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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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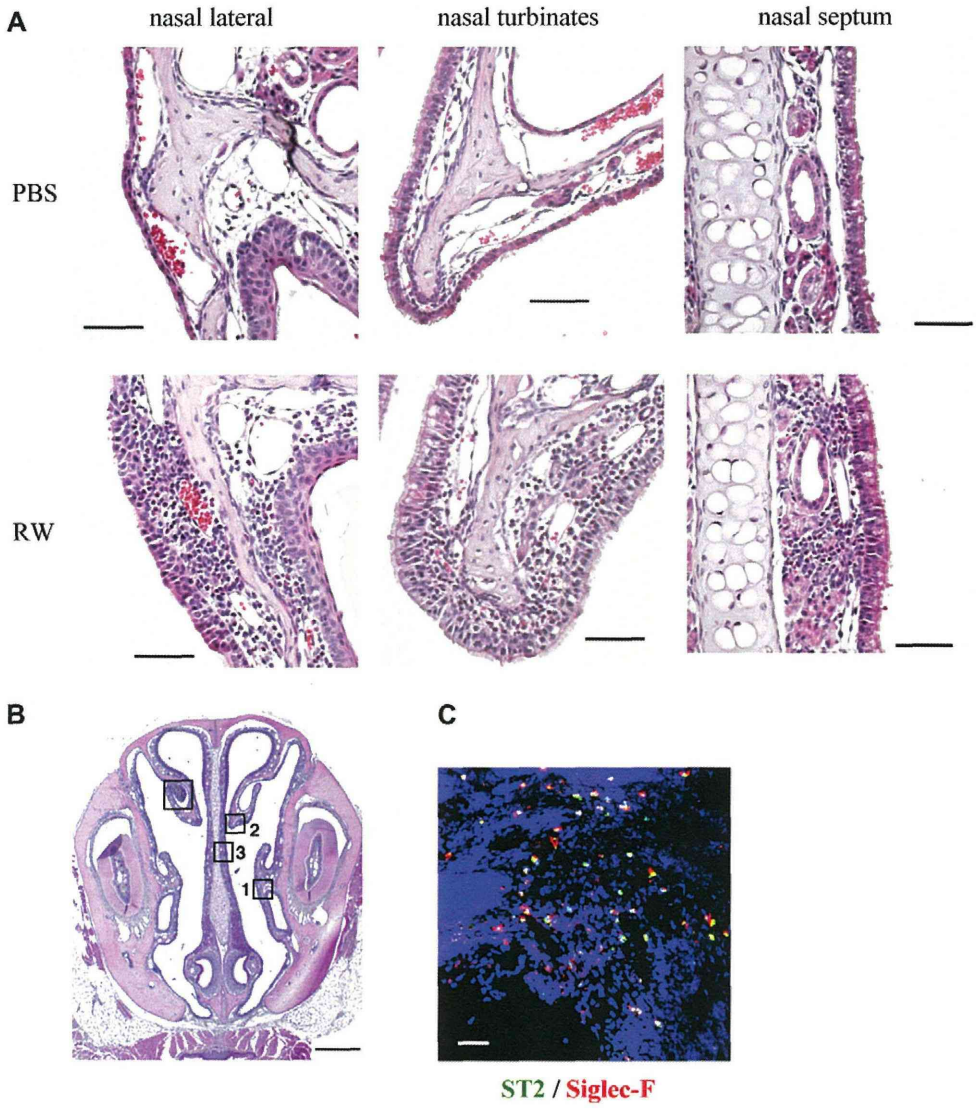

FIG E1. Ragweed *(RW)*–immunized and ragweed-challenged mice induce nasal accumulation of eosinophils. **A,** Coronal sections of noses from *il33*$^{+/+}$ mice immunized with ragweed and nasally challenged with PBS *(top row)* or ragweed *(bottom row)* were stained with hematoxylin and eosin. Nasal 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.

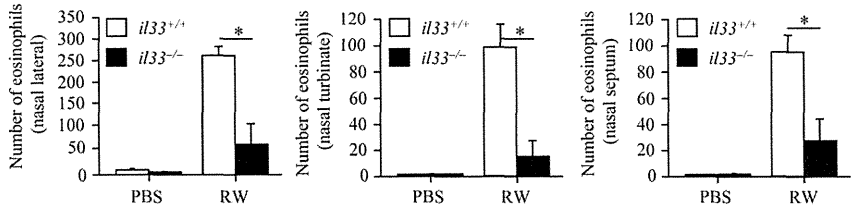

FIG E2. *il33*$^{-/-}$ mice diminish nasal accumulation of eosinophils. *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. The number of eosinophils in the nasal lateral mucosa, nasal turbinate, and nasal septal mucosa was counted. Data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

