Contribution of IL-33–activated type II innate lymphoid cells to pulmonary eosinophilia in intestinal nematode-infected mice

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When animals are infected with helminthic parasites, resistant hosts show type II helper T immune responses to expel worms. Recently, natural helper (NH) cells or nuocytes, newly identified type II innate lymphoid cells, are shown to express ST2 (IL-33 receptor) and produce IL-5 and IL-13 when stimulated with IL-33. Here we show the relevant roles of endogenous IL-33 for Strongyloides venezuelensis infection-induced lung eosinophilic inflammation by using Il33−/− mice. Alveolar epithelial type II cells (ATII) express IL-33 in their nucleus. Infection with S. venezuelensis or intranasal administration of chitin increases the number of ATII cells and the level of IL-33. S. venezuelensis infection induces pulmonary accumulation of NH cells, which, after being stimulated with IL-33, proliferate and produce IL-5 and IL-13. Furthermore, S. venezuelensis infected Rag2−/− mice increase the number of ATII cells, NH cells, and eosinophils and the expression of IL-33 in their lungs. Finally, IL-33–stimulated NH cells induce lung eosinophilic inflammation and might aid to expel infected worms in the lungs.

IL-33 Is Induced in the Lungs after S. venezuelensis Infection. We examined histological differences of the lungs before and after S. venezuelensis infection. C57BL/6 (B6) WT mice, infected with third-stage larvae (L3) of S. venezuelensis, developed eosinophil-dominated leukocyte infiltration at days 5 and 7 (Fig. 1 A and B). Immunohistochemical analysis of lung tissues revealed that there were a small number of cells that expressed IL-33 in their nucleus even before infection (Fig. 1 A). S. venezuelensis infection increased the number of these IL-33+ cells particularly at days 5 and 7 (Fig. 1 A). These kinetics seemed to be proportional to that of induction of eosinophil infiltration in the bronchoalveolar lavage fluid (BALF) and of IL-33 protein production in the lung (Fig. 1 B and C). Next, we performed kinetic study of Il33 mRNA expression in the lungs after infection. We found S. venezuelensis infection increased the expression of mRNA for Il33 at day 4 and elevated further this expression at day 7 (Fig. 1 D). These kinetics parallel well with that for Il5 or Il13, and the appearance of lung inflammation after S. venezuelensis infection (Fig. 1 A). Nippostrongylus brasiliensis infection induced a similar kinetics of induction of Il33, Il5, and Il13 mRNA in BALB/c mice (Fig. S1 A). Next we sought the IL-33–producing cells in the lung. DAPI staining data confirmed that IL-33 is present in the nucleus. These IL-33+ cells are ATII cells because they were also positively stained by anti-IL5 and anti-IL13 antibodies (Fig. S1 B). These data suggest that Il33+ cells are ATII cells and contain IL-33.

Supporting Online Material

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stained for prosurfactant protein C, a specific marker for ATII cells (22) (Fig. 1E, Left). To determine whether other types of cells, such as macrophages, also express IL-33, we stained macrophages with anti–IL-33 antibody and anti-F4/80 antibody, and found that they do not express IL-33 (Fig. 1E, Right). As chitin is a component of the outer membrane of parasites (23), we examined the capacity of chitin to induce IL-33 in the lungs. Immunohistochemical analysis of lung tissues revealed that an intranasal administration of chitin promptly increased the number of IL-33–ATII cells in the lungs (Fig. 1F). This treatment promptly increased the IL-33 level in the BALF (Fig. S2A). We examined whether administration of chitin indeed increased the number of ATII cells. We counted the number of cells negative for T1α, CD16/32, and CD45.2 and positive for MHC class II as ATII cells (22, 24) (Fig. S3) and concluded that chitin treatment increased the number of ATII cells at least 72 h after treatment (Fig. 1F). Taken together, these results indicate that S. venezuelensis infection induces IL-33 production in the lung possibly by the action of chitin.

