The roles of epithelial cell derived type 2 initiating cytokines in experimental allergic conjunctivitis

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Abstract

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

Methods: IL-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. The clinical scores and eosinophil infiltration were evaluated. Serum IgE and cytokine expression in the conjunctival tissue were quantified and immunohistochemical analysis was carried out.

Results: Significant reductions of the clinical scores and the numbers of infiltrating eosinophils were observed in the RW-EAC models using IL-33 KO mice. There were no significant differences in the clinical scores and the 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. Il4, il5, il13 and ccl5 mRNA expression was diminished in the conjunctivae of RW-EAC models using IL-33 KO mice compared to those of wild type mice. IL-33
expression was upregulated as early as one hour after RW eye drop challenge. The
numbers of infiltrated basophils were diminished in the conjunctivae of RW-EAC
models using IL-33 KO mice compared to those of wild type mice.

**Conclusions:** Among the type-2 initiating cytokines, IL-33 plays major roles for
conjunctival inflammation in RW-EAC model.

**Key words**

Type 2 initiating cytokines, IL-25, IL-33, Thymic stromal lymphopoietin, Experimental
allergic conjunctivitis.
INTRODUCTION

Type 2 immune responses are inflammatory conditions associated with parasite infections, or with atopic diseases like asthma, atopic dermatitis and atopic keratoconjunctivitis. Type 2 immune responses are characterized by the activation of CD4+ T helper type 2 (Th2) cells along with the production of typical type-2 immunity-associated cytokines, [i.e. interleukin-4 (IL-4), IL-5, and IL-13]. Although various external stimuli (including pollens, house dust mites, food allergens and parasites) could induce type 2 responses, these antigens can not directly activate Th2 cells because they are too large to be phagocytosed by antigen-presenting cells. The roles of epithelial cell-derived type 2 initiating cytokines [IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)] were characterized recently as indispensable cytokines for initiating type 2 immune responses stimulated by these type 2 immunity-related antigens.

We previously reported the expression of IL-33 and TSLP mRNA and protein in the giant papillae tissue obtained from patients of vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC). On the other hand, our study group established IL-33 knockout (KO) mouse and reported that IL-33 have essential roles in papain-induced lung inflammation, which considered to be innate immune system dependent type 2 inflammation. The roles of IL-33 were also reported using ovalbumin (OVA)-induced
Asthma model\textsuperscript{7-9}, an established model for acquired immune system dependent type 2 inflammation. Ragweed (RW)-induced experimental allergic conjunctivitis (EAC) had been used as a common model for T cell (acquired immunity)-dependent allergic conjunctivitis.\textsuperscript{10} Matsuba-Kitamura et al. reported that addition of recombinant IL-33 at the time of RW eyedrop challenge developed augmented eosinophil infiltration in the conjunctival tissue in their RW-EAC models.\textsuperscript{11}

TSLP is produced by epithelial cells in response to various protein allergens (i.e. OVA) and protease allergens (i.e. pollens and papain).\textsuperscript{12} TSLP activates dendritic cells (DCs) through TSLP receptor (TSLPR)-IL-7R\textsubscript{α} receptor heterodimer complex.\textsuperscript{13} TSLP-activated DCs express OX40L, which initiate T cell mediated type 2 inflammation by activating OX40 positive T cells.\textsuperscript{13} The upregulation of TSLP, TSLPR, and OX40L mRNA expression in RW-EAC models was reported by Zheng et al.\textsuperscript{14} IL-25 was originally discovered in cDNA libraries from highly polarized Th2 cells.\textsuperscript{15} IL-25 was also produced by epithelial cell, mast cells, eosinophils, macrophages.\textsuperscript{5} The importance of IL-25 for type 2 inflammation was demonstrated by the experiments using mouse OVA-induced asthma models, in which attenuated airway inflammation and airway hyper-responsiveness (AHR) were observed by deletion of IL-25 gene.\textsuperscript{16} There was no
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report concerning the expression of IL-25 in the conjunctival tissue and its roles for 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 using IL-25 KO, IL-33 KO, TSLPR KO mice and congenic wild type mice.

