

Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity

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Background: Asthma has been considered an immunologic disease mediated by T_H2 cells and adaptive immunity. However, clinical and experimental observations suggest that additional pathways might regulate asthma, particularly in its nonallergic forms, such as asthma associated with air pollution, stress, obesity, and infection.

Objectives: Our goal was to understand T_H2 cell-independent conditions that might lead to airway hyperreactivity (AHR), a cardinal feature of asthma.

Methods: We examined a murine model of experimental asthma in which AHR was induced with glycolipid antigens, which activate natural killer T (NKT) cells.

Results: In this model AHR developed rapidly when mice were treated with NKT cell-activating glycolipid antigens, even in the absence of conventional CD4⁺ T cells. The activated NKT cells directly induced alveolar macrophages to produce IL-33, which in turn activated NKT cells, as well as natural helper cells, a newly described non-T, non-B, innate lymphoid cell type, to increase production of IL-13. Surprisingly, this glycolipid-induced AHR pathway required not only IL-13 but also IL-33 and its receptor, ST2, because it was blocked by an anti-ST2 mAb and was greatly reduced in ST2^{-/-} mice. When adoptively transferred into IL-13^{-/-} mice, both wild-type natural helper cells and NKT cells were sufficient for the development of glycolipid-induced AHR.

Conclusion: Because plant pollens, house dust, and some bacteria contain glycolipids that can directly activate NKT cells, these studies suggest that AHR and asthma can fully develop or be greatly enhanced through innate immune mechanisms involving IL-33, natural helper cells, and NKT cells. (*J Allergy Clin Immunol* 2011;■■■:■■■-■■■.)

Key words: Innate, natural killer T, natural helper cells, T_H2, IL-33, IL-13, glycolipid, asthma

Asthma, which has increased dramatically in prevalence over the past 2 to 3 decades, is a major public health problem that affects 300 million persons worldwide.¹ Although allergic inflammation guided by CD4⁺ T_H2 cells and eosinophils is thought to play a dominant role in the pathogenesis of asthma, several clinical and experimental observations suggest that additional pathologic mechanisms might underlie the development of at least some forms of asthma.² For example, nonallergic asthma triggered by environmental factors, such as air pollutants (eg, smoke, diesel particles, and ozone), stress, obesity, and infection, appears to develop independently of T_H2 cells.³⁻⁷ In addition, non-T_H2 factors, such as IFN- γ , IL-17, and neutrophils, are frequently found in the lungs of patients with asthma, particularly in the lungs of patients with severe asthma or corticosteroid-resistant asthma. Moreover, T_H2-targeted therapies, including anti-IL-4 mAb, anti-IL-5 mAb, and IL-13 antagonists, have not been uniformly effective in many clinical trials of asthma.⁸ These findings suggest that asthma is heterogeneous and that other cell types and pathways that include innate immune cells and their associated cytokines might also regulate the development of asthma.⁹

To further understand the conditions under which airway hyperreactivity (AHR), a cardinal feature of asthma, might occur in the absence of T_H2 cells and adaptive immunity, we examined a model of experimental asthma in which natural killer T (NKT) cells activated with glycolipid antigens induced AHR.¹⁰ In systems in which AHR is induced with allergen, the presence of both IL-13-producing T_H2 cells and NKT cells is required.¹¹ In contrast, when induced directly by glycolipid-activated NKT cells, AHR occurred rapidly in the absence of T_H2 cells and adaptive immunity, such as in MHC class II^{-/-} mice treated with NKT cell-activating glycolipid antigens, including glycolipid antigens isolated from bacteria. We now show, surprisingly, that glycolipid antigen-induced AHR required IL-33 and its receptor, ST2, because it was blocked by an anti-ST2 mAb and was greatly reduced in ST2^{-/-} mice. Importantly, in the lungs a newly described non-T, non-B, innate lymphoid cell type called natural helper cells or nuocytes responded to IL-33 produced by alveolar macrophages and dendritic cells (DCs) that directly interacted with NKT cells. Both natural helper cells and NKT cells responded to IL-33 by producing significant quantities of IL-13, which induced AHR.^{12,13} When adoptively transferred to IL-13^{-/-} mice, both wild-type NKT cells and natural helper cells were sufficient for the development of glycolipid-induced AHR. Because plant pollens,¹⁴ house dust,¹⁵

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Disclosure of potential conflict of interest: P. B. Savage has received research support from the National Institutes of Health. J. B. Rottman is employed by and has received research support from Amgen, Inc. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication June 24, 2011; revised October 12, 2011; accepted for publication October 14, 2011.

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0091-6749/\$36.00

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doi:10.1016/j.jaci.2011.10.036

Abbreviations used

AHR:	Airway hyperreactivity
α -GalCer:	α -Galactosylceramide
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
Lin:	Lineage
NKT:	Natural killer T
PE:	Phycoerythrin
Rag:	Recombination-activating gene

and some bacteria, including bacteria found in the lungs of patients with poorly controlled asthma,¹⁶⁻¹⁸ contain glycolipids that can directly activate NKT cells, we suggest that AHR and airway inflammation can develop in the complete absence of T_H2 cells or be greatly enhanced through innate immune mechanisms involving IL-33, natural helper cells, and NKT cells.

METHODS**Mice**

Wild-type BALB/c ByJ recombination-activating gene (Rag) 2^{-/-} mice were generated by Dr Shizuo Akira, and B6.129-H2 dAb1-Ea/J (MHC II^{-/-}) mice on the C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, Me). CD1d^{-/-} and J α 18^{-/-} mice were gifts from Michael Grusby (Harvard School of Public Health) and Masaru Taniguchi/Toshinori Nakayama (Chiba University), respectively. ST2^{-/-} and IL-4^{-/-}/IL-13^{-/-} mice were generated by Andrew McKenzie (Cambridge, United Kingdom). Female mice were studied at approximately 6 to 8 weeks of age and were age matched. The Animal Care and Use Committee, Children's Hospital Boston, approved all animal protocols.

Antibodies and reagents

α -Galactosylceramide (α -GalCer), *Sphingomonas* species glycolipid (PS-30), and its corresponding vehicle control were synthesized by Paul B. Savage (Brigham Young University, Provo, Utah). Recombinant human IL-33, anti-mouse ST2 blocking antibody, and the isotype control rat IgG1_k antibody were generated at Amgen (Thousand Oaks, Calif).

Measurement of AHR

Mice were anesthetized with pentobarbital (7.5-10 mg per mouse), and AHR was assessed by means of invasive measurement of airway resistance, in which anesthetized and tracheostomized mice were mechanically ventilated by using a modified version of a described method (Buxco Electronics, Wilmington, NC).¹¹

ELISA

Total lung homogenate or cell-culture supernatants were collected and measured with an IL-33 ELISA kit (eBioscience, San Diego, Calif).

Statistical tests

Data are presented as means $\pm$ SEMs and were analyzed by means of ANOVA or unpaired Student *t* tests (2-tailed; Prism 4; GraphPad Software Inc, San Diego, Calif).

RESULTS **α -GalCer-induced AHR is IL-33 dependent**

Administration of α -GalCer to wild-type BALB/c mice rapidly (within 24 hours) induced a robust AHR response associated with

significant airway inflammation. The rapid development (within 24 hours) of α -GalCer-induced AHR suggested that innate rather than adaptive mechanisms were involved. We therefore assessed the role of an IL-33-ST2 (IL-33 receptor) axis and showed that AHR and airway inflammation induced by α -GalCer were blocked by treatment with an ST2 blocking mAb (Fig 1, A-C). These results contrast with those for allergen-induced AHR, which occurs independently of ST2 and IL-33.^{19,20} The requirement for IL-33 and ST2 in α -GalCer-induced AHR was confirmed by challenging ST2^{-/-} mice with α -GalCer, which resulted in a reduced AHR and airway inflammatory response compared with that observed in wild-type mice (Fig 1, D and E). Histologic evaluation of the lungs confirmed the significant reduction in airway inflammation in the ST2^{-/-} mice treated with α -GalCer compared with that seen in the wild-type BALB/c mice (Fig 1, F), indicating that the induction of AHR by α -GalCer occurred in an ST2-dependent and presumably IL-33-dependent manner.

 α -GalCer increases production of IL-33 in the lung

The development of α -GalCer-induced AHR was associated with a rapid increase in IL-33 levels in extracts of the lung in terms of both mRNA (Fig 2, A) and protein (Fig 2, B). Assessment of IL-33 by means of immunohistochemistry indicated that IL-33 was produced by alveolar macrophages and type II pneumocytes (identified as surfactant protein C positive) after α -GalCer stimulation (Fig 2, C and D). Furthermore, IL-33 immunoreactivity was weakly detected in airway epithelial cells and might have increased after α -GalCer stimulation (Fig 2, C). To confirm the apparent IL-33 expression in these populations, we assessed IL-33 by means of intracellular cytokine staining of dispersed lung cells. IL-33 was found in alveolar macrophages (F4/80⁺CD11c⁺), interstitial macrophages (F4/80⁺CD11c⁻),²¹ and DCs (F4/80⁻CD11c⁺), and numbers of these cells greatly increased after challenge with α -GalCer (Fig 2, E and F). Taken together, these results indicated that IL-33 production in the lungs by alveolar macrophages, DCs, and type II pneumocytes significantly increased after α -GalCer challenge. Note that IL-33 expression by type II pneumocytes was nuclear in location, as has been previously described, possibly functioning as a transcriptional repressor.^{22,23}

NKT cells, but not T_H2 cells, induce IL-33 in macrophages and DCs

To determine the mechanism by which α -GalCer induced IL-33 production in the antigen-presenting cells, we cocultured NKT cells with macrophages, DCs, an airway epithelial cell line (MLE12), and a type II pneumocyte line (A549). Coculture of NKT cells with alveolar macrophages or CD11c⁺ lung DCs greatly increased IL-33 mRNA levels compared with those seen in macrophages or DCs cultured alone, and this was further increased by the addition of α -GalCer (Fig 3, A and B). Culture of NKT cells with the airway epithelial cell line MLE did not increase IL-33 mRNA expression (Fig 3, C), but culture of human NKT cells with a type II pneumocyte epithelial cell line, A549, which expresses CD1d (see Fig E1 in this article's Online Repository at www.jacionline.org), greatly increased IL-33 mRNA expression (Fig 3, D). The induction of IL-33 mRNA by NKT cells in alveolar macrophages and DCs was specific and CD1d dependent because it was blocked by anti-CD1d mAb (HB323; Fig 3, A, B, and D) but not by anti-CD40 ligand or

