Roles of Epithelial Cell–Derived Type 2–Initiating Cytokines in Experimental Allergic Conjunctivitis

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Purpose. To clarify the possible involvement of the type 2–initiating cytokines interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP) in the pathophysiology of allergic conjunctivitis, we evaluated ragweed (RW)-induced experimental allergic conjunctivitis (EAC) models by using IL-25 knockout (KO), IL-33 KO, and TSLP receptor (TSLPR) KO mice.

Methods. Interleukin-25 KO, IL-33 KO, TSLPR KO, and BALB/c wild-type mice were sensitized twice with RW in alum and then challenged with RW in eye drops. Clinical scores and eosinophil infiltration were evaluated. Expression levels of serum immunoglobulin E (IgE) and cytokines in the conjunctival tissues were quantified and immunohistochemical analysis was carried out.

Results. Significant reductions in clinical scores and numbers of infiltrating eosinophils were observed in the RW-EAC model using IL-33 KO mice. There were no significant differences in clinical scores and numbers of infiltrating eosinophils among IL-25KO, TSLPR KO, and wild-type mice. Serum IgE concentration was upregulated after RW challenges, and there were no differences among the mouse genotypes. Expression levels of of *il4*, *il5*, *il13*, and *ccl5* mRNA were diminished in the conjunctivae of the RW-EAC model using IL-33 KO mice compared to those in wild-type mice. Interleukin-33 expression was upregulated as early as 1 hour after RW eye-drop challenge. The number of infiltrating basophils in the conjunctivae of the RW-EAC model using IL-33 KO mice was diminished compared to that in wild-type mice.

Conclusions. Among the type 2–initiating cytokines, IL-33 may play a major role in conjunctival inflammation in an RW-EAC model.

Keywords: experimental allergic conjunctivitis, IL-25, IL-33, thymic stromal lymphopoietin, type 2–initiating cytokines

Type 2 immune responses are inflammatory conditions associated with parasitic infections[1] and atopic diseases like asthma, atopic dermatitis, and atopic keratoconjunctivitis (AKC).[2] These type 2 immune responses are characterized by activation of $CD4^+$ T-helper type 2 (Th2) cells and production of typical type 2 immunity–associated cytokines (e.g., interleukin-4 [IL-4], IL-5, and IL-13). Although various external stimuli (including pollen, house dust mites [HDM], food allergens, and parasites) can induce type 2 responses, these antigens cannot directly activate Th2 cells because they are too large to be phagocytosed by antigen-presenting cells.[3] Epithelial cell–derived type 2–initiating cytokines (IL-25, IL-33, and thymic stromal lymphopoietin [TSLP]) were characterized recently as indispensable for initiating type 2 immune responses stimulated by these type 2 immunity–related antigens.[3]

We previously reported the expression of IL-33[4] and TSLP[5] mRNA and protein in giant papillae obtained from patients with vernal keratoconjunctivitis (VKC) and AKC. Our study group also established an IL-33 knockout (KO) mouse and reported that IL-33 has an essential role in papain-induced lung inflammation, which is considered to be an innate immune system-dependent type 2 inflammation.[6] The role of IL-33 was also reported in an ovalbumin (OVA)-induced asthma model,[7–9] an established model for acquired immune system-dependent type 2 inflammation. Ragweed (RW)-induced experimental allergic conjunctivitis (EAC) has been used as a common model for T-cell (acquired immunity)–dependent allergic conjunctivitis.[10] Matsuba-Kitamura et al.[11] reported that addition of recombinant IL-33 at the time of RW eye-drop challenge augmented eosinophil infiltration in the conjunctival tissue in their RW-EAC model.

Thymic stromal lymphopoietin is produced by epithelial cells in response to various protein allergens (e.g., OVA) and protease allergens (e.g., pollen and papain).[12] It activates dendritic cells through the TSLP receptor (TSLPR)–IL-7Rα receptor heterodimer complex.[13] Thymic stromal lymphopoie-

tin–activated dendritic cells express tumor necrosis factor superfamily, member 4 (OX40L), which initiates T-cell–mediated type 2 inflammation by activating OX40-positive T cells.[13] Upregulation of TSLP, TSLPR, and OX40L mRNA in RW-EAC models was reported by Zheng et al.[14] Interleukin-25 was originally discovered in cDNA libraries from highly polarized Th2 cells.[15] It is also produced by epithelial cells, mast cells, eosinophils, and macrophages.[3] The importance of IL-25 for type 2 inflammation was demonstrated by experiments using mouse OVA-induced asthma models, in which attenuated airway inflammation and airway hyperresponsiveness were observed after deletion of the IL-25 gene.[16] To date, there have been no published data concerning the expression of IL-25 in conjunctival tissue and its role in the pathophysiology of allergic conjunctivitis. In this study, we investigated the roles of type 2–initiating cytokines in the pathophysiology of allergic conjunctivitis by evaluating RW-EAC models by using IL-25 KO, IL-33 KO, and TSLPR KO mice and congenic wild-type mice.

Materials and Methods

IL-25 KO, IL-33 KO, and TSLPR KO Mice

Interleukin-33–deficient (IL-33 KO) mice,[6] IL-25 KO mice,[17] and TSLPR KO mice[18] were generated as previously reported. BALB/c wild-type mice purchased from Japan SLC (Shizuoka, Japan) and KO mice were backcrossed with them for at least seven generations to establish congenic IL-33 KO, IL-25 KO, and TSLPR KO mice. All animal experiments conformed to the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

Alum-RW Experimental Allergic Conjunctivitis

Mouse EAC was induced as previously described,[10] with slight modifications. Briefly, short RW pollen (Polysciences, Warrington, PA, USA) was emulsified using Imject Alum adjuvant (Thermo Scientific, Rockford, IL, USA). At day 0, 50 μL emulsified RW (50 μg RW with 50 μL alum) was injected into the left hind footpad and tail base, and serum was collected from the tail vein. Two weeks later, a second immunization was carried out using the right hind footpad. From days 26 to 29, the eyes of immunized mice were challenged daily with RW in phosphate-buffered saline (PBS) (2 mg in 10 μL per eye) or with PBS alone. Twenty minutes after the last eye-drop challenge, the EAC was evaluated using the scores for chemosis, redness, lid edema, tearing, discharge, and scratching behavior based on criteria described by Magone et al.[19] (Supplementary Table S1). At 24 hours after the last eye-drop challenge, the eyeballs (with lids and conjunctival tissue) were collected for histological analyses and quantification of cytokine expression. For IL-33 protein expression analysis, we made an ex vivo culture model of resected conjunctivae from eyes challenged with RW eye drops only once at day 26. Blood samples were also collected for the measurement of serum immunoglobulin E (IgE) levels at day 30.