**Generation and Immunological Investigation of Il33−/− Mice.** We wished to determine whether endogenous IL-33 is critically required for establishment of lung eosinophilic inflammation in S. venezuelensis infected mice. For this purpose, we generated Il33 gene-deficient mice (Fig. S4 A and B) and examined their immunological properties. RT-PCR and Western blot analysis showed that the expression of IL-33 was completely abrogated in their lung tissues (Fig. S4 C and D). Proportions of T cells, B cells, dendritic cells, neutrophils, and eosinophils in the spleens of Il33−/− mice were comparable to those of Il33+/+ and Il33+/− mice (Fig. S4E). Anti-CD3–induced cytokine production responses revealed no skewing of splenic CD4+ T cells into Th1 or Th2 phenotype (Fig. S4F). Next, we examined the susceptibility of Il33−/− mice to S. venezuelensis infection. We simultaneously measured their systemic Th2/IgE response and mucosal mast cell activation in vivo. CD4+ T cells prepared from mesenteric lymph nodes of Il33−/− mice exhibited normal differentiation into Th2 cells (Fig. S5A). However, the measurement of serum levels of IgE and mouse mast cell protease 1, an activation marker of mucosal mast cells, indicated that the absence of IL-33 partly but significantly diminished these responses (Fig. S5 B and C). Furthermore, their capacity to expel S. venezuelensis was also modestly impaired (Fig. S5D). Thus, IL-33 is partly involved in the host defense against S. venezuelensis infection.

**Il33−/− Mice Show Reduced Accumulation of Eosinophils in the Lungs After S. venezuelensis Infection.** After S. venezuelensis infection, Il33+/+ mice developed eosinophilic inflammation and goblet-cell...
hyperplasia in the lungs at day 7, but \textit{Il33}−/− mice only modestly developed these changes (Fig. 2A), suggesting critical involvement of IL-33 in these responses. Consistent with this modest eosinophilic inflammation, expressions of mRNA for eosinophil markers (25) Epx (eosinophil peroxidase) and Prg2 (major basic protein) in the lungs were significantly lower in \textit{Il33}+/+ mice than those in \textit{Il33}−/− mice (Fig. 2B). Because the development and the recruitment of eosinophils are regulated by IL-5, IL-13, and chemokines (e.g., CCL11) (26), respectively, we measured their mRNA expressions. We also measured the mRNA expression for the epithelial cells-derived cytokines, IL-18, IL-33, thymic stromal lymphopoietin and IL-25, all of which are shown to up-regulate allergic inflammation (27, 28). The expressions of IL5, IL13, and CCL11 were strongly increased in the lungs of \textit{Il33}+/+ mice, but significantly diminished in those of \textit{Il33}−/− mice, suggesting that IL-33 is responsible for the productions of IL-5, IL-13, and CCL11, which in turn stimulate eosinophils to grow and infiltrate into the lung (Fig. 2B). Simultaneous measurement of epithelial cytokines revealed that \textit{S. venezuelensis} infection selectively increased the expression of IL33 mRNA among these cytokines (Figs. 1D and 2B). These results strongly indicated the \textit{Il33}−/−/− dependent production of IL-5, IL-13, and CCL11 is essential for goblet-cell hyperplasia and eosinophilic inflammation in the lung after \textit{S. venezuelensis} infection. Next we examined the proportion of eosinophils in the BALFs. There were very few eosinophils in the BALFs of uninfected mice. However, at day 7 after \textit{S. venezuelensis} infection, we observed high proportion of eosinophils (Fig. 2C and Fig. S5E) in the BALFs from \textit{Il33}+/+ mice. In contrast, this proportion in the BALFs from \textit{Il33}−/− mice was relatively low (Fig. 2C and Fig. S5E). As chitin is shown to induce IL-33 production in the lung (Fig. 1E and Fig. S2A), we examined whether \textit{Il33}−/− mice developed eosinophilia after treatment with chitin. We found that mice treated with chitin, displayed infiltration of inflammatory cells around the chitin particles and marked goblet-cell hyperplasia at 72 h (Fig. S2B). In contrast, chitin-treated \textit{Il33}−/− mice failed to develop these changes (Fig. S2C). Furthermore, chitin treatment increased the number of eosinophils in the BALF and the expression of \textit{Il5} and \textit{Il13} mRNA by BALF cells in an \textit{Il33}−/−-dependent manner (Fig. S2D and E).

