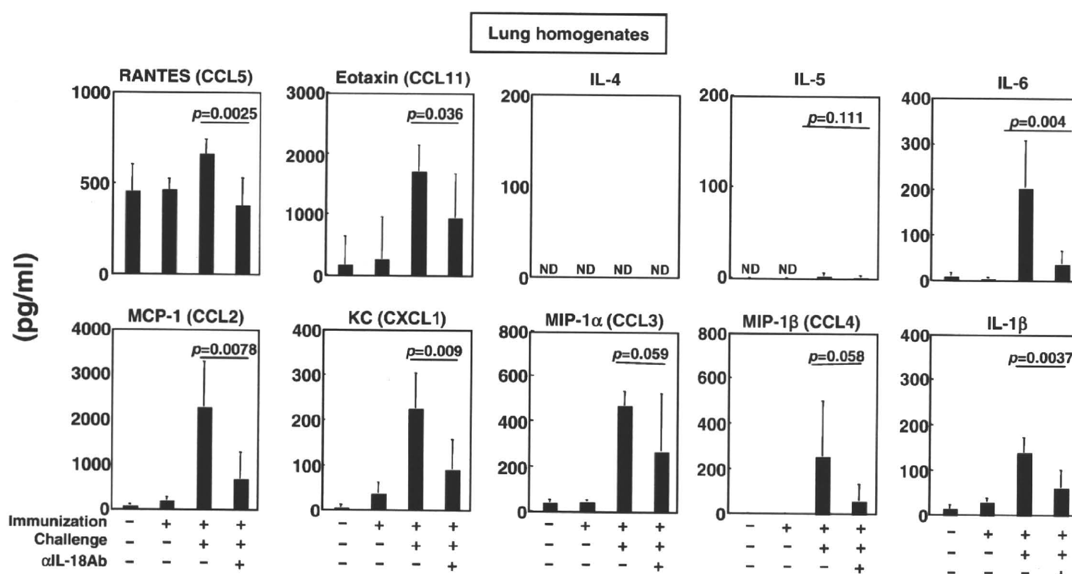

Fig. 3. IL-18 involvement in the induction of chemokines for neutrophils and eosinophils. Lung homogenates were prepared from the mice of three experimental groups (five mice per group) as shown in the legend to Fig. 1A and naive mice (five mice per group). The concentration of CCL2 (MCP-2), CCL3 (MIP1α), CCL4 (MIP1β), CCL5 (RANTES), CCL11 (Eotaxin), CXCL1 (KC), IL-1β, IL-4, IL-5 and IL-6 were measured by BioPlex®. Data are representative of three independent experiments.

available at *International Immunology* Online). Taken together, these results suggest that blocking the action of IL-18 is a potent therapeutic regimen for human airway inflammation initiated and/or exacerbated by bacterial infection.

Discussion

This study showed that endogenous IL-18 is critical for the development of SpA-induced asthmatic inflammation in mice. Upon intranasal exposure to SpA, mice immunized with SpA developed bronchial asthma-like airway inflammation (Fig. 1), concomitant with super T_h1-cell development and elevation of lung pro-inflammatory cytokine/chemokine production (Figs 2A–B and 3). Notably, lung IL-18 levels were significantly elevated in the SpA-induced asthmatic mice (Fig. 2C), and all these responses were prevented by inhibiting the action of IL-18 (Figs 1–3). Thus, IL-18 released from the lung exposed to SpA, together with SpA-presenting APC, might enter into mesenteric lymph node, in which SpA-specific T_h1 cells might develop toward super T_h1 cells. Furthermore, SpA-activated $CD4^+$ T cells, when transferred into naive mice, prepared the host mice to be highly responsive to intranasal administration of SpA, inducing bronchial asthma-like symptoms dependent upon endogenous IL-18 (Fig. 4). Finally, the humanized mice developed airway inflammation in a manner dependent on human IL-18 after intranasal SpA challenge (Fig. 6). IL-18 is important for *S. aureus*-associated asthmatic inflammation in mice and perhaps in humans.

We generated an airway inflammation model of temporally humanized mice by intranasal challenge with the bacterial protein, SpA. *Rag2*$^{-/-}$*C*$\gamma^{-/-}$ mice injected with human PBMC transiently possessed human hematopoietic cells in their peripheral lymphoid organs (Fig. 5). Due of their lack of T, B and NK cells, the recipient mice could not recognize human donor cells as antigens. In contrast, human donor cells consisting of those types of lymphocytes had the potential to be activated by recognizing xenogeneic recipient cells in host mice. In fact, mice having received human PBMCs spontaneously exhibited non-specific inflammatory changes in their lungs and livers as compared with control *Rag2*$^{-/-}$*C*$\gamma^{-/-}$ mice (Figs 1C and 6A; Supplementary Figure 6 is available at *International Immunology* Online). Despite apparent infiltration with human hematopoietic cells in the steady state (Fig. 6B), the basal lung inflammatory change was minimal (Fig. 6A), and the host mice survived without ill effects until sacrificed. This may be partly due to the inability of host cells to respond to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 signals. Therefore, it could be said that these transiently humanized mice have limited inflammatory responses without SpA challenge. However, intranasal SpA challenge induced severe airway inflammation in the mice that received SpA-stimulated PBMCs (Fig. 6C and D). Human $CD4^+$ T cells were densely recruited into airway after SpA challenge (Fig. 6C). Administration of neutralizing anti-human IL-18 significantly inhibited the development of airway inflammation by diminishing the accumulation of the donor cells (Fig. 6E and F). SpA-specific human $CD4^+$ T cells activated by both SpA and IL-18 likely induced the development of airway inflammation by releasing human cytokines and chemokines that recruit human PBMCs. Additionally, some of human chemokines [e.g. CCL5 (RANTES)] might act on murine cells to migrate as well. Further study is required to identify the human factors involved in this airway inflammation. Nonetheless, our results strongly suggest that IL-18 is a potent clinical target for the treatment of bronchial asthma associated with *S. aureus* colonization or infection.