**MATERIAL and METHODS**

**IL-25 KO, IL-33 KO, TSLPR KO mice**

IL-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 at least seven generations to establish congenic IL-33 KO, IL-25 KO and TSLPR KO mice. All the animal experiments conformed to the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

**Alam-ragweed immunized experimental allergic conjunctivitis**

Mouse experimental allergic conjunctivitis (EAC) was prepared as previously described\(^{10}\) with slight modifications. In brief, short ragweed pollen (RW, purchased from Polysciences, Warrington, PA) was emulsified on Imject Alam\(^\circledR\) (Thermo Scientific, Rockford, IL). At day 0, 50 \(\mu\)l of emulsified RW (50ug of RW with 50 \(\mu\)l of
alum) was injected into the left hind-footpad and the tail base, and blood was collected from tail vein. Two weeks later, second immunization was carried out using the right hind-footpad. From day 26 to 29, the eyes of immunized mice were challenged by RW in phosphate-buffered saline (PBS) (2mg in 10ul per eye) or by PBS alone daily for 4 days. 20 minutes after last eye drop challenge, clinical scores for EAC was evaluated by chemosis, redness, lid edema, tearing, discharge and scratching behavior, based on the criteria described by Magone et al. (Supplementary Table 1).\textsuperscript{19} 24 hours after the last eye drop challenge, the eye balls with lids and conjunctival tissue were collected for histological analyses and for quantification of cytokine expression. For IL-33 protein expression analysis, we made ex vivo culture model of resected conjunctivae from the eye challenged by RW eye drop only once at day 26. Blood samples were also collected for the measurement of serum IgE levels at day 30.

**Measurement of serum IgE levels**

Total IgE levels in the sera at day 0 and day 30 were quantified using ELISA MAX mouse IgE ELISA kit (Biolegend, San Diego, CA), according to the manufacturer’s protocol.

**Histological analysis**

The eye balls were dissected with conjunctival tissue and eye lids, and fixed in 4%
paraformaldehyde (PFA) in PBS. Vertical 2 μm-thick paraffin sections were made and
stained with Giemsa staining. Infiltrating eosinophils in the lamina propria mucosae of
the tarsal and bulbar conjunctivases throughout each section were counted at 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 mice eyes was immediately submerged in RNA
Later solution (Ambion, Austin, TX) to protect RNA. Total RNA was extracted from the
tissue using a NucleoSpin® II RNA isolation kit (Macherey-Nagel GmbH, Duren,
Germany). 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 mRNA were designed by QuantPrime (http://quantprime.mpimp-golm.mpg.de/),
and summarized in the Supplementary Table 2. Real-time PCR analysis was performed
with the ABI PRISM 7300 HT Sequence detection system using FAST-SYBER green
master mix (Life Technology Japan, Tokyo, Japan). The relative expression of il4, il5,
il13, il33, ccl5, and ccl11 was quantified by comparative Ct methods using gapdh
mRNA expression in the same cDNA as the internal controls.
Immunohistochemistry

Immunofluorescent staining was performed to examine the expression of IL-33 in the conjunctival tissue obtained from experimental conjunctivitis. Goat anti-mouse IL-33 polyclonal antibody was purchased from R&D systems (Minneapolis, MN), rat anti-mouse F4/80 antibody (clone CI:A3-1) was from BioLegends (San Diego, CA), and sheep anti-mouse mast cell protease (mcp)1 antibody (clone MS-RM8) was from Moredun Scientific (Midlothian, UK). Basophil specific rat anti-mouse mcp 8 antibody was from BioLegends, and rat anti-mouse major basic protein (MBP) antibody was provided from Dr. J Lee (Mayo Clinic).

