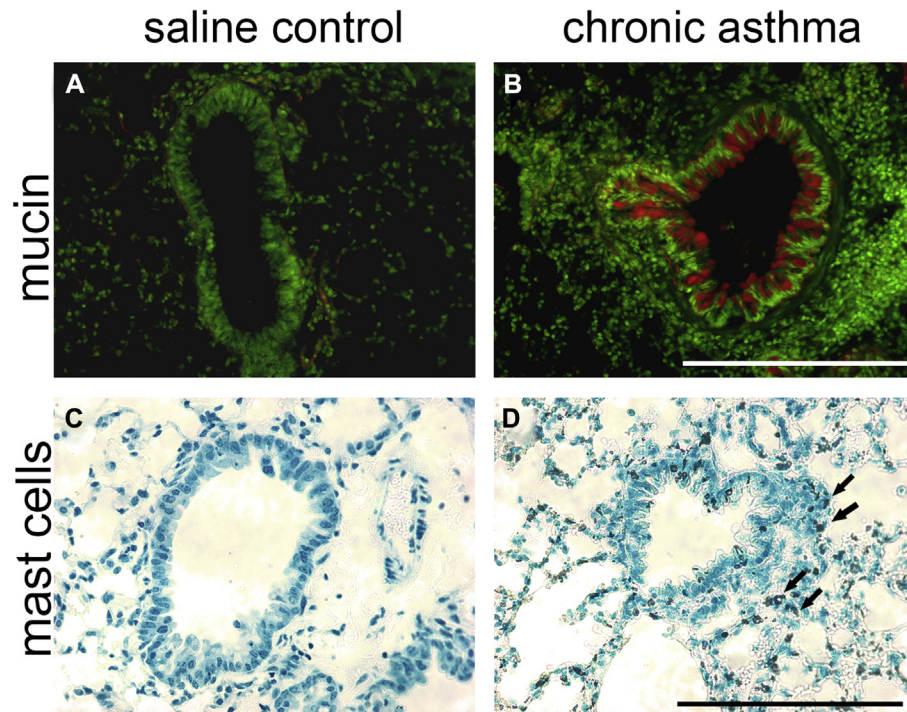


FIG E1. Expression of mucin (*red*) expression and toluidine-positive (*dark brown*) mast cells in airways of mice from the chronic asthma and saline control groups.