Measurement of Serum IgE Levels

Total IgE levels in sera at days 0 and 30 were quantified using mouse IgE ELISA kits (ELISA MAX; Biolegend, San Diego, CA, USA), according to the manufacturer's protocol.

Histological Analysis

Eyeballs (with conjunctival tissues and eye lids) were dissected and fixed in 4% paraformaldehyde in PBS. Vertical 2-μ-thick paraffin sections were made and stained with Giemsa stain (Merk, Darmstadt, Germany). Throughout each section, infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivae were counted in the central portion of the eye, which included the pupil and optic nerve head, as described previously.[20]

Real-Time PCR Analysis

Conjunctival tissue obtained from the mouse eye was immediately submerged in RNA Later solution (Ambion, Austin, TX, USA) to protect the RNA. Total RNA was extracted from the tissue using an RNA isolation kit (NucleoSpin II; Macherey-Nagel GmbH, Duren, Germany). Complementary DNAs (cDNAs) were prepared using random primers and ReverTra Ace reverse transcriptase (both from Toyobo, Osaka, Japan), according to the manufacturer's protocol. Real-time PCR primers specific for mouse *il4*, *il5*, *il13*, *il33*, *ccl5*, *ccl11*, and *gapdh* mRNAs were designed by QuantPrime (Universitat Potsdam, Potsdam, Germany) and are summarized in Supplementary Table S2. Real-time PCR analysis was performed using a PRISM model 7300 HT (Applied Biosystems, Grand Island, NY, USA) sequence detection system with Fast SYBR green master mix (Life Technologies Japan, Tokyo, Japan). The relative expression levels of *il4*, *il5*, *il13*, *il33*, *ccl5*, and *ccl11* were quantified by comparative cycle threshold (C_t) methods using *gapdh* mRNA expression in the same cDNA as the internal controls.

Immunohistochemistry

Immunofluorescent staining was performed to examine IL-33 expression in the conjunctival tissue obtained from experimental conjunctivitis. A goat anti-mouse IL-33 polyclonal antibody was purchased from R&D Systems (Minneapolis, MN, USA), a rat anti-mouse F4/80 antibody (clone CI:A3-1; BioLegends), and a sheep anti-mouse mast cell protease (mcp) 1 antibody (clone MS-RM8; Moredun Scientific, Midlothian, UK). A basophil-specific rat anti-mouse mcp8 antibody was obtained from BioLegends,[21] and a rat anti-mouse major basic protein (MBP) antibody was provided by J. Lee (Mayo Clinic, Rochester, MN, USA).[22] Frozen sections (5 μm) were cut and then immunostained with the anti-IL-33, anti-MBP, anti-F4/80, and anti-mcp1 antibodies. Anti-mcp8 immunohistochemical staining was carried out using 2-μm paraffin sections. Stained slides were observed using confocal laser scanning microscopy (FV-1000; Olympus Corp., Tokyo, Japan). Negative control specimens were immunostained with control goat IgG or rat IgG antibodies (all from Santa Cruz Biotechnology, Santa Cruz, Dallas, TX, USA) instead of the primary antibodies. Double-immunostaining was carried out using goat anti-IL-33 antibody with the rat anti-MBP antibody, the rat anti-F4/80 antibody, or the sheep anti-mcp1 antibody. A donkey–Alexa 488-conjugated anti-rat IgG antibody, donkey–Alexa 594-conjugated anti-goat IgG antibody, and donkey–Alexa 488-conjugated anti-sheep IgG antibody (all from Life Technologies Japan) were used as secondary antibodies.

Measurement of IL-33 Protein Concentration in Supernatant of Ex Vivo Conjunctival Tissue Culture

At the indicated times (1, 3, 6, and 12 hours) after the RW eye-drop challenge, the mice were killed, and conjunctival tissues were sampled. Tissues were cultured in 1.5-mL sterile microcentrifuge tubes (Eppendorf Japan, Tokyo, Japan) for 60 minutes, using 200 μL of serum-free culture medium (OPTI-MEM; Life Technologies Japan) with protease inhibitors (Complete Mini; Roche Diagnostics GmbH, Mannheim, Ger-

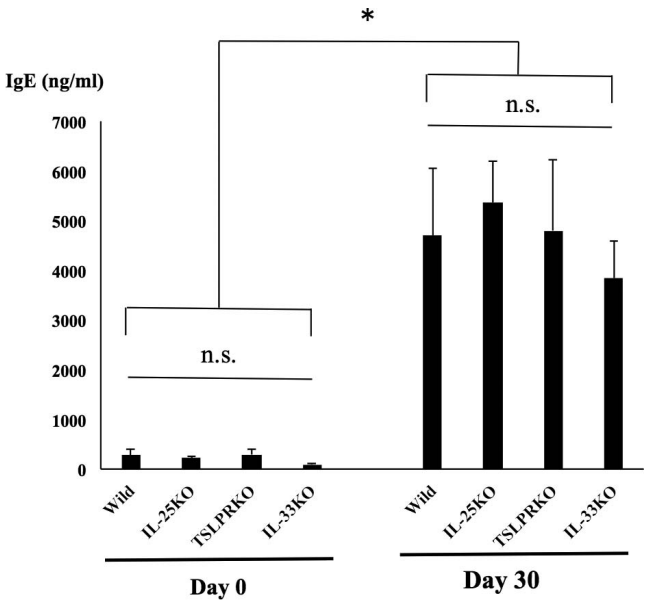

FIGURE 1. Total serum IgE measurement before and after RW-immunization. Total serum IgE concentration was quantified using ELISA at day 0 (before RW immunization) and on day 30 (after RW eye-drop challenges). A statistically significant increase (*$P < 0.01$, by Mann-Whitney U test) in IgE concentration was observed in day 0 samples compared to that in day 30 samples obtained from the same types of mice. There were no significant differences between day 0 and day 30 samples from the same types of mice (n.s., no significant differences). Data are triplicate measurements using five mice per each group and show mean ± SD IgE concentrations (ng/mL).

many). The IL-33 concentration in the supernatant of ex vivo tissue culture was quantified using mouse IL-33 Ready-SET-GO ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol.