5 μm frozen sections were made and then immunostained with the anti-IL-33, anti-MBP, anti-F4/80, anti-mcp1 antibodies. Anti-mcp8 immunohistochemical staining was carried out using 2 μm paraffin sections. The stained slides were scanned by confocal microscope (FV-1000; Olympus Corporation, Tokyo, Japan). Negative control specimens were immunostained with control goat IgG or rat IgG antibodies (all from Santa Cruz biotechnology, Santa Cruz, CA) instead of primary antibodies. Double-immunostaining was carried out on pairs of the goat anti-IL-33 antibody with the rat anti-MBP antibody, with the rat anti-F4/80 antibody or with the sheep anti-mcp1 antibody. Donkey Alexa 488-conjugated anti-rat IgG antibody, donkey Alexa 594-conjugated anti-goat IgG antibody, donkey Alexa 488
conjugated anti-sheep IgG antibody (all from Life Technology Japan, Tokyo, Japan) were used as secondary antibodies.

**Measurement of IL-33 protein concentration in the supernatant of ex vivo conjunctival tissue culture**

At indicated time period (1hr, 3hr, 6hr, and 12 hr) after the RW eye drop challenge, the mice were sacrificed and conjunctival tissues were sampled. The tissues were cultured in 1.5ml sterile Eppendorf tube (Eppendorf Japan, Tokyo, Japan) for 60 minutes using 200 μl of serum free culture medium (OPTI-MEM, Life Technologies Japan, Tokyo Japan) with protease inhibitors (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). IL-33 concentration in the supernatant of ex vivo tissue culture was quantified using Mouse IL-33 ELISA Ready-SET-GO (eBioscience, San Diego, CA), according to the manufacturer’s protocol.

**Statistical analysis**

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

**RESULTS**
Serum IgE concentration in the RW-EAC model

To evaluate the effect of RW-sensitization and the roles of type 2 initiating cytokines during sensitization process, we measured the serum IgE level of the day 0 (before sensitization) and day 30 (after eye drop challenge) by ELISA. Significant increase of total serum IgE at day 30 was observed in all type of mice (including wild type and IL-25 KO, IL-33 KO and TSLPR KO). There was no significant difference among the groups (Figure 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 day 0 and day 14, and they were challenged from day 26 using RW or PBS eye drops daily for 4 days. 20 min after 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 described in Supplementary Table 1. Clinical score of IL-33KO mice is significantly smaller than that of wild type mice. (Figure 2A and 2B, n=5 per group). There was no significant difference among the clinical scores of IL-25 KO, TSLPR KO and wild type mice (Figure 2B).

IL-33 deletion diminished the number of infiltrated eosinophils in the conjunctivae
of RW-induced EAC.

24 hours after the last RW eye drop challenge, the eyes collected and the number of eosinophils was counted using Gimsa stained slides (Figure 3A). The numbers of infiltrating eosinophils in the conjunctivae of IL-33 KO mice were significantly smaller than that of wild type mice. (Figure 3B, n=5 per group). There was no significant difference among the numbers of infiltrating eosinophils of IL-25 KO, TSLPR KO and wild type mice (Figure 3B).

Quantification of cytokine expression in the mouse conjunctival tissue obtained from RW-EAC models.

The conjunctival tissues were sampled 24 hour after the last RW eye drop challenge, their cytokine expression was quantified. Significant upregulation of il4, il5, il13, and ccl5 mRNA expression was observed in the conjunctival tissue of RW-induced EAC using wild type mice compared to those of PBS-challenged conjunctival tissue of the wild mice (Figure 4, asterisks). No significant upregulation was observed in ccl11-mRNA expression by RW eye drop challenges. Significant attenuation of il4, il5, il13, and ccl5 mRNA expression was observed in IL-33 KO mice compared to those of the wild type mice (Figure 4, double asterisks). Comparable amounts of cytokine expression was observed in the conjunctivae of TSLPR KO mice (Supplementary
Figure 1) and of IL-25 KO mice (Supplementary Figure 2) compared to those of the wild type mice, except significantly attenuated \textit{il5} mRNA expression in both TSLPR KO mice and IL-25 KO mice (Supplementary Figure 1 and 2, asterisks). In addition, significantly augmented \textit{il13} mRNA expression in the conjunctivae of TSLPR KO mice was observed compared to that of wild type mice (Supplementary Figure 1, double asterisks).

