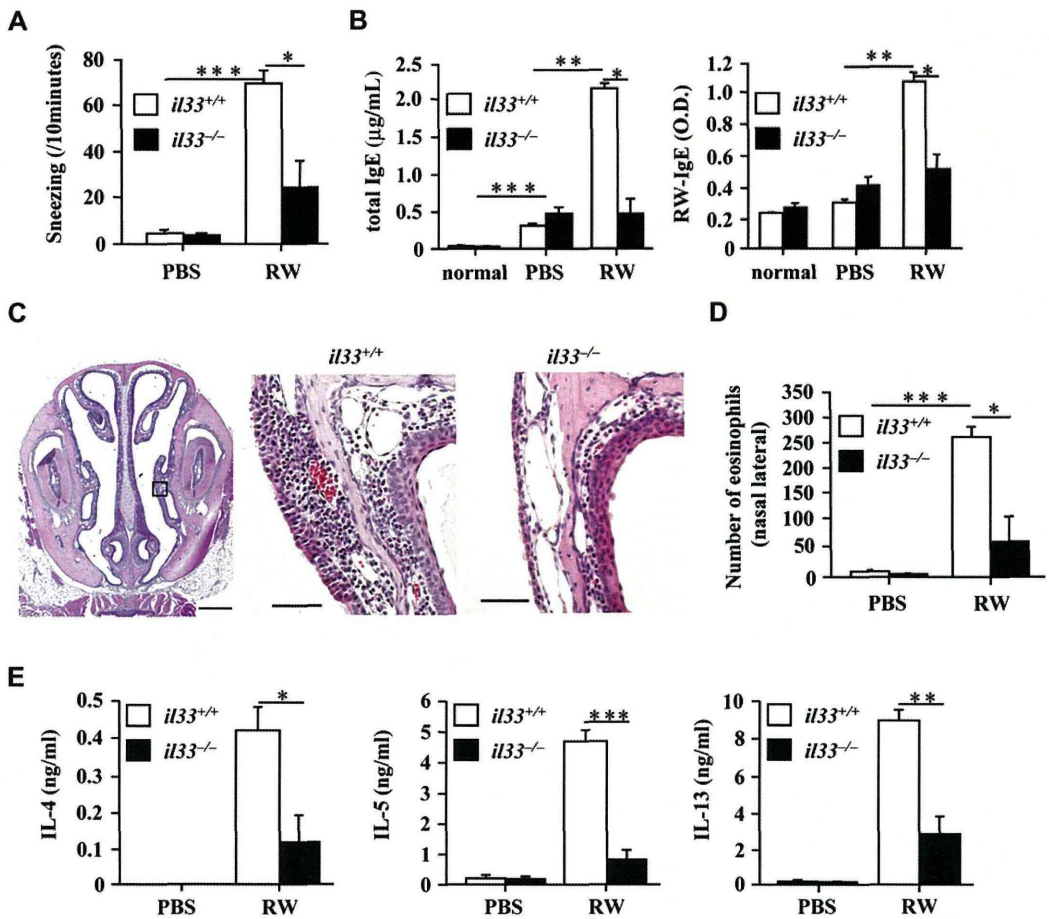

FIG 1. *il33*$^{-/-}$ mice do not induce ragweed *(RW)*–induced AR. Ragweed-immunized mice were nasally challenged with PBS or ragweed. **A,** Number of sneezes. **B,** Total and ragweed-specific IgE levels in serum. **C,** Hematoxylin and eosin staining of the nose from ragweed-immunized ragweed-challenged mice. **D,** Number of eosinophils in nasal mucosa. **E,** Cytokine production by cervical lymph node cells. Data are representative of 3 independent experiments (means and SEMs of 5 mice). *$P < .05$, **$P < .005$, and ***$P < .001$. *Bar* = 500 μm (Fig 1, *C, left*) and 50 μm (Fig 1, *C, middle* and *right*).

compared with those seen in nonimmunized *il33*$^{-/-}$ mice (Fig 1, *B*), suggesting that *il33*$^{-/-}$ mice have the capacity to develop T_H2/IgE responses on immunization but have a markedly diminished capacity to mount T_H2/IgE responses on ragweed challenge. Indeed, cervical lymph node cells from ragweed-challenged *il33*$^{-/-}$ mice showed a markedly diminished production of IL-4, IL-5, and IL-13 on stimulation with ragweed extract protein *in vitro* compared with those from *il33*$^{+/+}$ mice (Fig 1, *E*). These results clearly indicate that endogenous IL-33 contributes to induction of both early- and late-phase AR manifestation.

Exposure to ragweed pollen induced IL-33 release from the nasal epithelium

We next examined the expression of IL-33 in nasal epithelial cells and the secretion of IL-33 in response to nasal exposure to ragweed pollen. Immunohistochemical analysis revealed that IL-33 is constitutively expressed in the nucleus of nasal epithelial cells (Fig 2, *A* and *B*). IL-33 was not detected in the nasal mucosa of *il33*$^{-/-}$ mice, which indicates the specificity of this staining for IL-33 protein (Fig 2, *A*). The expression of IL-33 in the nucleus of nasal epithelial cells from mice that had been continuously challenged with ragweed pollen for 4 days was considerably lower than that from mice challenged with PBS (Fig 2, *C*), suggesting that exposure to ragweed pollen reduced IL-33 expression by causing epithelial cells to secrete IL-33.

To examine this possibility, we performed a time-course analysis of IL-33 expression in nasal epithelial cells after ragweed challenge. We found that nasal IL-33 expression was promptly downregulated and became very faint 1 hour after ragweed administration, but it recovered gradually thereafter (Fig 2, *D* and *E*). Consistent with this observation, IL-33 protein levels increased promptly in nasal lavage fluid of naive wild-type (WT) mice but not *il33*$^{-/-}$ mice after ragweed administration (Fig 2, *F*, and data not shown). Thus nasal exposure to ragweed pollen promptly induces endogenous IL-33 production from nasal epithelial cells, and resultant IL-33 subsequently contributes to induction and augmentation of AR manifestation.

IL-33 stimulated FcεRI$^+$ mast cells to increase histamine release

In patients with AR, histamine released from activated mast cells and basophils has an important role in the induction of sneezing.[29,30] Thus we next examined the role of mast cells and

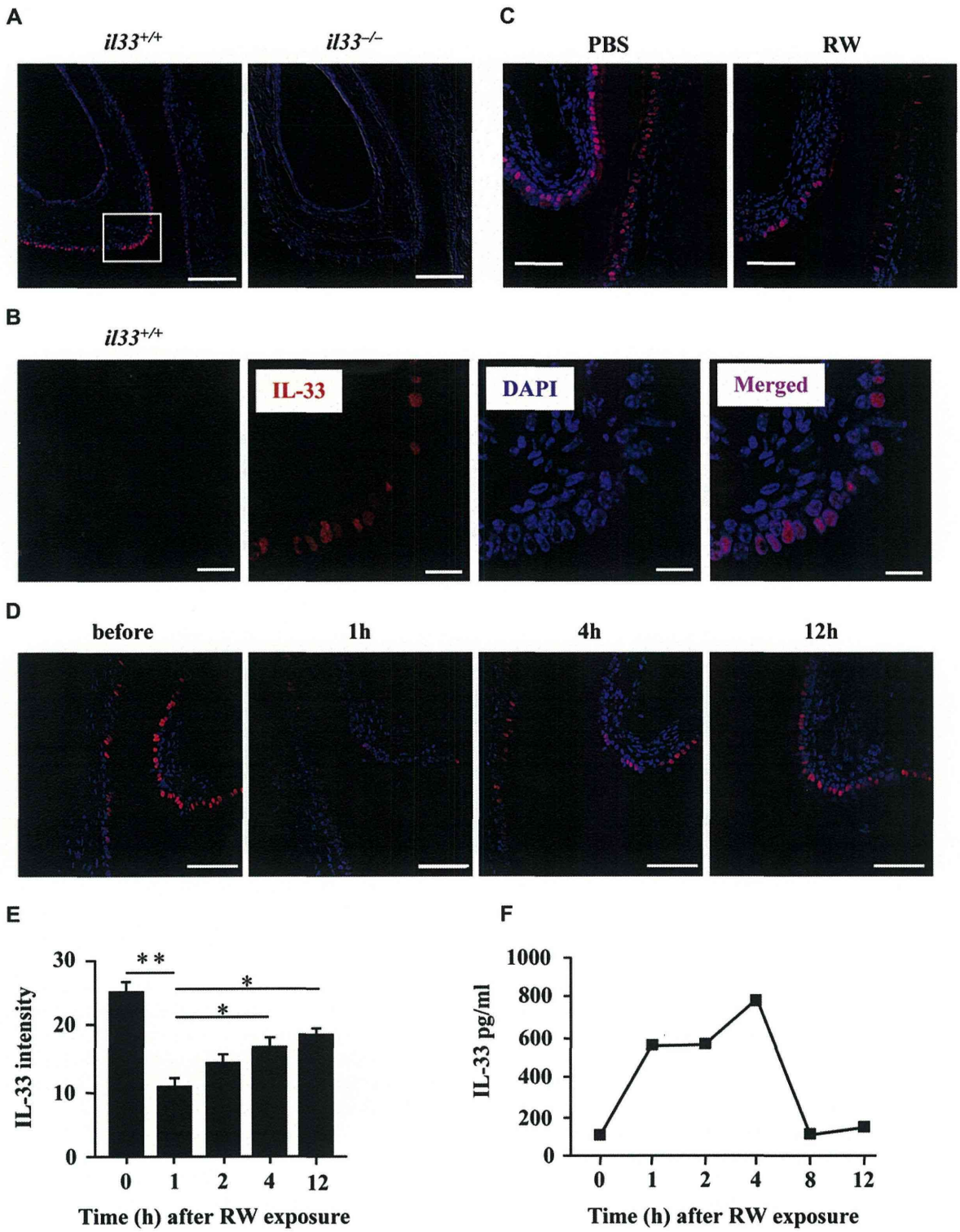

FIG 2. Nasal administration of ragweed *(RW)* pollen induces IL-33 release from nasal epithelial cells. **A-C,** Immunofluorescence staining of nose stained for IL-33 *(red)* and 4′-6-diamidino-2-phenylindole dihydrochloride *(blue)*. Fig 2, *A* and *B*, Naive nice. Fig 2, *C*, Ragweed-immunized and PBS- or ragweed-challenged mice. **D-F,** Naive WT mice were nasally administered single ragweed challenge and killed at the indicated time. Fig 2, *D*, Staining of IL-33. Fig 2, *E*, Quantitative image analysis of stained IL-33. Fig 2, *F*, IL-33 protein level in nasal lavage fluid. Data are representative of 3 independent experiments (3 mice per time point). $*P < .005$ and $**P < .0001$. *Bar* = 50 μm.