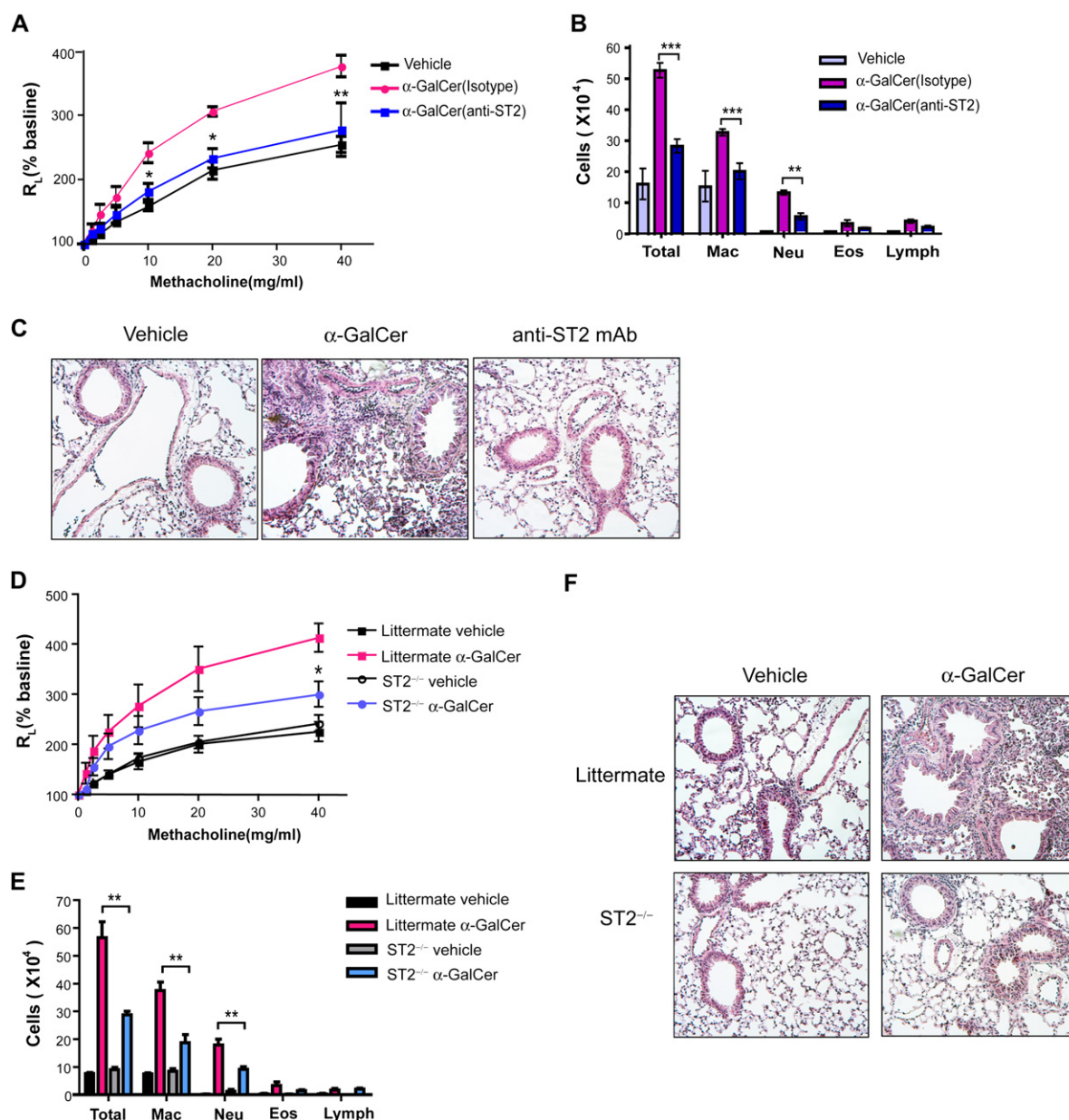


FIG 1. Blockade of the IL-33 receptor ST2 abrogates α -GalCer-induced AHR. **A**, Anti-mouse ST2 blocking antibody or rat IgG1_k isotype control antibody was administered intravenously to the mice 24 hours before intranasal administration of 0.5 μ g of α -GalCer or vehicle. These data show the mean $\pm$ SEM percentages of saline values and are representative of 3 experiments. The α -GalCer plus anti-ST2 mAb-treated group was compared with the α -GalCer plus isotype control mAb-treated group. * P < .05 and ** P < .01. R_L , Airway resistance. **B**, Data represent the number of cells per milliliter in bronchoalveolar lavage fluid. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. ** P < .01 and *** P < .001. **C**, Twenty-four hours after intranasal α -GalCer challenge, lung tissues from each group were sectioned and stained with hematoxylin and eosin ($\times 40$ magnification). **D**, Littermate control and ST2^{-/-} mice were challenged with α -GalCer or vehicle, and AHR was determined by means of invasive measurement of airway resistance (R_L), as in Fig 1, A. * P < .05. **E**, Numbers of cells in bronchoalveolar lavage fluid were counted as in Fig 1, B. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils. * P < .05 and ** P < .01. **F**, Lung tissues from each group were stained with hematoxylin and eosin, as in Fig 1, C. Data are representative of at least 3 experiments.

anti-ST2 mAb (Fig 3, E and F). The response of the alveolar macrophages cultured with NKT cells in the absence of α -GalCer was most likely due to the presence of an endogenous glycolipid present in the antigen-presenting cell. The dependence on CD1d was

further demonstrated by coculture of the NKT cells with alveolar macrophages or lung DCs from CD1d^{-/-} mice, which did not produce IL-33 protein (Fig 3, G and H). The secretion of IL-33 protein required direct contact between the NKT cell and

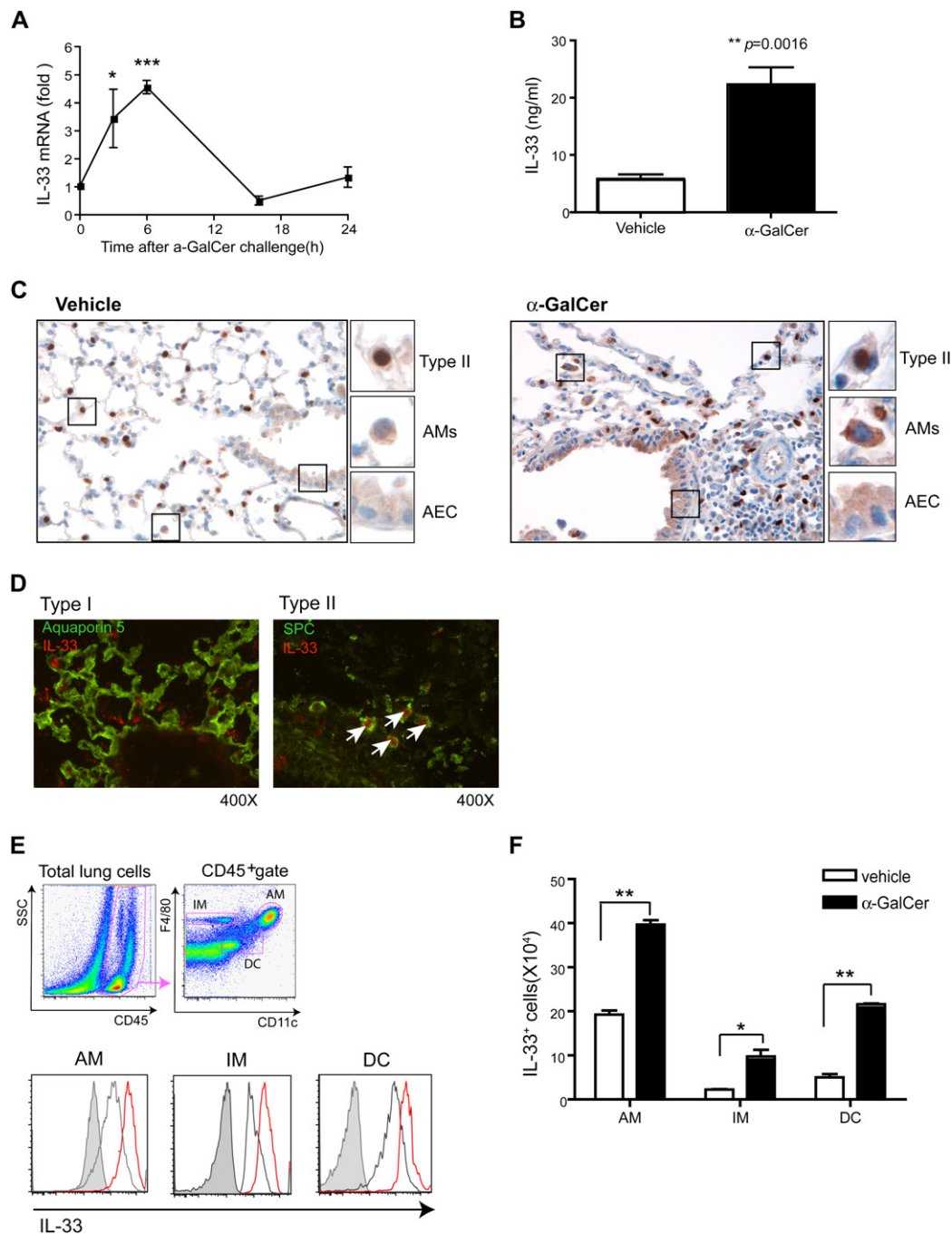


FIG 2. α -GalCer induces AHR by enhancing IL-33 production in the lung. **A**, IL-33 mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase, and relative mRNA levels were calculated as the fold increase over vehicle control. $*P < .05$ and $***P < .001$. **B**, Total lung homogenates were taken from vehicle- or α -GalCer-treated mice, and IL-33 protein levels were tested by using ELISA. $**P < .01$. **C**, Lung tissues from vehicle-treated mice (left) or α -GalCer-treated mice (right) were evaluated by means of immunohistochemistry for IL-33 expression and immunolocalization. The higher-power images on the right of each panel show type II pneumocytes, alveolar macrophages (AMs), and an airway epithelial cell (AEC; Original magnification $\times 200$). **D**, IL-33 expression by type I (left, Aquaporin 5 positive) or type II (right, surfactant protein C positive) pneumocytes was identified by using lung tissues. Arrows indicate IL-33⁺ cells, which are surfactant protein C positive (type II pneumocytes). **E**, IL-33 expression was examined by means of intracellular cytokine staining. Shaded, Isotype control; black line, vehicle; red line, α -GalCer treatment. IM, Interstitial macrophage; SSC, side scatter. **F**, The graph represents the total numbers of IL-33-producing cells in the lung. $*P < .05$ and $**P < .01$ ($n \geq 9$).

