Contribution of IL-33 to induction and augmentation of experimental allergic conjunctivitis

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Abstract

IL-33, a member of the IL-1 family of cytokines, is the ligand for ST2 (IL-33Ra chain). IL-33 has the capacity to induce T₄,₂ cytokine production from T₄,₂ cells, mast cells and basophils, indicating that IL-33 has the potential to induce T₄,₂ cytokine-mediated allergic inflammation of the eye. Thus, we tested the pathological role of IL-33 in allergic conjunctivitis (AC). As reported elsewhere, animals immunized with ragweed pollen (RW)/alum and boosted with RW/PBS developed AC promptly (within 15 min) and conjunctival eosinophilic inflammation after a delay (within 24 h) in response to eye drop challenge with RW. Furthermore, RW-immunized mice, when topically challenged with both RW and IL-33, developed more striking eosinophilia in their conjunctiva without exacerbation of the clinical AC score. This in vivo IL-33 treatment significantly increased the capacity of T cells in the cervical lymph nodes of RW-immunized mice to produce IL-4, IL-5 and IL-13 upon challenge with anti-CD3 and anti-CD28 antibodies in vitro. Furthermore, the infiltrating cells were largely eosinophils and a small proportion of CD4⁺ T cells, both of which express ST2. We also found that even splenic eosinophils express ST2 and show increased expression in response to IL-5, granulocyte–macrophage colony-stimulating factor (GM-CSF) or IL-33. Eosinophils, stimulated with IL-5 and/or GM-CSF, are responsive to IL-33, which induces production of IL-4 and chemokines. Finally, we showed that conjunctival tissues constitutively express biologically active IL-33, suggesting that IL-33 might play a crucial role in the induction and augmentation of AC.

Keywords: allergen, allergic conjunctivitis, chemokines, eosinophils, eotaxin, eye, IL-33, rodent, ST2, T₄,₂ cells

Introduction

Allergic conjunctivitis (AC) is a common ocular inflammatory disease. In developed countries, 20–30% of the population has experienced allergies, and 50% of these individuals suffer from ocular allergies (1, 2). AC can occur as mild transient inflammation such as seasonal AC or more severe chronic forms such as vernal keratoconjunctivitis (3, 4). AC is induced by a hypersensitivity response after exposure to an allergen. This response comprises two stages: an IgE-dependent early-phase response (within 15 min after exposure) and a T₄,₂ cytokine-dependent late-phase (12–24 h after exposure) response. Clinical symptoms and signs, such as itching, conjunctival swelling (chemosis) and congestion, occur as a result of the early-phase response. The late-phase response can involve conjunctival eosinophilic infiltration at 8–24 h after exposure to an allergen. Eosinophilic inflammation is not only a hallmark of AC but also a major cause of tissue injury and remodeling (5). Induction of the late-phase response is dependent on the accumulation of antigen-activated T₄,₂ cells (6–8), which produce IL-4, IL-5, IL-9, IL-13 and chemokines. However, the precise mechanisms by which T₄,₂ cells promote the pathogenic immune responses in AC are still unclear. Indeed, it has not been clearly demonstrated that antigen-specific T₄,₂ cells actually infiltrate the conjunctiva. Furthermore, the mechanisms underlying the onset of AC and the progression to severe AC pathologies, such as vernal keratoconjunctivitis, remain unclear.
Recently, IL-33 was cloned and shown to be the ligand of ST2 (9). Initially, IL-33, like other members of the IL-1 family (10), was thought to be changed into its active form after cleavage with caspase-1 (9). However, a very recent study revealed that an even larger (31 kDa) form of IL-33 has strong biological activity and loses its activity after cleavage with caspase-1 (11). Our laboratory and others reported that T<sub>h</sub>2 and T<sub>h</sub>1 cells preferentially express IL-18R<sub>α</sub> chain and ST2, respectively (12–14). In the same way that functional IL-18R is composed of an IL-18R<sub>α</sub> and an IL-18R<sub>β</sub> chain (15, 16), functional IL-33R consists of an IL-33R<sub>α</sub> (ST2) and an IL-33R<sub>β</sub> (IL-1R<sub>β</sub>) chain (17, 18). Although T<sub>h</sub>2 cells preferentially express IL-33R, ST2 deficiency does not affect the development of T<sub>h</sub>2 cells in vitro (19). Furthermore, inoculation with gastrointestinal nematodes normally induces IgE in ST2<sup>−/−</sup> mice (19). These results suggest the possibility that IL-33 principally augments allergic inflammation by enhancing T<sub>h</sub>2 cytokine production from T<sub>h</sub>2 cells (12). However, basophils and mast cells, when stimulated with IL-3 and IL-33, also produce large amounts of T<sub>h</sub>2 cytokines (12, 20). Furthermore, we recently demonstrated that administration of IL-33 into naive mice induces ST2/MyD88-dependent airway hyperresponsiveness (AHR), goblet cell hyperplasia and eosinophilia by induction of IL-4, IL-5 and IL-13 in the lungs even in the absence of T cells (12). These results clearly suggest that IL-33 is an important cytokine that induces and augments T<sub>h</sub>2 cytokine-mediated allergic inflammation by activation of T<sub>h</sub>2 cells and possibly mast cells and basophils.