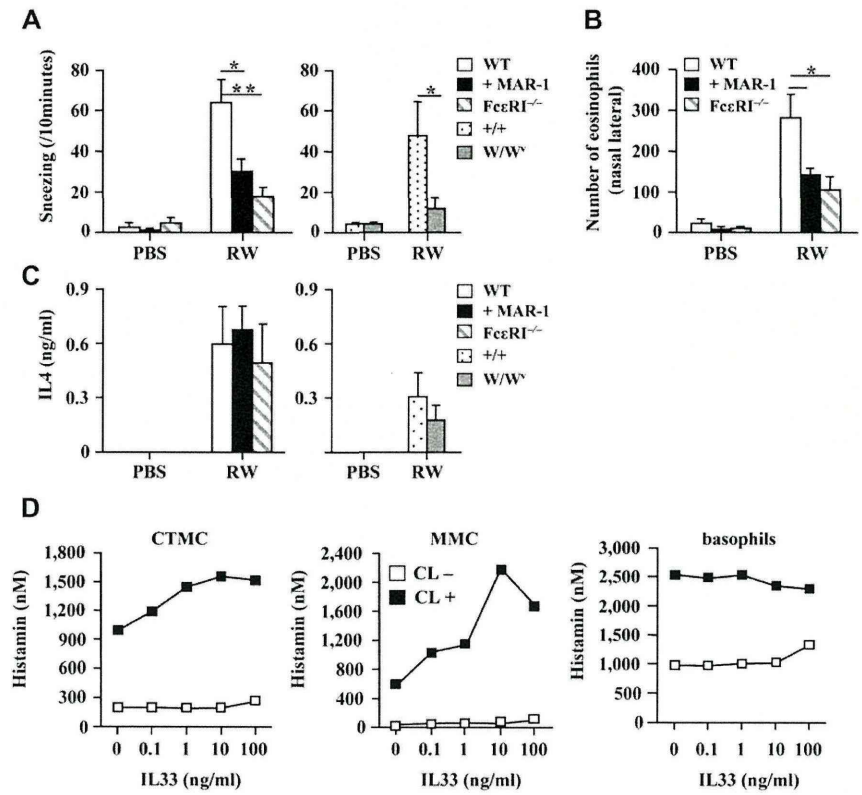

FIG 3. IL-33 induces histamine release from FcεR1$^+$ cells. **A-C,** Ragweed *(RW)*–immunized WT, basophil-depleted *(+MAR-1)*, FcεRI$^{-/-}$, WBB6F1-$^{+/+}$ *(+/+)*, and WBB6F1-W/W^v *(W/W^v)* mice were challenged with PBS or ragweed. Fig 3, *A*, Number of sneezes. Fig 3, *B*, Number of eosinophils in nasal mucosa. Fig 3, *C*, IL-4 production by cervical lymph node cells. Data are representative of 2 independent experiments (means and SEMs of 5 mice). *$P < .05$ and **$P < .01$. **D,** ELISA of histamine. *CL+*, With FcεRI cross-linkage; *CL−*, without FcεRI cross-linkage. Data are representative of 5 independent experiments.

basophils in experimental AR. For this purpose, we used mast cell–deficient WBB6F1-W/W^v mice, basophil-depleted mice,[28] and FcεRI$^{-/-}$ mice. Compared with ragweed-immunized control mice, ragweed immunization of all of these mice evidenced a significant diminishing of sneezing frequency after nasal exposure to ragweed pollen (Fig 3, *A*). In addition to sneezing, eosinophilic accumulation in the nasal mucosa was also significantly reduced in basophil-depleted and FcεRI$^{-/-}$ mice, suggesting the importance of basophils or basophils plus mast cells for eosinophilic accumulation (Fig 3, *B*). Nevertheless, cervical lymph node cells from ragweed-challenged basophil-depleted mice, FcεRI$^{-/-}$ mice, or WBB6F1-W/W^v mice produced amounts of T_H2 cytokines comparable with those seen in control mice on stimulation *in vitro* (Fig 3, *C*). These results suggest that in addition to T_H2 cells, activated FcεRI$^+$ mast cells and basophils might contribute to both early-phase (sneezing) and late-phase (eosinophilic accumulation) responses in AR.

To reveal the mechanism whereby *il33*$^{-/-}$ mice suppressed the induction of sneezing (Fig 1, *A*), we examined the role of IL-33 in histamine release from mast cells and basophils. Mast cells are generally classified into 2 populations, CTMCs and MMCs, both of which exist in the nasal membranes of patients with AR.[31-36] It is controversial whether mast cells (and if so which type) or basophils play the crucial role in AR.[31,33,35,37,38] Thus we developed and purified CTMCs, MMCs, and basophils from murine bone marrow cells (see Fig E5 in this article's Online Repository at www.jacionline.org) and examined their capacity to release histamine in response to IL-33 *in vitro*. Stimulation with IL-3 induced production of histamine from CTMCs and basophils but not from MMCs; additional IL-33 stimulation did not enhance histamine release from these cells (Fig 3, *D*). In contrast, cross-linkage of FcεRI significantly enhanced histamine release from CTMCs, MMCs, and basophils. Furthermore, IL-33 dose-dependently augmented histamine release from CTMCs and MMCs but not from basophils, although all these cells express IL-33Rα (Fig 3, *D*, and see Fig E5). These results suggest that ragweed pollen–induced endogenous IL-33 stimulates CTMCs and MMCs to increase histamine release under the condition of cross-linkage of FcεRI with IgE–ragweed pollen.

il33$^{-/-}$ mice showed diminished ragweed-induced nasal accumulation of basophils

Histologic examination revealed that there were very few basophils in the nasal mucosa of ragweed-immunized PBS-challenged mice. However, ragweed challenge significantly increased the number of basophils in the nasal mucosa at 24 hours after the final challenge, particularly in the posterior section of the nose (18.5 ± 4.5 in the anterior section compared with 34.5 ± 10.6 in the posterior section) of ragweed-immunized mice, as illustrated by immunohistochemical staining with an mAb specific for murine basophils (Fig 4, *A* and *B*).[39] We found that the degree of basophil accumulation in the nasal mucosa of *il33*$^{-/-}$ or FcεRI$^{-/-}$ mice was significantly lower than in control mice (Fig 4, *B*).