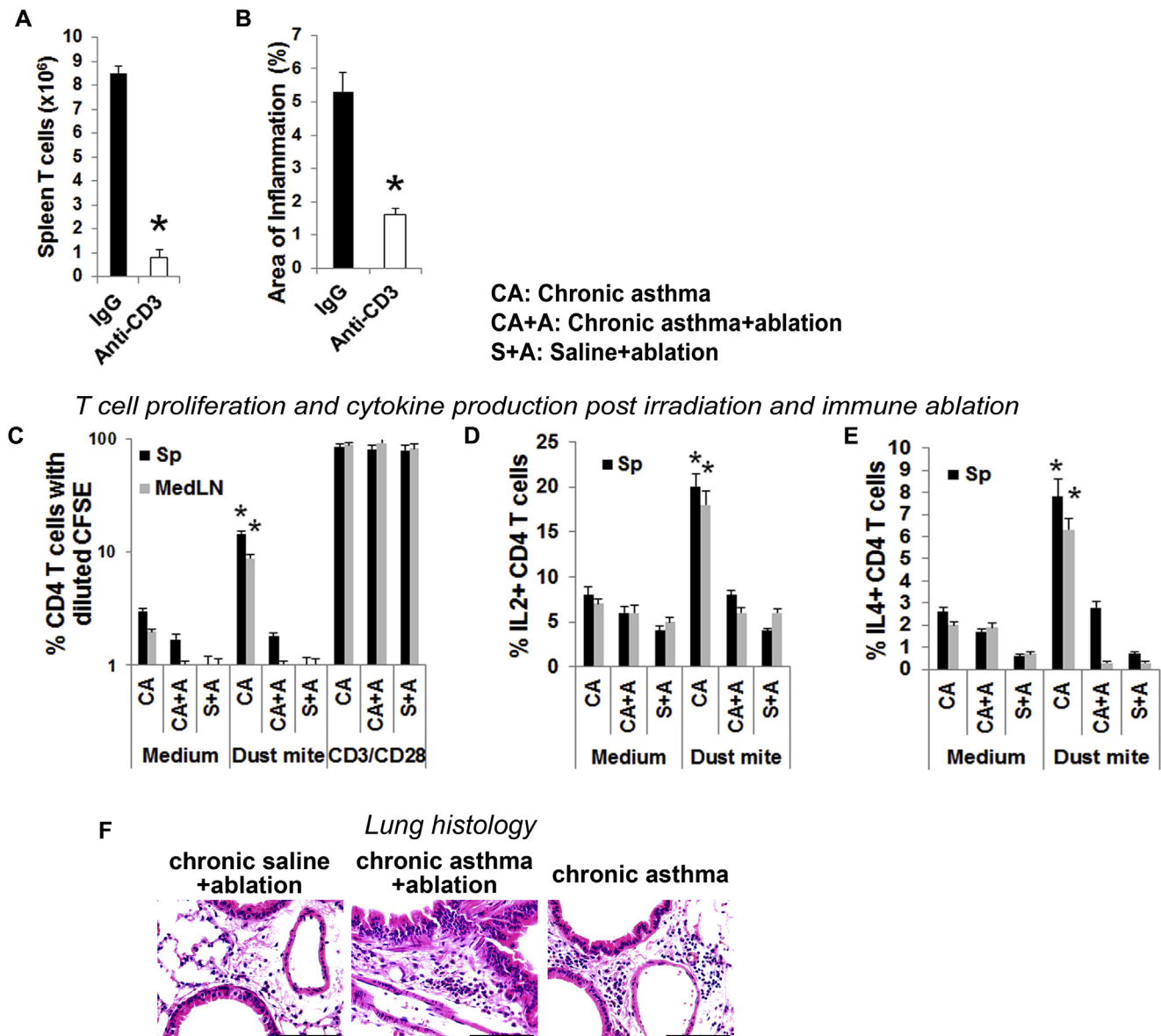


FIG E2. **A** and **B**, Effect of an anti-CD3 ϵ or an isotype control antibody on spleen TCR- β^+ T cells (Fig E2, **A**) and airway inflammation (Fig E2, **B**). Three doses (200 μ g per dose) of the antibodies were administered on alternate days at week 10 ($n = 5$). * $P = .02$. **C-E**, T-cell proliferation and cytokine production 6 weeks after irradiation at week 9. Spleen (*sp*) and mediastinal lymph node (*medLN*) CD4 $^+$ T cells were obtained from 3 study groups: chronic asthma (CA), chronic asthma plus ablation (CA+A), and saline control plus ablation (S+A). T cells (2×10^6 cells per well) were stimulated with medium, dust mite (10 μ g/mL), and anti-CD3 (1 μ g/mL) plus anti-CD28 (1 μ g/mL) antibodies for 96 hours. Proliferation was assessed by means of flow cytometric analysis of carboxyfluorescein succinimidyl ester (CFSE) dilution. For flow cytometric detection of intracellular IL-2 (Fig E2, **D**) and IL-4 (Fig E2, **E**), cells were treated with monensin 6 hours before conclusion of the culture. Twenty thousand events (live cells) per well were sampled for flow cytometric analyses ($n = 4$). * $P < .05$ compared with medium control. **F**, Effect of irradiation and immune ablation on lung inflammation. Representative histologic images of the lung sections from the study mice are as shown in Fig 2, **D**. Scale bar = 100 μ m.