In this study, we examined the pathological role of IL-33 in the development of AC. Ragweed pollen (RW)-immunized mice develop early-phase AC manifestation and late-phase conjunctival eosinophilic inflammation after challenge with RW. Additional IL-33 challenge significantly increased the late-phase response without affecting the early-phase response. We found that antigen challenge induced recruitment of ST2<sup>+</sup>CD4<sup>+</sup> T cells and ST2<sup>+</sup> eosinophils into the conjunctiva and additional IL-33 challenge significantly enhanced these responses. We also found that IL-5 induced IL-33R expression on eosinophils and that these IL-5-stimulated eosinophils produced IL-4 and chemokines in response to IL-33. Finally, we demonstrated that IL-33 is constitutively expressed in epithelial cells in the conjunctiva, suggesting its important role in induction and augmentation of AC.

**Methods**

**Mice**

BALB/c mice were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan). All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care Committee of Hyogo College of Medicine.

**Reagents**

Recombinant human IL-33 and recombinant mouse IL-33 (rmIL-33) were made by Hokudo Co., Ltd (Sapporo, Japan) as described in our previous report (12). Purified antibody against mouse CD3 (2C11) was prepared in our laboratory. PE-anti-mouse CD4 ( GK1.5), PE-anti-mouse Siglec-F (E50-2440) and biotin-anti-mouse IgE (R35-118) were purchased from BD Biosciences (San Diego, CA, USA). FITC-anti-mouse T1/ST2 was purchased from MD Biosciences (St Paul, MN, USA). Anti-CD28 and anti-CD16/32 were purchased from BioLegend (San Diego, CA, USA). Rat anti-mouse IgE (23G3) and affinity-purified goat anti-mouse IgG1 were purchased from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). RW was purchased from Polysciences (Niles, IL, USA). RW extract was purchased from LSL Co. Ltd. (Tokyo, Japan). Mouse IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Genetics Institute Inc. (Cambridge, MA, USA).

**Experimental AC by active immunization**

Mice were immunized with a mixture of RW (100 μg in 200 μl) and aluminum hydroxide hydrate gel (1 mg in 200 μl) (Sigma Aldrich, St Louis, MO, USA) by subcutaneous (s.c.) injection on day 0 and with RW/PBS (100 μg in 200 μl) by intraperitoneal (i.p.) injection on day 14. A week after the boost, mice (five mice per group) were challenged by topical administration of eye drops of RW (1 mg in 5 μl PBS per eye) or PBS (5 μl per eye). For IL-33 treatment, mice (five mice per group) were treated with IL-33 (1 μg in 5 μl PBS per eye) by topical administration of eye drops 1 h before and 2, 4 and 6 h after challenge with PBS or RW. A clinical score for AC was determined within 15–30 min after eye drop challenge with RW by examining chemosis, redness, lid edema, tearing, discharge and scratching behavior, based on the criteria described by Ozaki et al. (8) (Table 1). Two observers, one of whom was an experienced ophthalmologist, carried out a blind test to evaluate clinical appearances and photographs. Scratching behavior was monitored for 30 s, and the frequency of scratching was counted and evaluated as follows: one to three times, mild; four to six times, moderate, and more than seven times, severe. The final AC score was calculated as the sum of the values for both eyes for each mouse. After 24 h, eyes were isolated for histological analysis, and the number of infiltrating cells was counted in the conjunctiva.