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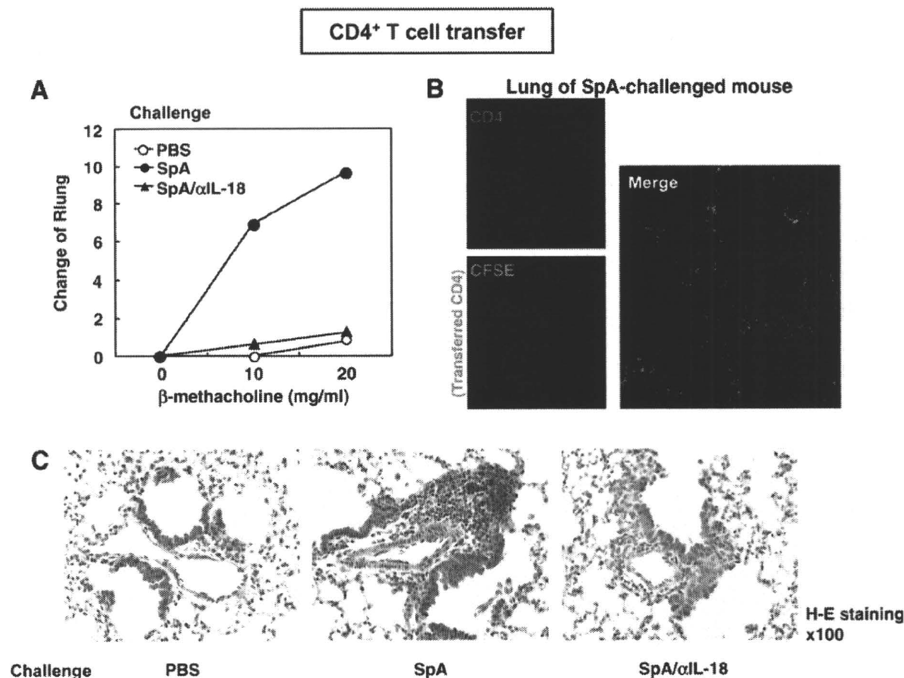

Fig. 4. SpA-sensitized CD4$^+$ T cells can transfer disease susceptibility. CD4$^+$ DLN cells isolated from mice immunized and boosted with SpA were labeled with CFSE and transferred into naive mice (1 × 10^7 per mouse). Recipient mice received intranasal challenge with SpA (250 μg/50 μl) (SpA), PBS or SpA and neutralizing anti-IL-18 antibodies (500 μg) (SpA/αIL-18). Twenty-four hours after the last SpA challenge, invasive measurement of AHR (A), confocal microscopic analysis for pulmonary CFSE (green) and CD4 (red) expression (B) and histological analysis (H&E) (C) were performed. Data are representative of three independent experiments with five mice.

It is unclear how human T cells recognize SpA in the transiently humanized mice. After *in vitro* stimulation with SpA, human PBMCs produced IFN-γ and IL-13 (Supplementary Figure 3 is available at *International Immunology* Online), and the CD4$^+$ T-cell population expanded (data not shown), suggesting that human APCs possess the potential to present SpA to the CD4$^+$ T cells in order to activate them. It was assumed that APCs included in the human donor cell preparation might serve as APCs for the SpA-specific human effector CD4$^+$ T cells in humanized mice as well. Alternatively, SpA-specific human effector cells might recognize SpA presented by xenogeneic mouse APCs by the mechanisms currently poorly understood.

We did not measure AHR of transiently humanized mice for a number of reasons. First, the recipients are *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice, which lack responsiveness to IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Second, in general, mice are poor responders to human cytokines. Third, C57BL/6 mice, a background of *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice, are resistant to T$_h$2 type and super T$_h$1 cell type asthma (16). Fourth, is that transiently humanized mice are not homogeneous in terms of the degree of repopulation with human CD4$^+$ T cells.