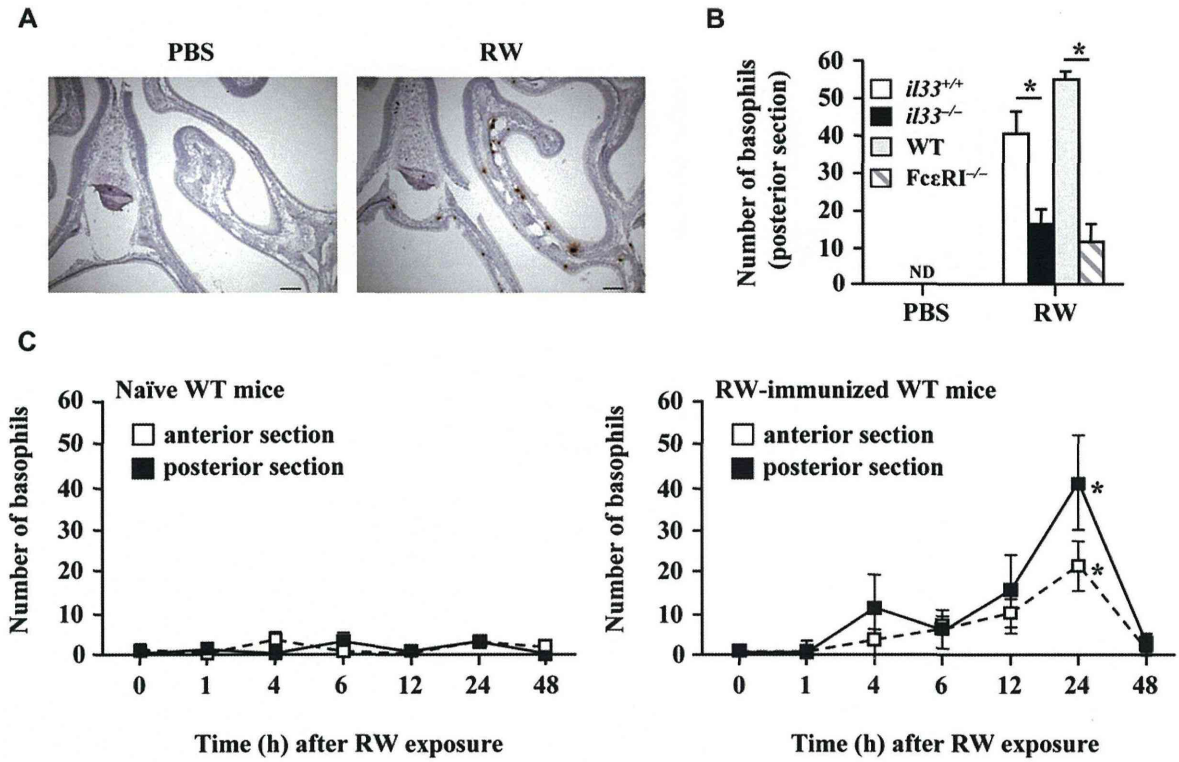

FIG 4. Ragweed *(RW)*–induced endogenous IL-33 regulates nasal accumulation of basophils. **A** and **B,** Ragweed-immunized mice were nasally challenged with PBS or ragweed. Fig 4, *A,* Immunohistochemical staining for basophils in the nose from *il33*$^{+/+}$ mice. *Bar* = 100 μm. Fig 4, *B,* Number of basophils in the nose. Fig 4, *C,* Naive or ragweed-immunized WT mice were nasally administered single ragweed. Kinetics of the number of basophils in the nose (3 mice per time point) are shown. Data are representative of 2 or 3 independent experiments. Means and SEMs of 3 mice are shown. Fig 4, *B,* **P* < .05. Fig 4, *C,* **P* < .05 compared with before ragweed exposure (0 hours).

We studied the kinetics of accumulation of basophils in the nasal mucosa after challenge with ragweed pollen. Ragweed-immunized WT mice had significantly increased basophil accumulation in the nasal mucosa, especially in the posterior section of the nose, at 4 hours, and this peaked at 24 hours after provocation; naive mice did not show this (Fig 4, *C*). Similar to basophils, the number of eosinophils in the nasal mucosa increased, whereas the number of CTMCs or MMCs decreased after nasal administration of ragweed pollen into ragweed-immunized mice (see Fig E6 in this article's Online Repository at www.jacionline.org). However, we were able to detect degranulated CTMCs in the nasal mucosa at 4 hours after ragweed challenge (data not shown), which suggests that both basophils and mast cells might be the early IL-33–responding cells. Taken together, these results suggest that basophil accumulation in AR is regulated by ragweed-induced endogenous IL-33, the IgE/FcεRI pathway, or both.

il33$^{-/-}$ mice developed AR on ragweed plus IL-33 challenge

Ragweed-immunized *il33*$^{-/-}$ mice were nasally challenged with ragweed and IL-33 to examine the role of FcεRI^{+} cells stimulated by IL-33 plus FcεRI cross-linkage *in vivo*. As seen in Fig 1, *B*, ragweed-immunized and ragweed-challenged *il33*$^{-/-}$ mice evidenced a considerably increased total IgE level but did not have AR responses (Fig 5). In contrast, ragweed-immunized and ragweed plus IL-33–challenged *il33*$^{-/-}$ mice manifested early- and late-phase nasal responses and mounted T_H2 responses (Fig 5). These results further substantiate that IL-33 plus FcεRI cross-linkage is essential to the development of AR.

IL-33 stimulated FcεRI^{+} cells to produce chemoattractants for both eosinophils and basophils

Accumulation of both eosinophils and basophils in the nasal mucosa in ragweed-immunized and ragweed-challenged *il33*$^{-/-}$ or FcεRI$^{-/-}$ mice was significantly lower than that seen in control mice (Fig 1, *D*, Fig 3, *B*, and Fig 4, *B*), which suggests that both IL-33 and FcεRI^{+} cells are essential for the recruitment of eosinophils, basophils, or both. The role of cytokines and chemokines in the accumulation of eosinophils and basophils in inflamed tissue has been well studied.[40,41] Thus we examined the capacity of CTMCs, MMCs, and basophils to produce cytokines and chemokines in response to IL-33 plus cross-linkage of FcεRI. Cross-linkage of FcεRI on CTMCs and MMCs in the presence of IL-3 markedly induced production of IL-1β and eotaxin (Fig 6). However, CTMCs and MMCs increased production of IL-9, IL-13, GM-CSF, RANTES, macrophage inflammatory protein 1α (MIP-1α), and monocyte chemotactic protein 1 (MCP-1) when additionally stimulated with IL-33 (Fig 6). As reported in our previous article,[16,28] basophils strongly produced all the cytokines and chemokines that we measured. Because one set of chemokines (eotaxin, RANTES, and MIP-1α) and the other set (RANTES, MIP-1α, and MCP-1) have been shown to act,

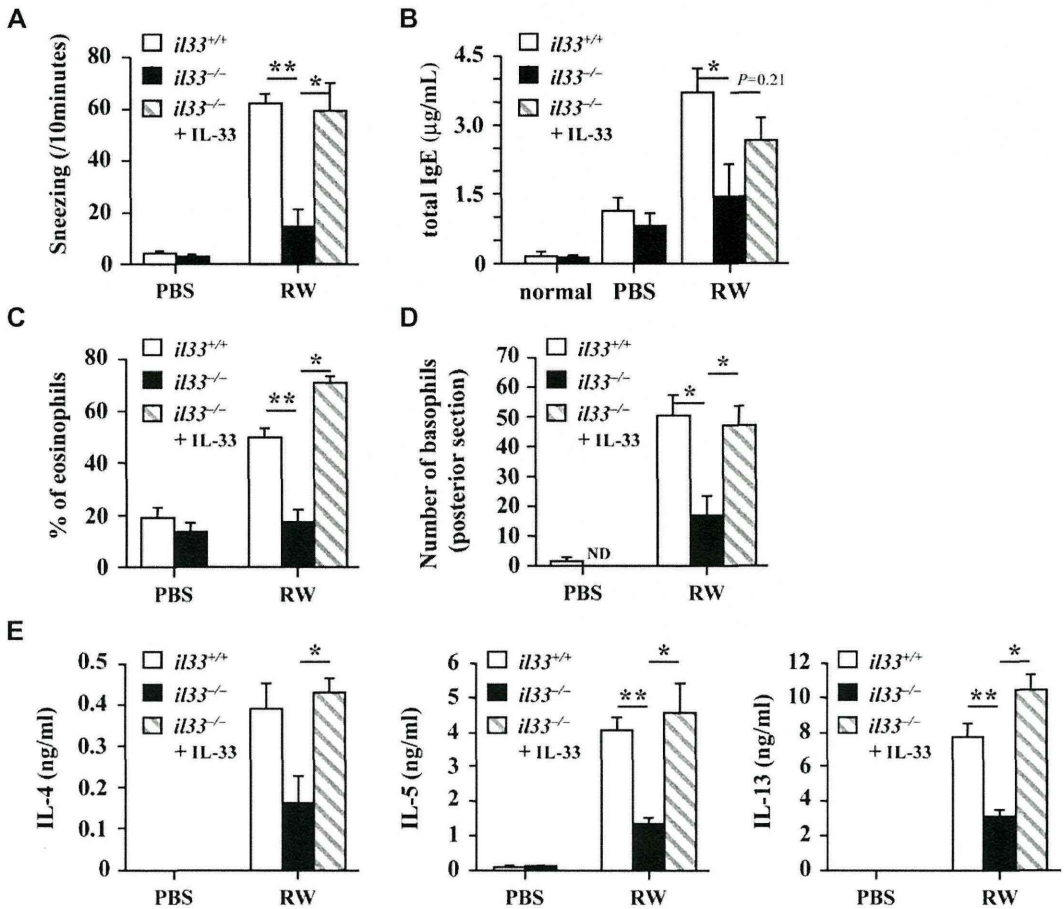

FIG 5. Ragweed *(RW)* plus IL-33–challenged $il33^{-/-}$ mice developed AR. Ragweed-immunized mice were nasally challenged with PBS or ragweed ± IL-33 (1 μg). **A,** Number of sneezes. **B,** Total IgE levels in serum. **C,** Percentage of eosinophils in cervical lymph nodes. **D,** Number of basophils in nasal mucosa. **E,** Cytokine production by cervical lymph node cells. Data are means and SEMs of 5 mice. $*P < .05$ and $**P < .005$.

respectively, as eosinophil[41,42] and basophil[40,43,44] chemotactic factors, ragweed pollen–driven endogenous IL-33 seems to play an important role in the recruitment of eosinophils and basophils by inducing the production of chemoattractants for both these types of cells. In addition, ragweed-immunized WT mice induced the expression of genes encoding MIP-1α and MCP-1 in the nasal mucosa, which peaked at 4 hours and decreased gradually after the ragweed challenge, whereas ragweed-immunized $il33^{-/-}$ mice did not do so (data not shown). These results further substantiate that ragweed pollen–driven endogenous IL-33 contributes to the temporal recruitment of inflammatory cells into the nasal mucosa through the induction of chemoattractants.