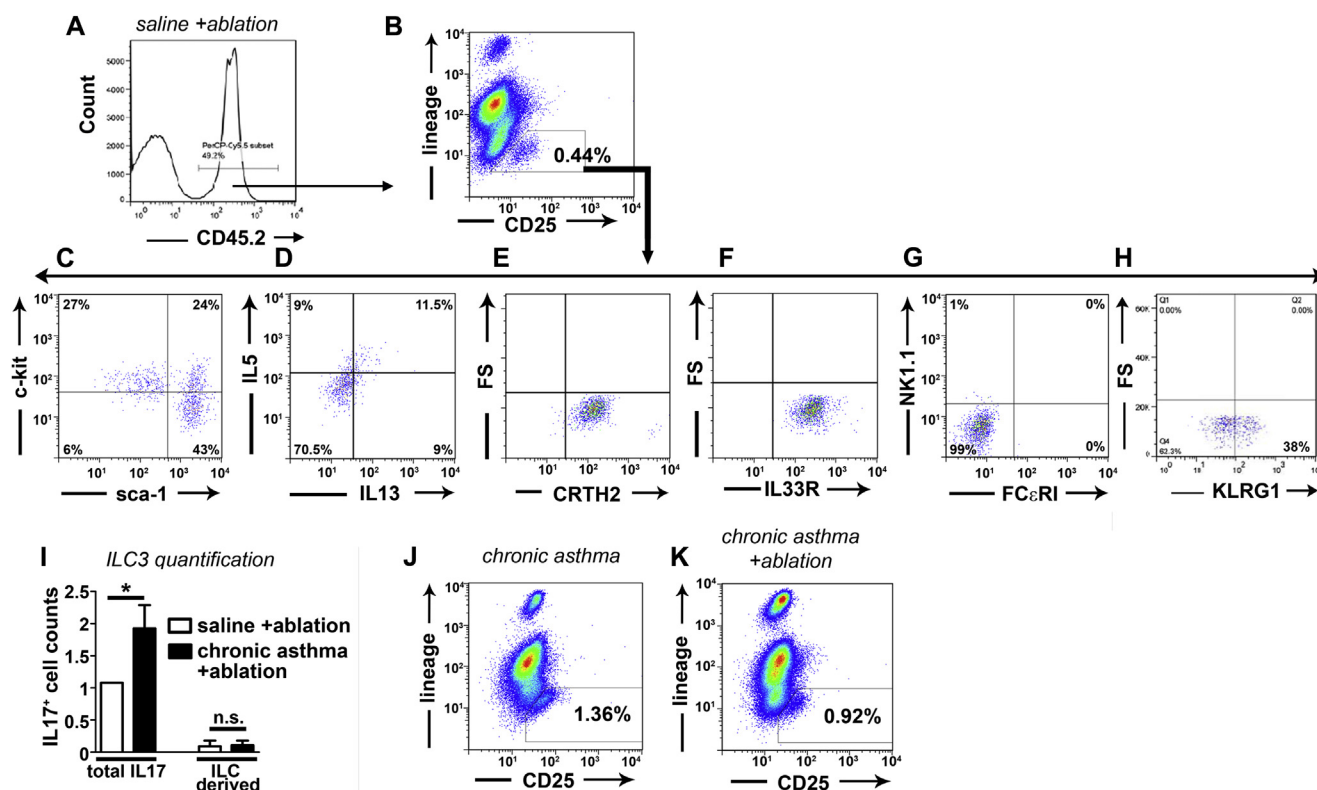


FIG E3. Characterization of lung ILCs. **A** and **B**, Collagenase-digested single lung cells were gated on live cells through forward scatter/side scatter and then selected for the hematopoietic CD45⁺ population. The ILCs were identified as a lineage (CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/erythroid cells)-negative and CD25⁺ population. **C-H**, This subpopulation was positive for ILC2 markers, including c-kit, Sca-1, intra-cellular IL-13 and IL-5, and cell-surface CRTH2, IL-33R, and killer cell lectin-like receptor G superfamily, member 1 (KLRG1) but negative for the natural killer cell marker NK1.1 or mast cell/basophil marker FcεRI. **I**, Total IL-17⁺ lung cells in the chronic asthma and saline control groups, as detected by using flow cytometry. Lung ILCs (lin⁻CD25⁺) were negative for IL-17 (n = 3). **J** and **K**, Comparison of ILCs between the chronic asthma and chronic asthma after immune ablation groups.