Bronchial asthma is now recognized to have diverse immunopathogenesis. Recently, we demonstrated that intranasal challenge with OVA, plus the IL-18-inducible bacterial LPS, initiates robust bronchial asthma in mice immunized with OVA/CFA. Neutralization of IL-18 during OVA plus LPS challenges inhibits AHR in OVA-specific T$_h$1 cell-bearing mice, suggesting that OVA plus LPS activates T$_h$1 cells via endogenous IL-18. In contrast, the role of endogenous IL-18 in allergic T$_h$2/IgE-dependent asthma is not clearly defined. One report demonstrated only partial contribution of IL-18 to OVA inhalation-induced chronic allergic airway inflammation with remodeling (20). Another report demonstrated that IL-18 does not affect AHR and airway inflammation in allergic bronchial asthma (21), contrasting strikingly to the clear pathological role of endogenous IL-18 in non-allergic super T$_h$1 cell-dependent asthma (17).

Various types of cells, including macrophages and epithelial cells, can produce IL-18 (28). In this study, we observed elevation of lung IL-18 in the SpA-immunized mice only after intranasal SpA challenge (Fig. 2B), suggesting that IL-18 might be derived from airway constituents, such as respiratory epithelial cells and/or alveolar macrophages. Despite our intensive efforts, we could not observe obvious release of IL-18 from either type of cells after *in vitro* stimulation with SpA.

Recent reports have clearly shown that respiratory epithelial cells play a pivotal role in the development of T$_h$2 type murine asthma induced by airway exposure to the house dust mite, a common allergen of human asthma (29–31). It was believed that an antigen-specific T$_h$2-cell response develops only under the limited condition of immunization with the protein in combination with T$_h$2-cell adjuvant. Beyond this dogma, it was shown that multiple intra-tracheal challenges with house dust mite alone could trigger a T$_h$2-cell response without prior immunization with antigen/T$_h$2 adjuvant complex. Mice lacking TLR4 expression on their respiratory epithelial cells, but not hematopoietic cells, were able to evade T$_h$2 type asthma. Because house dust mites possess intrinsic TLR4 agonists, TLR4 on respiratory

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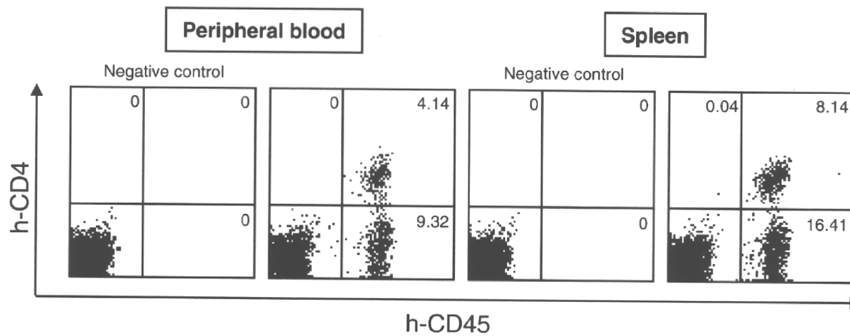

Fig. 5. Mice injected with human PBMCs. Seven days after SpA-stimulated PBMC (1×10^7) were injected into mice, the *Rag2*$^{-/-}$*C*$\gamma^{-/-}$ mice were analyzed for repopulation with human lymphocytes in their peripheral blood and spleen. Cells were incubated with PE-conjugated anti-human CD4 and FITC-labeled anti-human CD45 or a corresponding isotype-matched mAb (negative control). Data are representative of four independent experiments with five mice.

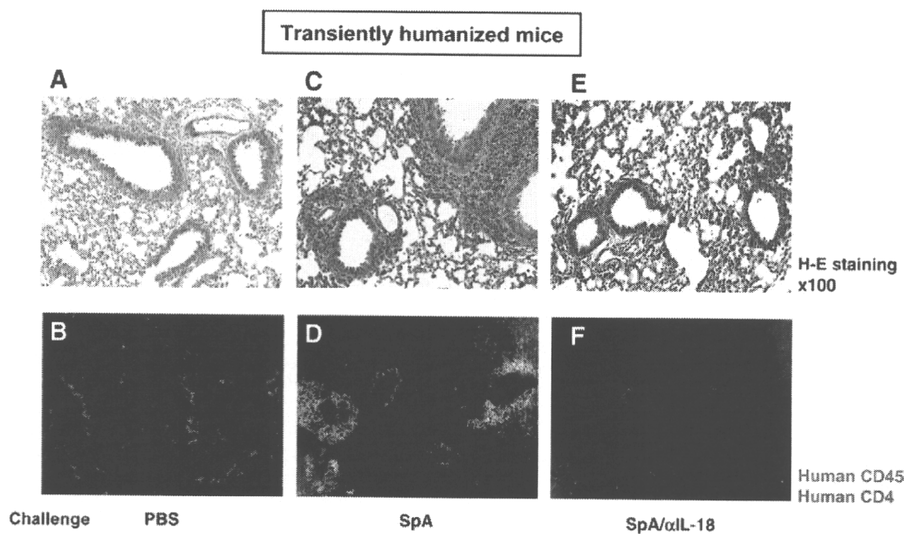

Fig. 6. Blocking the action of human IL-18 prevents SpA-induced airway inflammation in transiently humanized mice. Mice having received SpA-stimulated PBMC (1×10^7) were intranasally treated with PBS (A and B) or challenged with SpA (250 μg/50 μl) (SpA; C and D) or with SpA and neutralizing anti-human IL-18 antibody (300 μg) (SpA/αIL-18; E and F). Twenty-four hours after the last SpA challenge, lung specimens were sampled for histological analysis [hematoxylin and eosin (H&E)] (A, C and E) and for localization analysis of human $CD4^+CD45^+$ T cells (B, D and F). Data are representative of two independent experiments with five mice.