IL-33 expression was diminished in nasal epithelial cells of patients with AR

Finally, IL-33 expression was analyzed in nasal epithelial cells taken from patients with AR and healthy control subjects to determine the relevance of the findings in the AR murine model to patients with AR (see Tables E1 and E2). Although IL-33 protein was strongly expressed in the nucleus of nasal epithelial cells from healthy subjects, diminished or even undetectable expression of IL-33 was found in nasal epithelial cells in patients with AR (Fig 7, *A*). A quantitative analysis showed significantly reduced IL-33 expression in nasal epithelial cells in patients with AR (Fig 7, *B*). On the other hand, compared with healthy control subjects, IL-33 mRNA expression in nasal epithelial cells from patients with AR significantly increased during the pollen season (Fig 7, *C*). These results indicate that as in the AR murine model, IL-33 protein expression was significantly reduced in inflamed nasal epithelial cells in patients with AR. However, IL-33 mRNA expression in nasal epithelial cells from patients with AR was considerably upregulated. Taken together, these results indicate the involvement of nasal IL-33 in the induction of AR.

DISCUSSION

We first demonstrated that compared with control mice, ragweed-immunized and ragweed-challenged $il33^{-/-}$ mice showed a significant reduction in the frequency of sneezing, total and ragweed-specific IgE response, and accumulation of eosinophils and basophils in the nasal mucosa. These mice evidenced a diminished capacity of their cervical lymph node T cells to produce T_H2 cytokines *in vitro*. Furthermore, histologic examination revealed only modest changes in the noses of ragweed-immunized, ragweed-challenged $il33^{-/-}$ mice. Thus IL-33 is an essential molecule in the development of ragweed-induced AR. In addition to these results, endogenous IL-33 is critically involved in the development of ovalbumin (OVA)–specific

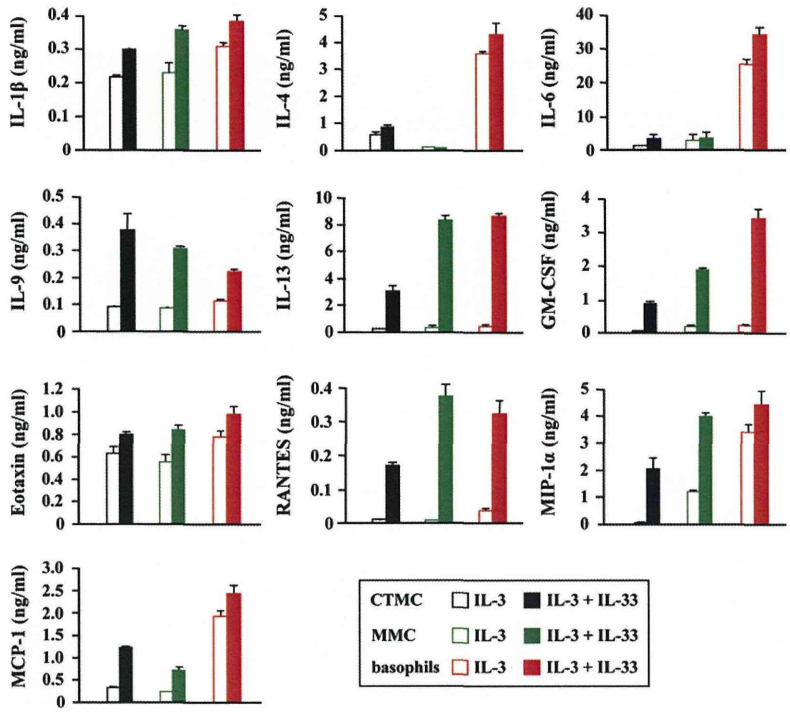

FIG 6. IL-33 induces the production of chemoattractants for eosinophils and basophils from FcεRI$^+$ cells. Cytokines and chemokines produced by CTMCs, MMCs, or basophils stimulated for 16 hours with IL-3 alone or IL-3 plus IL-33 with FcεRI cross-linkage. Data are representative of 3 independent experiments (means and SEMs).

T_H2-type immune responses,[45,46] which suggests that IL-33 is not specific to the ragweed-induced response.

Recruitment of T_H2 cells to the site of allergen challenge is a key step in the induction of AR. We found that AR mice showed increased capacity of their cervical lymph node T cells to produce T_H2 cytokines. Furthermore, we could detect $CD4^+ST2^+$ T cells (T_H2 cells)[47] in the nasal mucosa in ragweed-immunized, ragweed-challenged *il33*$^{+/+}$ mice but not in *il33*$^{-/-}$ mice (data not shown). The mechanism underlying reduced T_H2/IgE responses on ragweed pollen challenge in *il33*$^{-/-}$ mice is not clear. We propose the possibility that ragweed-induced endogenous IL-33 might enhance interaction between antigen-presenting cells and T_H2 cells in the cervical lymph nodes. Alternatively, IL-33 might enhance recruitment of T_H2 cells into the cervical lymph nodes. Indeed, it has been reported that IL-33 is a selective T_H2 cell chemoattractant.[48]

Next, we demonstrated that IL-33 protein is constitutively expressed in the nucleus of nasal epithelial cells and that these IL-33 expressions diminished within 1 hour after exposure to ragweed pollen. By contrast, IL-33 protein levels promptly increased in nasal lavage fluid. This is the first report to demonstrate that pollen grains induce IL-33 protein *in vivo*. At the same time, we were able to reveal constitutive IL-33 expression in the nasal epithelial cells of healthy control subjects and downregulated expression of IL-33 in inflamed nasal epithelial cells of patients with AR. However, IL-33 mRNA expression in nasal epithelial cells from patients with AR was not reduced but significantly upregulated during the pollen season, suggesting that enhanced extracellular IL-33 release was associated with reduced IL-33 protein expression in inflamed nasal epithelial cells, as in the AR murine model. We previously reported that the serum level of IL-33 is significantly increased in patients with AR,[25] which suggests the importance of measuring IL-33 levels in nasal lavage fluid after provocation of allergens in patients with AR.

Unlike IL-1β and IL-18, full-length IL-33 has biological activity and loses its activity after cleavage with caspases.[11] It is believed that epithelial cells produce IL-33 when they become necrotic or injured.[12] At present, we have no data about necrosis of epithelial cells by ragweed pollen. Recently, Kouzaki et al[49] demonstrated that in response to a fungal antigen, *Alternaria alternata*, bronchial epithelial cells translocate nuclear IL-33 and actively release full–length proform IL-33. Ragweed pollen is known to contain antigenic and enzymatic proteins.[50] Further study is needed to define the mechanisms for IL-33 release by ragweed pollen.

We demonstrated the importance of basophils and mast cells in the induction of AR. Histamine released from activated mast cells and basophils has been recognized as one of the most important chemical mediators for sneezing in patients with AR.[29,30] We demonstrated that IL-33, together with cross-linkage of FcεRI, dose-dependently increased the production of histamine by CTMCs and MMCs but not by basophils. However, human basophils increase further histamine release on additional IL-33 stimulation.[51] Thus endogenous IL-33 enhances AR by stimulation of FcεRI$^+$ cells, and it becomes a therapeutic target molecule. Indeed, we found that treatment with recombinant human ST2-Fc chimera protein (the decoy receptor of IL-33)[9,52] into ragweed-immunized mice during ragweed challenge significantly reduced the frequency of sneezing (data not shown).

Several reports have shown that the numbers of CTMCs, MMCs, and basophils are increased, decreased, or unchanged in the nasal mucosa of patients with AR examined after nasal allergen provocation or during the pollen season.[31,33,35,37,38] In our AR murine model the numbers of CTMCs or MMCs in the

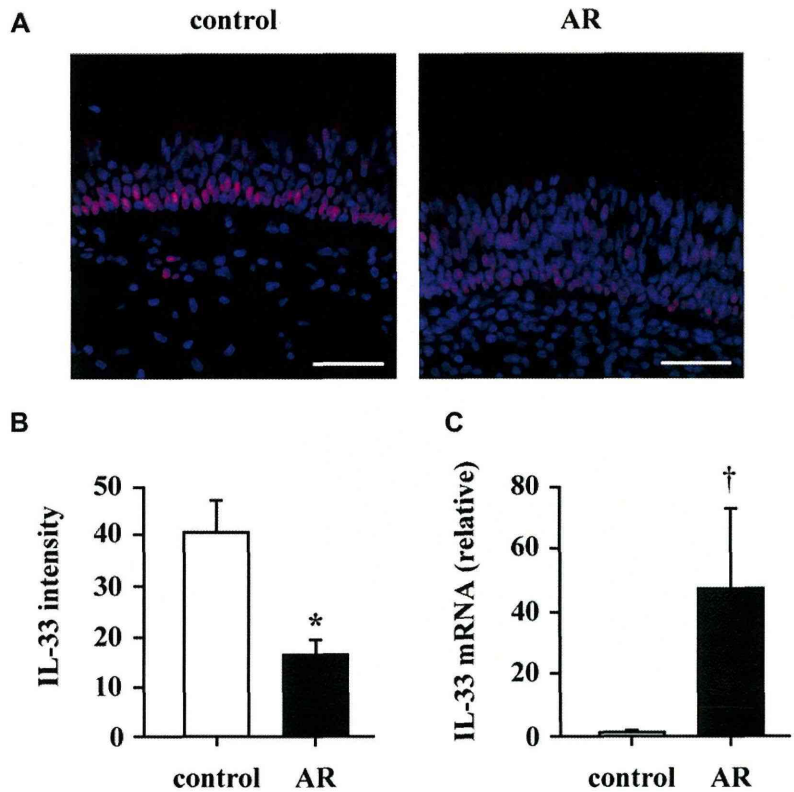

FIG 7. IL-33 is released from nasal epithelial cells in patients with AR. **A,** Immunofluorescence staining of nasal mucosal specimens from control subjects and patients with AR (see Table E1) stained for IL-33 *(red)* and 4′-6-diamidino-2-phenylindole dihydrochloride *(blue)*. *Bar* = 50 μm. Representative results from 5 healthy subjects and 10 patients with AR are shown. **B,** Quantitative image analysis of stained IL-33 in nasal mucosa. **P* < .05. **C,** Relative IL-33 mRNA expression in nasal epithelial cells from control subjects and patients with AR (see Table E2), as determined by using real-time PCR. †*P* < .02 compared with control subjects (Mann-Whitney *U* test).