Quantification of ILC

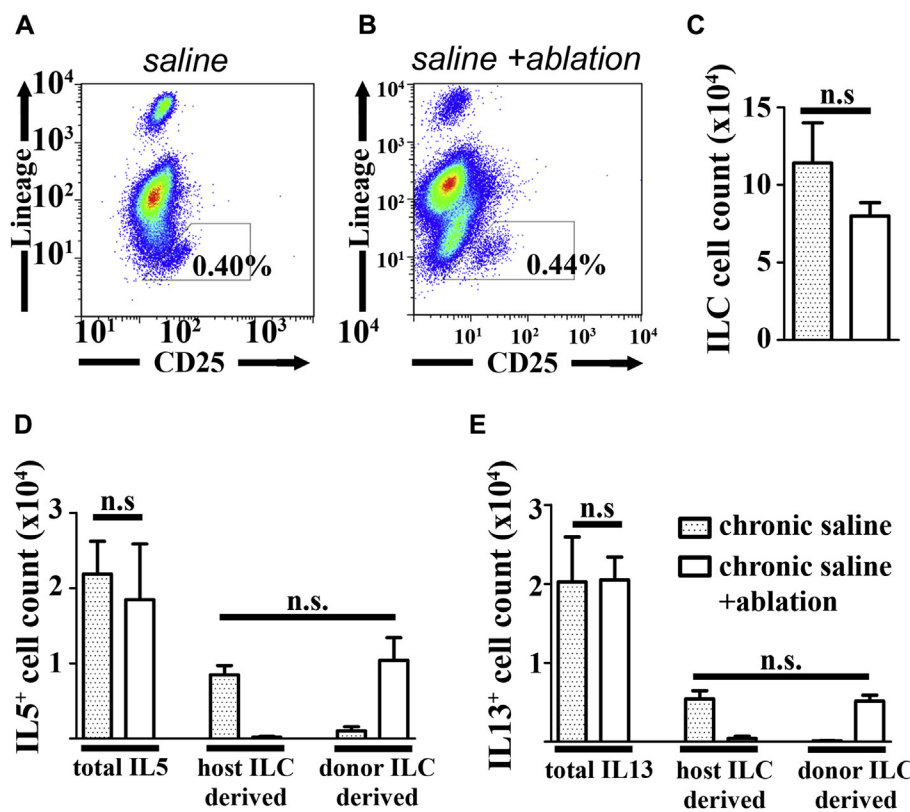


FIG E4. Effect of irradiation on ILCs. **A** and **B**, Frequency of ILCs ($\text{lin}^- \text{CD25}^+ \text{CD45}^+$ cells) in the lung digest from mice treated with saline alone (control for chronic asthma) and saline followed by irradiation and transplantation of bone marrow from a naive mouse. Measurements were done 6 weeks after bone marrow transplantation. **C**, Comparison of total lung ILC numbers between the 2 groups. **D**, Comparison of total lung IL-5⁺ cell numbers and host- and donor-derived IL-5⁺ ILC2 numbers. **E**, Comparison of total lung IL-13⁺ cell numbers and host- and donor-derived IL13⁺ ILC2 numbers. There were 5 mice per group for Fig E4, C through E. n.s., Not significant.