epithelial cells might recognize the agonist and produce T_h2-activating cytokines, such as IL-25, TSLP and IL-33 (29, 32, 33), eventually resulting in the development of T_h2 type asthma. As previously reported, daily topical application of SpA without T_h1 adjuvant can induce atopic dermatitis, in which super T_h1-cell development plays a critical role (19). Intriguingly, murine epidermal cells can release super T_h1 cell-activating IL-18 in response to SpA (34). Thus, SpA, like house dust mites in the airway, seems to exert dual actions as a T-cell antigen and adjuvant in the skin. These observations together with our present results suggest that respiratory mucosa and skin, particularly respiratory epithelial cells and epidermal cells, respectively, are the sites required for activation of T_h2 and/or super T_h1 cells. A similar mechanism might also be responsible for the airway asthmatic inflammation induced by SpA.

Accumulated evidence suggests the involvement of IL-18 in atopic diseases in humans. Patients with atopic dermatitis and bronchial asthma have higher levels of serum IL-18 than healthy volunteers (35, 36). In particular, serum IL-18 levels are shown to coincide with the disease severity of atopic dermatitis. Furthermore, gain-of-function polymorphisms of *IL-18* are observed in patients with atopic dermatitis and bronchial asthma (37–39). This implies that IL-18 is preferentially produced after airway and/or dermal colonization with microbes in those patients, eventually leading to the development of exposure site-specific super T_h1 cell-dependent allergic diseases.

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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-33Rα chain). IL-33 has the capacity to induce T_h2 cytokine production from T_h2 cells, mast cells and basophils, indicating that IL-33 has the potential to induce T_h2 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, T_h2 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 T_h2 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 T_h2 cells (6–8), which produce IL-4, IL-5, IL-6, IL-9, IL-13 and chemokines. However, the precise mechanisms by which T_h2 cells promote the pathogenic immune responses in AC are still unclear. Indeed, it has not been clearly demonstrated that antigen-specific T_h2 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_h1 and T_h2 cells preferentially express IL-18Rα chain and ST2, respectively (12–14). In the same way that functional IL-18R is composed of an IL-18Rα and an IL-18Rβ chain (15, 16), functional IL-33R consists of an IL-33Rα (ST2) and an IL-33Rβ (IL-1Rβ) chain (17, 18). Although T_h2 cells preferentially express IL-33R, ST2 deficiency does not affect the development of T_h2 cells *in vitro* (19). Furthermore, inoculation with gastrointestinal nematodes normally induces IgE in $ST2^{-/-}$ mice (19). These results suggest the possibility that IL-33 principally augments allergic inflammation by enhancing T_h2 cytokine production from T_h2 cells (12). However, basophils and mast cells, when stimulated with IL-3 and IL-33, also produce large amounts of T_h2 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_h2 cytokine-mediated allergic inflammation by activation of T_h2 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 $ST2^+CD4^+$ T cells and $ST2^+$ 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 PolyScience (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 who 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 2×10^5 0.2 ml^{-1} per well under stimulation with immobilized anti-CD3 and anti-CD28 (each 5 μg ml^{-1} for coating) in RPMI 1640 supplemented with 10% fetal bovine serum, 2-ME (50 μM), L-glutamine (2 mM), penicillin (100 U ml^{-1}) and

Table 1. Clinical evaluation of AC

	Absent	Mild	Moderate	Severe
Chemosis	0	1	2	3
Conjunctival redness	0	1	2	3
Lid edema	0	1	2	3
Tear and discharge	0	1	2	3
Scratching	0	1	2	3

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.

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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).

Flow cytometry and cell purification

Spleen cells (2 × 10^6 ml^{-1}) from naive BALB/c mice 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}) 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 $scatter^{high}$ (SSC^{high}), 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 and blocked with StartingBlock™ blocking buffer (Thermo Scientific, Rockford, IL, USA). Mouse IL-33 was detected with biotin-conjugated IL-33 mAb (clone; Nessy-1, AXXORA, San Diego, CA, USA) and streptavidin–HRP (BD Biosciences). The ELISA system was specific for mouse IL-33 and did not detect any other cytokines tested, including mouse IL-1β, IL-2, IL-4, IL-12, IL-18, tumor necrosis factor-α, IFN-γ, GM-CSF and human IL-33. IL-33 levels were determined by standard curves obtained using known amounts of rmIL-33. Total IgE was measured by ELISA as described previously (21). To detect RW-specific IgE in sera, biotin-conjugated RW extract was prepared in our laboratory.

Bioassay for mouse IL-33

Sorted bone marrow-derived basophils (10^5 0.2 ml^{-1} per well), obtained as described previously (12), were stimulated in the presence of IL-3 (20 U ml^{-1}) with rmIL-33 (0–100 ng ml^{-1}) or the soluble fraction (0–320 μg ml^{-1}) of homogenized conjunctival tissue with or without affinity-purified anti-IL-33 polyclonal antibody (20 μg ml). After 24 h, supernatants were harvested and tested for IL-6 by ELISA.