nasal mucosa were somewhat decreased after nasal administration of ragweed pollen. To examine the number of basophils in the nasal mucosa of AR mice, we used TUG8, a recently established mAb that recognizes basophil-specific murine mast cell protease 8 (mMCP-8).[39] We found that the number of mucosal $TUG8^+$ basophils was significantly increased after nasal ragweed challenge; this suggests a recruitment of basophils from the circulating blood or memory basophils in the bone marrow. It has been reported in patients with seasonal AR after allergen challenge that the number of basophils increased significantly in the nasal mucosa, whereas the number of blood basophils decreased,[31,33] which supports an influx of basophils from the blood into the nasal mucosa. Importantly, the nasal accumulation of basophils was observed in ragweed-immunized mice but not in naive mice after provocation by ragweed pollen; this further substantiates that $Fc\varepsilon RI^+$ cells stimulated by IL-33 plus FcεRI cross-linkage are essential to the recruitment of basophils by producing chemoattractants for basophils.

Recently, 3 groups, including ours, have independently demonstrated that basophils are antigen-presenting cells that are necessary and sufficient for T_H2 priming both *in vitro* and *in vivo*.[28,53,54] In the present study, however, cervical lymph node cells from ragweed-challenged basophil-depleted mice produced almost the same amounts of IL-4 as control mice on stimulation *in vitro*, which suggests that basophils are not required for ragweed-induced T_H2 cell differentiation under these circumstances.

In summary, we established a novel ragweed-specific AR murine model, which could be very useful in the development of antiallergic drugs for AR. Also, we demonstrated that IL-33, promptly released from nasal epithelial cells in response to exposure to ragweed pollen, is essential for sneezing and the accumulation of eosinophils and basophils in the nasal mucosa by increasing histamine release and inducing production of chemoattractants from $Fc\varepsilon RI^+$ mast cells and basophils, respectively. IL-13 produced by IL-33–stimulated T_H2 cells, CTMCs, MMCs, and basophils induces goblet cell hyperplasia (see Fig E7 in this article's Online Repository at www.jacionline.org). This process, together with the contribution of IL-33 to stimulation of eosinophils,[19,20,55] basophils, and mast cells[16,18,51] to produce allergic inflammatory mediators, might lead to the recurrent seizures and irreversible mucosal hypertrophy seen in patients with AR. Thus IL-33 might present an important therapeutic target for the prevention of AR.

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Clinical implications: The discovery of ragweed pollen–driven endogenous IL-33 as a critical factor for the development of early- and late-phase responses in patients with AR might create a new therapeutic strategy for AR.

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METHODS

Mice

BALB/c mice were purchased from Charles River Laboratories Japan, Inc (Yokohama, Japan). The generation of *il33*$^{-/-}$ mice is detailed in our separate report.[E1] *il33*$^{-/-}$ mice (129SvJ × C57BL/6) were backcrossed for 7 generations onto BALB/c mice, and their littermate controls (*il33*$^{+/+}$) were used for the experiments. Mice with a disruption in the α subunit of the high-affinity IgE receptor (FcεRI$^{-/-}$ mice, BALB/c background mice)[E2] were purchased from Jackson Laboratories (Bar Harbor, Me). Mast cell–deficient WBB6F1-W/W^{v} mice and littermate control WBB6F1$^{+/+}$ mice were purchased from SLC Japan (Hamamatsu, Japan). All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine.

Human nasal mucosal specimens

Normal nasal mucosal specimens were obtained from the inferior turbinates of 5 patients who had undergone submucosal turbinectomy, rhinoplasty, total maxillectomy, or extirpation of the inferior turbinates. Allergic nasal mucosal specimens were obtained by means of submucosal turbinectomy from 10 patients with AR. The specimens were fixed in 10% buffered formalin at room temperature overnight and then embedded in paraffin. Patients with AR were recruited from the University Hospital, Kyoto Prefectural University of Medicine. Specific IgE to 7 aeroallergens, *Dermatophagoides pteronyssinus* (house dust mite), Japanese cedar (JC), Japanese cypress, cocksfoot, gray alder, common ragweed, and mugwort, were measured with the Pharmacia CAP System (Pharmacia CAP, Uppsala, Sweden; Table E1). Positive sensitization was signified by an allergen-specific serum IgE level of greater than 0.7 (CAP RAST score, 2). Demographic and clinical characteristics of the patients are summarized in Table E1. All patients had a history of persistent (>2 years) perennial nasal symptoms (sneezing, nasal discharge, and nasal obstruction) and specific IgE against house dust mites in their sera. Healthy subjects who had never had symptoms of AR and indicated no sensitization to the allergens were recruited (Table E1). All subjects were Japanese and provided written informed consent to participate in the study, according to the rules of the process committee at the Kyoto Prefectural University of Medicine.

Human nasal epithelial cells

Human nasal epithelial cells were collected from 11 control subjects and 13 patients with JC pollinosis by brushing the inferior turbinates with a CytoSoft Cytology Brush (Medical Packaging, Camarillo, Calif) without anesthesia. The CytoSoft brush with the scraped cells was rinsed with Lysis buffer of the RNeasy Mini Kit (Qiagen, Germantown, Mass), and the total RNA was immediately isolated. All the subjects were residents of Eiheiji-cho, Fukui Prefecture, Japan, and were recruited in mid-March 2010. Specific IgE to 7 aeroallergens, JC, *D pteronyssinus* (house dust mite), *Dermatophagoides farinae*, cocksfoot, *Candida albicans*, *Aspergillus fumigatus*, and common ragweed, were measured with the Pharmacia CAP System (Table E2). Positive sensitization was signified by an allergen-specific serum IgE level of greater than 0.7 (CAP RAST score, 2). Diagnosis of JC pollinosis was confirmed by symptoms of allergic rhinoconjunctivitis during the JC pollinosis season and specific IgE against JC in the sera. Healthy subjects were recruited who had never had symptoms of AR and indicated no sensitization to the allergens (Table E2). All subjects were Japanese and provided written informed consent to participate in the study in accordance with the rules of the process committee at the School of Medicine, University of Fukui.

Reagents

Recombinant murine IL-33 and polyclonal rabbit IgG antibody to murine IL-33, which we prepared in our laboratory, as described previously,[E3,E4] were used. Polyclonal rabbit IgG antibody to human IL-33 was purchased from MBL (Nagoya, Japan). Phycoerythrin (PE)–anti-mouse c-Kit, PE–anti-mouse Siglec-F (E50-2440), and biotin–anti-mouse IgE (R35-118) were purchased from BD Biosciences (San Diego, Calif). Fluorescein isothiocyanate (FITC)–anti-mouse ST2 (IL-33Rα chain) was purchased from MD Biosciences (St Paul, Minn). Biotin–anti-mouse FcεR1α (MAR-1) and streptavidin-allophycocyanin were purchased from eBioscience (San Diego, Calif). Anti-CD16/32 (FcγRII/III) was purchased from BioLegend (San Diego, Calif). Rat anti-mouse IgE (23G3) and affinity-purified goat anti-mouse IgG_1 were purchased from Southern Biotechnology Associates, Inc (Birmingham, Ala). An mAb specific for murine mMCP-8 (clone, TUG8)[E5] was kindly provided by Professor Hajime Karasuyama (Tokyo Medical and Dental University Graduate School, Tokyo, Japan). Ragweed pollen was purchased from PolyScience (Niles, Ill). Ragweed extract was purchased from LSL Co Ltd (Tokyo, Japan). Murine IL-3, IL-4, and stem cell factor and FITC–anti-mouse CCR3 were purchased from R&D Systems (Minneapolis, Minn). Anti–2,4-dinitrophenyl (DNP) IgE mAb and OVA (grade V) were purchased from Sigma-Aldrich (St Louis, Mo). DNP-conjugated OVA was prepared in our laboratory.

Nasal lavage fluid

Naive or ragweed-immunized mice were nasally administered with single ragweed pollen (1 mg in 20 μL of PBS) and then killed 1, 2, 4, 8, 12, 24, and 48 hours after exposure for histologic examination and collection of nasal lavage fluid. The noses were lavaged with 150 μL of PBS twice, and supernatants of the nasal lavage fluid were prepared by means of centrifugation and stored at −80°C until further analysis.