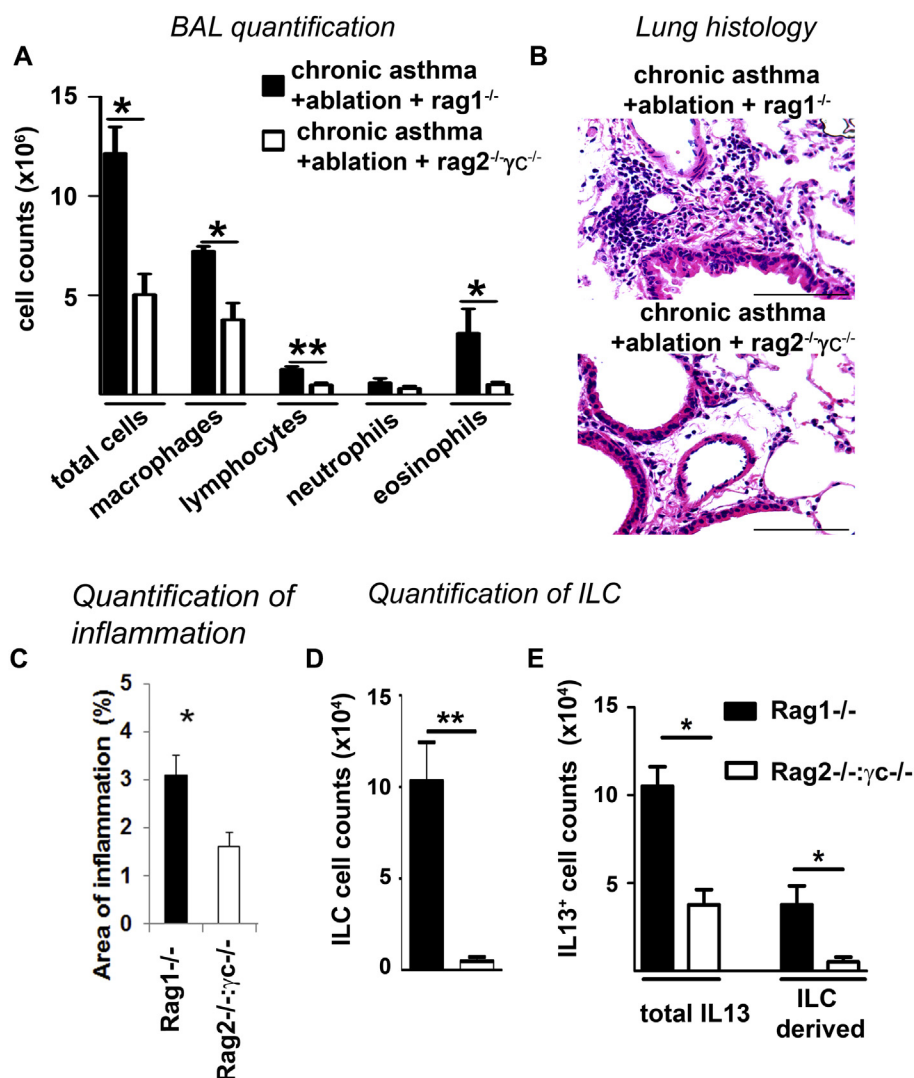


FIG E5. Effect of marrow transplantation on the persistence of inflammation. **A** and **B**, Chronic asthma was induced as per Fig 2, A, and bone marrow from either Rag1^{-/-} or Rag2^{-/-}γc^{-/-} mice was transferred to mice after irradiation. Outcomes were measured 6 weeks after irradiation at week 15. Fig E5, A, Differential cell counts in BAL fluid. Fig E5, B, Representative lung sections stained with H&E from the mice represented in Fig E5, A. Scale bar = 10μm. **C**, Peribronchial and perivascular inflammation as a percentage of total area. **D** and **E**, Flow cytometric analysis of lung digests from Rag1^{-/-} and Rag2^{-/-}γc^{-/-} bone marrow recipient mice showing changes in total ILC (Fig E5, D) and IL-13⁺ ILC (Fig E5, E) numbers. **P* < .05 and ***P* < .01. There were 5 mice per group.

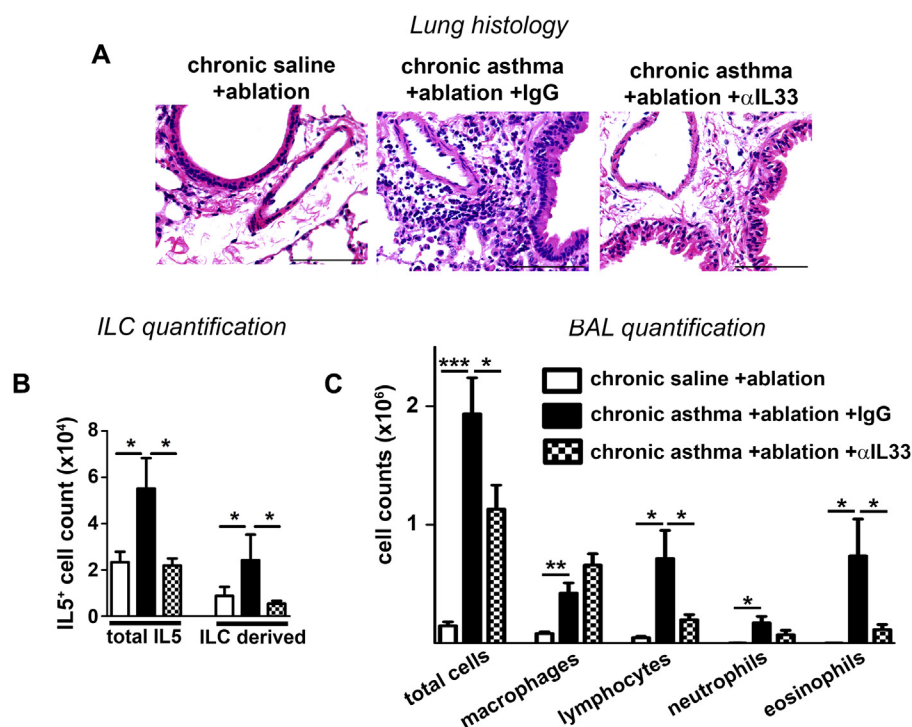


FIG E6. **A** and **B**, Effect of anti-IL-33 on the persistence of airway inflammation ($n = 4$). Fig E6, **A**, Representative lung sections stained with H&E. Scale bar = 100 $\mu\text{mol/L}$. Fig E6, **B**, Differential cell counts in BAL fluid. **C**, Effect of intranasal anti-IL-33 antibody on airway inflammation. Representative lung sections were stained with H&E. Scale bar = 100 $\mu\text{mol/L}$. * $P < .05$, ** $P < .01$, and *** $P < .001$.