\textbf{S. venezuelensis} Infection Induces Pulmonary Eosinophilia Even in the Absence of Acquired Immune Cells. We wished to directly demonstrate that \textit{S. venezuelensis} infection induces pulmonary eosinophilia without help from Th2 cells. We infected WT, \textit{Rag2}−/−, or \textit{γc}−/−\textit{Rag2}−/− mice with \textit{S. venezuelensis}. All of these mice increased the number of IL-33+ ATII cells in their lungs (Fig. 3A). Expectedly, like WT mice, \textit{Rag2}−/− mice developed pulmonary eosinophilia (Fig. 3B). WT mice and \textit{Rag2}−/− mice also increased the number of eosinophils in the BALFs and the expression of \textit{Il5} mRNA and \textit{Il5} mRNA in their lungs (Fig. 3C and E). As innate cells, such as NH cells, were reported to produce IL-5 in response to IL-33 stimulation (29), we tried to show the presence of these innate cells in the BALF cells. Expectedly, these cells appeared as Sca-1+ ST2+ cells in the FSClowSSc̅cd11c̅Lin− cells in the BALF cells from WT and \textit{Rag2}−/− mice after infection (Fig. 3D). To further identify the phenotype of Lin−ST2+ cells, we examined the expression of other surface markers; then, we found they expressed Sca-1, Thy1.2, IL-7Rα, CD25, c-Kit, and ICOS and had limited expression of MHC class II, as described in NH cells (Fig. S6) (30). In contrast to WT and \textit{Rag2}−/−, \textit{γc}−/−\textit{Rag2}−/− mice, which have no NH cells in mesenteric tissues (15), failed to develop these changes, suggesting the importance of the expression of the γc chain for the induction of pulmonary eosinophilia, NH cell proliferation, and IL-5 expression. NH cells emerged around day 7 after infection and expanded at least until day 14 in WT mice (Fig. 3F). Along with their expansion, degree of eosinophilia and expression of IL-5 and IL-13 are simultaneously up-regulated (Fig. 1B and D). Similar increases in the number of NH cells in the lungs were also observed at day 7 after \textit{N. brasiliensis} infection (Fig. S1B).

\textbf{NH Cells Are Induced in \textit{S. venezuelensis} Infected Mice in an \textit{Il33}−/−-Dependent Manner.} We demonstrated that \textit{S. venezuelensis} infected \textit{Il33}+/+ mice but not \textit{Il33}−/− mice markedly increased the expression of \textit{Il5} and \textit{Il13} mRNA in their lungs (Fig. 2B). Thus, we examined whether \textit{S. venezuelensis} infection increased the number of NH cells by induction of IL-33 production in the lungs. Compared with \textit{Il33}+/+ mice, \textit{Il33}−/− mice exhibited significantly reduced number of NH cells in the BALFs (Fig. 4A), suggesting the importance of endogenous IL-33 for the induction of NH cells. To determine whether IL-33 is directly responsible for increasing NH cells and IL-5 expression, we examined the effects of IL-33 on these responses by intranasal administration. Intranasal administration of IL-33 strongly increased the number of NH cells and eosinophils in the BALF of \textit{Il33}−/− mice (Fig. 4B). At the same time, this treatment strongly increased the expression of \textit{Il5} mRNA in the lungs.