**In vitro cytokine production**

Cervical lymph node cells were isolated from mice and cultured at 2 × 10<sup>5</sup> 0.2 ml<sup>−1</sup> per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg ml<sup>−1</sup> for coating) in RPMI 1640 supplemented with 10% fetal bovine serum, 2-ME (50 μM), l-glutamine (2 mM), penicillin (100 U ml<sup>−1</sup>) and streptomycin (100 μg ml<sup>−1</sup>) in 5% CO<sub>2</sub>.

**Table 1. Clinical evaluation of AC**

<table>
<thead>
<tr>
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<th>Absent</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
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<tbody>
<tr>
<td>Chemosis</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Conjunctival redness</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Lid edema</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Tear and discharge</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Scratching</td>
<td>0</td>
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Animals were examined clinically for signs of an early-phase response 15 min after topical application of RW. Chemosis, conjunctival redness, lid edema, tearing and discharge and scratching behavior were graded based on the grading table. Clinical appearances were evaluated blind by two observers. A score was given for each eye, and the final results show the sum of these scores for both eyes of each mouse. Scores shown in the figures are the average values for each mouse.
Flow cytometry and cell purification

Spleen cells (2 × 10^6 ml^-1) from naïve BALB/c mice were stimulated with medium alone, IL-5 (40 ng ml^-1) and/or IL-33 (100 ng ml^-1) in 24-well plates for 24 h. After incubation, cells were harvested and examined for their expression of IL-33Rα chain and gated as side scatterhigh (SSChigh), Siglec-F-, non-B and non-T cells by FACSCalibur (BD Biosciences). For preparation of splenic eosinophils, spleen cells from BALB/c mice were first depleted of Thy1.2^+ T cells and B220^+ cells using the MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) and then residual cells were stained and separated into Siglec-F^-CCR3^- cells using a fluorescence cell sorter (FACS Aria; BD Biosciences). The purity of sorted eosinophils was >99%.

**ELISA assay**

ELISA kits for IL-4, IL-5, IL-6, IL-13 and eotaxin (R&D Systems Inc., Minneapolis, MN, USA) were used. To measure IL-33 protein levels, we constructed an ELISA system to quantify mouse IL-33 protein levels. We made polyclonal rabbit IgG antibody to IL-33, which were further purified using a cyanogen bromide-activated Sepharose 4B column conjugated with rmIL-33 (10 mg). A 96-well plate (Coster 9018; Corning Incorporated, Corning, NY, USA) was coated with this affinity-purified anti-IL-33 polyclonal antibody (rabbit IgG) and blocked with StartingBlockTM blocking buffer (Thermo Scientific, Rockford, IL, USA). Mouse IL-33 cells were isolated from each mouse and homogenized with PBS containing streptomycin (100 µg ml^-1). After 48 h stimulation, supernatants were harvested and the concentration of IL-4, IL-5 and IL-13 was tested using an ELISA kit. Eosinophils (1 × 10^5 0.2 ml^-1 per well), sorted as described below, were stimulated with medium alone, IL-5 (40 ng ml^-1) and/or IL-33 (100 ng ml^-1) in the presence or absence of GM-CSF (50 ng ml^-1) for 24 h. Supernatants were harvested and tested for cytokines and chemokines using the Bio-Plex System (Bio-Rad, Hercules, CA, USA) as previously described (12).