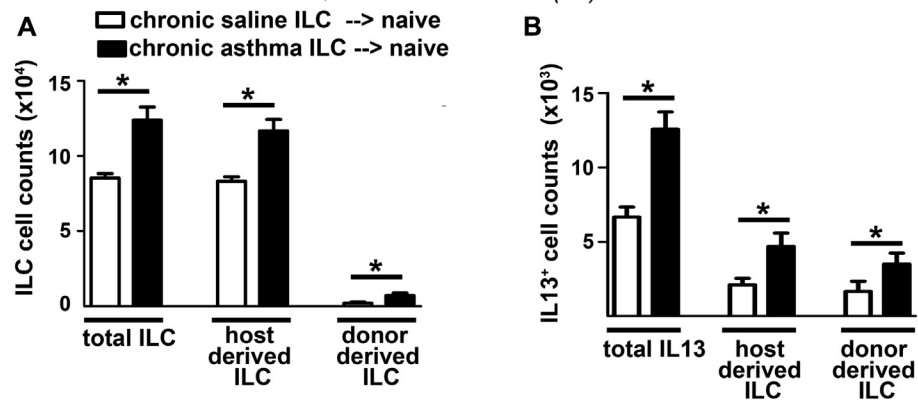
*Effect of adoptive transfer of ILC**Quantification of ILC (h-i)*

FIG E7. Effect of adoptive transfer of ILCs. CD45⁺lin[−]CD25⁺ cells from the lung digest obtained from the chronic asthma and saline control models were adoptively transferred to naive mice. The presence of host- and donor-derived total ILCs (**A**) and IL-13⁺ ILC2s (**B**) in the lungs was analyzed on day 21 after transfer (n = 5 per group). **P* < .05.