**Time course of \textit{il33} mRNA and IL-33 protein expression in RW-EAC models**

Significant \textit{il33} mRNA upregulation was observed in the conjunctival tissue obtained from the wild type mice after 1 hour from the last RW eye drop challenge (Figure 5A). IL-33 protein levels in the ex vivo culture supernatant was significantly upregulated at the conjunctival tissue sample obtained 1 hour after the first RW eye drop challenge (Figure 5B). No IL-33 protein was detected in the conjunctival tissue obtained from RW eye drop challenged IL-33 KO mice (data not shown).

**Immunohistochemical analysis of EAC**

Immunofluorescent staining was performed to examine the expression of IL-33, and immunolocalization of eosinophils, macrophages, mast cells and basophils in the conjunctival tissue obtained from EAC. Anti-mcp8 (basophil marker) immunostaining of the RW-EAC models showed basophil infiltration under subepithelial region of
RW-challenged eye (Figure 6A and 6B), and the number of infiltrated basophils are significantly higher in RW-EAC models of wild-type mice compared to that of IL-33 KO mice (Figure 6C).

RW-challenged conjunctivae of wild type mice showed IL-33 protein expression in the conjunctival epithelial cells and in the infiltrating cells of substantia propria of the conjunctival tissue at the vicinity of MBP-positive eosinophils (Figure 7, top row). The PBS-challenged conjunctival tissue of wild type mice showed IL-33 positive immunoreactivity at the epithelial cell layer, and sparse infiltration of eosinophils in subepithelial region (Figure 7, second row). Conjunctival tissue of RW-EAC model using IL-33KO mice had less MBP-positive eosinophil infiltration compared to those of wild type mice and no IL-33 positive immunostaining was observed (Figure 7, bottom row). Double immunohistochemical staining using anti-IL-33 antibody and the macrophage marker F4/80 antibody showed that some of the IL-33 positive cells in the substantia propria are also positive for F4/80 antigen (Figure 8 top row, arrows).

Similarly, some of the IL-33 positive cells in the substantia propria are also positive for the mast cell marker (mcp1) (Figure 8 bottom row, arrowheads).

**DISCUSSION**
To explore the roles of type 2 initiating cytokines (IL-25, IL-33, TSLP) in the pathophysiology of allergic conjunctivitis, we made RW-EAC models using IL-25 KO, IL-33 KO and TSLPR KO mice. The measurement of serum total IgE showed clear upregulation of serum IgE in RW-EAC models and no significant difference among the mice groups (Figure 1). The results are consistent with a previous report showing 10 folds upregulation of serum IgE in RW-EAC model using BALB/c mice, and another report showing the increase of total serum IgE in RW-induced rhinitis models using IL-33 KO mice. Similarly, house dust mite (HDM) immunization and subsequent intranasal HDM challenges using TSLPR KO mice or IL-17RB (IL-25 receptor) KO mice showed comparable serum IgE increases to those of wild type mice. Taken together, we concluded that the type 2 initiating cytokines do not affect IgE responses on RW-EAC models.