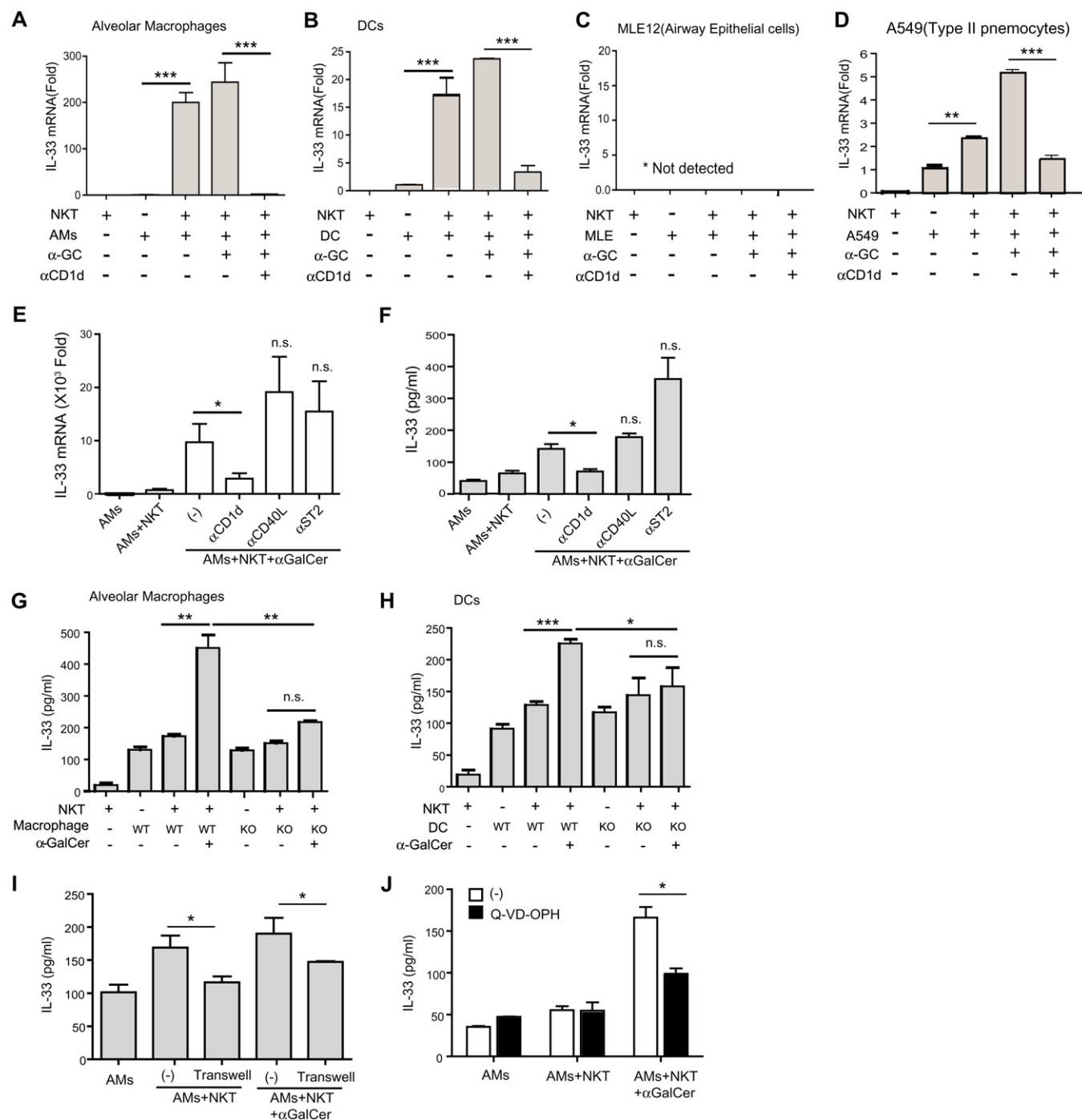


FIG 3. NKT cells induce IL-33 production from alveolar macrophages (AMs), DCs, and type II pneumocytes. **A-C**, Murine NKT cell lines were cocultured with alveolar macrophages (Fig 3, A), DCs (Fig 3, B), or murine airway epithelial cell lines (MLE12; Fig 3, C). Relative mRNA levels were calculated as the fold increase over alveolar macrophages (Fig 3, A) or DCs (Fig 3, B). For CD1d blocking, alveolar macrophages or DCs were treated with 10 μ g/mL anti-CD1d mAb (HB323) 1 hour before coculture. Data are presented as means $\pm$ SEMs and are representative of 3 experiments. *** P < .001. **D**, Human NKT cells (10^5 /96-well plate) were cocultured with human type II pneumocyte epithelial cell lines (A549, 5×10^4 /96-well plate). Anti-CD1d mAb (42.1; 10 μ g/mL) was used for CD1d blocking experiments. Relative mRNA levels were calculated as the fold increase over type II pneumocytes. * P < .05 and *** P < .001. **E** and **F**, Interaction between NKT cells and alveolar macrophages was blocked by anti-CD1d mAb, anti-CD40 ligand mAb, and anti-ST2 mAb (10 μ g/mL). IL-33 expression was measured by using mRNA expression (Fig 3, E) or ELISA (Fig 3, F). * P < .05. **G** and **H**, Alveolar macrophages (Fig 3, G) or DCs (Fig 3, H) from wild-type or CD1d $^{-/-}$ mice cocultured with NKT cells for 72 hours. * P < .05, ** P < .01, and *** P < .001. **I**, NKT cells were placed in the upper chamber, and alveolar macrophages were placed in the lower chamber of transwell chambers to block the NKT cell-macrophage cell-cell contact. * P < .05. **J**, Apoptosis inhibitor (Q-VD-OPH; 10 μ g/mL) was added to macrophages. * P < .05. Data are representative of at least 3 experiments. *n.s.*, Not significant.

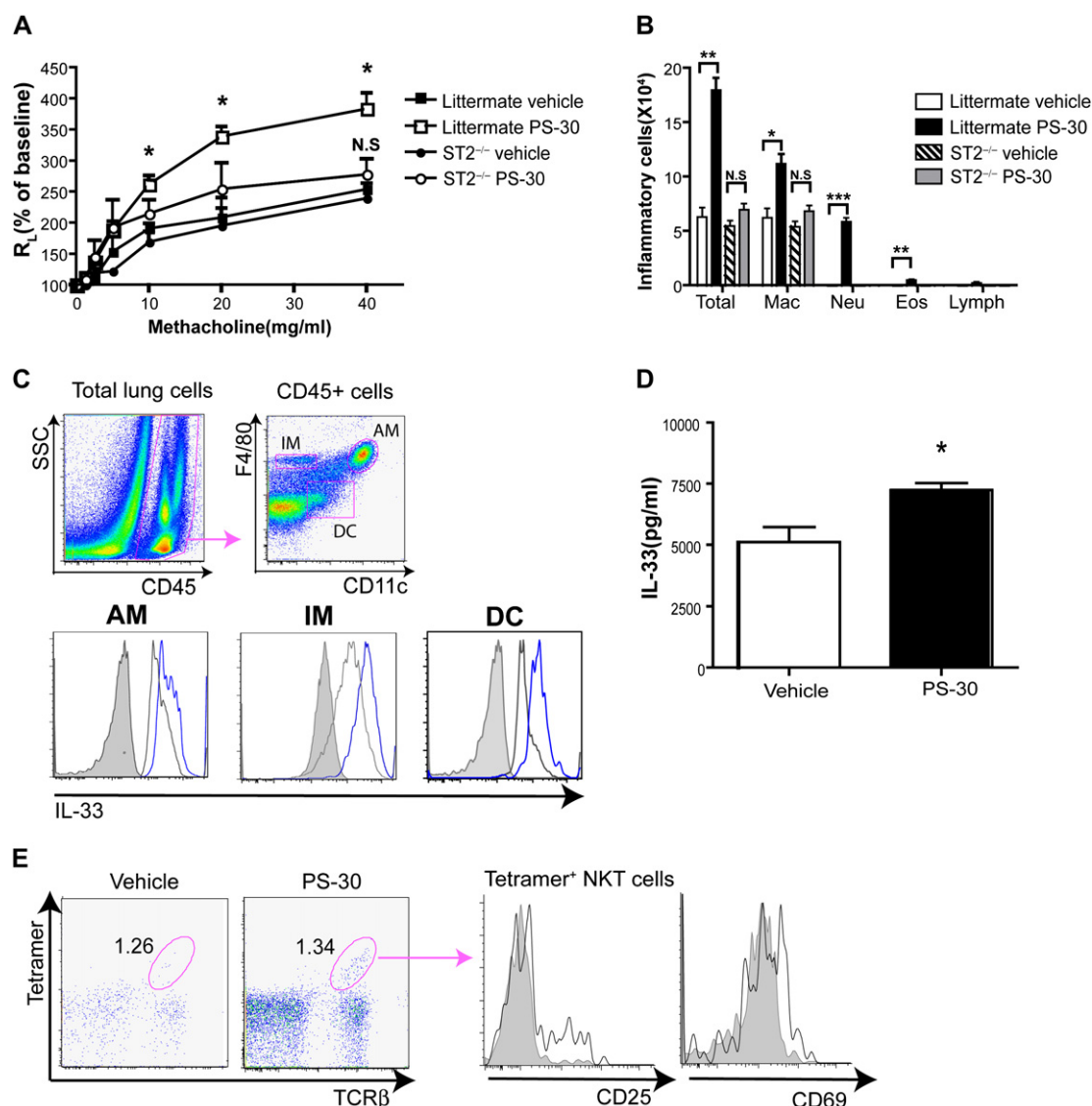


FIG 4. Glycolipid from *Sphingomonas* species induces AHR by producing IL-33. **A**, Ten micrograms of *Sphingomonas* species glycolipid (PS-30) was administered to littermate control or ST2^{-/-} mice. Twenty-four hours after glycolipid challenge, AHR was measured as in Fig 1, A (n ≥ 6). **P* < .05. N.S., Not significant. **B**, Data represent the number of cells per milliliter in bronchoalveolar lavage fluid and are presented as means ± SEMs. **P* < .05, ***P* < .01, and ****P* < .001. Eos, Eosinophils; Lymph, lymphocytes; Mac, macrophage; Neu, neutrophils. **C**, Lung cells were stained as in Fig 2, D, 24 hours after *Sphingomonas* species glycolipid (PS-30) challenge. Shaded histogram, Isotype control; black line, vehicle; blue line, PS-30-treated group. AM, Alveolar macrophages; IM, interstitial macrophage; SSC, side scatter. **D**, IL-33 protein levels from lungs of mice at 24 hours after treatment with vehicle or PS-30, as measured by means of ELISA. **P* < .05. **E**, Bronchoalveolar lavage fluid was collected 24 hours after PS-30 treatment, and NKT cells were analyzed. Shaded histogram, Vehicle control; black line, PS-30-exposed NKT cells. The mean fluorescence intensity for CD25 was 147 (control, 94.7), and the mean fluorescence intensity for CD69 was 1236 (control, 885). Data are representative of at least 3 experiments (n ≥ 6).