This treatment also significantly accelerated the worm expulsion in \textit{Il33}−/− mice (Fig. 4B, Right). Finally, we tried to identify the cells that produce IL-5 in response to IL-33 in the lungs of \textit{S. venezuelensis} infected mice. We prepared BALF cells from WT mice at day 7 after infection and divided them into two fractions: lineage marker- (CD3, CD4, CD8, CD19, NK1.1, Gr-1, siglec F, IgE) positive and negative fractions. We could not find IL-5-producing cells in Lin+ cells, thus excluding the presence of IL-
We demonstrated that intranasal administration of chitin also induced similar pulmonary eosinophilia through their production of IL-5 and IL-13. In this article, we showed that Staphylococcus aureus infection failed to induce IL-33 production in the lungs. Data are expressed as the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001 versus corresponding values for PBS-treated mice (Student’s t test). (A) Confocal microscopic analysis of the IL-33 (red) expression in the lungs. (Scale bars, 50 μm.) (B) Histological analysis (H&E) of lungs. (Scale bar, 100 μm.) (C and D) Flow cytometric analysis of the BALF cells. Numbers indicate proportion of eosinophils (C) or NH cells (D). Cells were gated on the CD45+ fraction (C) or FSC<sup>-</sup>SSC<sup>-</sup>Lin<sup>-</sup> fraction (D). (E) qPCR analysis of the expression levels of mRNA for Il5 and Il13 in the lungs. Data are expressed as the means ± SD *P < 0.01, **P < 0.05 (Student’s t test). (F) The numbers of NH cells in the BALFs from mice in Fig. 1B are shown. Data are expressed as the means ± SD *P < 0.05, **P < 0.01 versus corresponding values for PBS-treated mice (Student’s t test). (C) Intracellular staining of IL-5 in Lin<sup>-</sup>ST2<sup>+</sup> cells in BALF cells. Cells were stained as described in SI Materials and Methods. Numbers indicate the proportion of ST2<sup>+</sup>IL-5<sup>+</sup> cells. Data are representative of two independent experiments.

**Discussion**

We demonstrated that S. venezuelensis infection of mice induced severe eosinophilic inflammation, goblet-cell hyperplasia, and accumulation of NH cells, and increased the number of IL-33–producing ATII cells and the expressions of mRNA for Il5 and Il13 in the lungs, even without help from acquired immune cells. Intranasal administration of chitin also induced similar pulmonary eosinophilia through their production of IL-5 and IL-13.

S–producing Th2 cells in the BALF (Fig. 4C). However, we found a substantial proportion of ST2<sup>+</sup> cells in Lin<sup>-</sup> fraction produced IL-5 (Fig. 4C). Taking these data together, IL-33 contributes to the induction of NH cells, which in turn protect host against S. venezuelensis infection by inducing lung eosinophilia through their production of IL-5 and IL-13.

IL-33-dependent induction of NH cells in S. venezuelensis infected mice. (A) Flow cytometric analysis of NH cells in BALF cells from uninfected or S. venezuelensis infected (Sv) Il33<sup>−/−</sup> or Il33<sup>+/+</sup> mice. Cells were gated on the FSC<sup>-</sup>SSC<sup>-</sup>Lin<sup>-</sup> fraction. (Right) The numbers of NH cells in total BALF cells from Il33<sup>−/−</sup> (n = 11) or Il33<sup>+/+</sup> (n = 5) mice. Data are expressed as the means ± SD *P < 0.005 (Student’s t test). (B) IL-33<sup>+/+</sup> (n = 7) and IL-33<sup>−/−</sup> mice (PBS; n = 4, IL-33<sup>+/−</sup>; n = 6) were infected with S. venezuelensis at day 0 and treated daily with 30 μL of PBS or 4 μg rhIL-33 intranasally from day 1 to 3. (Upper) Flow cytometry of NH cells in FSC<sup>-</sup>SSC<sup>-</sup>Lin<sup>-</sup> fraction of BALF cells and the proportion of NH cells in total BALF cells at 8 dpi post infection. (Lower Left) The numbers of eosinophils (eo), neutrophils (neu), lymphocytes (lym), or monocytes (mo) in BALF cells. (Lower Center) The expression of Il5 mRNA in the lungs. (Lower Right) The numbers of eggs per gram feces from each group at day 6, 7, 8 post infection. Data are expressed as the means ± SD; *P < 0.001, **P < 0.05 versus corresponding values for Il33<sup>−/−</sup> mice with PBS treatment (Student’s t test). (C) Intracellular staining of IL-5 in Lin<sup>-</sup>ST2<sup>+</sup> cells in BALF cells. Cells were stained as described in SI Materials and Methods. Numbers indicate the proportion of ST2<sup>+</sup>IL-5<sup>+</sup> cells. Data are representative of five mice and of two independent experiments.

changes, suggesting that S. venezuelensis infection induced these alterations principally by the action of chitin. In contrast, Il33<sup>−/−</sup> mice infected with S. venezuelensis failed to develop these pathological changes. Thus, IL-33 plays a critical role in induction of eosinophilia and goblet-cell hyperplasia in the lungs.