In vitro cytokine and histamine production

Cervical lymph node cells were isolated from mice and cultured for 5 days in 96-well plates at 2×10^5 per 0.2 mL per well with IL-2 (100 pmol/L) and ragweed extract (5 μg/mL) in the presence of 1×10^5 irradiated conventional antigen-presenting cells (T cell–depleted BALB/c splenic cells) in RPMI 1640 supplemented with 10% FBS, 2–mercaptoethanol (50 μmol/L), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL). Bone marrow–derived CTMCs, MMCs, or basophils (5×10^4 per 0.2 mL per well), prepared and sorted as described below, were stimulated with IL-3 (20 U/mL) alone or IL-3 plus IL-33 (100 ng/mL) with or without FcεRI cross-linkage. For FcεRI cross-linkage, sorted CTMCs, MMCs, and basophils were sensitized with a murine IgE anti–DNP (1 μg/mL) for 1 hour and cultured with DNP-OVA (10 μg/mL) in the presence of IL-3 alone or IL-3 plus IL-33 for 5 (for histamine release) or 16 (for cytokine production) hours. Supernatants were collected, and cytokine or histamine production was assessed by using ELISA kits (R&D Systems) or the Bio-Plex System (Bio-Rad, Hercules, Calif) or the histamine ELISA kit (Immunotech, Marseille, France), respectively.

Flow cytometry and cell purification

Bone marrow–derived CTMCs, MMCs, and basophils were prepared as described previously.[E3,E6,E7] Briefly, bone marrow cells cultured with IL-4 (10 ng/mL) plus stem cell factor (100 ng/mL) or IL-3 (10 U/mL) in complete RPMI 1640 medium for 30 or 14 days, respectively, were washed twice. Cells were first treated for 30 minutes at 4°C with anti-FcγRII/III (10 μg/mL), followed by treatment for 2 hours at 4°C with biotin–anti-mouse FcεRIα (5 μg/mL) in staining buffer (1% [vol/vol] FCS in PBS). After being washed twice, cells were stained for 30 minutes with streptavidin-allophycocyanin and PE–anti-mouse c-Kit. Samples were separated into FcεRI^{+}c-Kit^{+} cells (mast cells) or FcεRI^{+}c-Kit^{-} cells (basophils) by using a fluorescence cell sorter (FACS Aria; BD Biosciences). The purity of each population was greater than 97%. Resultant populations were further stained with FITC–anti-mouse ST2 (IL-33Rα chain). For analysis of eosinophils in the cervical lymph nodes, cells were harvested and examined for their expression of Siglec-F^{+}CCR3^{+} cells gated on side scatter (SSC)^{high} cells[E4] by FACSCalibur (BD Biosciences).

Histology

After stripping the facial skin, the mouse heads were severed between the upper and lower jaws, and noses were removed. Samples were immediately fixed in 4% paraformaldehyde at 4°C for 3 days and decalcified in 0.12 mol/L EDTA solution (pH 6.5) for 7 days at room temperature. The EDTA solution was changed daily. After decalcification, tissues were embedded in paraffin, cut into 4-μm coronal sections, and stained with hematoxylin and eosin, periodic acid–Schiff, toluidine blue, or Alcian blue. Cytospin preparations of sorted CTMCs, MMCs, and basophils were stained with Diff–Quik or toluidine blue. Immunohistochemical staining for basophils was carried out according to a previous report.[E5] Tissue sections were first treated with

microwave radiation, followed by incubation with methanol containing 0.3% H_2O_2 to inhibit endogenous peroxidase and with a protein-blocking solution containing 0.25% casein (Dako, Carpinteria, Calif) to prevent the nonspecific binding of antibodies. Then they were incubated with anti–mMCP-8 (TUG8; 2.5 μg/mL) at 4°C overnight, followed by horseradish peroxidase–conjugated goat anti-rat IgG (diluted 1:500). The sections were subsequently incubated in 3′-diaminobenzidene tetrahydrochloride solution (Dako) and counterstained with hematoxylin.

Confocal microscopy

Frozen sections from freshly isolated nasal specimens were fixed and incubated with FITC–anti-mouse ST2 and PE–anti-mouse Siglec-F at 4°C overnight. For IL-33 protein staining, samples were incubated in 4% paraformaldehyde at 4°C overnight. Paraffin-embedded sections (4 μm thick) of the nose were deparaffinized, heated in a microwave oven (500 W for 5 minutes 3 times) in citrate buffer (pH 6.0) for antigen retrieval, and then cooled at room temperature for 50 minutes before blocking. The sections were incubated in PBS containing 1.0% BSA and 0.05% Tween 20 for blocking. The sections were incubated with primary antibody, purified polyclonal rabbit IgG antibody to murine IL-33 or human IL-33 at 4°C overnight, and then secondary antibody, biotin-conjugated goat antibody against rabbit IgG (Vector Laboratory, Burlingame, Calif), at room temperature for 30 minutes. Sections were stained with a tertiary antibody, Alexa Fluor 555–conjugated streptavidin (Invitrogen, Carlsbad, Calif), at room temperature for 30 minutes. Cover slips were applied along with mounting medium containing 4′-6-diamidino-2-phenylindole dihydrochloride (Invitrogen), and the sections were examined under a Zeiss LSM 510 microscope (Carl Zeiss, Thornwood, NY). Computer software, Zeiss LSM 510 ver. 3.2, was used for image processing and analysis.

Quantification of immunohistochemical examination

Quantitative image analysis of immunohistochemically stained IL-33 in the nasal membrane was performed as described previously.[E8] Briefly, the images of IL-33 staining in murine nasal turbinates captured by the Zeiss LSM 510 microscope were stored in monotonous color. The mean gray value of 10 randomly selected areas (20 μm × 20 μm) per sample was measured with the freeware ImageJ 1.42q downloaded from the National Institutes of Health Web site (http://rsb.info.nih.gov/ij) and analyzed as the mean ± SEM.

ELISA assay

Total IgE levels were measured by using ELISA, as described previously.[E9] Biotin-conjugated ragweed extract was prepared in our laboratory to detect ragweed-specific IgE in sera.[E4] IL-33 in the nasal fluid was analyzed with ELISA (R&D Systems), according to the manufacturer's directions.

Quantitative PCR analysis

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen), and the cDNA was synthesized with SuperScript III RNase H Reverse Transcriptase (Invitrogen, Carlsbad, Calif). For quantitative PCR analysis, cDNA fragments were amplified with the TaqMan Universal Master Mix and the Assay-on-Demand Gene Expression Kit (Applied Biosystems, Foster City, Calif). Gene expression was quantified with the TaqMan Gene Expression Assay (Applied Biosystems). The IL-33 mRNA expression level in each subject was normalized to the expression level of the gene encoding glyceraldehyde-3-phosphate dehydrogenase. The values were shown as relative to the average IL-33 mRNA expression of control.

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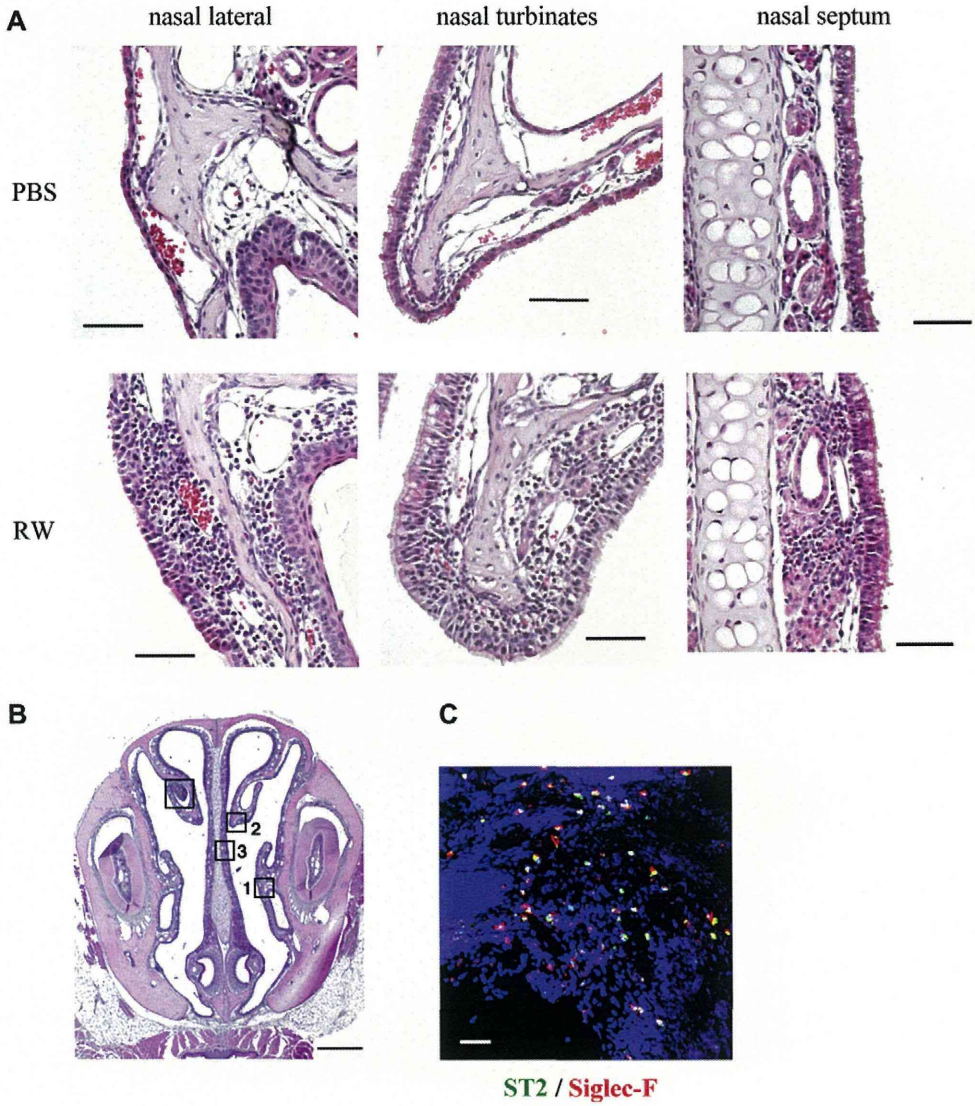

FIG E1. Ragweed *(RW)*–immunized and ragweed-challenged mice induce nasal accumulation of eosinophils. **A,** Coronal sections of noses from *il33*$^{+/+}$ mice immunized with ragweed and nasally challenged with PBS *(top row)* or ragweed *(bottom row)* were stained with hematoxylin and eosin. Nasal sections from the lateral mucosa *(left)*, turbinates *(middle)*, and septum *(right)* are shown. *Bar* = 50 μm. Representative results from 15 to 20 noses per group are shown. **B,** Coronal section of nose from a ragweed-immunized, ragweed-challenged *il33*$^{+/+}$ mouse was stained with hematoxylin and eosin. The numbered squares indicate, the areas of the nasal lateral mucosa *(1)*, nasal turbinates *(2)*, and nasal septum *(3)*, respectively. *Bar* = 500 μm. **C,** Immunofluorescence staining of a frozen nose from a ragweed-immunized, ragweed-challenged *il33*$^{+/+}$ mouse stained for ST2 *(green)* and Siglec-F *(red)*. *Bar* = 100 μm.