alveolar macrophage (Fig 3, I, and see Fig E2, A, in this article's Online Repository at www.jacionline.org) and was partially reduced by blockade of macrophage apoptosis (Fig 3, J). The induction of IL-33 required NKT cells and not T_H2 cells because OVA-specific T_H2 cells (generated from T cells from DO11.10 transgenic mice) cocultured with alveolar macrophages, DCs, or airway epithelial cell lines and OVA caused only minimal induction of IL-33 mRNA in these cells (see Fig E2, B-D). These results suggest that NKT cells activated by α-GalCer, but not

OVA-activated T_H2 cells, induced alveolar macrophages, DCs, and type II pneumocytes to express increased amounts of IL-33.

***Sphingomonas* species glycolipid also induces AHR in an ST2-dependent manner**

The list of glycolipid antigens recognized by NKT cells is growing rapidly and includes several endogenous glycolipids²⁴ and glycolipids found in pollen,¹⁴ house dust,¹⁵ and a number of

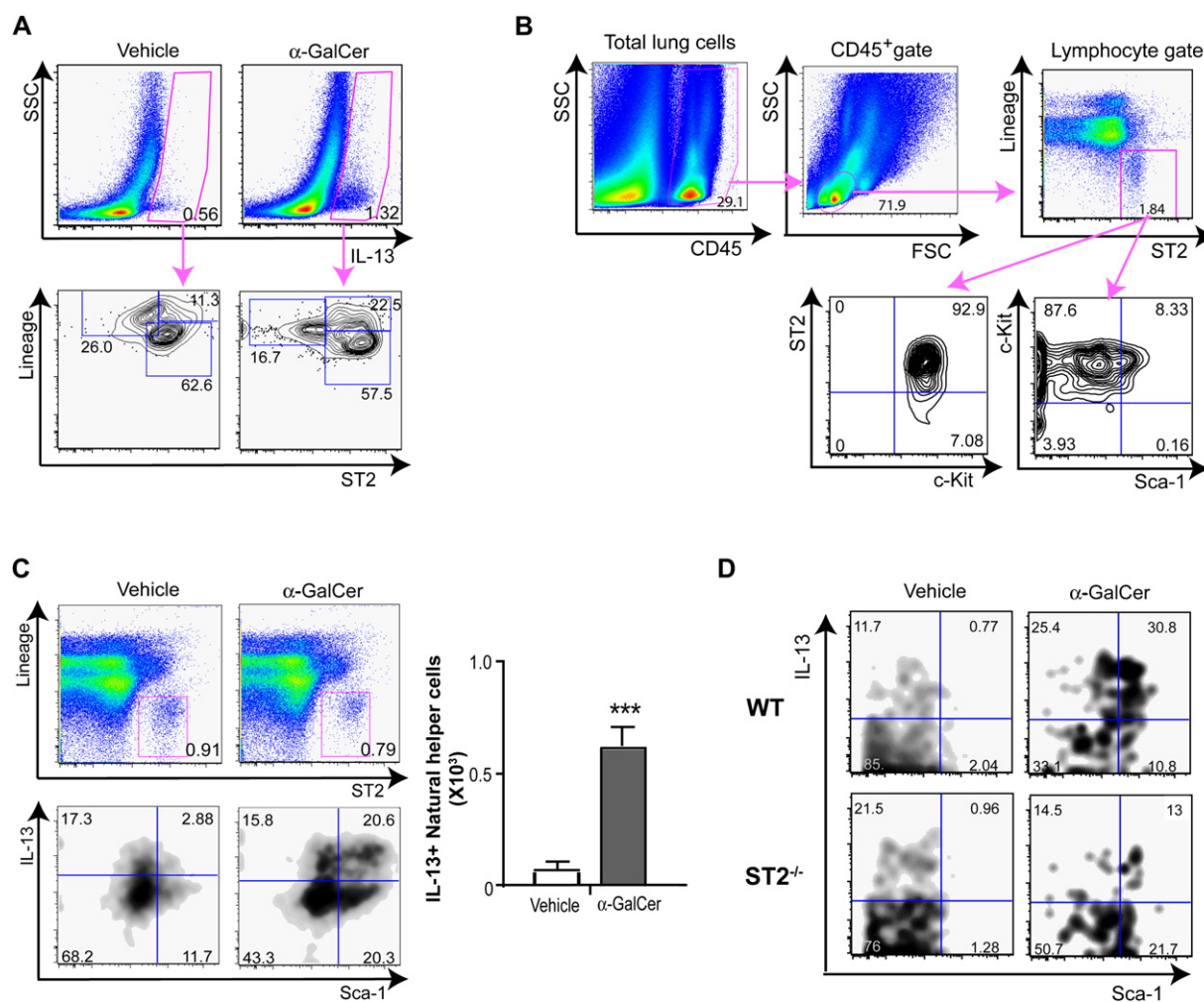


FIG 5. α -GalCer induces IL-13-producing natural helper cells in the lung. **A**, Total lung cells were obtained from vehicle- or α -GalCer-treated mice, and the level of IL-13 on CD45⁺ cells was detected by means of intracellular staining (upper panel). Total IL-13⁺ cells were further analyzed by using antibodies against Lin and ST2 (lower panel). SSC, Side scatter. **B**, Natural helper cell (Lin⁻ ST2⁺) subsets were gated from CD45⁺ cells and then assessed based on the expression of c-Kit and Sca-1. **C**, Natural helper cells (CD45⁺ Lin⁻ ST2⁺) were gated as shown in Fig 5, B, and the expressions of Sca-1 and IL-13 were further analyzed. The graph represents the number of Lin⁻ ST2⁺ c-Kit⁺ Sca-1⁺ IL-13⁺ natural helper cells in the lung (mean fluorescence intensity for vehicle, 78.6 ± 19.1; mean fluorescence intensity for α -GalCer, 641 ± 128.5). ***P < .001. Data are representative of at least 3 experiments. **D**, IL-13-producing natural helper cells from wild-type (WT) or ST2^{-/-} mice were compared as in Fig 5, C (n ≥ 4 mice in each group in this experiment).

bacteria.^{16,17} Fig 4, A, shows that one glycolipid from a *Sphingomonas* species, PS-30, which is known to activate NKT cells, induced significant AHR and airway inflammation in wild-type BALB/c mice but not in ST2^{-/-} mice (Fig 4, A and B), indicating a requirement for the IL-33/ST2 axis. The importance of PS-30 is highlighted by recent studies showing that bacteria in the Sphingomonadaceae family, which include *Sphingomonas* species, are commonly found in the lungs of patients with poorly controlled asthma and are associated with the presence of AHR.¹⁸ Moreover, administration of PS-30 to mice increased IL-33 expression by CD11c⁺ DCs and F4/80⁺ macrophages (Fig 4, C) and increased production of IL-33 in the lung (Fig 4, D). In addition, PS-30 activated NKT cells, which increased expression of CD25 and CD69 (Fig 4, E). These data suggest that glycolipids from bacteria can

activate NKT cells to induce AHR in an IL-33/ST2-dependent fashion.

α -GalCer-induced AHR is associated with an increase in natural helper cell numbers

Because IL-13 is required for α -GalCer-induced AHR,¹² we asked which cell types were required. After the administration of α -GalCer, we found that a large fraction of non-T, non-B innate cells produced the bulk of IL-13 (Fig 5, A). Recently, a novel innate lymphoid cell type called natural helper cells, nuocytes, multipotent progenitor cells, or innate type 2 cells has been identified that responds to IL-33 by producing large quantities of IL-13.²⁵⁻²⁸ These innate lymphocytes are present in fat-associated lymphoid