Next, we compared clinical scores (Figure 2) and the numbers of infiltrated eosinophils (Figure 3) in the RW-EAC models. The results showed that IL-33 deletion caused attenuated clinical severities and the diminished numbers of eosinophil infiltration in the conjunctivae of RW-EAC models. Attenuation of inflammation of RW-EAC in IL-33 KO mice in both early-phase clinical scores and delayed-phase eosinophile infiltration was consistent with the previous report showing roles of IL-33
during antigen challenge phase of RW-EAC model.\textsuperscript{11} We found no differences among wild type mice, TSLPR KO mice, IL-25 KO mice concerning clinical severities and the numbers of eosinophil infiltration in the conjunctivae of RW-EAC models (Figure 2 and 3). The results of inflammatory cytokine quantification showed attenuated \textit{il5} mRNA expression and but no differential expression of \textit{il4}, \textit{ccl5} or \textit{ccl11} mRNA in the conjunctival tissue of RW-EAC models using TSLPR-KO mice/IL-25 KO mice compared to wild type mice (Supplementary Figure 1 and 2). Schleimer et al.\textsuperscript{25} reported the effects of IL-5, CCL5 and CCL11 for the transendothelial migration of eosinophils. According to their results, IL-5 itself showed minimum effects for eosinophil migration but synergetic effects for eosinophil migration with CCL5 or with CCL11. Absence of differential expression of \textit{ccl5} and \textit{ccl11} mRNA may account for the lack of difference for eosinophil infiltration among TSLPR KO mice, IL-25 KO mice and wild type mice. To further clarify the roles of IL-33 for the pathophysiology of RW-EAC, chronological changes of IL-33 expression in the conjunctival tissue was examined. Upregulation of \textit{il33} mRNA expression, peaked at 1hr after RW eyedrop challenge, was observed (Figure 5A). To quantify IL-33 protein expression at ocular surface in RW-EAC model, we tried to detect IL-33 protein by simply collecting ocular surface
exudate using small amount of PBS, however, the IL-33 concentration in the exudates were below the detection level (data not shown). So we measured IL-33 protein concentration of ex vivo culture supernatant of conjunctival tissue obtained after RW-challenge. Significant increase of IL-33 protein in the culture supernatant from 1hr until 12hr after RW eye drop challenge (Figure 5B) suggested continuous IL-33 protein release from IL-33 producing cells. These rapid upregulations of IL-33 mRNA/protein were consistent with the results of RW-induced experimental rhinitis model\textsuperscript{23} and mixture of allergens (HDM, \textit{Aspergillus, Alternaria})-induced lung inflammation. We observed significant attenuation of \textit{il4} mRNA expression in the conjunctivae of RW-EAC models using IL-33 KO mice compared to those of wild type mice (Figure 4). IL-4 is a key cytokine for type 2 immune responses mediated by adaptive immunity. IL-4 stimulates Th2 cell differentiation from naïve T cell, and induces IL-5 and IL-13 expression.\textsuperscript{27} The effects of IL-33 deletion for \textit{il4} mRNA expression were significant even between the eyes with mock eye drop challenges, but it became more apparent when challenged by RW eyedrops (Figure 4). We observed diminished numbers of infiltrating basophils, which is known as the producers of large amount of IL-4\textsuperscript{28}, in the conjunctivae of RW-EAC models using IL-33 KO mice compared to those of wild type
mice (Figure 6). These results suggested that IL-33 might augment the expression of IL-4 in RW-EAC model by promoting systemic basophil expansion or by promoting basophil infiltration into the EAC tissue. Our hypothesis is further supported by the report showing IL-33 induces murine basophil expansion, and by the report showing diminished numbers of basophils in the nasal tissue of RW-induced rhinitis model using IL-33 KO mice.

Differential upregulation of ccl5 (encoding RANTES) mRNA but not of ccl11 (encoding eotaxin) mRNA in the RW-EAC models in IL-33 KO mice suggested IL-33 could induce ccl5 expression (Figure 4). These results were consistent with the report of Haenuki et al., showing IL-33 stimulation upregulate CCL5 protein expression but showing marginal effects for CCL11 expression in mast cells and in basophils. In RW-EAC model, we observed infiltration of basophils (Figure 6) and mast cells (Figure 8) in the conjunctival tissue, therefore IL-33 activated mast cells and basophils may play some roles for eosinophil infiltration in the RW-EAC model through the effect of CCL5, a well-known eosinophil chemoattractant.