Homogenized conjunctival tissue

Bulbar conjunctiva, palpebral conjunctiva and the eyelid were isolated from each mouse and homogenized with PBS using Bead Smash 12 (Wakenyaku, Kyoto, Japan) for 1 min, five times. The homogenates were then centrifuged at 20 000 *g* for 5 min at 4°C. Supernatants (soluble fraction) were harvested and stored at −80°C prior to use.

Histology

Eyes were enucleated from mice, fixed in 4% PFA, embedded in paraffin, cut into 4-μm vertical plane sections including the optic nerve and stained with hematoxylin and eosin. Cytospan preparations of sorted Siglec-F^+ $CCR3^+$ (eosinophils) cells were stained with Wright–Giemsa.

Confocal 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, samples 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-phenylindole (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_h2 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_h2 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_h2 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-33Rα 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-33Rα 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_h2 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_h2 cells were able to act in combination to induce recruitment of eosinophils via eotaxin production in the conjunctival tissue (Fig. 2C).

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

Recruitment of T_h2 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_h2 cells. Since T_h2 cells express IL-33Rα (ST2) (12, 25), we examined T_h2 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_h2 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_h2 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-33Rα chain on eosinophils

Since RW challenge induced accumulation of T_h2 cells, which produce IL-4, IL-5, IL-13 and GM-CSF, we next determined

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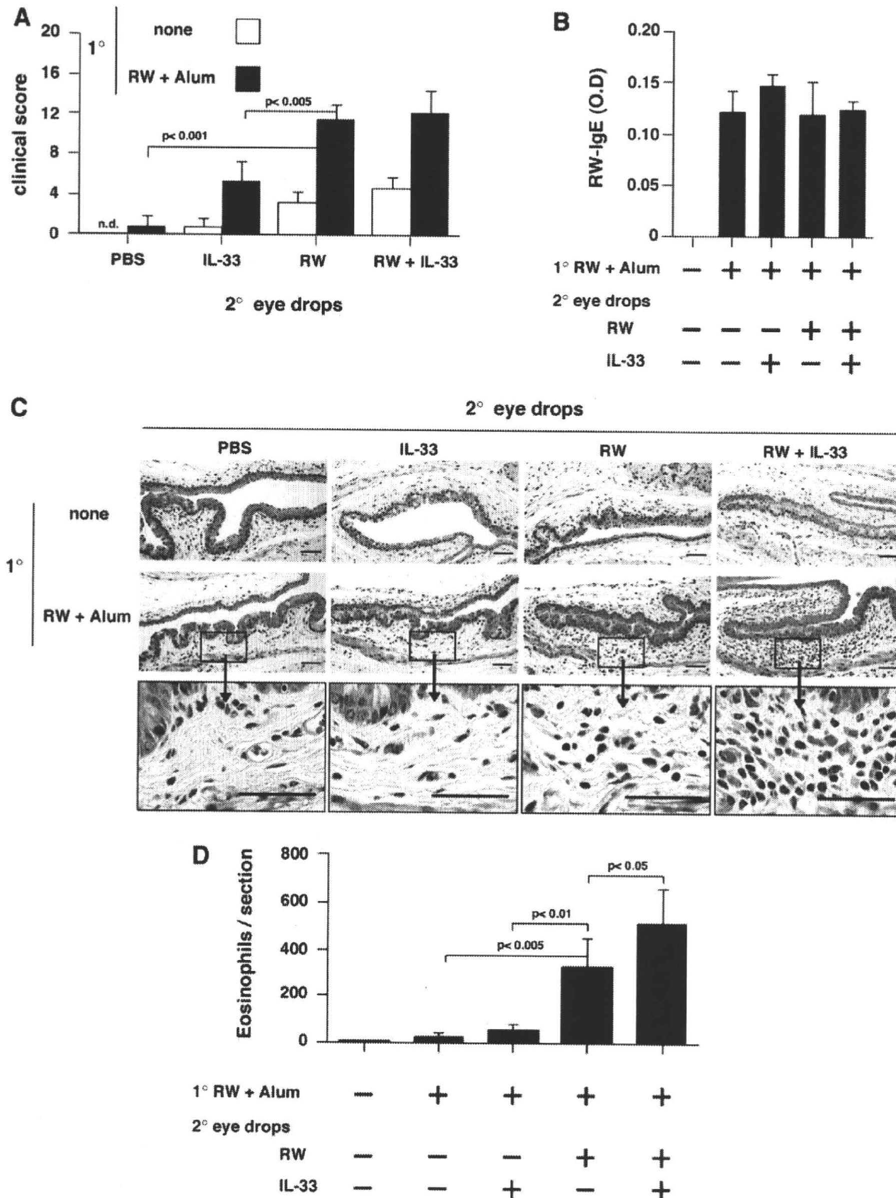

Fig. 1. Induction of AC and conjunctival eosinophilic inflammation by RW or RW plus IL-33 in RW-immunized mice. BALB/c mice were immunized with RW as described in Methods. Naive mice or RW-immunized mice were topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and/or IL-33 (1 μg 5 μl^{-1} PBS per eye). We administered IL-33 1 h before and 2, 4 and 6 h after challenge with PBS or RW. (A) Clinical score (as described in Methods) in the early-phase response, 15–30 min after challenge. (B) RW-specific IgE levels in the serum. (C) Twenty-four hours after challenge, eyes were enucleated from each group of mice (five mice per group), fixed in PFA, cut into 4-μm vertical plane sections including the optic nerve and stained with hematoxylin and eosin. Representative results from three independent experiments are shown; scale bar, 50 μm. (D) The number of eosinophils in the conjunctiva was counted. Results are shown as the mean ± SD of five animals per group and are representative of more than three independent experiments; n.d., not detected. Statistical differences between samples were determined using the Student's *t*-test (A and D).