**Confluent microscopy**

Frozen sections from freshly isolated conjunctival specimens were fixed and incubated with FITC-anti-mouse T1/ST2 and PE-anti-mouse CD4 or FITC-anti-mouse T1/ST2 and PE-anti-mouse Siglec-F at 4°C overnight. For IL-33 protein staining, sections were incubated in 4% PFA PBS (Wako, Osaka, Japan) at 4°C overnight. Paraffin-embedded sections (4-µm thick) of the conjunctiva were deparaffinized, heated in a microwave (500 W for 5 min, three times) in citrate buffer (pH 6.0) for antigen retrieval and then cooled at room temperature for 50 min before blocking. The sections were incubated in PBS containing 1.0% BSA and 0.05% Tween 20 for blocking. The sections were incubated with purified anti-IL-33 polyclonal antibody (rabbit IgG), at 4°C overnight, and then secondary antibody, biotin-conjugated goat antibody against rabbit IgG (Vector Laboratory, Burlingame, CA, USA), at room temperature for 30 min. Sections were then stained with a tertiary antibody, Alexa Fluor 555-conjugated streptavidin (Invitrogen, Carlsbad, CA, USA), at room temperature for 30 min. Coverslips were applied along with mounting medium containing 4',6-diamidino-2-phenyindole (Invitrogen) and the sections were examined under a microscope Zeiss LSM 510 (Carl Zeiss, Thornwood, NY, USA). Computer software, Zeiss LSM 510 ver. 3.2 (Carl Zeiss), was used for image processing and analysis.

**Quantitative real-time PCR**

Total RNA was extracted from cervical lymph nodes or conjunctiva using the RNeasy Plus Mini Kit (Qiagen, Germantown, MA, USA) and the cDNA was synthesized using SuperScript III RNase H Reverse Transcriptase (Invitrogen). The expression of the gene was quantified with the TaqMan Gene Expression Assay (Applied Biosystems, Foster, CA, USA). The results were presented as relative expression values standardized with the expression of the gene encoding eukaryotic 18S ribosomal RNA (rRNA) (18S). Specific primers used for quantitative real-time PCR were ST2 (IL1RL1, interleukin 1 receptor-like 1) (Assay ID: Mm00516117_m1), IL-33 (Il33) (Assay ID: Mm00505403_m1) and 18S rRNA (18S) (Assay ID: Hs99999901_s1).

**Statistics**

Data are presented as means ± SDs. Statistical comparisons between two experimental groups were determined by the paired Student’s t-test performed using GraphPad Instat.
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Software (San Diego, CA, USA). P-values <0.05 were considered statistically significant.

Results

Exogenous IL-33 fails to augment RW-induced immediate type AC

We first examined whether exogenous IL-33 has the capacity to enhance the early-phase response of RW-induced allergic inflammation (i.e. AC). We immunized BALB/c mice with RW by sequential s.c. injection of RW/alum, followed by i.p. injection of RW/PBS. Then, we challenged their eyes by topical administration of RW and/or IL-33. At 15 min after challenge, we scored the severity of AC (see Methods) by measuring the degree of chemosis, conjunctival redness, lid edema, tearing, discharge and scratching as described in Table 1. As reported elsewhere (8), naive mice after being challenged with RW developed AC-like manifestations (score, 3.3 ± 1.2) (Fig. 1A), suggesting that RW has the capacity to irritate conjunctiva in a non-specific manner. Compared with non-immunized and subsequently PBS- or RW-challenged control mice, RW-immunized mice developed severe AC (score, 11.6 ± 1.7; P < 0.001) (Fig. 1A) at 15 min after the challenge with RW, suggesting that RW challenge induces immediate type AC possibly in an IgE-dependent manner. Indeed, RW-immunized mice displayed RW-specific IgE in their sera (Fig. 1B). Eye drop challenge with RW and/or IL-33 did not change the level of RW-specific IgE in sera. We also found that this additional IL-33 challenge was unable to augment RW-induced AC, suggesting that RW is solely responsible for inducing AC manifestations after the challenge with RW and IL-33, although we cannot exclude the contribution of endogenous IL-33.