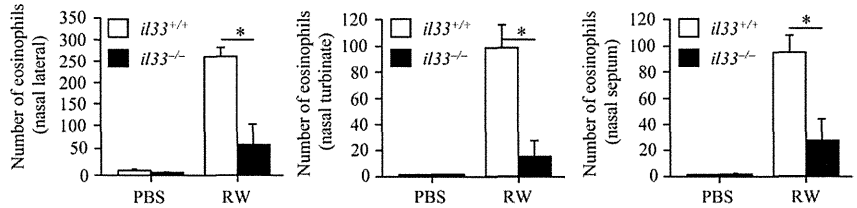

FIG E2. *il33*$^{-/-}$ mice diminish nasal accumulation of eosinophils. *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. The number of eosinophils in the nasal lateral mucosa, nasal turbinate, and nasal septal mucosa was counted. Data are representative of 3 independent experiments (means and SEMs of 5 mice). *$P < .05$.

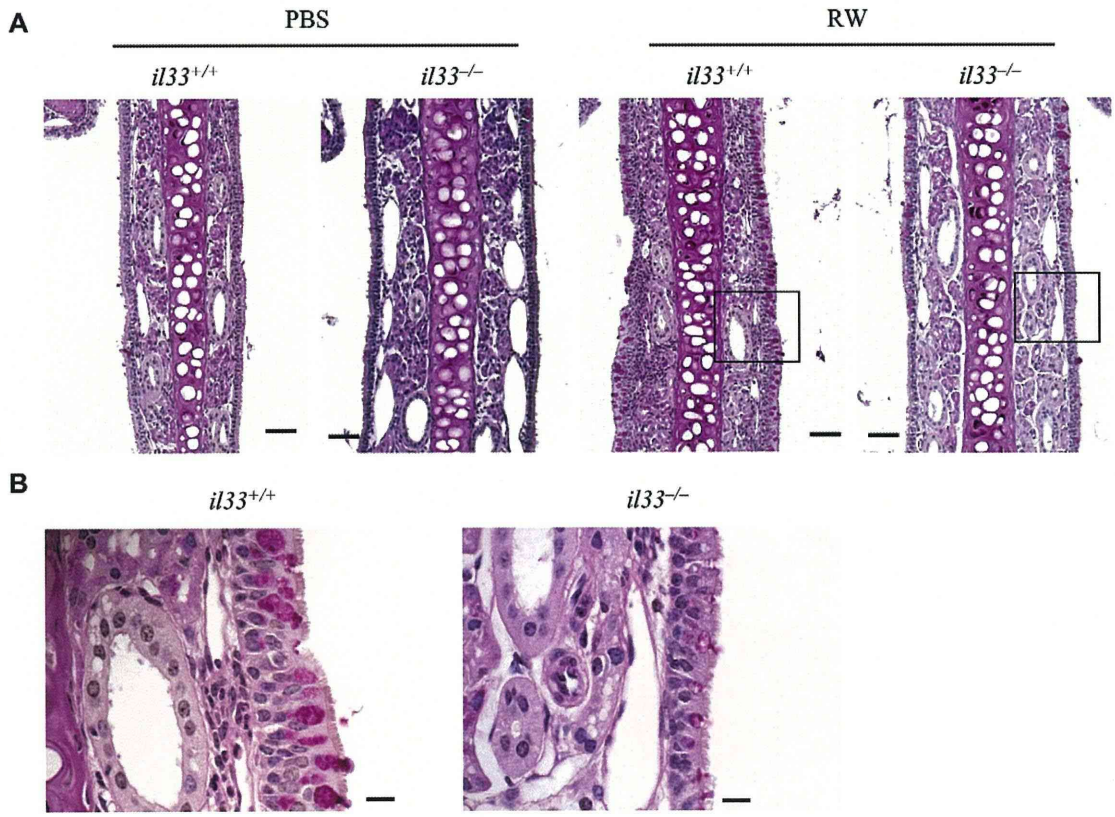

FIG E3. *il33*$^{-/-}$ mice diminish the degree of multilayered epithelial formation and goblet cell hyperplasia in the nasal mucosa. **A** and **B,** *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. Twenty-four hours after the last challenge, noses were removed from each group of mice, fixed in paraformaldehyde, decalcified, cut into 4-μm coronal sections, and stained with periodic acid–Schiff. Fig E3, *A*, *Bar* = 50 μm. Fig E3, *B*, Image zoomed from indicated areas. *Bar* = 10 μm.

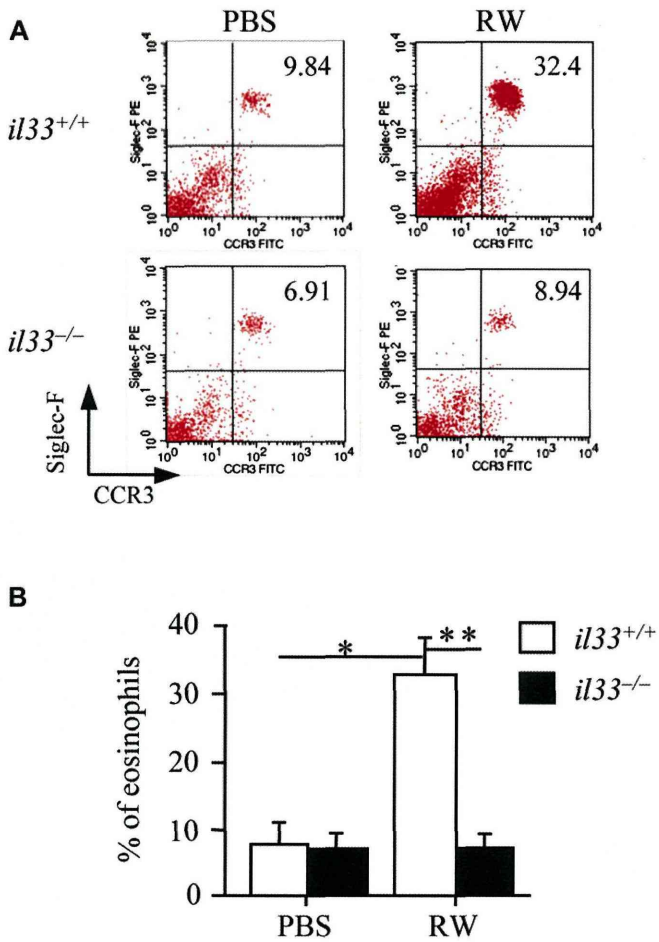

FIG E4. *il33*$^{-/-}$ mice do not induce eosinophilic accumulation in cervical lymph nodes. *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. **A,** Flow cytometric analysis of SSC^{high}Siglec-F^{+}CCR3^{+} eosinophils in cervical lymph node cells. Numbers indicate the percentage of SSC^{high}Siglec-F^{+}CCR3^{+} cells. Representative results from 16 to 20 cervical lymph nodes per group are shown. **B,** Percentage of SSC^{high}Siglec-F^{+}CCR3^{+} eosinophils in cervical lymph node cells. Data are representative of 3 independent experiments (means and SEMs of 5 mice). *$P < .005$ and **$P < .001$.