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^{high}, Siglec-F^{+}, 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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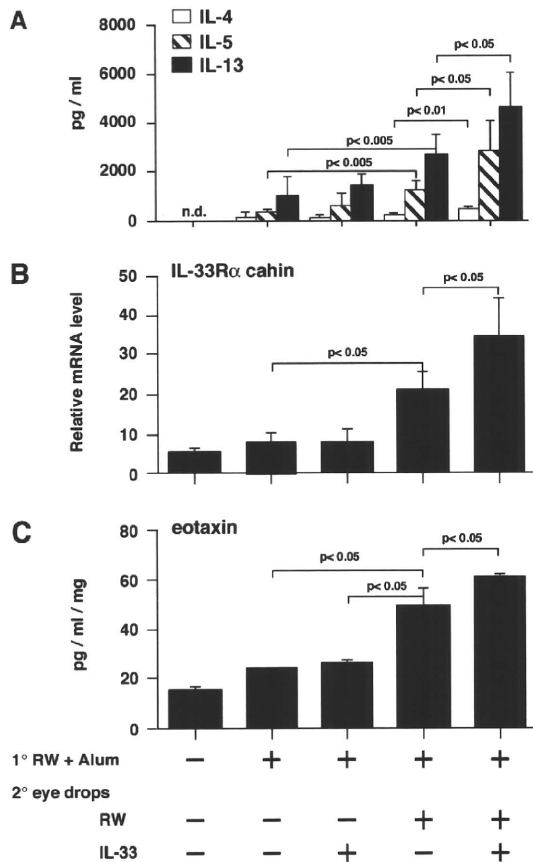

Fig. 2. Enhancement of T_h2 cytokine production and IL-33R expression by cervical lymph node cells after eye drop challenge with RW or RW plus IL-33. BALB/c mice were immunized with RW and topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and/or IL-33 (1 μg 5 μl^{-1} PBS per eye). Cervical lymph nodes cells were isolated from mice 24 h after challenge. Cell suspensions were cultured at 2×10^5 0.2 ml^{-1} per well under stimulation with immobilized anti-CD3 and anti-CD28 antibodies (each 5 μg ml^{-1} for coating). After 48 h of culture, supernatants were harvested and tested for IL-4, IL-5 and IL-13 by ELISA (A). The relative mRNA expression levels of IL-33Rα chain were determined by real-time PCR (B). (C) At 24 h post-challenge, eyes were enucleated from mice, homogenized and centrifuged as described in Methods. The obtained supernatants were tested for eotaxin by ELISA. Results shown are the mean ± SD of five animals per group and are representative of more than three independent experiments; n.d., not detected. Statistical differences between samples were determined using the Student's *t*-test.

stimulation with IL-5, IL-33 or IL-5 plus IL-33 strongly increased IL-33Rα expression (70.5, 69.8 and 84.7%, respectively) (Fig. 4A). Furthermore, stimulation with GM-CSF or GM-CSF plus IL-33 or with the combination of GM-CSF, IL-5 and IL-33 also strongly up-regulated IL-33Rα expression (66.0, 79.2 and 86.2%, respectively) (Fig. 4A). Thus, T_h2 cytokines, IL-4 and IL-13, play a critical role in recruitment of eosinophils via eotaxin production from conjunctival tissue and IL-5 and GM-CSF induce ST2 expression on eosinophils.

Next, we examined whether eosinophils become responsive to IL-33 after stimulation with IL-5, GM-CSF or IL-5 plus 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.

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).

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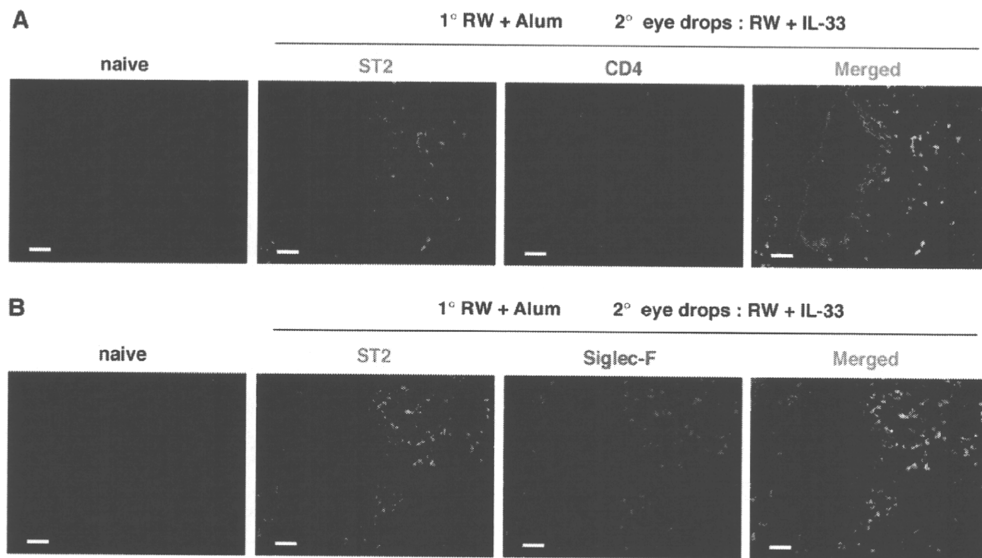