Statistical Analysis

Statistical evaluations of cell numbers, cytokine expression, serum IgE levels, and histological analysis were performed with the two-tail unpaired Mann-Whitney U test. A P value of <0.05 was considered statistically significant. All experiments were repeated at least three times, and representative data are shown.

RESULTS

Sera IgE Concentrations in RW-EAC Models

To evaluate the effect of RW sensitization and roles of type 2–initiating cytokines during the sensitization process, we measured sera IgE levels at day 0 (before sensitization) and at day 30 (after eye-drop challenge) by ELISA. Significant increases ($P < 0.01$) of total sera IgE at day 30 were observed in all types of mice (wild-type, IL-25 KO, IL-33 KO, and TSLPR KO) compared to those from day 0 samples of the same type of mice. There were no significant differences within day 0 samples and day 30 samples among the types of mice (Fig. 1).

Attenuated Clinical Symptoms of RW-Induced EAC in IL-33 KO Mice But Not in IL-25 KO or TSLPR KO Mice

Mice were immunized with RW in alum at days 0 and 14 and challenged from day 26, using RW or PBS eye drops daily for 4 days. Twenty minutes after the last challenge, we photographed and scored the severity of EAC by measuring the degree of chemosis, conjunctival redness, lid edema, tearing, discharge, and scratching, as shown in Supplementary Table S1. The clinical score for IL-33KO mice was significantly lower ($P = 0.011$) than that for wild-type mice (Figs. 2A, 2B, $n = 5$ per group). There were no significant differences among clinical scores for IL-25 KO, TSLPR KO, and wild-type mice (Fig. 2B).

IL-33 Deletion Diminished the Number of Eosinophils Infiltrating Conjunctivae of RW-Induced EAC

Twenty-four hours after the last RW eye-drop challenge, the eyes were collected, and the number of eosinophils was counted using Giemsa-stained slides (Fig. 3A). The number of eosinophils infiltrating the conjunctivae of IL-33 KO mice was significantly lower ($P = 0.011$) than those in wild-type mice (Fig. 3B, $n = 5$ per group). There were no significant

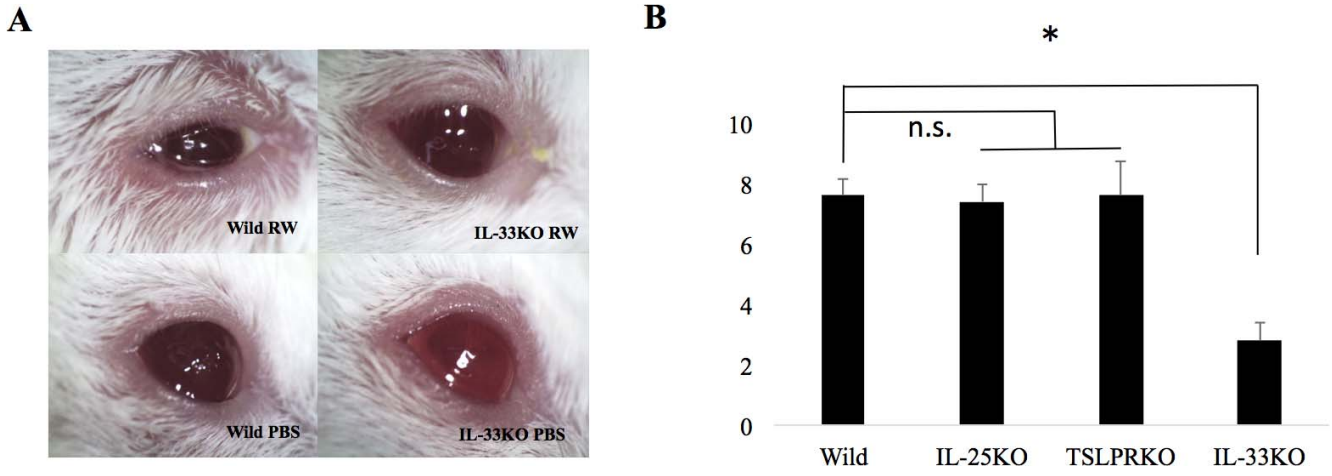

FIGURE 2. Clinical evaluation of RW-EAC. Representative photographs show RW-EAC models using wild-type and IL-33 KO mice challenged with either RW-PBS (*upper row*) or PBS alone (*lower row*), taken 20 minutes after the last eye-drop challenge (**A**). Clinical scores of the RW-challenged EAC models are shown (**B**). Data are representative mean ± SD clinical scores of five mice for each group (*$P < 0.05$, Mann-Whitney U test).