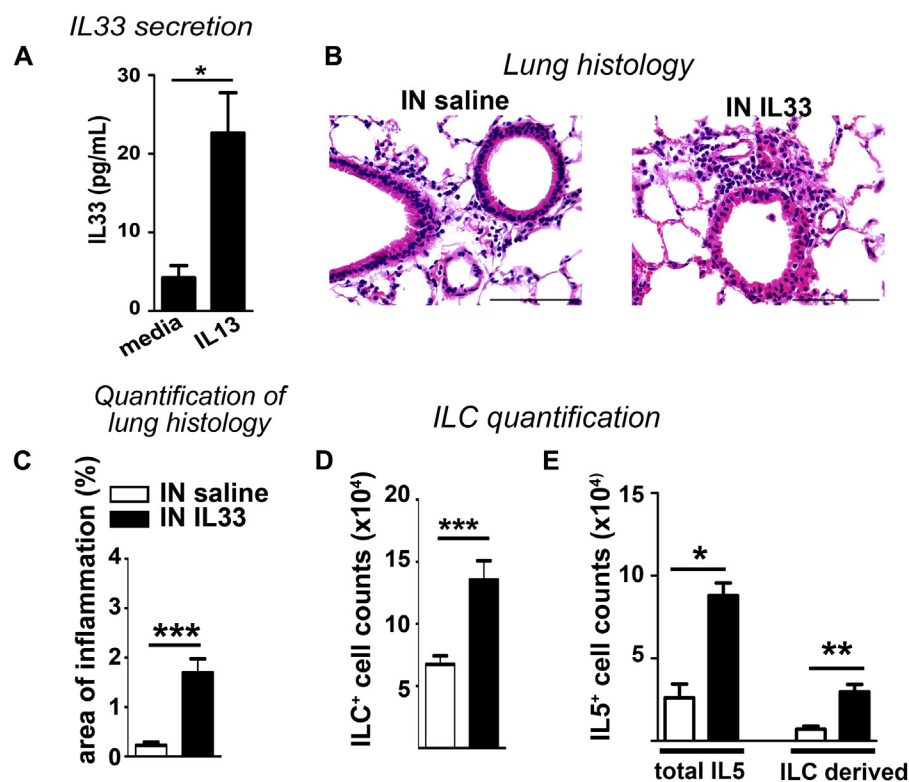


FIG E8. **A**, Effect of IL-13 on IL-33 secretion. A549 cells were treated overnight with medium or IL-13 (20 ng/mL) and ATP (100 μ mol/L), and the released IL-33 was measured by using ELISA ($n = 6$). $*P = .04$. **B-E**, Direct effect of IL-33 on airway hyperreactivity and lung ILCs. C57Bl/6 mice were treated intranasally for 3 consecutive days with 400 ng per dose of IL-33. Outcome measures were evaluated 15 days after final exposure. Fig E8, **B** and **C**, Airway inflammation and its quantification as a percentage of total area. **D** and **E**, Effect on lung ILCs: flow cytometric analysis of lung digests showing total ILCs (Fig E8, **D**) and total IL-5⁺ lung cells and IL-5⁺ ILC2s (Fig E8, **E**). $*P < .05$, $**P < .01$, and $***P < .001$. There were 5 mice per group.

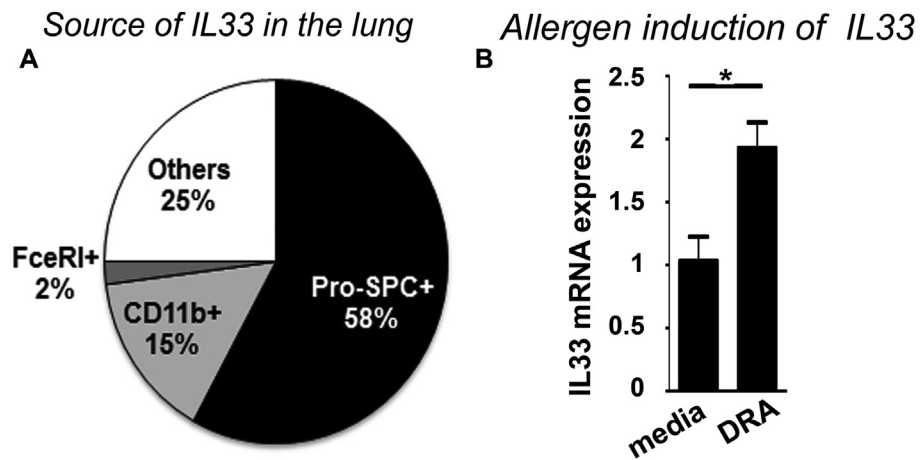


FIG E9. **A**, Lung digest from the chronic asthma model was stained for IL-33 and pro-surfactant protein C (*Pro-SPC*), CD11b, or FcεRI ($n = 5$). **B**, A549 cells were treated overnight with DRA, and IL-33 mRNA was quantified by using real-time PCR. $*P = .04$. There were 5 mice per group.