The results of immunohistochemical staining revealed that positive IL-33 immunostaining not only in the cell nuclei of the conjunctival epithelium as shown in previous reports but also in the infiltrating cells located in substantia propria
In Figure 8, we also showed that some of the IL-33 positive cells are double positive with macrophage marker (F4/80) or with mast cell marker (mMCP1). These results are consistent with previous reports showing IL-33 is produced by mast cells and activated macrophages. Taken together, we concluded that not only epithelial cells but also macrophages and mast cells are the source of IL-33 in RW-EAC models. It also should be noted that there are no apparent IL-33 positive staining in the vascular endothelium of conjunctival tissue obtained from RW-EAC models (Figure 7), whereas in human conjunctival tissue obtained from AKC/VKC patients, the vascular endothelium (especially high endothelial venules) was immunopositive for IL-33. The limitation of present study is feasibility of RW-induced allergic conjunctivitis as a model for severe human chronic allergic conjunctivitis in which exposure to multiple, and divergent allergens (i.e. HDM, pollens, animal derived antigens) caused severe chronic allergic conjunctivitis, and also the differences of IL-33 expression between human and mice tissue, especially at vascular endothelium.

In conclusion, among the epithelial cell derived type 2 initiating cytokines, we showed indispensable roles of IL-33 for the pathophysiology of RW-EAC. Targeting IL-33 signaling cascades at ocular surface using the decoy receptor (soluble ST2) for IL-33 will be one of the promising therapeutic targets for ocular allergic diseases.
Figure 1

IgE (ng/ml)

Day 0
Wild IL-25KO TSLPRKO IL-33KO

Day 30
Wild IL-25KO TSLPRKO IL-33KO
Figure 2

A

B

Figure 3

A

B

Numbers of amoeboid beige
Figure 4

[Graphs showing relative mRNA expression levels for various cytokines and chemokines under different conditions.]
Figure 5
A

(fold)

Relative IL-33 mRNA expression

Time after RW challenge

PBS challenge 1hr 3hr 6hr 12hr

B

(pg/ml)

Time after RW challenge

PBS challenge 1hr 3hr 6hr 12hr
Figure 6

Figure 7
Figure 8
Figure Legends

Figure 1. Total serum IgE measurement before and after RW-immunizations

Total serum IgE concentration was quantified using ELISA at day 0 (before RW-immunization) and on day 30 (after RW eyedrop challenges). The data is a representative data measured in triplicate using five mice per each group, and shown by mean IgE concentration (ng/ml) ± standard deviation (SD).

Figure 2. Clinical evaluation of RW-EAC

Representative photographs of RW-EAC models, using wild type mice and IL-33 KO mice challenged either by RW-PBS (up row) or by PBS alone (down row) were taken 20 minutes after the last eye drop challenge (A). Clinical score of RW-challenged EAC models was shown (B). The data is a representative data of mean ± SD clinical score of 5 mice for each group. (*P<0.05, Mann Whitney’s U-test)

Figure 3. Eosinophil infiltration in the conjunctivae of RW-EAC

The eye of RW-EAC models were sampled 24 hours after last RW challenges and the numbers of infiltrating eosinophils (arrows) in the substantia propria of the conjunctival tissue were counted using Gimsa stained slides (A). The data is a representative data of mean ± SD numbers of the infiltrated eosinophil per slides counting the conjunctivae of 5 mice for each group (B). (*P<0.05, Mann Whitney’s U-test)
Figure 4. Quantification of cytokine mRNA expression in the conjunctivae of RW-EAC models

Expression of inflammatory cytokines/chemokines (\textit{il}4, \textit{il}5, \textit{il}13, \textit{ccl}5, \textit{ccl}11) mRNA were quantified by realtime PCR. Relative mRNA expression was shown as fold changes to mRNA expression levels of PBS-challenged conjunctival tissue. The data is normalized by the expression of \textit{gapdh} mRNA of the same cDNA samples. Significantly elevated \textit{il}4, \textit{il}5, \textit{il}13, \textit{ccl}5 mRNA expression was observed in the RW challenged conjunctivae of wild type mice compared to PBS challenged conjunctivae (asterisks, *\textit{P}<0.05). Attenuated \textit{il}4, \textit{il}5, \textit{il}13, \textit{ccl}5 mRNA expression was observed in the RW-challenged conjunctivae of IL-33 KO mice compared to those of wild type mice (double asterisks, **\textit{P}<0.05). No differential expression was observed for \textit{ccl}11 mRNA expression. A representative data (mean fold expression ± SD) measured in triplicate was shown.