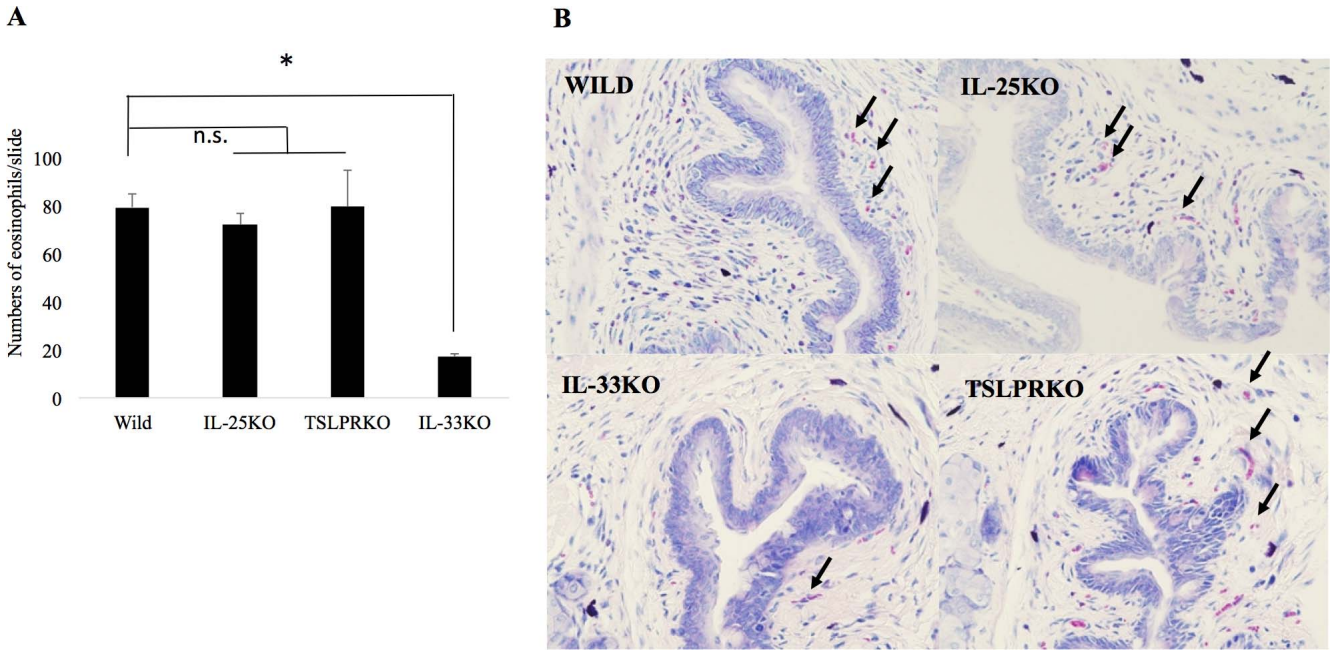

FIGURE 3. Eosinophil infiltration in conjunctivae of RW-EAC mice. Eyes of RW-EAC models were sampled 24 hours after the last RW challenge, and the numbers of eosinophils (*arrows*) infiltrating the substantia propria of the conjunctival tissues were counted using Giemsa-stained slides (**A**). Data are representative mean ± SD numbers of infiltrating eosinophils per slide counted in conjunctivae of five mice per group (**B**) (*$P < 0.05$, Mann-Whitney *U* test).

differences among the numbers of infiltrating eosinophils for IL-25 KO, TSLPR KO, and wild-type mice (Fig. 3B).

Quantification of Cytokine Expression in Mouse Conjunctival Tissues Obtained From RW-EAC Models

Conjunctival tissues were sampled 24 hours after the last RW eye-drop challenge, and their cytokine expression levels were quantified. Significant upregulation of *il4*, *il5*, *il13*, and *ccl5* mRNA expression ($P < 0.05$) was observed in the conjunctival tissue of RW-induced EAC in wild-type mice compared to that in PBS-challenged conjunctival tissue of wild-type mice (Fig. 4, asterisks). No significant upregulation of *ccl11* mRNA was induced by the RW eye-drop challenge. Significant attenuation of *il4*, *il5*, *il13*, and *ccl5* mRNA expression ($P < 0.01$) was observed in the IL-33 KO mice compared to that in wild-type mice (Fig. 4, double asterisks).

Time Courses of *il33* mRNA and IL-33 Protein Expression in RW-EAC Models

The IL-33 protein level in the ex vivo culture supernatant was significantly upregulated ($P < 0.05$) in the conjunctival tissue samples obtained at 1, 3, 6, and 12 hours after the first RW eye-drop challenge (Fig. 5A). No IL-33 protein was detected in the conjunctival tissue obtained from RW eye-drop-challenged IL-33 KO mice (data not shown). Significant *il33* mRNA upregulation ($P < 0.05$) was observed in the conjunctival tissue obtained from wild-type mice 1 hour after the last RW eye-drop challenge compared to that in PBS-challenged control tissue (Fig. 5B).

Immunohistochemical Analysis of EAC

Immunofluorescent staining was performed to examine the expression of IL-33 and the immunolocalization of eosinophils, macrophages, mast cells, and basophils in the conjunctival tissue obtained from EAC mice. Anti-mcp8 (basophil marker) immunostaining of the RW-EAC models showed basophil infiltration in the subepithelial regions of the RW-challenged eyes (Figs. 6A, 6B), and the number of infiltrating basophils was significantly higher (*$P < 0.05$) in the RW-EAC models of the wild-type mice than in IL-33 KO mice (Fig. 6E).

Ragweed-challenged conjunctivae of wild-type mice showed IL-33 protein expression in conjunctival epithelial cells and infiltrating cells of the substantia propria of the conjunctival tissue in the vicinity of MBP-positive eosinophils (Figs. 7B, 7C, arrowheads). Phosphate-buffered saline–challenged conjunctival tissue of wild-type mice showed IL-33-positive immunoreactivity in the epithelial cell layer (Fig. 7E, asterisk) and sparse infiltration of eosinophils in the subepithelial region (Fig. 7D). Conjunctival tissue of the RW-EAC model using IL-33KO mice had less MBP-positive eosinophil infiltration than in wild-type mice (Fig. 7G) and no IL-33-positive immunostaining was observed (Fig. 7H). Double immunohistochemical staining using the anti-IL-33 antibody and macrophage marker F4/80 antibody showed that some of the IL-33–positive cells in the substantia propria were also positive for F4/80 antigen (Figs. 8A–C, arrows). Similarly, some of the IL-33–positive cells in the substantia propria were also positive for the mast cell marker (mcp1) (Figs. 8D–F, arrowheads).