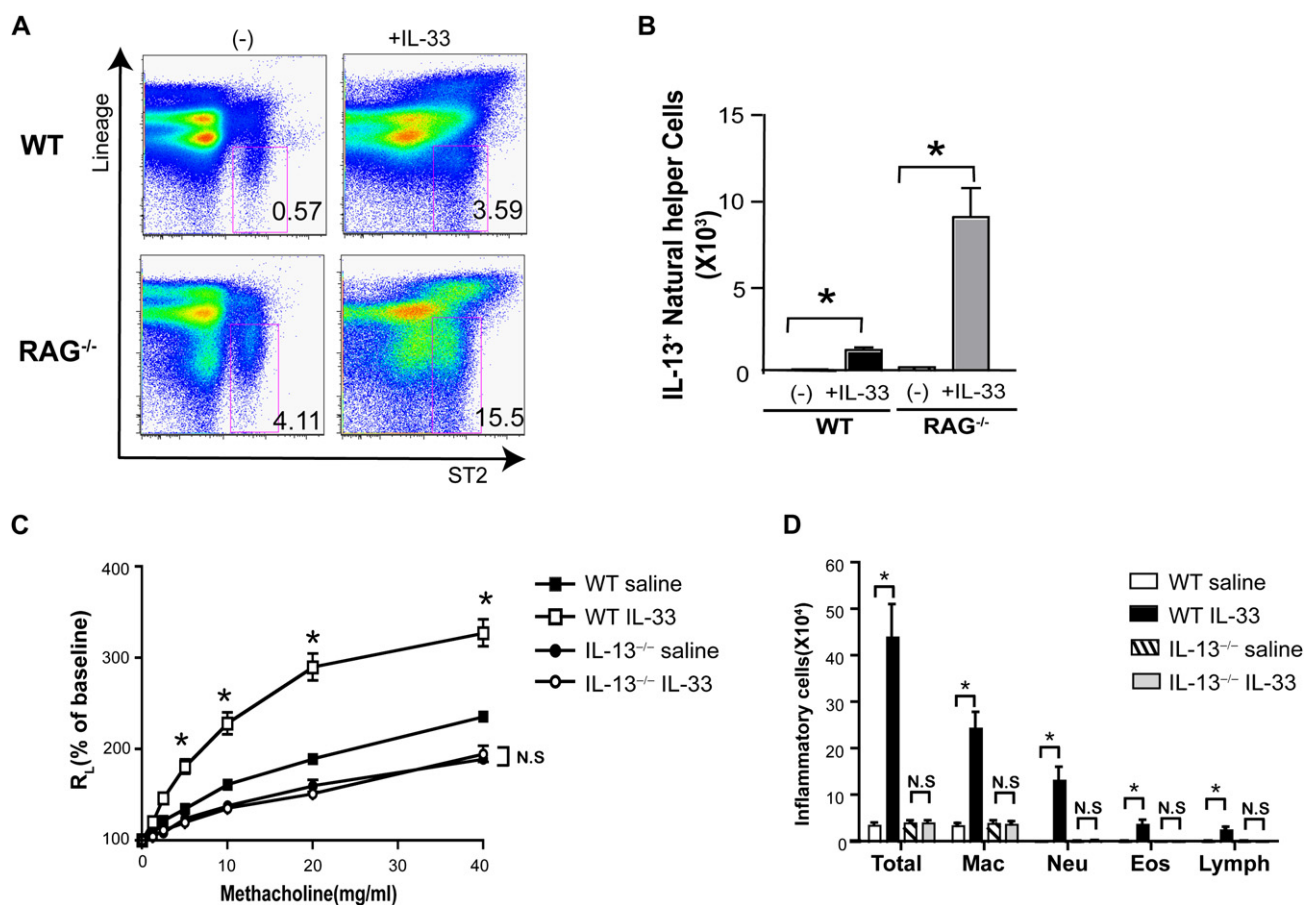


FIG 6. IL-33 induces AHR by increasing IL-13-producing natural helper cells. **A**, Recombinant IL-33 (0.1 μ g) administrated into wild-type or Rag^{-/-} mice for 3 consecutive days. The number of natural helper cells (CD45⁺Lin⁻ST2⁺) was assessed by means of flow cytometry. **B**, The number of IL-13-producing natural helper cells (Lin⁻ST2⁺c-Kit⁺Sca-1⁺IL-13⁺) was calculated after saline or IL-33 challenge (wild-type saline, 69.1 $\pm$ 11.5; wild-type IL-33, 1265 $\pm$ 248.7; Rag^{-/-} saline, 138.6 $\pm$ 25.4; and Rag^{-/-} IL-33, 927.2 $\pm$ 196.8). * P < .05. **C**, Wild-type BALB/c mice or IL-13^{-/-} mice were treated with 0.1 μ g of IL-33 for 3 consecutive days, and AHR was measured. Data represent the mean $\pm$ SEM percentages of saline values ($n \geq 4$). * P < .05. R_L , Airway resistance. **D**, Bronchoalveolar lavage fluid from mice in Fig 6, C, was analyzed for airway inflammatory cells, as shown in Fig 1, B. Data represent the number of cells (means $\pm$ SEMs) and are representative of 3 experiments. * P < .05. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils; WT, wild-type.

clusters²⁵ and in the mesenteric lymph nodes of helminth-infected mice^{26,27} and the lungs of influenza A virus-infected mice.⁷ Surprisingly, we found natural helper cells/nuocytes in the lung and identified them as lineage (Lin)⁻negative (CD3, CD19, Fc ϵ RI α , CD11b, CD11c, and CD49b), ST2⁺, and c-Kit⁺. Flow cytometric analysis showed that 1% to 2% of the total lung cells from naive mice were natural helper cells (Fig 5, B). After α -GalCer treatment, the total numbers of these ST2⁺c-Kit⁺ natural helper cells expressing Sca-1 and producing IL-13 greatly increased, although the percentage of Lin⁻ST2⁺ cells was slightly reduced (Fig 5, C). In addition, PS-30, a glycolipid from *Sphingomonas* species, also increased the number of Sca-1⁺ natural helper cells in the lung (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, treatment of ST2^{-/-} mice with α -GalCer did not increase IL-13 production in lung natural helper cells (Fig 5, D). Taken together, these results suggest that α -GalCer or *Sphingomonas* species glycolipid treatment induced AHR by activating NKT cells, which induced macrophages, DCs,

and type II pneumocytes to produce IL-33, which in turn activated natural helper cells to produce IL-13, a known inducer of AHR.^{12,13}

IL-33 induces AHR in the absence of adaptive immunity

Previous studies have reported that intranasal administration of IL-33 induces AHR and airway inflammation in Rag^{-/-} mice,²⁹ but the specific cells responding to the IL-33 was not clear, particularly because IL-33 was not required for allergen-induced AHR,^{7,19,20} although the resolution of AHR occurred more rapidly when IL-33 was neutralized.³⁰ We confirmed that administration of IL-33 to Rag2^{-/-} mice for 3 consecutive days resulted in the induction of AHR and airway inflammation without adaptive immune cells (see Fig E4 in this article's Online Repository at www.jacionline.org). Importantly, administration of IL-33 greatly increased the numbers of natural helper cells in the lungs

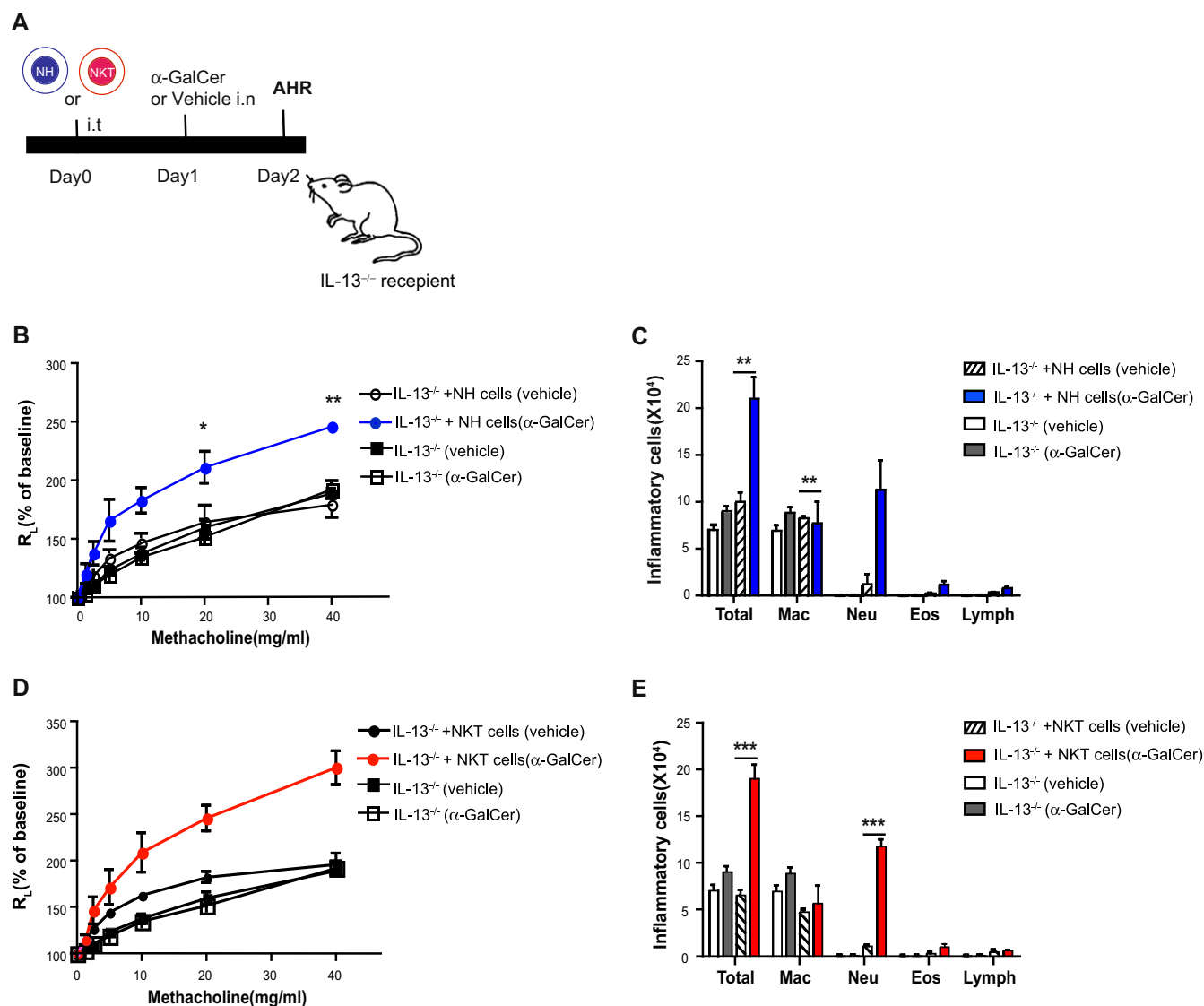


FIG 7. Natural helper cells and NKT cells are required for α -GalCer-induced AHR. **A**, Schematic showing the protocol for adoptive transfer of natural helper cells or NKT cells. **B** and **C**, Change in lung resistance (R_L ; Fig 7, **B**) and inflammatory cells in bronchoalveolar lavage fluid (Fig 7, **C**) in IL-13^{-/-} recipients ($n = 4$ per group) given purified natural helper cells (Lin⁻ST2⁺ subsets) from wild-type donors followed by vehicle or α -GalCer challenge for 24 hours. **D** and **E**, Change in lung resistance (R_L ; Fig 7, **D**) and inflammatory cells in bronchoalveolar lavage fluid (Fig 7, **E**) in IL-13^{-/-} recipients ($n = 4$ per group) given purified NKT cells from wild-type donors followed by vehicle or α -GalCer challenge for 24 hours. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils.

of both wild-type and Rag2^{-/-} mice (Fig 6, **A**). The natural helper cells produced large quantities of IL-13 (Fig 6, **B**), as well as IL-5 (data not shown), in response to IL-33. IL-13 production by the natural helper cells was required for IL-33-induced AHR because challenge of IL-13^{-/-} mice with IL-33 did not induce AHR (Fig 6, **C**) and airway inflammation (Fig 6, **D**). IL-5 production by natural helper cells did not result in airway eosinophilia, most likely because IL-33 induces epithelial cells to produce CXCL1(KC)/IL-8,^{31,32} a chemokine that attracts neutrophils, and because IL-33 directly activates neutrophils. Although IL-33 can activate mast cells to produce IL-4 and IL-13, which might induce AHR, administration of IL-33 to mast cell-deficient mice still resulted in AHR, suggesting that mast cells are not the primary target cell of IL-33 in asthmatic patients.²⁹ Taken

together, these results indicate that activation of natural helper cells producing IL-13 by IL-33 is sufficient for the induction of AHR in the absence of adaptive immunity.