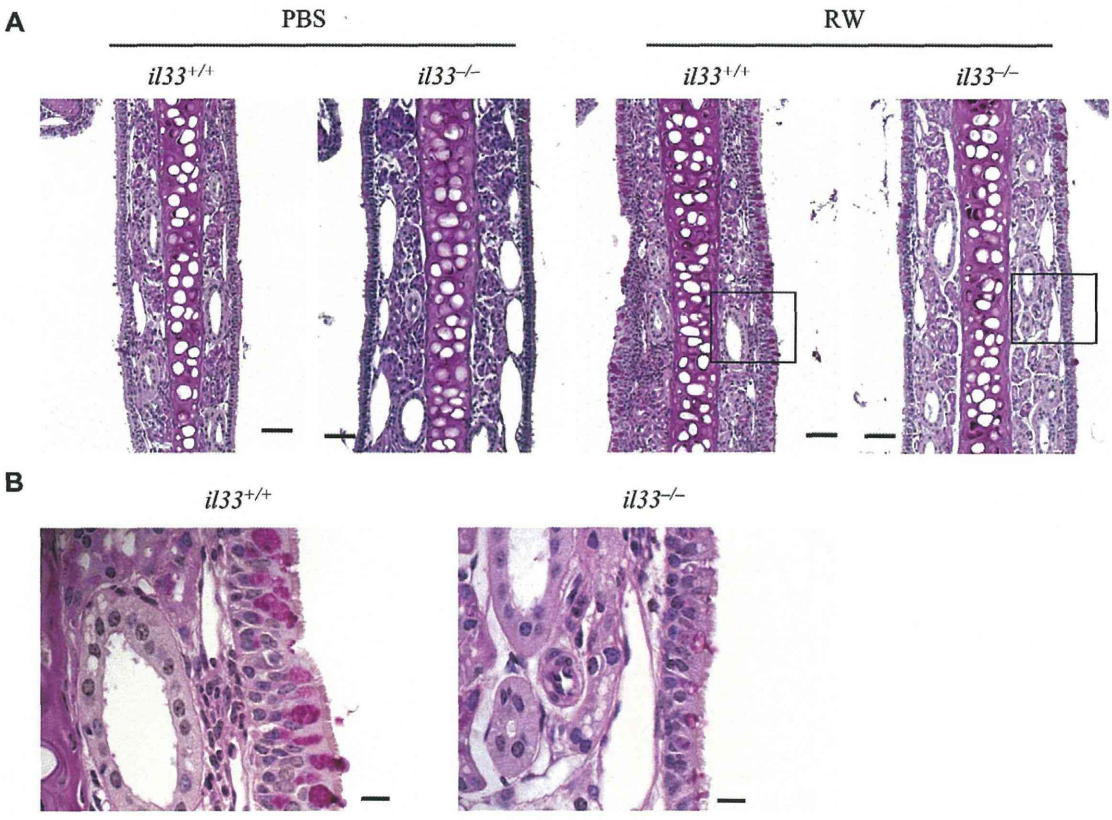

FIG E3. *il33*$^{-/-}$ mice diminish the degree of multilayered epithelial formation and goblet cell hyperplasia in the nasal mucosa. **A** and **B,** *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. Twenty-four hours after the last 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.