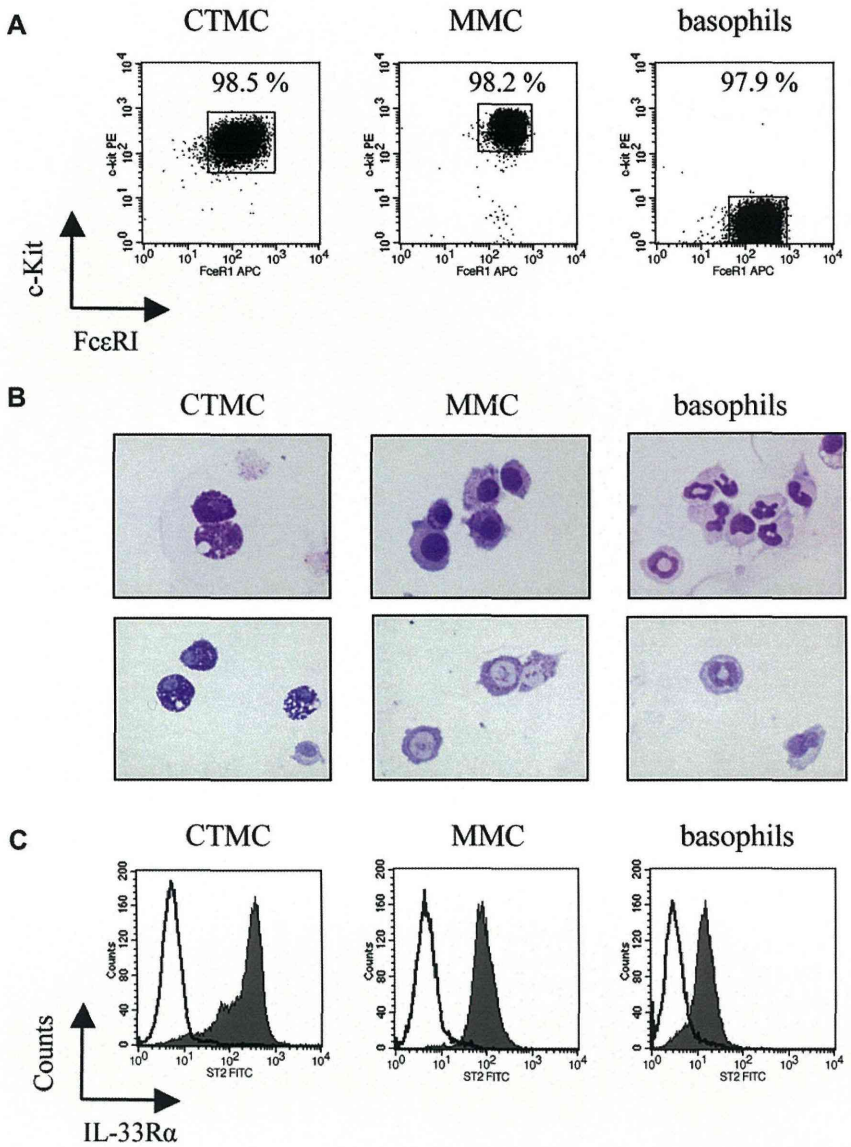

FIG E5. Preparation and characterization of bone marrow-derived CTMCs, MMCs, and basophils. **A** and **B,** Flow cytometric analysis of the expression of FcεRI and c-Kit by bone marrow–derived and sorted CTMCs, MMCs, and basophils. Fig E5, *A, Numbers above the outlined areas* indicate percentages of cells. Fig E5, *B,* Diff–Quik *(upper)* and toluidine blue *(lower)* staining of sorted CTMCs, MMCs, and basophils (original magnification ×400). **C,** Flow cytometric analysis of the expression of the IL-33Rα chain by sorted CTMCs, MMCs, and basophils. *Filled histograms,* IL-33Rα chain; *lines,* unstained cells.

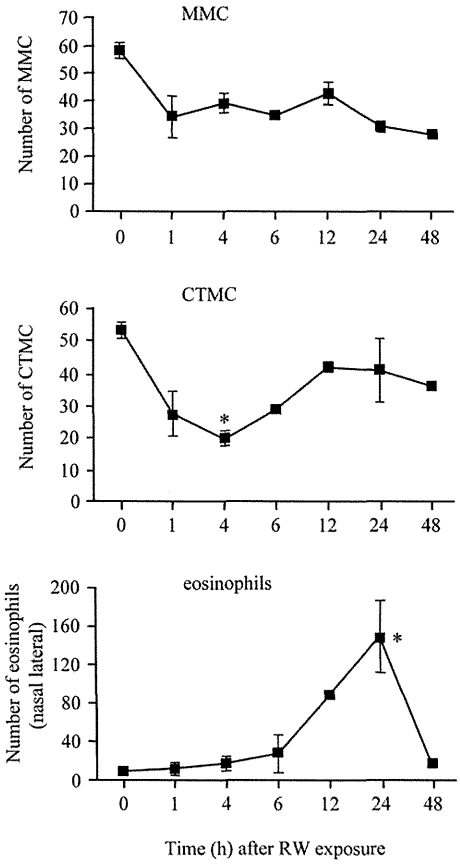

FIG E6. Kinetics of the number of MMCs, CTMCs, and eosinophils in the nose. Ragweed *(RW)*-immunized WT mice were nasally administered with single ragweed. Kinetics of the numbers of MMCs, CTMCs, and eosinophils in the nose (3 mice per time point) are shown. Data are representative of 2 independent experiments. Means and SEMs of 3 mice are shown. $*P < .05$ compared with before ragweed exposure (0 hour).

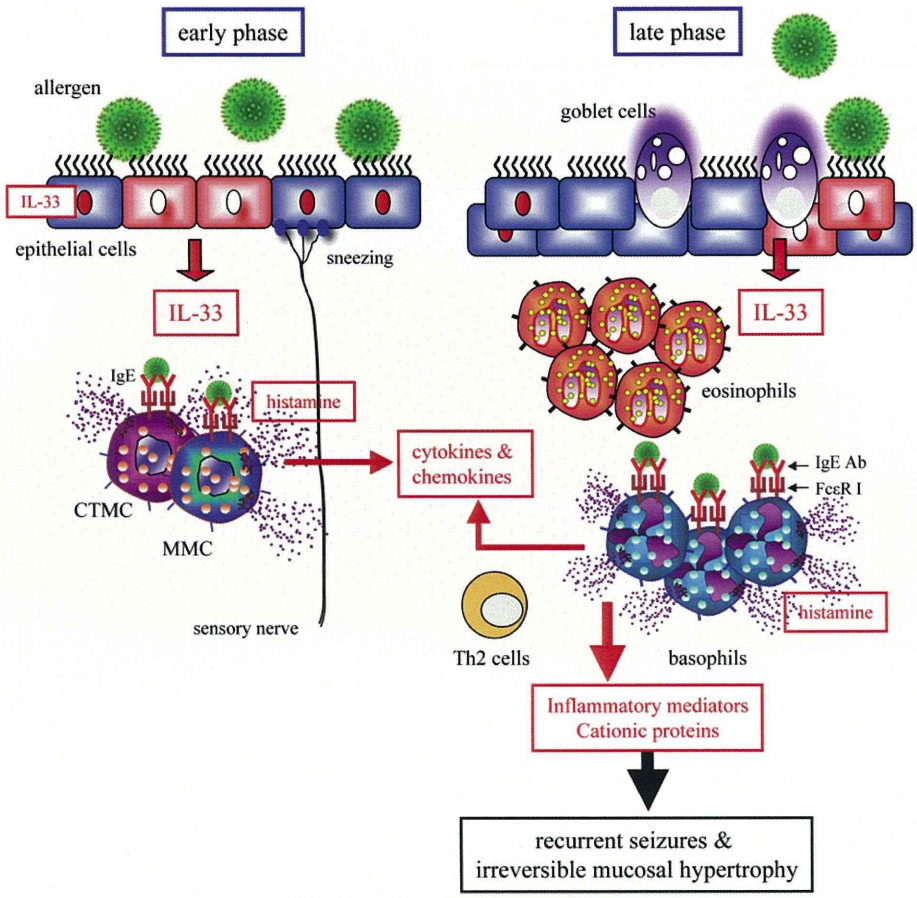

FIG E7. Schematic representation of contribution of IL-33 to allergic responses in AR. Ragweed pollen–driven endogenous IL-33 from nasal epithelial cells contributes to both early-phase (sneezing) and late-phase (nasal accumulation of eosinophils and basophils) responses in AR by increasing histamine release and inducing production of chemoattractants from mast cells and basophils, respectively. This process, together with the contribution of IL-33 to stimulate T_H2 cells, eosinophils, basophils, and mast cells to produce allergic inflammatory mediators, might lead to the recurrent seizures and irreversible mucosal hypertrophy seen in patients with AR.

TABLE E1. Characteristics of the patients with AR and control subjects

Characteristics	Cases	Control subjects
Age (y), mean (range)	39.9 (10-62)	44.5 (24-65)
Total subjects and sex (male/female)	10 (9/1)	5 (3/2)
Serum total IgE (IU/mL), mean ± SEM	478.8 ± 166.9	32.5 ± 7.8
Atopic sensitization (RAST), no. (%)		
Dermatophagoides pteronyssinus positive	10 (100)	0 (0)
JC pollen positive	5 (50)	0 (0)
Japanese cypress pollen positive	5 (50)	0 (0)
Cocksfoot pollen positive	3 (33.3)	0 (0)
Grey alder pollen positive	2 (20)	0 (0)
Common ragweed pollen positive	1 (10)	0 (0)
Mugwort positive	1 (10)	0 (0)

TABLE E2. Characteristics of the patients with JC pollinosis and control subjects

Characteristics	Cases	Control subjects
Age (y), mean (range)	27.8 (20-32)	23.8 (19-34)
Total subjects and sex (male/female)	13 (8/5)	11 (5/6)
Serum total IgE (IU/mL), mean ± SEM	93.8 ± 17.0	40.0 ± 10.5
Atopic sensitization (RAST), no. (%)		
JC pollen positive	13 (100)	0 (0)
Dermatophagoides pteronyssinus positive	0 (0)	0 (0)
Dermatophagoides farinae positive	0 (0)	0 (0)
Cocksfoot pollen positive	0 (0)	0 (0)
Candida albicans positive	0 (0)	0 (0)
Aspergillus fumigatus positive	0 (0)	0 (0)
Common ragweed pollen positive	0 (0)	0 (0)