DISCUSSION

To explore the roles of type 2–initiating cytokines (IL-25, IL-33, and TSLP) in the pathophysiology of allergic conjunctivitis, we made RW-EAC models by using IL-25 KO, IL-33 KO, and TSLPR KO mice. We first conducted preliminary studies to determine the intensity of allergic inflammation by changing the numbers of RW eye-drop challenges. We found that four RW eye-drop challenges produced appropriate and reproducible inflammation for further analysis. Measurement of serum total IgE showed clear upregulation of serum IgE in the RW-EAC models

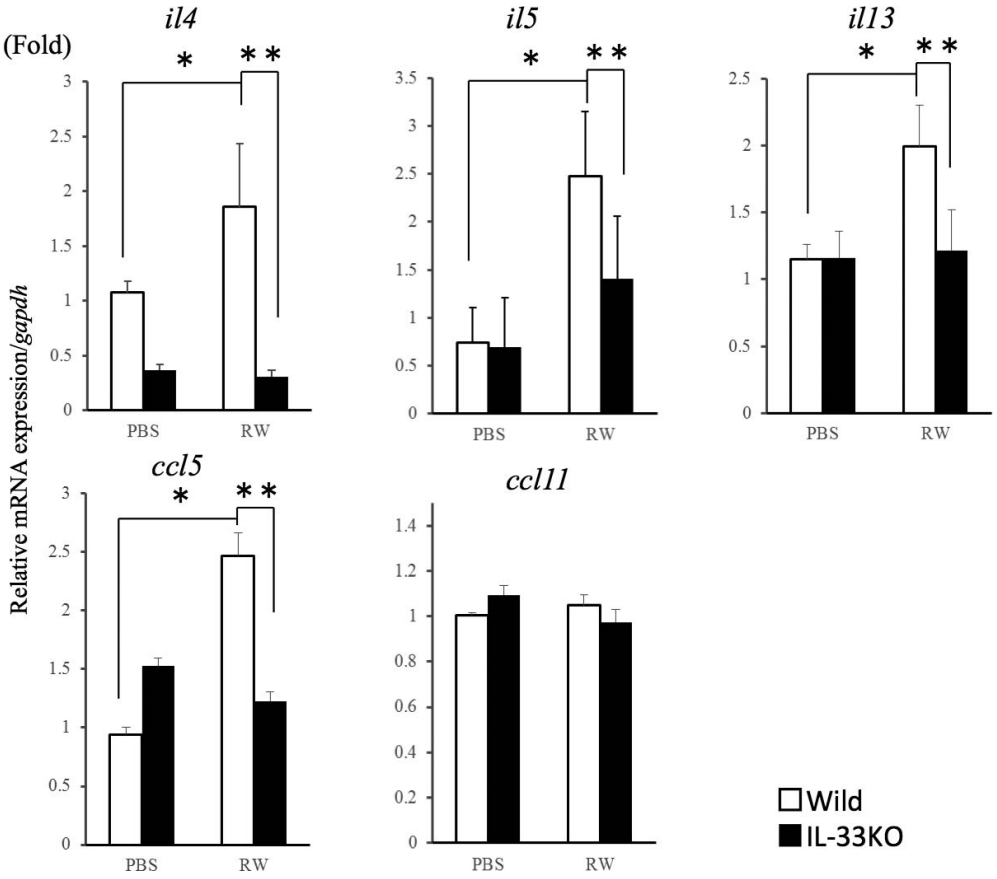

FIGURE 4. Quantification of cytokine mRNA expression in conjunctivae of RW-EAC models. Expression levels of inflammatory cytokine/chemokine (*il4*, *il5*, *il13*, *ccl5*, *ccl11*) mRNAs were quantified by real-time PCR. Relative mRNA expression is shown as fold changes of mRNA expression levels of PBS-challenged conjunctival tissues. Expression data were normalized to that of *gapdh* mRNA of the same cDNA samples. Significantly elevated expression levels of *il4*, *il5*, *il13*, and *ccl5* mRNAs were observed in RW-challenged conjunctivae of wild-type mice compared to those in PBS-challenged conjunctivae (*$P < 0.05$). Attenuated expression of *il4*, *il5*, *il13*, and *ccl5* mRNAs was observed in RW-challenged conjunctivae of IL-33 KO mice compared that in conjunctivae of wild-type mice (**$P < 0.05$). No differential *ccl11* mRNA expression levels were observed. Data are representative mean ± SD fold expression values of triplicate measurements.

(Fig. 1). The increase in serum IgE in our RW-EAC model was consistent with a previous report showing upregulation of serum IgE in an RW-EAC model[10] and in an RW-induced rhinitis model using BALB/c mice.[23] Nakinishi et al.[24] and Chu et al.[25] showed that there were no differences in increases of total serum IgE among IL-33 KO, IL-25 KO, TSLPR KO, and wild-type mice in an HDM-induced rhinitis model. Consistent with those reports, our results did not show significant differences in total sera IgE concentrations among the mouse types in the RW-EAC model (Fig. 1). On the other hand, Canbaz et al.[26] made an HDM-induced mouse airway inflammation model and showed that the total serum IgE level was IL-33 dependent in the case of HDM extracts with low endotoxin levels, whereas the IgE level was IL-33 independent in case of an HDM extract with high endotoxin levels. We consider that the type 2–initiating cytokines did not affect the IgE responses in our RW-EAC models, although the effect of IL-33 on IgE production was dependent on the model systems and the antigens used in the experiments.

Next, we compared clinical scores (Fig. 2; Supplementary Table S1) and numbers of infiltrating eosinophils (Fig. 3) in the RW-EAC models. Results showed that IL-33 deletion attenuated the clinical severity and diminished the eosinophil infiltration in the conjunctivae of the RW-EAC model. The attenuation of inflammation of RW-EAC in IL-33 KO mice in both the early phase clinical scores and the delayed phase eosinophilic infiltration were consistent with a previous report showing the role of IL-33 during the antigen challenge phase in an RW-EAC model.[11]

We found no significant differences among wild-type, TSLPR KO, and IL-25 KO mice with regard to clinical severity and numbers of eosinophils infiltrating the conjunctivae of the RW-EAC models (Figs. 2, 3). Results of inflammatory cytokine quantification showed attenuated *il5* mRNA expression but no differential expression of *il4*, *ccl5*, or *ccl11* mRNAs in the conjunctival tissues of the RW-EAC models using TSLPR-KO mice/IL-25 KO mice compared to those in wild-type mice (Supplementary Figs. S1, S2). Schleimer et al.[27] reported the effects of IL-5, CCL5, and CCL11 on the transendothelial migration of eosinophils. According to their results, IL-5 itself had a minimal effect on eosinophil migration but synergistic effects with CCL5 or with CCL11. The absence of differential expression of *ccl5* and *ccl11* mRNAs may account for the lack of a difference in eosinophil infiltration among TSLPR KO, IL-25 KO, and wild-type mice.