Exogenous IL-33 augments RW-driven conjunctival eosinophilic inflammation

We next compared the histological changes in the conjunctiva at 24 h after challenge with RW and/or IL-33 (Fig. 1C). Despite the failure of the additional IL-33 challenge to increase the AC score at 15 min (Fig. 1A), this treatment did significantly augment eosinophilic infiltration in the conjunctiva compared with that induced by RW challenge alone (P < 0.05; Fig. 1C and D). Thus, we investigated the mechanism behind exogenous IL-33-augmented eosinophilic infiltration. We compared the capacity of T cells from the cervical lymph nodes of RW-immunized mice at 24 h after challenge with RW and/or IL-33 to produce T helpers cytokines upon stimulation with anti-CD3 and anti-CD28 antibodies in vitro (Fig. 2A). Lymph node cells from RW-immunized mice produced IL-4, IL5 and IL-13 upon stimulation in vitro and lymph node cells from RW-immunized and RW-challenged mice produced the same cytokines but at higher levels. Furthermore, additional in vivo IL-33 challenge significantly increased the capacity of lymph node cells to produce IL-4, IL-5 and IL-13 upon stimulation in vitro, although IL-33 challenge alone failed to do so (Fig. 2A). In addition to T helpers cytokines, we simultaneously measured GM-CSF production from lymph node cells stimulated with anti-CD3 plus anti-CD28 in vitro. Although we could detect GM-CSF in the supernatants of lymph node cells from naive mice, the levels in RW-immunized mice after in vivo challenge with RW or RW plus IL-33 were significantly increased upon stimulation with anti-CD3 and anti-CD28 antibodies in vitro (P < 0.01 and P < 0.05, respectively) (Supplementary Figure 1 is available at International Immunology Online).

To clarify the mechanism of how RW and IL-33 synergistically increase T helpers cytokine production, we examined IL-33R expression by cervical lymph node cells in RW-immunized mice after challenge with RW and/or IL-33. We found that RW challenge markedly increased IL-33R mRNA expression by lymph node cells (P < 0.05; Fig. 2B) and additional IL-33 challenge further increased this mRNA expression (P < 0.05; Fig. 2B). Thus, cervical lymph node cells in RW-immunized and RW plus IL-33-challenged mice increased their IL-33 responsiveness by increasing IL-33R chain expression. We also examined local levels of eotaxin, a potent chemoattractant for eosinophils (22–24), after challenge with RW and/or IL-33. Although we could detect eotaxin in the supernatants of homogenates of conjunctiva from naive mice, the supernatants from RW-immunized mice showed significantly increased levels of eotaxin after challenge with RW or RW plus IL-33 (P < 0.05; Fig. 2C). Taken together, these results strongly indicated that when T helpers cells in cervical lymph nodes were stimulated with RW or RW plus IL-33, they were able to migrate to the conjunctiva and produce IL-4, IL-5 and IL-13 in the tissue. Then, IL-4 and IL-13 from T helpers cells were able to act in combination to induce recruitment of eosinophils via eotaxin production in the conjunctival tissue (Fig. 2D).

Accumulation of IL-33R chain-positive cells in the conjunctiva of AC mice

Recruitment of T helpers cells to the site of RW challenge is a key step in induction of AC. Thus, we tested whether topical RW application induces local accumulation of T helpers cells. Since T helpers cells express IL-33R chain (ST2) (12, 25), we examined T helpers cell accumulation by measuring ST2 expression. Topical challenge with PBS or IL-33 alone did not induce accumulation of ST2+ cells, while challenge with RW, particularly when combined with IL-33, induced marked accumulation of ST2+ cells in the conjunctiva of RW-immunized mice. We detected a substantial number of CD4+ T cells in the tissue and found that a large proportion (~70%) expressed ST2 (Fig. 3A). Thus, these challenges induced recruitment of RW-specific T helpers cells into the conjunctiva. We also detected a much larger number of ST2+ cells lacking CD4 compared with ST2+CD4+ T cells. By testing the cell type, we found that ~80% of these cells were eosinophils because they expressed Siglec-F (6, 26) (Fig. 3B). Taken together, these results indicated that, upon challenge with RW or RW plus IL-33, RW-specific T helpers cells infiltrated the conjunctiva and produced IL-4 and IL-13, which in turn induced accumulation of ST2+ eosinophils via local induction of eotaxin production in the conjunctiva.