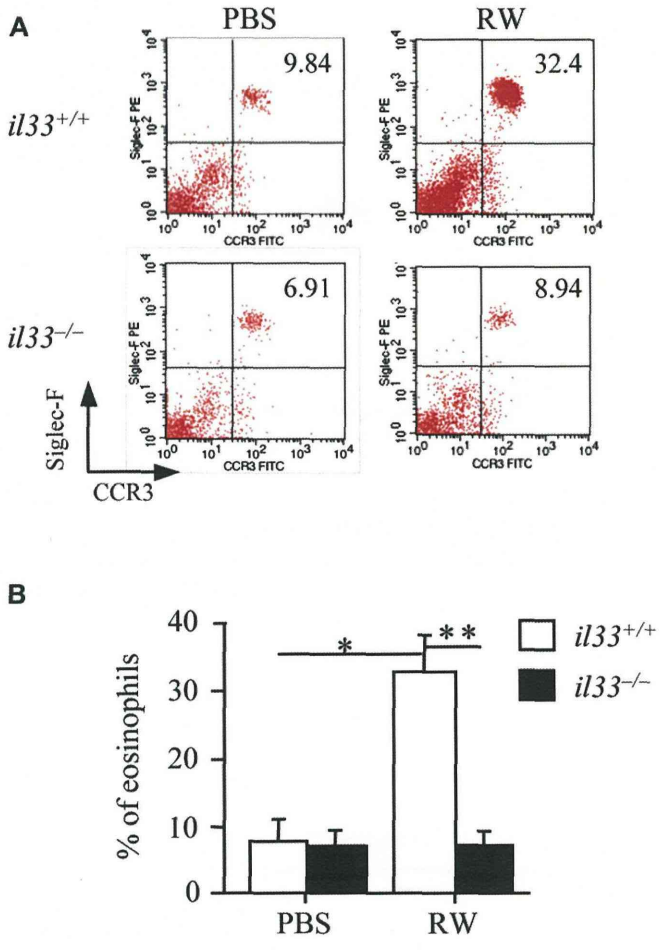

FIG E4. *il33*$^{-/-}$ mice do not induce eosinophilic accumulation in cervical lymph nodes. *il33*$^{+/+}$ and *il33*$^{-/-}$ mice were immunized with ragweed *(RW)* and nasally challenged with PBS or ragweed. **A,** Flow cytometric analysis of SSC^{high}Siglec-F^{+}CCR3^{+} eosinophils in cervical 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 $**P < .001$.

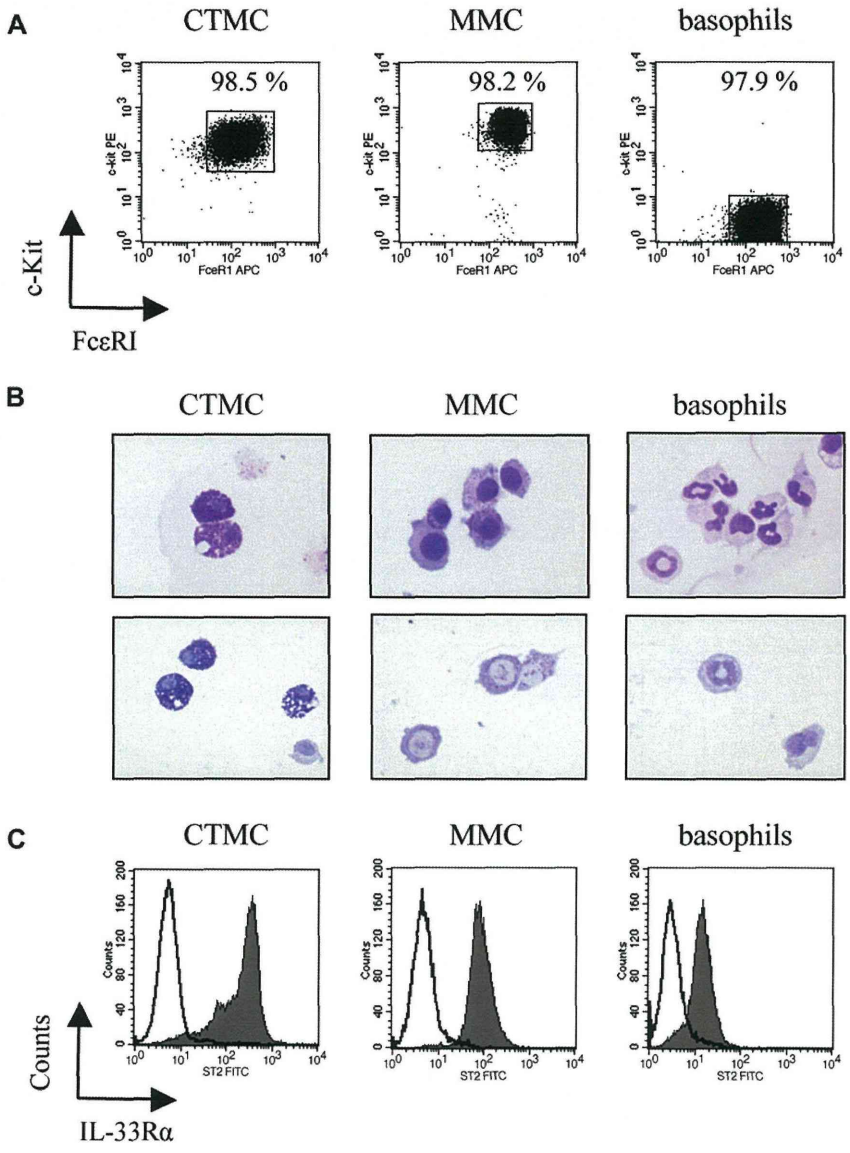

FIG E5. Preparation and characterization of bone marrow-derived CTMCs, MMCs, and basophils. **A** and **B,** Flow cytometric analysis of the expression of FcεRI and c-Kit by bone marrow–derived and sorted CTMCs, MMCs, and basophils. Fig E5, *A, Numbers above the outlined areas* indicate percentages of 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.