To further clarify the role of IL-33 in the pathophysiology of RW-EAC, chronological changes in IL-33 expression in the conjunctival tissue were examined. To quantify the IL-33 protein expression on the ocular surface in the RW-EAC model, we tried to detect IL-33 protein by simply collecting ocular surface exudate, using a small amount of PBS. However, the IL-33 concentration in the exudate was below

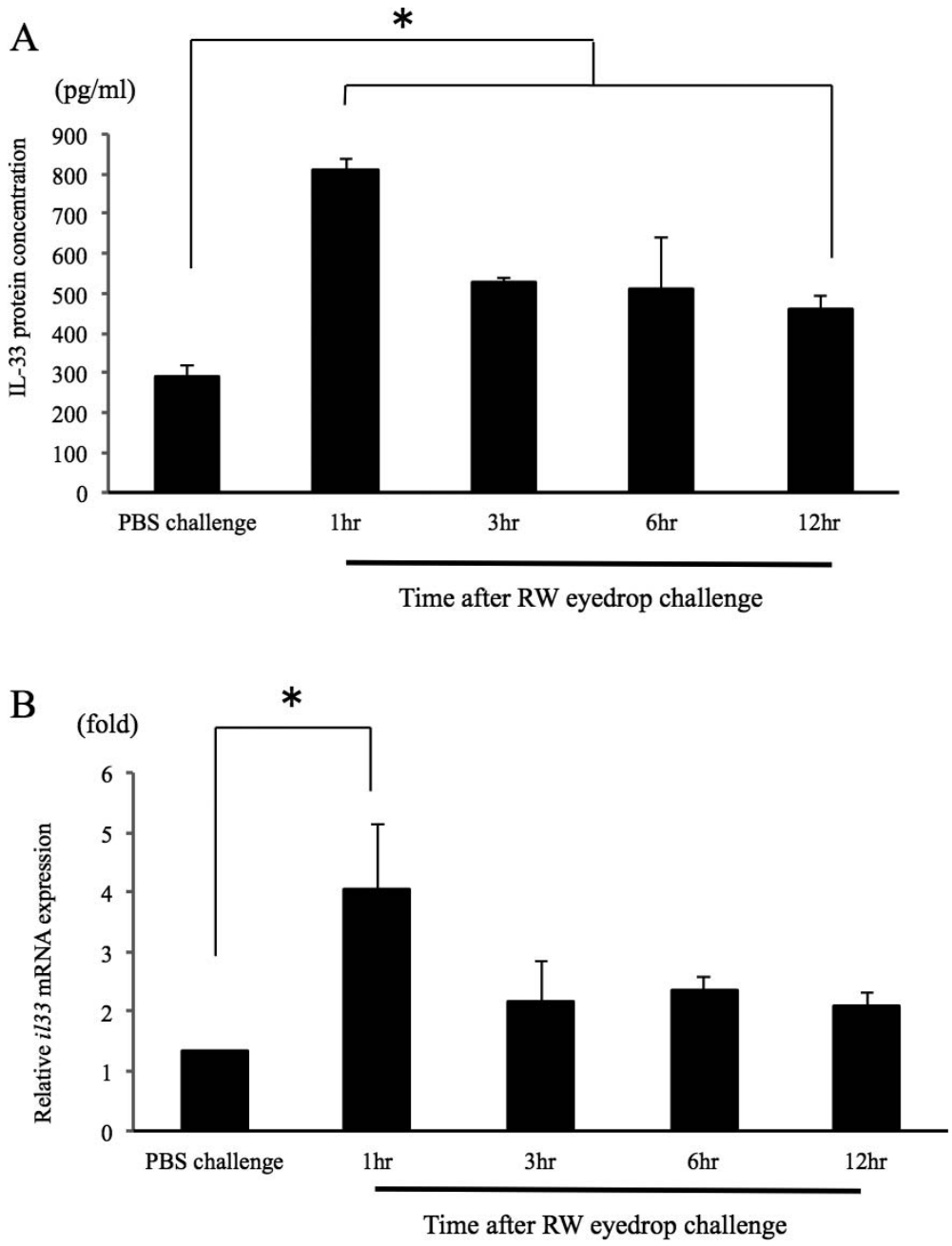

FIGURE 5. Time courses of IL-33 expression in the conjunctivae of RW-EAC models. Expression levels of IL-33 protein (**A**) and *il33* mRNA (**B**) at various time points (1, 3, 6, and 12 hours) after a single RW eye-drop challenge were quantified by ELISA and real-time PCR analysis, respectively. Interleukin-33 concentrations in culture supernatants of ex vivo–cultured conjunctivae tissues were quantified. Data are representative mean ± SD fold expression values using three conjunctivae for each time point, measured in duplicate. Significant upregulation was observed from 1 through 12 hours after eye-drop challenge (**A**). Relative mRNA expression is shown as fold change of mRNA expression levels in PBS-challenged conjunctival tissues. Data were normalized to the expression of *gapdh* mRNA of the same cDNA samples. Data are representative mean ± SD fold expression values of triplicate measurements. Significant upregulation was observed at 1 hour after eye-drop challenge but not at 3 to 12 hours (**B**) (*$P < 0.05$, Mann-Whitney U test).

the detection level (data not shown). Therefore, we measured the IL-33 protein concentrations in the ex vivo culture supernatant of conjunctival tissues obtained after RW challenge. A significant increase in IL-33 protein in the culture supernatants from 1, 3, 6, and 12 hours after RW eye-drop challenge peaked at 1 hour (Fig. 5A) suggested continuous IL-33 protein release from IL-33–producing cells. This rapid upregulation of IL-33 protein was consistent with the results from an RW-induced experimental rhinitis model in which IL-33 protein release was observed upon RW stimulation of nasal epithelium accompanied by temporal loss of IL-33 immunostaining.[23] Rapid release (1 hour after allergen challenge) of IL-33 protein was also observed in a model of lung inflammation induced by a mixture of allergens (HDM and *Aspergillus* and *Alternaria* spp.).[28] Transient upregulation of *il33* mRNA expression was observed at 1 hour after the RW eye-drop challenge (Fig. 5B). These results suggested that a rapid phase of IL-33 protein upregulation was