IL-5, GM-CSF or IL-33 stimulation up-regulates the expression of IL-33R chain on eosinophils

Since RW challenge-induced accumulation of T helpers cells, which produce IL-4, IL-5, IL-13 and GM-CSF, we next determined
which of these cytokines were responsible for inducing ST2 expression on eosinophils. We prepared splenic cells from naive mice and cultured them with medium alone or with IL-5, IL-33 or a combination of IL-5 and IL-33, in the presence or absence of GM-CSF for 24 h. We then compared IL-33Rα expression by eosinophils cultured under these various conditions. We selected eosinophils by gating SSC<sub>high</sub>, Siglec-F<sup>-</sup>, non-B and non-T cell fractions. Eosinophils cultured alone expressed IL-33Rα (23.6%). Neither IL-4 nor IL-13 stimulation increased IL-33Rα expression (data not shown). However,
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**IL-33 production by conjunctiva**

We finally examined whether conjunctiva contains biologically active IL-33. It has been reported that IL-33 is constitutively expressed in the nucleus of endothelial and epithelial cells (31, 32). Immunohistochemical analysis revealed that IL-33 is constitutively expressed in the nucleus of epithelial cells of the conjunctiva of naive mice (Fig. 5A). Measurement of IL-33 mRNA expression indicated that the conjunctiva of naive mice constitutively expressed IL-33 mRNA and increased this message moderately after challenge with topical RW (Fig. 5B). We also found that naive mice possess IL-33 protein in their conjunctiva (Fig. 5C) and also increase this protein content moderately after topical RW application (Fig. 5C). These results strongly suggested that IL-33 is constitutively expressed in conjunctiva and topical RW administration weakly but significantly increased IL-33 levels. Next, we examined whether this IL-33 protein was biologically active. For this purpose, we measured the capacity of IL-33 to induce production of IL-6 from bone marrow-derived basophils. Basophils incubated with IL-3 produced IL-6 in a dose-responsive manner upon challenge with various doses of IL-33 in vitro (12). Addition of anti-IL-33 antibody completely inhibited IL-6 production (Fig. 5D). We simultaneously stimulated basophils in the presence of IL-3 with various doses of supernatant from the homogenized conjunctiva of naive mice or RW-challenged mice. Basophils produced IL-6 in a dose-responsive manner in response to these supernatants. Addition of anti-IL-33 antibody completely inhibited IL-6 production, suggesting that these homogenates contain functionally active IL-33 (Fig. 5D). From the results of this bioassay, we could also estimate the level of biologically active IL-33 in the homogenized conjunctiva from naive mice and RW-challenged mice and revealed that they have similar IL-33 activity (naive mice, 23.7 ng mg⁻¹ protein, and RW-challenged mice, 25.5 ng mg⁻¹ protein).

**GM-CSF.** We highly purified Siglec-F⁺CCR3⁺ cells by cell sorting (Fig. 4B) and light and electron microscopic examination revealed that sorted Siglec-F⁺CCR3⁺ cells were mature eosinophils (27) (Fig. 4C). We examined the IL-33 responsiveness of these cells by measuring their production of cytokines, IL-4 and IL-13, and chemokines, MIP-1α and MIP-1β. Stimulation with IL-5, IL-33 or GM-CSF alone only modestly induced eosinophils to produce IL-4 and chemokines. However, when eosinophils were stimulated with IL-33 in the presence of IL-5 and/or GM-CSF, they could produce substantial amounts of cytokine (IL-4) and chemokines (MIP-1α and MIP-1β) (Fig. 4D). However, compared with basophils or mast cells (12, 20), eosinophils only modestly produced IL-13 in response to IL-33 (Fig. 4D). Thus, activated eosinophils alone further increase accumulation of eosinophils by production of IL-4, which induces eotaxin in the tissue, and MIP-1α and MIP-1β, potent chemoattractants for eosinophils (24, 28–30). Taken together, these results indicated that eosinophils might induce inflammation of conjunctiva when stimulated with IL-5, GM-CSF and IL-33 by the production of cytokines, chemokines and possibly chemical mediators.

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Discussion

The findings of previous studies strongly suggest that IL-33 is a powerful inducer of allergic inflammation (9, 12, 33–35). IL-33 stimulates antigen-stimulated Th2 cells to increase production of IL-4 modestly and IL-5 and IL-13 strongly (9, 12). IL-33 also induces mouse basophils and mast cells, which express IL-33R abundantly, to produce IL-4, IL-6, IL-9, IL-13, GM-CSF and chemokines (RANTES, MIP-1α, MIP-1β and MCP-1) (12). Intranasal administration of IL-33 induces AHR, goblet cell hyperplasia and eosinophilia in the lungs of mice even in the absence of acquired immunity, and this effect is entirely dependent on ST2, MyD88 and IL-13 (12). Human basophils also produce IL-4, IL-8 and IL-13 in response to IL-3 plus IL-33 (36, 37). These results indicate an important role for IL-33 and ST2 in allergic inflammatory responses.