In this article, we showed that S. venezuelensis infection normally induced the development of Th2 cells in mesenteric lymph nodes of Il33<sup>−/−</sup> mice, which did not develop severe pulmonary eosinophilia, confirming that there are some innate cells that produce IL-5 and IL-13 in response to IL-33 in the lungs. We could demonstrate that S. venezuelensis infection increased the number of NH cells by inducing IL-33 production in the lungs. Because we could not detect NH cells in the BALF before...
infection, we speculated that they existed in parenchyma and proliferated in response to IL-33. Consistent with this theory, NH cells were recently found in the normal lung and increased by infection with H5N1 subtype of influenza A virus (30), and were shown to proliferate in response to IL-33 and IL-2 in vitro (15). Influenza virus infection induces NH cells/IL-13-dependent airway hyper-reactivity in the lung, but cannot induce pulmonary eosinophilia. This finding is quite different from our case, that S. venezuelensis infection induces IL-33-dependent eosinophilia. We suspected that this discrepancy could come from IL-10 production in influenza virus-infected mice because IL-10 is shown to inhibit eosinophil accumulation (31, 32).

Pulmonary eosinophilia is a very common complication of helminth infections, such as Strongyloides, Ascaris, Toxocara, and Anclylostoma species (33). Nevertheless, the physiological relevance and the pathogenesis of pulmonary eosinophilia are still not thoroughly understood. Because helminth infection strongly increases and the pathogenesis of pulmonary eosinophilia are still not thoroughly understood. Because helminth infection strongly induces Th2 immune response (4), it is generally accepted that Th2 cells are responsible for inducing both systemic eosinophilia and local pulmonary eosinophilia (Loeffler syndrome) (34). On the other hand, it is well documented that most of the maternally cause pulmonary eosinophilia during larval migration through the lungs (35, 36). Indeed, we observed several hemorrhagic areas in the lung tissues of mice at day 5 after S. venezuelensis infection. Furthermore, nematode infection might induce pulmonary eosinophilia without systemic eosinophilia (37). In this article, we demonstrated lung eosinophilic inflammation seen with Löffler syndrome is induced by the action of NH cells even in the absence of conventional Th2 cells. However, compared with that in S. venezuelensis infected WT mice, the degree of lung eosinophilic inflammation in S. venezuelensis infected Rag2−/− mice seems relatively mild. Thus, innate immune cells might partly be responsible for increasing degree of lung inflammation by producing Th2 cytokines.

We demonstrated that mouse ATII cells, which express the surfactant proteins required for the expansion of the lung and produce cytokines and chemokines for immune regulation (24), began to express IL-33 in their nucleus at day 2 after infection with S. venezuelensis or at 6 h after treatment with chitin. It has been reported that bronchial epithelial cells or alveolar macrophages also express IL-33 (30, 38). Here we demonstrated that macrophages from S. venezuelensis infected mice did not express IL-33. We speculated that, because influenza virus activates Toll-like receptor 7 (TLR7) signal (39), macrophages may require TLR signaling for IL-33 expression.

There are two types of alveolar epithelial cells: ATI and ATII. ATII cells only cover 5% of the surface and the remaining 95% of the surface is covered by ATI cells (24). It may therefore be hypothesized that ATI cells might be more likely to be damaged by microbes, such as S. venezuelensis, than ATII cells. ATII cells have the potential to proliferate and develop into ATI cells eventually, suggesting that damaged ATI cells are replaced by ATII cells in this study. We demonstrated that, because chitin induces ATII cells to produce IL-1-like cytokines and activates TLR7 receptor (39), macrophages may require TLR signaling for IL-33 expression.


with the increased number of ATII cells, the production of IL-33 in the lung is rapidly increased. Subsequently, IL-33 stimulates NH cells to proliferate and to produce IL-5 and IL-13, which in combination induce severe eosinophilic inflammation and goblet-cell hyperplasia in the lung.