Figure 5. Time course of IL-33 expression in the conjunctivae of RW-EAC models

Expression of \textit{il}33 mRNA (A) and IL-33 protein (B) at various time points (1, 3, 6, 12 hour) after single RW eye drop challenge was quantified by realtime PCR and ELISA analysis, respectively. Relative mRNA expression was shown as fold changes to mRNA expression levels of PBS-challenged conjunctival tissue. The data is normalized by the
expression of \textit{gapdh} mRNA of the same cDNA samples. A representative data (mean fold expression ± SD) measured in triplicate was shown (A). IL-33 concentrations in the culture supernatants of ex vivo cultured conjunctivae tissues were quantified. A representative data (mean fold expression ± SD) using three conjunctivae for each time point measured in duplicate was shown (B). (*$P<0.05$, Mann Whitney’s U-test)

\textbf{Figure 6. Mcp8 positive basophils in the conjunctivae of RW-EAC models}

Higher numbers of mcp8 positive basophils (arrows) were found in the subepithelial regions of RW-EAC models in the wild type mice (A) compared to IL-33 KO mice (B). Original magnification x200. Numbers (mean ± SD) of the basophils per slides in the conjunctivae of 8 mice for each group was shown (C). (*$P<0.05$, Mann Whitney’s U-test)

\textbf{Figure 7. IL-33 expression of in the conjunctivae of RW-EAC models}

Anti-MBP and anti-IL-33 immunostaining of conjunctivae obtained from RW-EAC models were shown. Massive infiltration of MBP positive eosinophils (green) were observed in the substantia propria of RW-challenged EAC using wild type mice (top left). On the other hand, sparse infiltration of eosinophils was observed in PBS-challenged wild type mice and in RW-challenged IL-33 KO mice (middle and bottom left). IL-33 protein expression (red) was observed in the conjunctival epithelial
cells of wild type mice. The infiltrating cells of substantia propria at the vicinity of MBP
positive eosinophils (shown in the merged image) were also immunopositive for IL-33
(top right). No IL-33 positive cells were observed in the conjunctiva of IL-33 KO mice.
Original magnification x200.

**Figure 8. Colocalization of F4/80 and IL-33, as well as mMCP1 and IL-33 in the
substantia propria of RW-EAC model.**

Some of the IL-33 positive cells in the substantia propria are also immunopositive for
F4/80 (arrows) or mMCP1 (arrowheads). Original magnification x200.
References


Supplementary Figure 1

Supplementary Figure 2
Legends for Supplementary Figure 1

Expression of inflammatory cytokines/chemokines (il4, il5, il13, ccl5, ccl11) mRNA were quantified by realtime PCR analysis. Relative mRNA expression was shown as fold changes to mRNA expression levels of PBS-challenged conjunctival tissue. The data is normalized by the expression of gapdh mRNA of the same cDNA samples. Attenuated il5 mRNA expression (asterisk, *P<0.05) and augmented il13 mRNA expression (double asterisks, **P<0.05) was observed in the RW challenged conjunctivae of TSLPR KO mice compared to those of wild type mice. A representative data (mean fold expression ± SD) measured in triplicate was shown.

Legends for Supplementary Figure 2

Expression of inflammatory cytokines/chemokines (il4, il5, il13, ccl5, ccl11) mRNA were quantified by realtime PCR analysis. Relative mRNA expression was shown as fold changes to mRNA expression levels of PBS-challenged conjunctival tissue. The data is normalized by the expression of gapdh mRNA of the same cDNA samples. Attenuated il5 mRNA expression (asterisk, *P<0.05) was observed in the RW challenged conjunctivae of IL-25 KO mice compared to those of wild type mice. A representative data (mean fold expression ± SD) measured in triplicate was shown.