Fig. 3. Confocal microscopic examination of IL-33Rα chain$^+$ cells in the conjunctiva tissue of RW-immunized mice after challenge with RW and/or IL-33. BALB/c mice were immunized with RW and topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and IL-33 (1 μg 5 μl^{-1} PBS per eye). At 24 h post-challenge, eyes were enucleated from naive and treated mice (five mice per group) and frozen. Frozen sections were fixed and incubated with antibodies to mouse ST2 and CD4 (A) or mouse ST2 and Siglec-F (B) and then examined by confocal microscopy. (A) CD4, red; ST2, green, and co-localization, yellow (merged). (B) Siglec-F, red; ST2, green, and co-localization, yellow (merged); scale bar, 50 μm. Representative results from two independent experiments are shown.

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 T_h2 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 T_h2 cells in regional lymph nodes of RW-immunized mice to produce T_h2 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 T_h2 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 T_h2 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 future of this experimental AC model is the massive infiltration of IL-33Rα$^+$ eosinophils in the conjunctiva (Fig. 3B). This finding indicates that T_h2 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,

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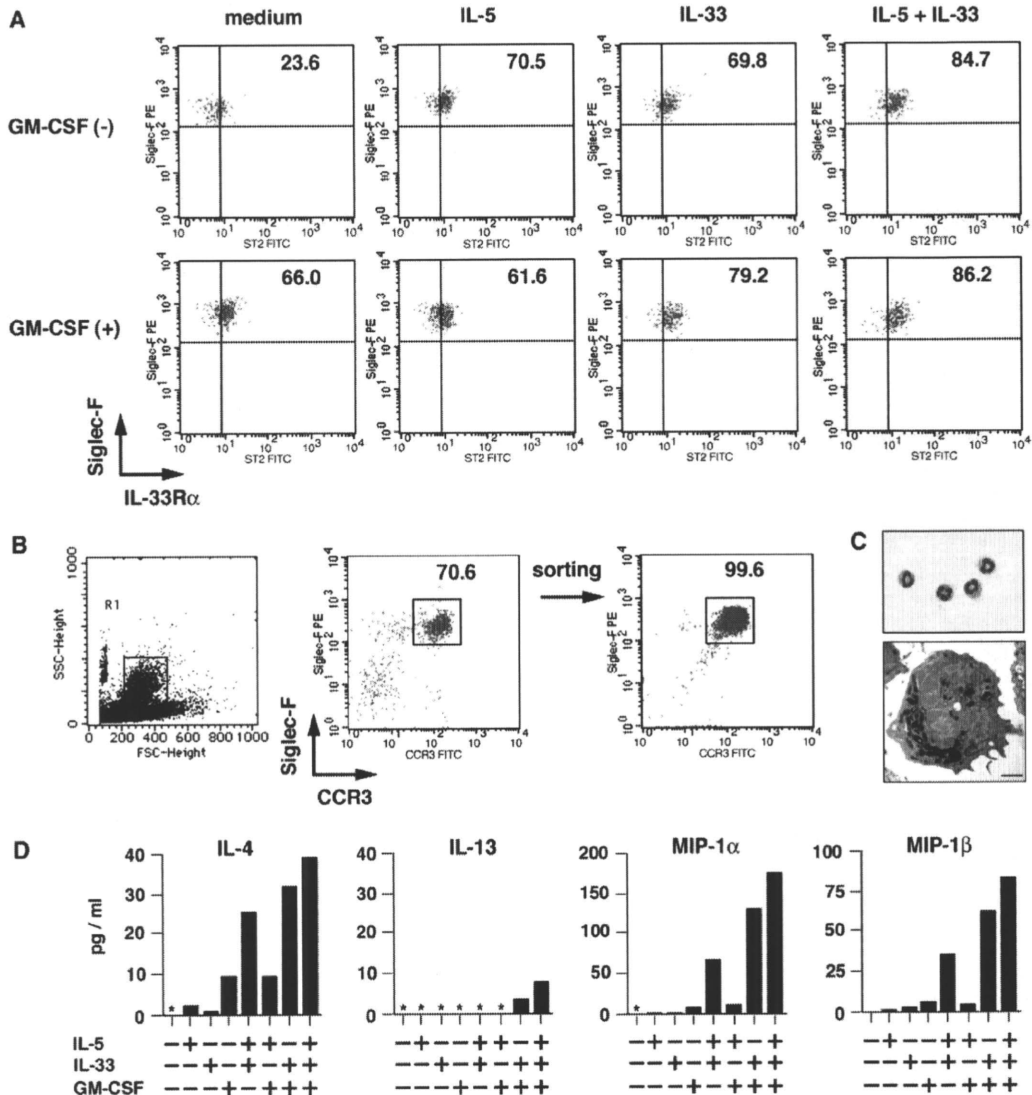