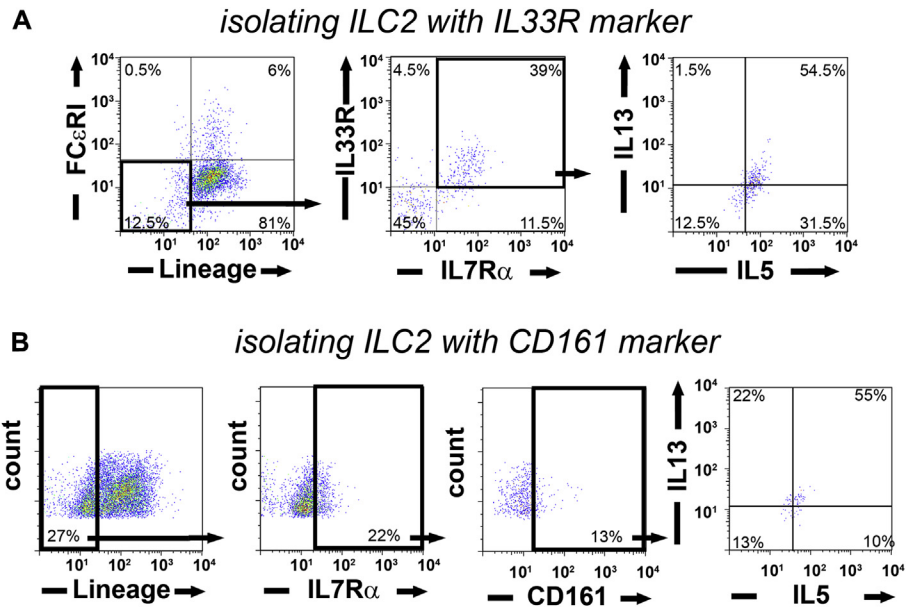


FIG E10. Isolation strategy of ILC2s from human BAL fluid. **A**, BAL cells were gated for lin (CD3, CD14, CD16, CD19, CD20, CD56)–negative and FcεRI⁺ cells. These lin[–]FcεRI⁺ cells were stained for IL-7Rα and IL-33R (suppressor of tumorigenicity). The IL-7Rα⁺IL-33R⁺ cells were identified as ILC2s. The majority of these cells were positive for IL-5 and IL-13. They were partially positive for CD161 (**B**). The gating strategy and threshold quadrants were based on isotype antibody controls (data not shown).

TABLE E1. Clinical characteristics of human subjects

Parameters	Asthmatic patients (n = 38)	Disease control subjects (n = 18)
Diagnosis and comorbidities	Thirty-eight patients with asthma; comorbidities: 30 patients with allergic rhinitis, 25 with chronic sinusitis, 29 with GERD, 3 with bronchiectasis, and 4 with aspiration	Seven patients with chronic cough and concurrent allergic rhinitis and GERD, 3 patients with bronchiectasis, 3 patients with MAC infection, 4 with chronic aspiration, and 1 with COPD
Age (y), mean $\pm$ SEM (median)	54 $\pm$ 4 (56)	56 $\pm$ 7 (58)
Sex (F/M)	22/16	11/7
BMI	28 $\pm$ 3 (27)	27 $\pm$ 3 (26)
FEV ₁ (%)	69 $\pm$ 4 (72)	82 $\pm$ 8 (76)
Reversibility	16 $\pm$ 3 (12)	5 $\pm$ 3 (6)
PC ₂₀ for methacholine	2.9 $\pm$ 1 (1.58)	Not done
Blood eosinophils	370 $\pm$ 80 (200)	116 $\pm$ 25 (100)
Medications	ICS + LABA (32)	ICS + LABA (12)
	Systemic glucocorticoids (3)	ICS (3)
	ICS (2)	Montelukast (0)
	Omalizumab (6)	Inhaled anticholinergics (3)
	Montelukast (8)	SABA (18)
	SABA (38)	

BMI, Body mass index; *COPD*, chronic obstructive pulmonary disease; *F*, female; *GERD*, gastroesophageal reflux disease; *ICS*, inhaled corticosteroid; *LABA*, long-acting bronchodilator; *M*, male; *MAC*, *Mycobacterium avium* complex; *SABA*, short-acting bronchodilator.

TABLE E2. Nucleotide sequence of primers used for real-time PCR

Primer name	Sequence 5' → 3'
mIL-4 F	TTGAGAGAGATCATCGGCATT
mIL-4 R	CTCACTCTCTGTGGTGTTC
mIL-7 F	CACACTCACGTCCAGATTAG
mIL-7 R	TCCTAGCCTGCCTTAGATC
mIL-25 F	CCAGCAAAGAGCAAGAAC
mIL-25 R	TTCAAGTCCCTGTCCAAC
mIL-33 F	TCCCAACAGAAGACCAAAG
mIL-33 R	GATACTGCCAAGCAAGGAT
mTSLP F	CTTCTCAGGAGCCTCTTCA
mTSLP R	AGCCAGGGATAGGATTGA
mST-2 F	GTGACACCTTACAAAACCCG
mST-2 R	TCAAGAACGTCCGGCAGAG
mGAPDH F	ACGGCCGCATCTTCTTGTGCA
mGAPDH R	AATGGCAGCCCTGGTGACCA
hIL-33 F	GTGACGGTGTTGATGGTAAGA
hIL-33 R	CTCCACAGAGTGTTCCTTGTT
hST-2 F	AACGAGTTACCAATACTTGCTC
hST R	CAGGCACTATTGCTTCTGGG
h18S F	CTGAGAAACGGCTACCACATC
h18S R	GCCTCGAAAGAGTCCTGTATTG

F, Forward; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *h*, human; *m*, mouse; *R*, reverse; *TSLP*, thymic stromal lymphopoietin.