Adoptive transfer of IL-13-producing natural helper cells or NKT cells

We assessed the role of natural helper cells or NKT cells in this model with reconstitution experiments (Fig 7, **A**). Adoptive transfer of a very limited number of lung natural helper cells (10⁴ per mouse) from wild-type mice into IL-13^{-/-} mice fully reconstituted α -GalCer-induced AHR and airway inflammation, whereas adoptive transfer of lung natural helper cells without α -GalCer stimulation did not reconstitute AHR (Fig 7, **B** and **C**). These

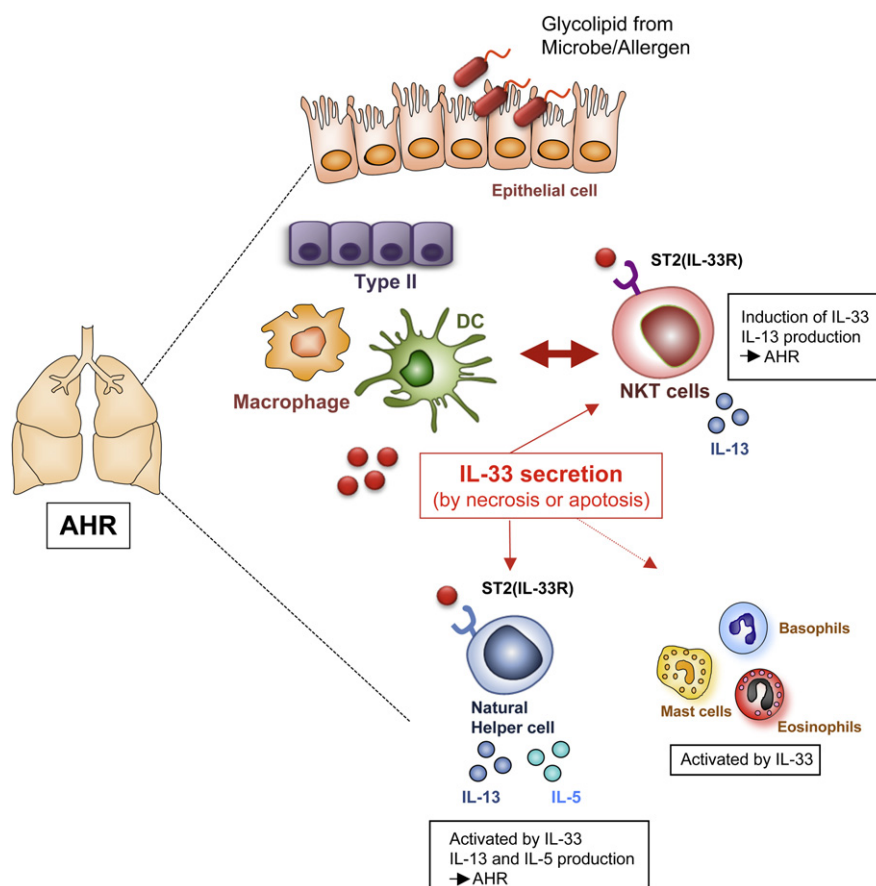


FIG 8. Schematic of the IL-33-ST2 axis in the development of AHR. On activation by glycolipid antigens, NKT cells induce macrophages, DCs, and type II pneumocytes to produce IL-33, which in turn activates natural helper and NKT cells to produce IL-13, resulting in the development of AHR. IL-33 can also activate mast cells, eosinophils, and basophils. *IL-33R*, IL-33 receptor.

data suggested that the IL-13-producing natural helper cells were sufficient for α -GalCer-induced AHR. In addition, the role of IL-13-producing NKT cells was confirmed by a separate adoptive transfer experiment, showing that adoptive transfer of purified wild-type NKT cells also reconstituted α -GalCer-induced AHR and airway inflammation (Fig 7, C and D). Furthermore, ST2 expression on pulmonary NKT cells or primary NKT cell lines was increased after intranasal challenge with α -GalCer (see Fig E5, A and B), and stimulation of NKT cells with both α -GalCer and IL-33 synergistically enhanced cytokine production and expression of activation markers from NKT cells (see Fig E5, C and D). Therefore these results suggest that α -GalCer stimulation increased NKT cell responsiveness to IL-33. Furthermore, these results suggest that either IL-13-producing natural helper cells or NKT cells are sufficient for the development of α -GalCer-induced AHR and that IL-33 plays an important role in the development of innate forms of asthma.

DISCUSSION

In these studies we found that innate lymphoid cells, including natural helper and NKT cells using an IL-33/ST2 receptor axis, could induce the development of AHR in the absence of T_H2 cells and adaptive immunity. In this pathway activation of NKT cells resulted in the induction of IL-33 production in alveolar

macrophages and DCs, which then drove the subsequent activation and expansion of natural helper cells, NKT cells, or both, producing IL-13 and the development of AHR (Fig 8). Because NKT cells can be directly activated by glycolipids from pollens and house dust,^{15,33} this pathway might greatly enhance the development of allergen-induced asthma. Moreover, this innate pathway might be very important in patients with nonallergic forms of asthma because this IL-33-mediated pathway could develop in the absence of T_H2 cells and adaptive immunity and because NKT cells can be activated by glycolipids expressed during viral infection⁶ and ozone exposure⁴ by pollen or bacteria,^{16,34-36} including *Sphingomonas* species, which are found in the lungs of patients with poorly controlled asthma and associated with AHR.¹⁸ Therefore these studies help to explain the heterogeneity in asthmatic patients, which appears to develop through several distinct pathogenic pathways, some involving allergic/adaptive mechanisms and others involving nonallergic/innate mechanisms.²

For example, although T_H2 cells have been shown to drive the development of AHR in experimental models of allergic asthma, in another model of asthma, Sendai virus infection precipitated chronic lung disease associated with AHR in the absence of T_H2 cells.⁶ This pathway involved alternatively activated alveolar macrophages interacting with NKT cells,^{6,37} although the role of natural helper cells was not evaluated. Exposure to air pollution might result in another T_H2 cell-independent pathway to asthma,

which can be modeled by exposing mice repeatedly to ozone, a major component of air pollution, resulting in severe AHR associated with airway neutrophils rather than eosinophils.⁴ This AHR response required NKT cells producing IL-17 but not adaptive immunity. Our current studies indicate that an additional innate pathway involving NKT cell-driven IL-33 production by alveolar macrophages and DCs can lead to the development of experimental asthma in the absence of adaptive immunity.

IL-33 is a member of the IL-1 family and has been shown to play an important role in mediating the inflammatory response required for parasite expulsion³⁸ and in several autoimmune and inflammatory disorders.^{39,40} Although studies of intestinal parasites suggest an important role of IL-33 in diseases associated with T_H2 cytokines, the precise role of IL-33 in the lungs and in asthmatic patients has not been fully investigated. IL-33 is present in the lungs of patients with severe asthma,⁴¹ is present in the blood of patients undergoing anaphylaxis,⁴² and can activate DCs to prime T cells to produce T_H2 cytokines.⁴³ In addition, *IL33* has been identified in genome-wide association studies in human subjects as a susceptibility gene for asthma.^{44,45} However, the conditions that result in IL-33 secretion in the lungs have not been clear, although IL-33 has been shown to be produced by airway epithelial cells activated by Toll-like receptor 4 ligation by dust mite allergen⁴⁶ or in the context of influenza A virus infection.⁷ Our studies now indicate that IL-33 can be produced by alveolar macrophages in the lungs after direct activation by NKT cells.

Although administration of recombinant IL-33 was previously shown to induce the development of AHR, even in the absence of T_H2 cells (eg, in Rag^{-/-} mice),^{29,47} the cell type in these models responding to IL-33 and required for AHR was not clear, although in retrospect it might have been natural helper cells. IL-33 binds to its receptor, ST2, which is expressed by T_H2 cells,⁴⁸ eosinophils,⁴⁹ mast cells, basophils,⁵⁰ and some NKT cells,⁵¹ as well as by natural helper cells (nuocytes or multipotent progenitor cells).²⁵⁻²⁷ Natural helper cells have only recently been described and have not been appreciated in the past,²⁵⁻²⁷ and the precise characteristics of natural helper cells are still being delineated. Natural helper cells do not express Lin markers but express c-Kit, IL-7 receptor, and, with activation, Sca-1 and might be related to another innate non-T, non-B lymphoid cell type that is Lin⁻c-Kit⁻, retinoic acid-related orphan receptor γ ⁺, IL-23 receptor-positive, IL-17⁺ and is involved in the development of colitis in mice⁵² and human subjects.⁵³ Natural helper cells described in mice might be similar to a non-T-cell, non-B-cell population found in the sputum of patients with asthma⁵⁴ and recently in human nasal polyp tissue.⁵⁵ We now show that natural helper cells are present in the lungs of mice and, in combination with NKT cells and alveolar macrophages, play an overlooked role in the development of AHR. Although IL-33 can affect several cell types that contribute to asthma (eosinophils, basophils, macrophages, and T_H2 cells), we demonstrated that IL-33 induced IL-13 production in natural helper and NKT cells, which then mediated the development of glycolipid-induced AHR.