We demonstrated that NH cells increased in the lungs of S. venezuelensis infected mice. NH cells and mucocytes have similarity in their expression patterns of surface antigens or the capacity for cytokine production. It has been demonstrated that mucocytes expand in response to both IL-25R-mediated and ST2-mediated signalings in *N. brasiliensis* infected mice (16). Here we reported that IL-33–deficient mice showed severely impaired accumulation of NH cells in the lungs after *S. venezuelensis* or *N. brasiliensis* infection compared with WT mice, although the expression of mRNA for IL25 did not differ in the lungs between WT and IL33−/− mice or before and after *S. venezuelensis* infection. At present, we cannot demonstrate that the expanded cells that produce IL-5 and IL-13 in the lungs of *S. venezuelensis* infected mice are either NH cells or mucocytes. On the other hand, we can exclude the contribution of multipotent progenitor (MMP) cells (40) or innate type-2 helper (IH2) cells (19), because MMP or IH2 cells have been shown to lack ST2 and Sca-1 expression, respectively (41).

Previous reports have shown that IL-33 is involved in eosinophil expansion (6). In agreement with this finding, IL-33–deficient mice showed significantly impaired accumulation of eosinophils in their lungs after *S. venezuelensis* infection. It has been also demonstrated that IL-33 can stimulate eosinophils to increase their survival, adhesion and production of cytokines and chemokines, and to produce superoxide anion and degranulation (5, 42, 43). These effects of IL-33 for the expansion and the activation of eosinophils might aid to expel infected worms in the lungs.

In conclusion, IL-33 is important not only for the expansion of NH cells but also for their production of IL-5, IL-13, and CCL11, which in turn induce the accumulation of eosinophils in *S. venezuelensis* infected mice.

**Materials and Methods**

**Helminths infection.** In vivo passage and animal infection of *S. venezuelensis* were shown previously (44). *N. brasiliensis* (Nb) has been maintained in male SD rat. Mice were inoculated subcutaneously with 500 L3 Nb. Details of analyses are described in SI Materials and Methods.

**Statistics.** All data are shown as the mean ± SD. The numerical data were analyzed using either Student’s t test or one-way ANOVA with Dunnett’s post test. P values less than 0.05 were considered significant.

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Supporting Information

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SI Materials and Methods

Mice. C57BL/6 were purchased from Oriental yeast, Rag2−/− and gc−/−Rag2−/− mice were from Taconic. All animal experiments were performed in accordance with guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine (Hyogo, Japan).

Reagents. Chitin was purchased from New England Biolabs. Recombinant human IL-33 (rhIL-33) was from MD Biosciences; and IgE (23G3) was from Southern Biotechnology Associates. Recombinant IL-33 was made by Hokudo Co.

Generation of Il33−/− Mice. The Il33 gene was isolated from genomic DNA extracted from ES cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 2.9-kb fragment encoding the Il33 ORF with a neomycin-resistance gene cassette (neo), and a herpes simplex virus thymidine kinase (HSV-TK) driven by the PGK promoter had been inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected, screened by PCR, and further confirmed by Southern blotting. Homologous recombinants were micro-injected into blastocysts from C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain Il33−/− mice. Il33−/− mice under 129Sv × C57BL/6 background were backcrossed to C57BL/6 for four generations and their littermate controls were used for the experiments.

Helminths Infection. At indicated time points after infection, the lungs were prepared for histology or analysis of protein or RNA after perfusing the mice via the right ventricle with 10 mL PBS under anesthesia. Bronchoalveolar lavage was performed and bronchoalveolar lavage fluid (BALF) cells were analyzed with flow cytometry. Sera were collected at 0, 1, 2, 7, and 14 d after infection and measured the concentrations of IgE and mouse mast cell protease 1 (mMCP-1) by ELISA (1, 2). Seven days after infection, mesenteric lymph nodes (mLN) were harvested and cell suspensions were enriched for CD4+ cells by positive sorting on AutoMACS (Miltenyi Biotech) with magnetic beads conjugated with anti-CD4 mAb. The CD4+ cells were stimulated with anti-CD3 and anti-CD28 antibodies. The culture supernatants were harvested 24 h later and measured the concentrations of IFN-γ, IL-4, and IL-5. In IL-33 reconstitution experiment, Il33−/− mice received intranasal administration of 4 μg rhIL-33 at 1 d before and 0, 1, 2, and 3 d after infection.

Chitin Administration. Mice were intranasally administered 50 μL of 0.75 mg chitin or 2 mg glass beads under anesthesia, and at indicated time points, histological and BALFs analysis were performed.