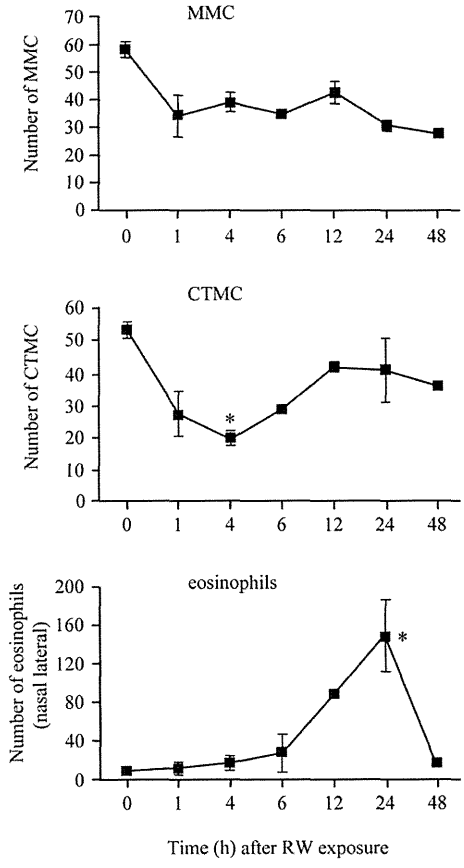

FIG E6. Kinetics of the number of MMCs, CTMCs, and eosinophils in the nose. Ragweed *(RW)*–immunized WT mice were nasally administered with single ragweed. Kinetics of the numbers of MMCs, CTMCs, and eosinophils in the nose (3 mice per time point) are shown. Data are representative of 2 independent experiments.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

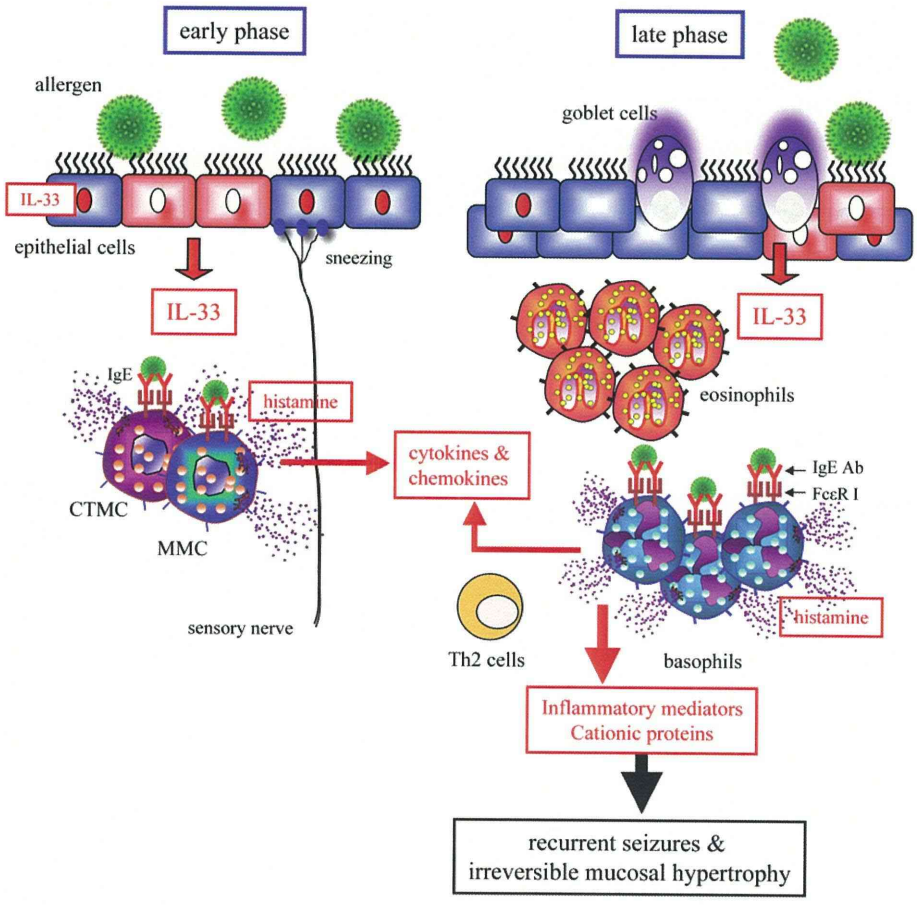

FIG E7. Schematic representation of contribution of IL-33 to allergic responses in AR. Ragweed pollen–driven endogenous IL-33 from nasal epithelial cells contributes to both early-phase (sneezing) and late-phase (nasal accumulation of eosinophils and basophils) responses in AR by increasing histamine 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)