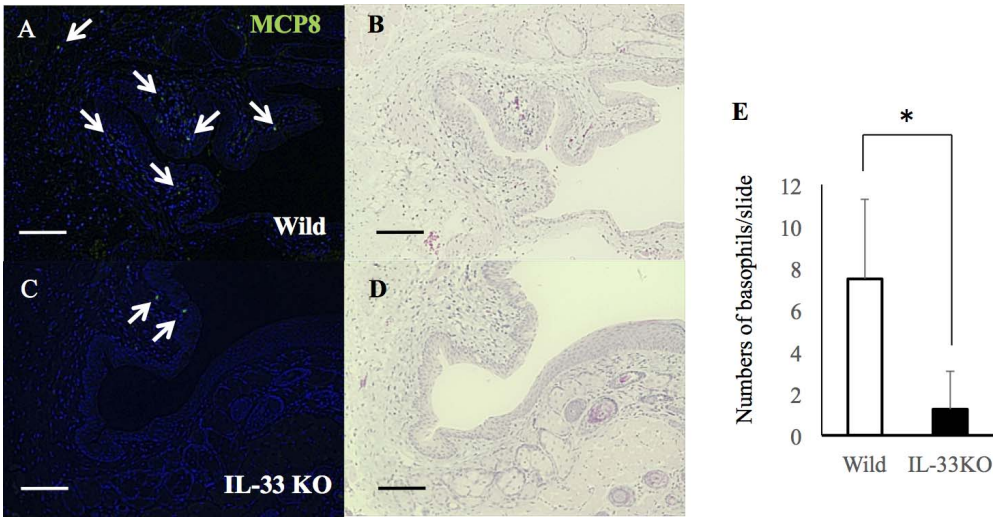

FIGURE 6. Mast cell protease 8–positive basophils in the conjunctivae of RW-EAC models. Mast cell protease 8 immunohistochemical staining of the conjunctiva showed higher numbers of mcp8-positive basophils (*arrows*) in the subepithelial regions of the RW-EAC model in wild-type mice (**A**) than in those of IL-33 KO mice (**C**). Hematoxylin and eosin staining of adjacent slides (**B**, **D**) is also shown. *Scale bars*: 100 μm. Mean ± SD numbers of basophils per slide in the conjunctivae of eight mice from each group are shown (**E**) (*$P < 0.05$, Mann-Whitney U test).

induced by the release of stored IL-33 from conjunctivae and that de novo IL-33 production might contribute later to continuous IL-33 release from conjunctivae.

We observed significant attenuation of *il4* mRNA expression in the conjunctivae of the RW-EAC model using IL-33 KO mice compared to that in wild-type mice (Fig. 4). Interleukin-4 is a key cytokine for type 2 immune responses mediated by adaptive immunity. It stimulates Th2 cell differentiation from naïve T cells and induces IL-5 and IL-13 expression.[29] The effects of IL-33 deletion on *il4* mRNA expression were significant even for eyes with mock eye-drop challenges, but it became more apparent with RW eye-drop challenge (Fig. 4). We observed diminished numbers of infiltrating basophils, which are known to produce a large amount of IL-4,[30] in the

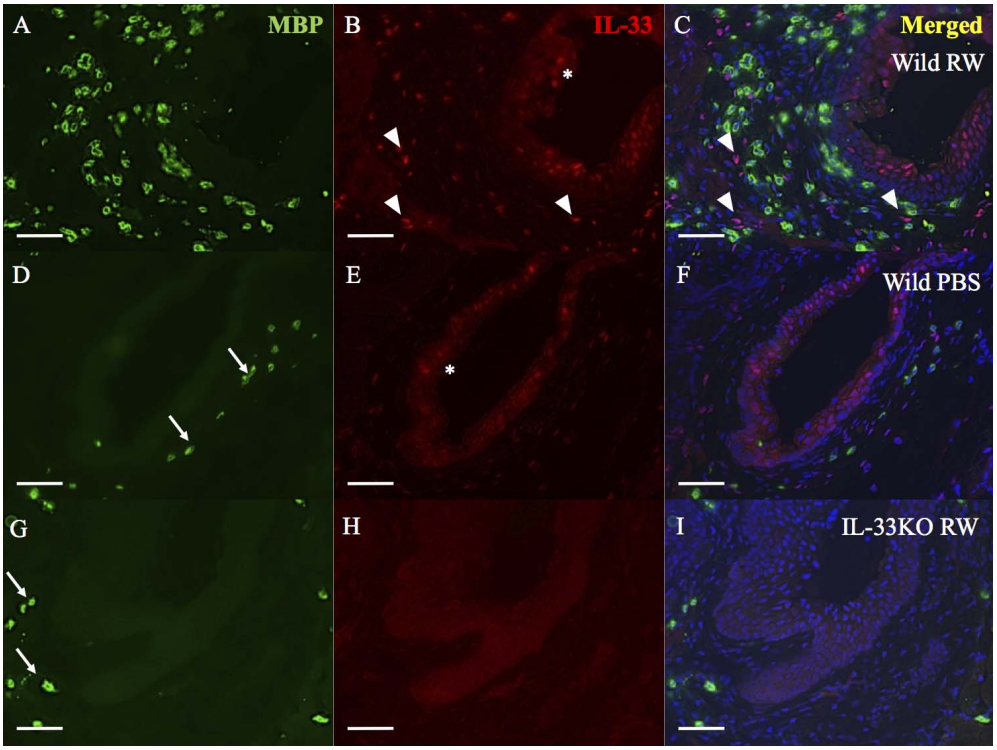

FIGURE 7. Interleukin-33 expression of in the conjunctivae of RW-EAC models. Anti-MBP (**A**, **D**, **G**) and anti-IL-33 (**B**, **E**, **H**) immunostaining of conjunctivae obtained from RW-EAC models are shown. Massive infiltration of MBP-positive eosinophils (*green*) is observed in substantia propria of RW-challenged EAC in wild-type mice (**A**). On the other hand, sparse infiltration of eosinophils was observed in PBS-challenged wild-type mice (**D**, **F**) and RW-challenged IL-33 KO mice (**G**). Interleukin-33 protein expression (*asterisks*) can be observed in the conjunctival epithelial cells of wild-type mice. Infiltrating cells of substantia propria in the vicinity of MBP-positive eosinophils (*arrowheads*) are also immunopositive for IL-33 (**B**, **C**). No IL-33–positive cells are observed in the conjunctivae of IL-33 KO mice (**H**, **I**). *Scale bars*: 50 μm.