Histological Analysis. Lungs were fixed with 4% (wt/vol) paraformaldehyde and embedded with paraaffin. Deparaffinized sections were microwave-heated in citrated buffer (pH 6.0) for antigen retrieval, then blocked with 1% BSA, stained with affinity-purified rabbit anti-mouse IL-33 polyclonal antibody, followed by biotinylated goat anti-rabbit antibody (Vector Labs) and Alexa-555–labeled streptavidin (Invitrogen). For the double-staining experiment, IL-33–stained sections were blocked and stained with anti-Pro surfactant Protein C antibody (Millipore), following biotinylated goat anti-rabbit antibody and Alexa-488–labeled streptavidin (Invitrogen), then mounted with Prolong Antifade Gold with DAPI (Invitrogen). For macrophage staining, frozen sections of freshly isolated lung specimens were incubated with F4/80 mAb, biotinylated anti-rat IgG, and then Alexa Fluor 488-conjugated streptavidin (Molecular Probes). Nuclei were stained with DAPI (KPL). The immunostaining of each section was evaluated under microscope Zeiss LSM 510 (Carl Zeiss). Computer software, Zeiss LSM 510 ver. 3.2 (Carl Zeiss), was used for image processing and analysis.

Flow Cytometry. BALF cells were stained with antibodies for CD45, CD3, B220, CCR3, and Gr-1, examined by FACS Calibur (BD Biosciences) and classified as follows. CD45+CD3+ B220−CCR3− cells: eosinophils; CD45+CD3+ B220−CCR3+ cells: lymphocytes; CD45+CD3+ B220+CCR3− Gr-1high cells: neutrophils; and CD45+CD3− B220−CCR3− Gr-1− cells with high background intensity: monocytes. Monocytes were further confirmed with CD11a expression. Natural helper (NH) cells in BALF were examined for the expressions of lineage markers (CD3, CD4, CD8, CD19, Gr-1, Siglec F, IgE, NK1.1), Sca-1, c-Kit, and ST2. For intracellular IL-5 staining, BALF cells were incubated in the culture medium [10% (vol/vol) FCS-RPMI containing 2-mercaptoethanol, l-glutamine, penicillin, and streptomycin] for 3 h and stained surface antigens in the presence of monensin. Cells were fixed with 4% PFA, permeabilized with 0.1% saponin buffer (PBS with 0.1% saponin, 1 mM Hepes and 0.1% BSA) and stained with anti–IL-5 or control Ab (rat IgG1; BD Biosciences).

Quantitative RT-PCR. Total RNA was extracted with RNeasy Mini Kit (Qiagen) and the cDNA was synthesized using SuperScript III (Invitrogen). The expressions of genes were quantified with TaqMan Gene Expression Assays (Applied Biosystems). The result were shown as relative expression standardized with the expression of the gene-encoding eukaryotic 18S rRNA or β-actin. Specific primers and probes used for quantitative RT-PCR were Il33, Il5, Il13, Eps, Ptg2, Ccl11, Il18, Tslp, Il25, Actin-b, and 18S rRNA (Applied Biosystems).


**Fig. S1.** IL-33 induction in *Nippostrongylus brasiliensis* infected mice. (A) Total RNA was prepared from the lungs of normal (cont) or *N. brasiliensis* infected BALB/c mice and the expression level of mRNA for *Il33*, *Il5*, or *Il13* was determined by quantitative RT-PCR. Data are representative of three independent experiments and expressed as the means ± SD. *P* < 0.01, **P* < 0.001 (one-way ANOVA with Dunnett’s posttest). (B) Flow cytometric analysis of the expression of Sca-1 and ST2 by BALF cells from *N. brasiliensis* infected B6 mice at 7 d postinfection. Cells were gated on the FSC<sub>low</sub>SSC<sub>low</sub>Lin<sup>−</sup> subset.