We have shown previously that the serum level of IL-33 is significantly elevated in patients with Japanese cedar pollinosis (35). In addition, IL-33 is reported to be markedly elevated in the sera of patients during anaphylactic shock (34). We have also shown a significant association between Japanese cedar pollinosis susceptibility and IL-33 polymorphism (rs1929992) (35). This was the first demonstration of the involvement of IL-33 in human allergic diseases. Subsequent studies also revealed that single-nucleotide polymorphisms within the genes encoding the ST2/IL-33Rα chain (rs1420101 on 2q12) and IL-33 (rs3939286 on 9p24) were significantly associated with blood eosinophil counts and allergic asthma (38). These studies prompted us to study the role of IL-33 in experimental AC.

In this study, we first demonstrated that RW challenge induced AC promptly and then eosinophilic inflammation in the conjunctiva of RW-immunized mice (Fig. 1A). Next, we demonstrated that additional IL-33 challenge significantly increased eosinophilic infiltration in the conjunctiva of RW-immunized mice at 24 h after challenge (Fig. 1C). Then, we investigated the mechanism of IL-33 activity and found that additional in vivo IL-33 treatment increased the capacity of Th2 cells in regional lymph nodes of RW-immunized mice to produce Th2 cytokines in response to anti-CD3 and anti-CD28 antibodies in vitro (Fig. 2A). Although further studies are required, we can speculate that IL-33, applied topically, and dendritic cells, pulsed with RW peptide, reach cervical lymph nodes via the afferent lymphatic vessel and in combination induce and activate RW-specific Th2 cells. We also found that cells in cervical lymph nodes increase their expression of IL-33Rα chain after challenge with RW or RW plus IL-33 (Fig. 2B), suggesting that antigenic stimulation, particularly with IL-33, up-regulates expression of IL-33Rα chain. We detected substantial numbers of IL-33Rα+ CD4+ T cells in the conjunctiva suggesting that Th2 cells, after challenge with RW or RW plus IL-33, migrate from cervical lymph nodes to the conjunctiva and play a critical role in the development of experimental AC (Fig. 3A).

Another striking feature of this experimental AC model is the massive infiltration of IL-33Rα+ eosinophils in the conjunctiva (Fig. 3B). This finding indicates that Th2 cells are responsible for inducing recruitment of IL-33Rα+ eosinophils in the conjunctiva. We found that topical application of RW and IL-33 strongly induces local production of eotaxin,
a chemoattractant for eosinophils, in the conjunctiva. Since IL-4 or IL-13 are known to induce production of eotaxin by fibroblasts (22–24), we propose that Th2 cells induce recruitment of eosinophils by production of IL-4 and IL-13 in the conjunctiva. It is also important to determine which Th2 cytokine can up-regulate IL-33Rα expression on eosinophils. We found that IL-5 strongly up-regulated IL-33Rα expression on eosinophils (Fig. 4A). Furthermore, IL-33 along with IL-5 stimulated eosinophils to produce IL-4, MIP-1α, and MIP-1β (Fig. 4D). We also demonstrated that, like IL-5, GM-CSF strongly up-regulates IL-33Rα expression on eosinophils and IL-33 along with GM-CSF stimulates eosinophils to produce IL-4, MIP-1α, and MIP-1β (Fig. 4A and D). Interestingly, IL-33 is able to up-regulate its own receptor (Fig. 4A).
However, IL-33 alone cannot induce production of IL-4 and chemokines. The receptors for IL-5 and GM-CSF are composed of a ligand-binding chain and a signal-transducing common β chain. Taken together, these results strongly indicated that two distinct signals, a common β-mediated signal (by IL-5 and/or GM-CSF) and an ST2/MyD88-mediated signal (by IL-33), are essential for induction of cytokine and chemokine production from eosinophils. A recent study indicated that ST2 expression by Th2 cells is regulated by GATA3 and STAT5 (39). IL-5 and GM-CSF are both STAT5 activators (40). These findings indicated that IL-33 and STAT5 activators increase ST2 expression not only by Th2 cells but also by eosinophils. Thus, our data strongly indicated that T_{h}2 cytokines play a crucial role in the recruitment and activation of eosinophils.