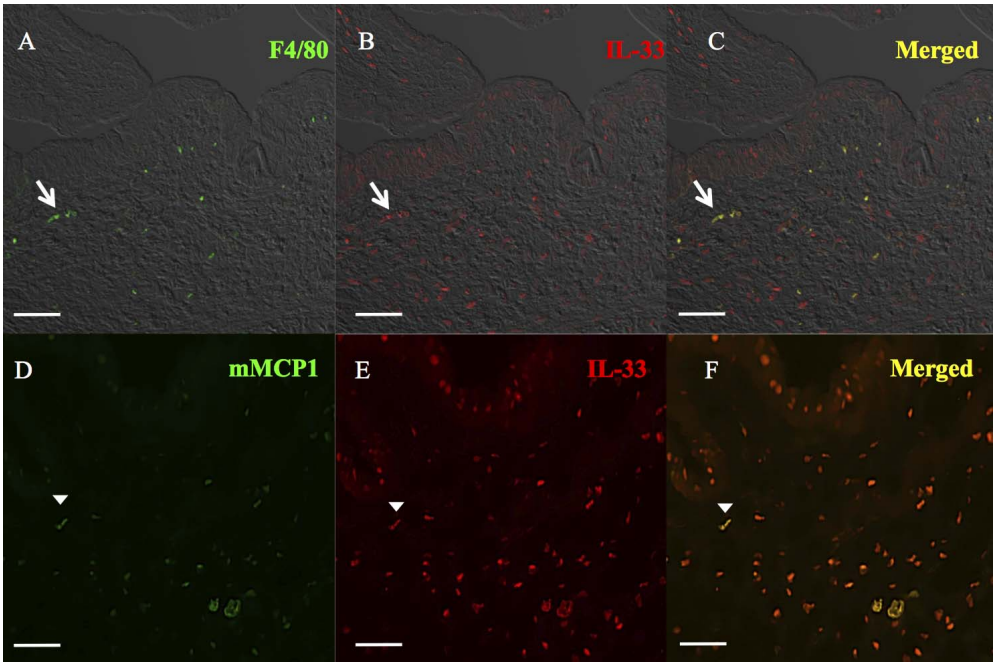

Figure 8. Colocalization of F4/80 and IL-33, as well as mMCP1 and IL-33, in substantia propria of the RW-EAC model. Some of the IL-33–positive cells (**B**, **E**) in substantia propria are also immunopositive for F4/80 ([**A**, **C**] *arrows*) or mMCP1 ([**D**, **F**] *arrowheads*). *Scale bars*: 50 μm.

conjunctivae of the RW-EAC model using IL-33 KO mice compared to the wild-type mice (Fig. 6). These results suggested that IL-33 might augment the expression of IL-4 in the RW-EAC model by promoting systemic basophil expansion or basophil infiltration into the EAC tissue. Our hypothesis is further supported by a report showing that IL-33 induces murine basophil expansion,[31] and another showing diminished numbers of basophils in the nasal tissue of an RW-induced rhinitis model using IL-33 KO mice.[23] We also found diminished *il4*, *il5*, and *il13* mRNA expression in cervical lymph node cells obtained from RW-EAC model IL-33 KO mice compared to those of the wild-type mice after in vitro stimulation using an RW extract for 48 hours (Supplementary Fig. S3). This result was consistent with a previous report on RW-induced rhinitis models using IL-33 KO,[23] and indicated diminished expression of cervical lymph node T cells in the IL-33 KO mice to produce Th2 cytokines.

The differential upregulation of *ccl5* (encoding the regulated on activation, normal T cell expressed and secreted [RANTES] protein) mRNA but not of *ccl11* (encoding eotaxin) mRNA, in the RW-EAC model in IL-33 KO mice suggested that IL-33 could induce *ccl5* expression (Fig. 4). These results were consistent with those in a report showing that IL-33 stimulation upregulated CCL5 protein expression but had only marginal effects on CCL11 expression in mast cells and in basophils.[23] In the RW-EAC model, we observed infiltration of basophils (Fig. 6) and mast cells (Fig. 8) in the conjunctival tissue; therefore IL-33 activated mast cells and basophils may play some roles in eosinophil infiltration in the RW-EAC model through the effect of CCL5, a well-known eosinophil chemoattractant.[32]

Immunohistochemical staining was positive for IL-33 not only in the cell nuclei of the conjunctival epithelium (as shown in previous reports[11,23]), but also in the infiltrating cells located in the substantia propria (Fig. 7). Figure 8 shows that some of the IL-33–positive cells were double-positive with a macrophage marker (F4/80) and mast cell marker (mMCP1). These results were consistent with previous reports showing that IL-33 is produced by mast cells[33] and activated macrophages.[34] Considering these results together, we concluded that not only epithelial cells but also macrophages and mast cells were the source of IL-33 in RW-EAC models.

It should also be noted that there was no apparent IL-33 positive staining in the vascular endothelia of conjunctival tissues obtained from the RW-EAC models (Fig. 7), whereas in human conjunctival tissues obtained from AKC/VKC patients, the vascular endothelium (especially high endothelial venules) is immunopositive for IL-33.[4] The limitations of the present study are the feasibility of RW-induced allergic conjunctivitis as a model for severe human chronic allergic conjunctivitis in which exposure to multiple, and divergent allergens (e.g., HDM, pollen, and animal-derived antigens) cause severe chronic allergic conjunctivitis, and the differences of IL-33 expression between human and mouse tissues, especially in the vascular endothelium.

In conclusion, among the epithelial cell–derived type 2–initiating cytokines, we demonstrated the indispensable role of IL-33 in the pathophysiology of RW-EAC. Targeting IL-33 signaling cascades on the ocular surface using a decoy receptor (soluble ST2) for IL-33 might be a promising therapeutic method for ocular allergic diseases.

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