The identification of a role for natural helper cells in AHR extends the range of cell types that can mediate AHR and asthma, perhaps reflecting the heterogeneous nature of asthma, which likely develops through several distinct pathways. T_H2 cells, which also produce IL-13 and IL-5, are present in the lungs of many patients with asthma and are essential in allergic asthma. Thus in experimental models T_H2 cells responding to allergen

are required for the induction of allergen-induced AHR, usually in combination with NKT cells,^{11,56} although T_H2 cells, when adoptively transferred in large numbers, are capable by themselves of inducing AHR.⁵⁷ NKT cells are also essential for the induction of AHR in some models independently of T_H2 cells, as in ozone-induced AHR by producing IL-17⁴ and in Sendai virus-induced AHR by producing IL-13.⁶ We now show that natural helper cells producing IL-13 are also sufficient for inducing the development of AHR independently of T_H2 cells but in partnership with NKT cells. Natural helper cells, when activated through a pathway involving influenza A virus, might also induce AHR independently of T_H2 or NKT cells.⁷ Although each of these pathways might mediate a distinct form of asthma and might occur independently of each other, it is possible that in some patients these distinct pathways might coexist, such as in patients with multiple triggers for asthma. In such patients these separate pathways could synergize and result in more severe disease.

In summary, we identified an immunologic pathway for AHR that occurs independently of T_H2 cells but that is dependent on NKT cells and natural helper cells. This pathway is also dependent on the IL-33 receptor ST2 and on IL-33 produced by alveolar macrophages, DCs, and type II pneumocytes. We suggest that some environmental agents, such as bacteria, pollens, and house dust antigen, which can directly activate NKT cells, might trigger the development of AHR and asthma through such an innate pathway. Because this innate pathway might be relatively resistant to current treatments for asthma (eg, corticosteroids), a greater understanding of NKT cells, natural helper cells, and IL-33 in this pathway could lead to improved therapies for this heterogeneous disorder.

Key messages

- This study examines a novel pathway for the development of experimental asthma that occurs in the absence of T_H2 cells and adaptive immunity.
- This pathway for AHR, a cardinal feature of asthma, is initiated by the activation of NKT cells, which induce alveolar macrophages to produce IL-33, which in turn stimulates NKT cells and natural helper cells, a newly described effector cell type, to increase production of IL-13, a cytokine critical for the development of AHR.
- Because allergens and bacteria in the lungs of asthmatic patients contain glycolipids that can activate NKT cells, these studies provide a mechanism by which NKT cells, natural helper cells, and IL-33 drive the development of AHR.

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METHODS

In vivo IL-33 administration and measurement of airway responsiveness

Mice were anesthetized with isoflurane, and 0.1 μ g of soluble recombinant human IL-33 (Amgen) was administered intranasally on 3 consecutive days. Twenty-four hours after the final dose, AHR responses were assessed in response to increasing doses of methacholine. AHR was assessed by means of invasive measurement of airway resistance in anesthetized, tracheostomized, and mechanically ventilated mice.

Bronchoalveolar lavage fluid and cytospin preparations

After measurement of AHR, murine tracheas were cannulated, lungs were lavaged twice with 1 mL of PBS 2% FCS, and the fluids were pooled. Cells in the lavage fluid were counted by using a hemocytometer, and bronchoalveolar lavage cell differential counts were determined on slide preparations stained with DiffQuik. At least 200 cells were differentiated on stained slides by means of light microscopy with conventional morphologic criteria. For some experiments, bronchoalveolar lavage fluid for each mouse or grouped bronchoalveolar lavage fluid was stained and analyzed by means of flow cytometry.

Lung tissue

After measurement of AHR and animal death, lungs were perfused with PBS 2% FCS. Lung tissue was removed, minced, and digested with 1.6 mg/mL collagenase type 4 (Worthington, Lakewood, NJ) and 0.1% DNase I (Fraction IX; Sigma-Aldrich, St Louis, Mo) at 37°C for 1 hour. The resulting lung cell suspension was then lysed for RBCs and stained for FACS analysis. Some lungs were fixed in 10% neutral buffered formalin (pH 7.4) for histologic analysis.

Preparation of alveolar macrophages and DCs

The lungs of naive mice underwent lavage twice with 1 mL of PBS 2% FCS. The fluid was used as the source for alveolar macrophages. Lungs from naive mice were minced and digested with 1.6 mg/mL collagenase type 4 (Worthington) and 0.1% DNase I (Fraction IX, Sigma-Aldrich) to obtain a single-cell suspension. DCs were isolated from this cell suspension by using CD11c microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) and an AutoMACS (Miltenyi Biotech).

Immunohistochemistry

After fixation, lung specimens were processed, embedded in paraffin, and sectioned at 4 μ m. Sections were immersed in antigen retrieval buffer (DIVA; Biocare Medical, Concord, Calif) and heated in a steamer for 60 minutes. After cooling, blocking reagent was applied (Sniper blocking reagent [Biocare Medical] plus 1% rabbit serum), and then sections were treated for 15 minutes to block endogenous biotin activity (Avidin-Biotin Blocking Kit, Biocare Medical). Subsequently, anti-mouse IL-33 antibody (AF2626; R&D Systems, Minneapolis, Minn) diluted 1:800 in DaVinci Green diluent (Biocare Medical) was applied, and sections were incubated for 2 hours at room temperature. Next, endogenous peroxidase activity was quenched (Peroxidized-1 Blocking Reagent, Biocare Medical), and secondary antibody (Goat IgG ABC kit; Vector Laboratories, Burlingame, Calif) was applied for 30 minutes. Slides were developed with Cardassian DAB (Biocare Medical), counterstained with hematoxylin, and placed under cover slips.

RT-PCR

Total RNA was isolated from the lung tissue or culture cells by using TRI reagent (Sigma-Aldrich). cDNA was reverse transcribed with the iScript cDNA synthesis kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's instruction. cDNA (0.5–1 μ g) was amplified in the presence of TaqMan universal master mix (Applied Biosystems, Foster City, Calif), gene-specific TaqMan probe, forward and reverse primers, and water. Gene-specific PCR products were measured with an Applied Biosystems 7500 Sequence Detection System. The levels of target gene expression were normalized to glyceraldehyde-3-phosphate dehydrogenase or 18S rRNA as an endogenous control. The following primers and probes were purchased from Applied Biosystems: murine glyceraldehyde-3-phosphate dehydrogenase (4352339E), human 18S rRNA (Hs99999901_s1), murine IL-33 (Mm00505403_m1), and human IL-33 (hs00369211_m1).

Flow cytometry

Cells were preincubated with anti-Fc γ R blocking mAb (2.4G2) and washed before staining. Hematopoietic cells were identified by gating on cells stained with anti-mouse PE-Texas red-conjugated CD45 mAb (Invitrogen). Total lung cells were stained with a combination of anti-mouse mAbs to F4/80, CD11b, and CD11c to identify macrophages. For natural helper cells, fluorescein isothiocyanate (FITC)-conjugated CD3, CD19, Fc ϵ RI, CD11b, CD11c, and CD49b; allophycocyanin-conjugated c-Kit; Alexa Fluor 700 Sca-1; and biotin-conjugated ST2 were used. ST2⁺ cells in various subpopulations were identified by using an FITC-conjugated anti-mouse T1/ST2 antibody (clone DJ8) from MD Bioproducts (St Paul, Minn) or rat IgG₁ FITC isotype controls. For intracellular staining, after fixation and permeabilization (Cytofix/Cytoperm kit; BD Biosciences, San Jose, Calif), cells were incubated with phycoerythrin (PE)-conjugated anti-IL-33. The respective isotype control was used for each experiment. Cells were collected on a FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo 8.3.3 software (TreeStar, Inc, Ashland, Ore).

Culture and *in vitro* Stimulation of NKT cells

NKT cells were purified from splenocytes of wild-type BALB/c mice. RBC-depleted splenic cell suspensions underwent negative selection by using biotin-conjugated mAbs to CD62 ligand, CD11b, Gr-1, and B220 to isolate NKT cells. The negative fraction was then stained with PE-conjugated CD1d tetramer loaded with PBS57 (National Institutes of Health/National Institute of Allergy and Infectious Diseases MHC tetramer core facility, Atlanta, Ga) and T-cell receptor β FITC, and NKT cells were purified with a Mo-Flo high-speed cell sorter (DakoCytomation, Glostrup, Denmark). Purified NKT cells were cultured with BALB/c ByJ-derived bone marrow-derived DCs in the presence of IL-15. Cells were harvested and "rested" overnight in the absence of bone marrow-derived DCs or IL-15 before being used for *in vitro* cell stimulation experiments. Rested NKT cells were cocultured with alveolar macrophages, DCs, type II pneumocytes, and MEL12 cells to measure the IL-33 mRNA or protein production.

Adoptive transfer of NKT cells and natural helper cells

NKT cells were positively selected from the spleens of BALB/c mice by using PE-conjugated PBS57-loaded CD1d tetramers and anti-PE beads. Lin[−]ST2⁺ natural helper cells were isolated from lungs and purified by using a Mo-Flo high-speed cell sorter (DakoCytomation). Purified NKT cells (3×10^5) or natural helper cells (1×10^4) were adoptively transferred into IL-13^{−/−} mice 1 day before α -GalCer challenge.

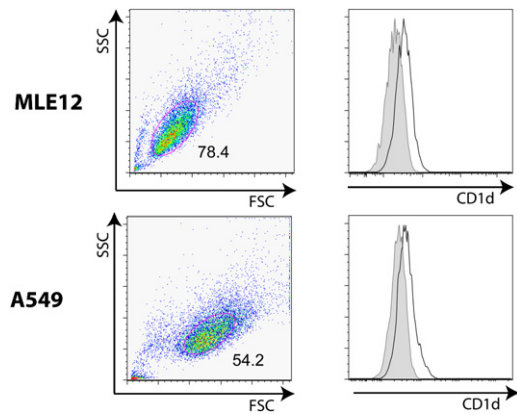


FIG E1. Expression of CD1d on lung cell lines. Surface expression of CD1d molecules in murine airway epithelial cells (MLE12) or human type II pneumocytes (A549) was assessed by using FACS. *FCS*, Forward scatter; *SSC*, side scatter.