In contrast to murine splenic eosinophils, as shown in this report, freshly isolated human peripheral blood eosinophils do not express ST2 on their cell surface, although they express ST2 mRNA (41, 42). However, human peripheral blood eosinophils start to express ST2 molecules on their cell surface after incubation with medium alone for 24 h and ST2 expression is increased further after incubation with GM-CSF (41) but not with IL-33 (42). Human eosinophils can produce IL-8 when stimulated with IL-33 in the presence of IL-5 or GM-CSF (36, 41). Furthermore, IL-33 potently activates and induces superoxide production and degranulation in human eosinophils (41). It is intriguing to speculate that topical challenge with RW and IL-33 which in combination stimulate T_{h}2 cells to produce T_{h}2 cytokines in the conjunctiva and IL-5 from T_{h}2 cells in combination with IL-33 stimulate these IL-33R_{A}+ eosinophils in the conjunctiva to produce cytokines, chemokines and eosinophil-derived cationic proteins, resulting in the exacerbation of AC.

Recent studies by our laboratory and others (43–45) suggest the importance of basophils in the induction of the T_{h}2 response against protease antigens, antigen–IgE complexes or intestinal parasites. Based on the findings of these studies, we could suspect that RW–IgE complexes might enhance uptake of RW by basophils via the receptor Fc epsilon receptor I and the resulting RW-pulsed basophils...
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might induce or enhance development of RW-specific T_{h2} cells in vivo. Thus, it is crucial to clarify the antigen-presenting cell function of basophils in the induction of pollen-specific T_{h2} cells in RW-immunized mice.

We further revealed that IL-33 is constitutively expressed in epithelial cells in the conjunctiva of normal mice by immunohistochemical staining (Fig. 5A). Furthermore, a biological assay of IL-33 clearly revealed that biologically active IL-33 is constitutively expressed in the conjunctiva (Fig. 5D) and that the level of this molecule is significantly increased by exposure of the conjunctiva to RW (Fig. 5B and C). In general, members of the IL-1 cytokine family, including IL-1α, IL-1β and IL-18, are widely expressed in hematopoietic cells and are important for inflammatory responses and host defenses (16, 46). In addition, human IL-33 is expressed in the nucleus of epithelial cells, including those of the skin and gastrointestinal tract, where pathogens, allergens and other environmental agents are frequently encountered (32). Thus, IL-33, in a similar way to the prototype ‘alarmin’ high-mobility group box 1 (47), may work as an endogenous danger signal (32, 48). Indeed, IL-33 can be released after endothelial cell damage or injury (11). Pollen grains contain allergen proteins, enzymes (49) and bioactive lipids (50), the latter two of which might be involved in the pathogenesis of allergic diseases via an IgE-independent mechanism. Furthermore, RW releases serine and cysteine endopeptidases (51, 52). In addition, RW contains nicotinamide adenine dinucleotide phosphate oxidase that can generate reactive oxygen species in the epithelial cells of the conjunctiva (53). Thus, IL-33 could be increased and released when epithelial cells are stimulated or damaged by RW-derived serine and cysteine endopeptidases or by RW-mediated oxidative stress. It is possible that scratching further induces the production of IL-33 from conjunctiva. Once IL-33 is released by epithelial cells, like exogenous IL-33, this endogenous IL-33 together with RW-pulsed dendritic cells enters lymph nodes via the afferent lymphatic vessel and stimulates RW-specific T_{h2} cells to develop into cells that migrate and produce IL-4, IL-5 and IL-13 in the conjunctiva. Thus, our results strongly suggest the presence of endogenous IL-33 to the activation of T_{h2} cells and eosinophils, which in combination induce AC. In this way, IL-33 might represent an important therapeutic target for the treatment of AC.

Supplementary data

Supplementary Figure 1 is available at International Immunology Online.

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References

14 Yoshimoto, T., Takeda, K., Tanaka, T. et al. 1998. IL-12 up regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: synergy with IL-18 for IFN-gamma production. J. Immunol. 161:3400.

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