**Fig. S2.** Intranasal administration of chitin induces IL-33 production in the lungs. (A) Chitin was intranasally administered into WT mice. IL-33 concentrations in the BALFs at indicated time points. The concentrations of IL-33 in the BALFs were normalized by the total protein concentration (*n* = 5). Data are representative of two independent experiments. (B and C) Chitin was intranasally administered into WT (*n* = 4), *Il33<sup>++</sup>* (*n* = 4), or *Il33<sup>−/−</sup>* (*n* = 5) mice. Histological analysis of the lungs was performed at the indicated time points. (Scale bars, 50 μm.) (D) Number of eosinophils in BALF cells from chitin- or glass bead- (2 mg) treated mice at indicated time points (*n* = 5). (E) The expression levels of *Il5* or *Il13* mRNA in BALF cells from *Il33<sup>++</sup>* or *Il33<sup>−/−</sup>* mice (*n* = 3). Data are expressed as the means ± SD. *P* < 0.05 (Student’s *t* test).
Fig. S3. Characterization of alveolar epithelial type II (ATII) cells. Flow cytometric analysis of the expressions of CD45, C16/32, and T1α by freshly prepared lung cells from naive B6 mice. MHC class II expression by cells in each gate was shown. CD45\(^-\)CD16/32\(^-\)T1α\(^-\) cells in red gate were sorted and stained with anti-SPC antibody and DAPI, and subjected to confocal microscopic examination. (Scale bar, 10 \(\mu\)m.)
Fig. S4. Generation of IL33−/− mice. (A) Structure of the mouse IL33 gene, the targeting construct and the disrupted gene. (B) Southern blot analysis of offspring from the heterozygote intercrosses. Genomic DNA was extracted from mouse tails, digested with PstI, separated by electrophoresis, and hybridized with the radiolabeled probe indicated in A. (C) RT-PCR for analysis of the expression levels of IL33 mRNA in the lungs from mice of indicated genotypes. Total RNAs isolated from the lungs were subjected to RT-PCR for the expression of IL33 and Actin-b. (D) Immunoblotting for analysis of the levels of IL-33 protein in the lungs from mice of indicated genotypes. Lung homogenates were immunoprecipitated with polyclonal anti–IL-33 antibody followed by immunoblotting with anti-IL-33 antibody (Nessy-1). The same homogenates were subjected to immunoblotting with anti-ERK antibody. (E) The comparison of cell populations of each subset for T cells, B cells, granulocytes, and dendritic cells in splenocytes of IL33+/+, IL33−/+ mice. (F) Splenic CD4+ T cells from IL33+/+ and IL33−/− mice were stimulated with anti-CD3 and anti-CD28. Twenty-four hours later, the culture supernatants were collected and measured concentration of IFN-γ, IL-4, and IL-13 by ELISA.
**Fig. S5.** Il33−/− mice show normal Th2 differentiation in the mLNs but reduced systemic Th2 responses to *Strongyloides venezuelensis* infection. (A) CD4+ T cells in mLNs from *S. venezuelensis* infected mice were labeled with anti-CD4 magnetic beads, purified with AutoMACS, and stimulated with plate bound anti-CD3 and anti-CD28 antibody for 24 h. Supernatants were harvested and tested for IFN-γ, IL-4, and IL-5 production by ELISA. (B and C) Il33+/+ (n = 8) and Il33−/− (n = 7) mice were infected with *S. venezuelensis*. IgE (B) or mMCP-1 (C) concentrations in the sera at indicated time points were measured by ELISA. Data are expressed as the means ± SD. *P < 0.05, **P < 0.01 (Student's T-test). (D) The numbers of eggs per gram feces were counted daily from day 6 after *S. venezuelensis* infection. Data are representative of two independent experiments and expressed as the means ± SD. *P < 0.05, **P < 0.01 (Student's t test). (E) Il33+/+ (n = 4) and Il33−/− (n = 5) mice were infected with *S. venezuelensis*. Flow cytometric analysis of CD45+ BALF cells from uninfected or *S. venezuelensis* infected mice (Sv). Numbers indicate proportion of eosinophils (box).

**Fig. S6.** Flow cytometry of BALF cells from noninfected or *S. venezuelensis* infected WT mice (n = 5). Cells were pooled in each group and gated on FSClowSSClowLin− fraction and were stained for ST2 and Sca-1. Cells in ST2+Sca-1+ fraction were examined for their expression of indicated antigens (filled histograms). Empty histograms: control antibody.