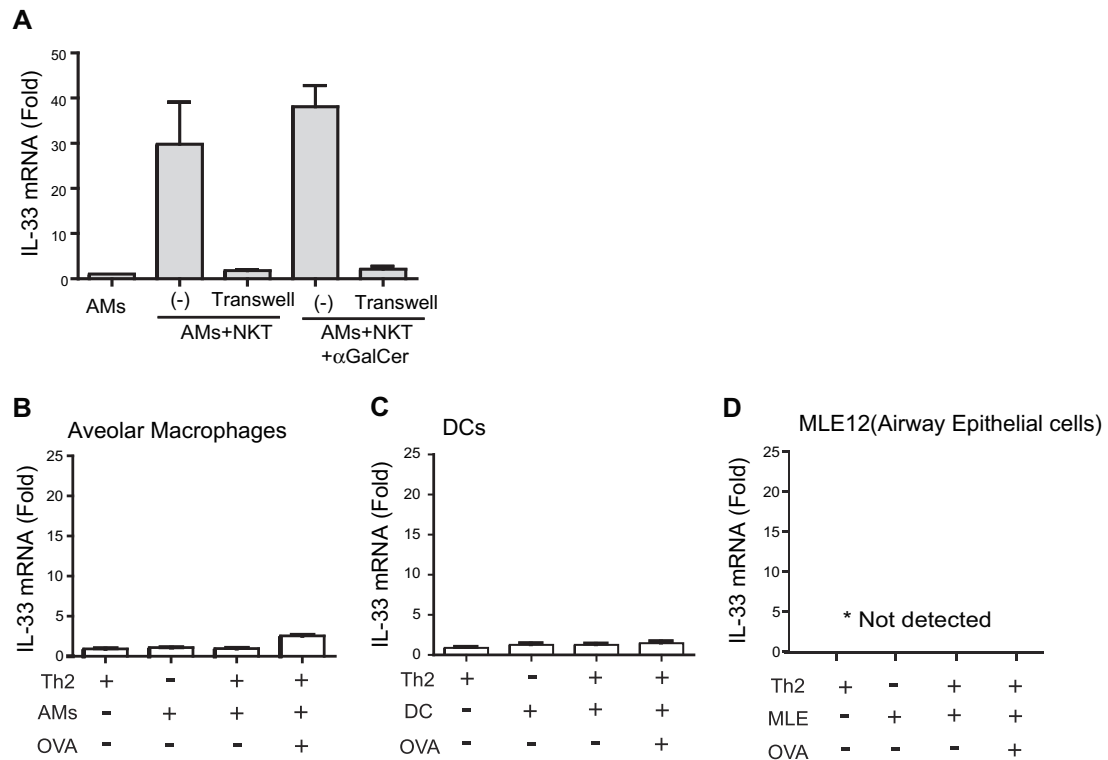


FIG E2. IL-33 mRNA expression in antigen-presenting cell cocultures with NKT or T_H2 cells. **A**, Cocultured cells from Fig 3, I, were collected for determinations of IL-33 mRNA expression. **B-D**, The polarized ovalbumin (OVA)-specific T_H2 cell line ($10^5/96$ -well plate) was generated from DO11.10 mice and then cocultured with alveolar macrophages (AMs; $5 \times 10^4/96$ -well plate; Fig E2, B), DCs ($5 \times 10^4/96$ -well plate; Fig E2, C), or murine airway epithelial cell lines (MLE12; $5 \times 10^4/96$ well plate; Fig E2, D) or without ovalbumin stimulation. Relative mRNA levels were calculated as the fold increase over the DO11.10 T_H2 cell line.

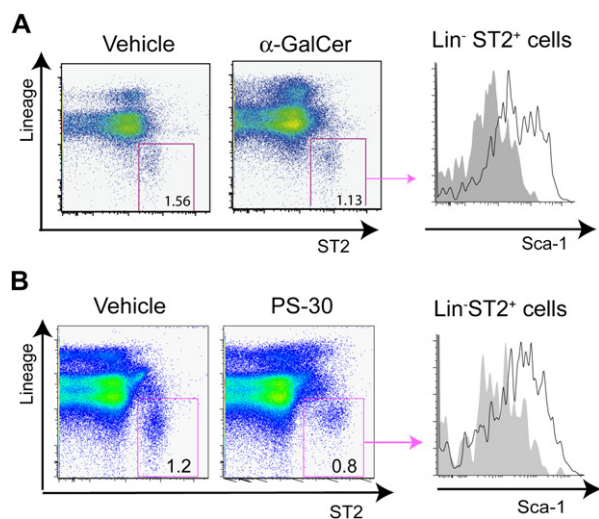


FIG E3. PS-30-induced Sca-1 expression on natural helper cells. Twenty-four hours after α -GalCer (**A**) or PS-30 (**B**) administration, the natural helper cells from the lungs of BALB/c mice were stained as described in Fig 5, B. Lin⁻ST2⁺ cells were further analyzed for Sca-1 expression. The mean fluorescence intensity for Sca-1 after α -GalCer (Fig E3, A) was 5605 (vehicle, 954), and that after PS-30 (Fig E3, B) was 2957 (vehicle, 1502).

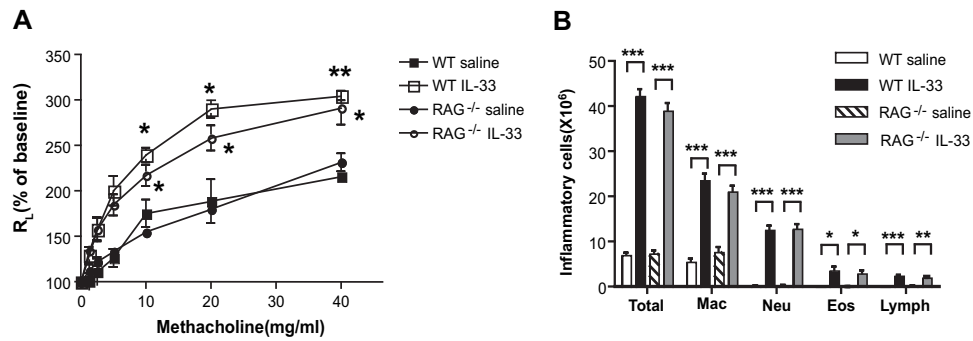


FIG E4. IL-33 induced the development of AHR in Rag2^{-/-} mice. **A**, Wild-type BALB/c or Rag2^{-/-} mice were treated with 0.1 μ g of IL-33 for 3 consecutive days, and AHR was measured. Data represent the mean $\pm$ SEM percentages of saline values ($n \geq 5$). * $P < .05$ and ** $P < .01$. R_L , Airway resistance. **B**, The numbers of inflammatory cells in bronchoalveolar lavage fluid were counted. *Eos*, Eosinophils; *Lymph*, lymphocytes; *Mac*, macrophage; *Neu*, neutrophils; *WT*, wild-type. * $P < .05$, ** $P < .01$, and *** $P < .001$.

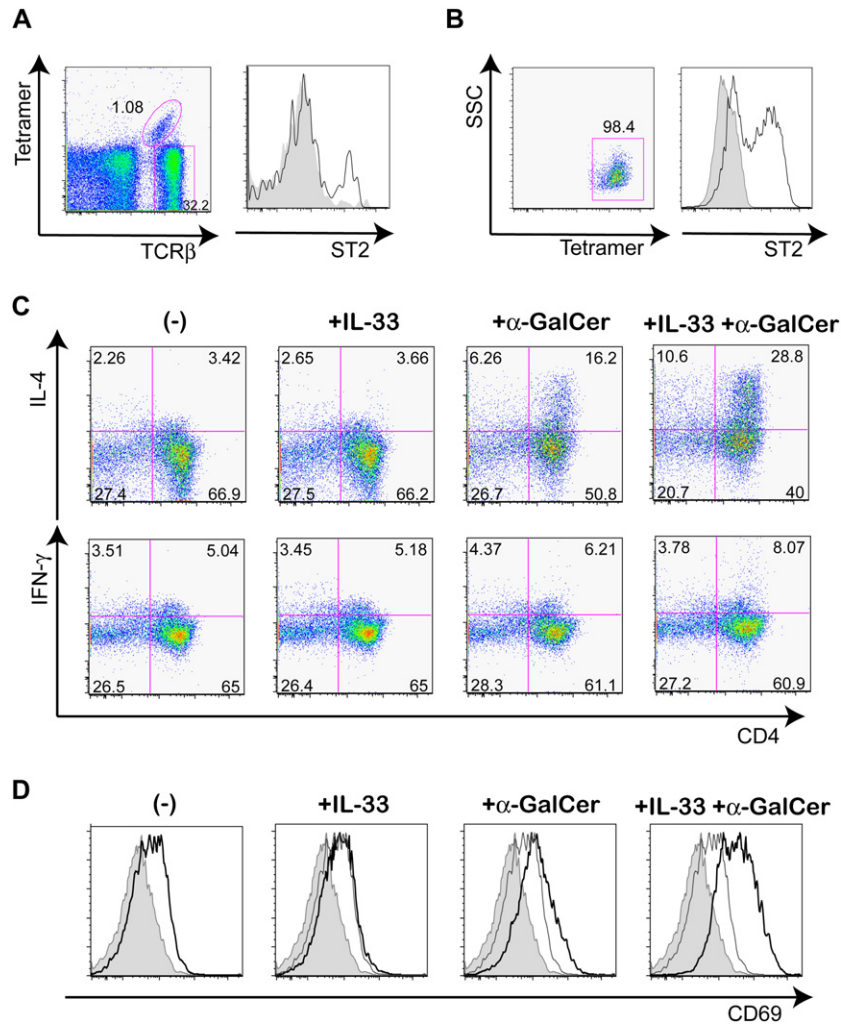


FIG E5. IL-33 and α -GalCer synergistically activated NKT cells. **A**, NKT cells from lungs were identified by first gating on anti-CD45 mAb-positive cells and then gating on lymphocytes. In the dot plots CD1d tetramers loaded with PBS57 and T-cell receptor β double-positive NKT cells are indicated by the red circle. ST2⁺ NKT cells were analyzed after vehicle or α -GalCer stimulation. Shaded histogram, Vehicle; black line, α -GalCer. **B**, Murine NKT cells were generated and stained for ST2 expression. Vehicle-treated (shaded histogram) and α -GalCer-treated (black line) NKT cells are as in Fig E5, A. The purity of the murine NKT cell line was greater than 98%. SSC, Side scatter. **C**, Murine NKT cells line were treated with IL-33, α -GalCer, or IL-33 plus α -GalCer and stained for activation markers or intracellular IL-4 and IFN- γ . The numbers shown in each dot plot represent the percentage of cytokine-producing NKT cells after gating on CD1d tetramer-positive cells. **D**, CD69 expression on NKT cells is presented as a histogram. Shaded histogram, Isotype control; gray line, no stimulation; black line, NKT cells after stimulation.