Fig. 4. Eosinophils stimulated with IL-5 increase IL-33Rα chain expression and become responsive to IL-33. (A) Spleen cells (2×10^6 ml^{-1}) from naive BALB/c mice were stimulated with medium alone, IL-5 (40 ng ml^{-1}) and/or IL-33 (100 ng ml^{-1}) in the presence or absence of GM-CSF (50 ng ml^{-1}) in 24-well plates for 24 h. Cultured cells, gated as SSC^{high}, Siglec-F$^+$, non-B and non-T cells, were examined for expression of IL-33Rα chain by flow cytometry. Numbers indicate the percentage of Siglec-F$^+$ IL-33Rα chain$^+$ cells. (B) Flow cytometric analysis of the expression of SSC and forward scatter (left) or Siglec-F and CCR3 (middle) by freshly prepared splenic non-B and non-T cells from naive BALB/c mice. Expression of Siglec-F and CCR3 by sorted cells is also shown (right). The numbers above the outlined areas indicate the percentage of Siglec-F$^+$ CCR3$^+$ cells. (C) Sorted Siglec-F$^+$ CCR3$^+$ (eosinophils) cell populations were stained by Wright–Giemsa staining (×100) (upper) and subjected to electron microscopic examination (lower); scale bar, 1 μm. (D) The sorted eosinophils (1×10^5 0.2 ml^{-1} per well) 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 IL-4, IL-13, MIP-1α and MIP-1β using the Bio-Plex System. Asterisk indicates not detected. Representative results from three (A) or two (C–D) independent experiments are shown.

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 T_h2 cells induce recruitment of eosinophils by production of IL-4 and IL-13 in the conjunctiva. It is also important to determine which T_h2 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).

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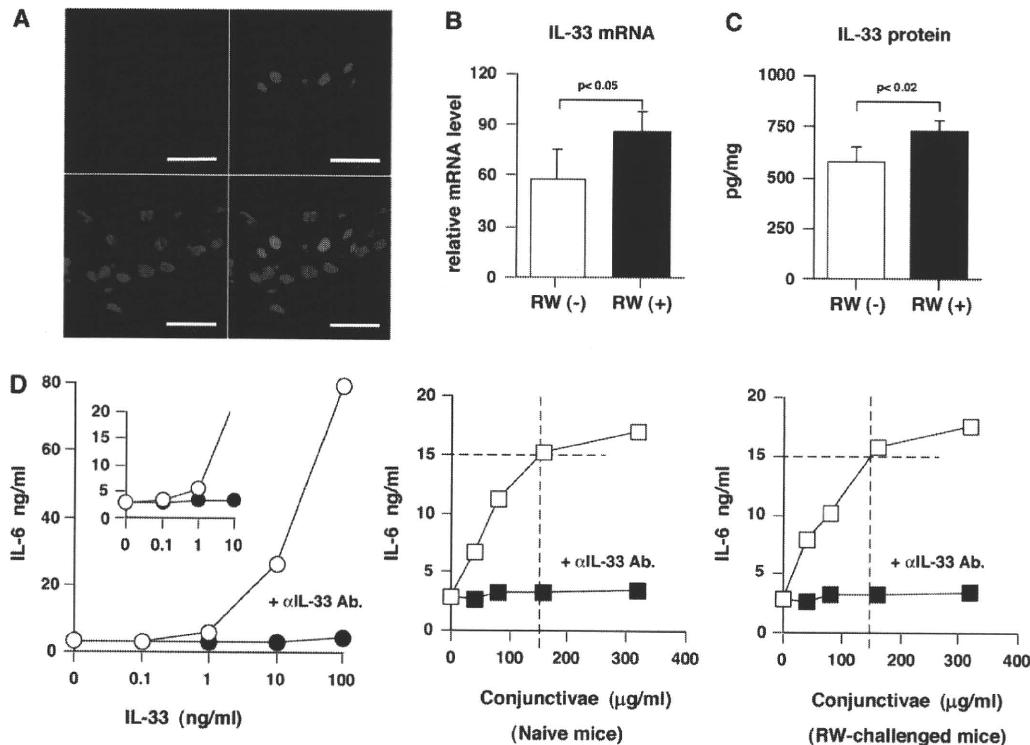

Fig. 5. Expression of IL-33 by the conjunctiva of mice before and after eye drop challenge with RW. (A) Eyes were enucleated from naive BALB/c mice, fixed in 4% PFA, embedded in paraffin and cut into 4-μm vertical plane sections including the optic nerve. Immunofluorescence staining was performed using an anti-IL-33 polyclonal antibody made in our laboratory. IL-33 expression was detected by red staining and DNA was counterstained with 4′,6-diamidino-2-phenylindole (blue); scale bar, 20 μm. (B and C) IL-33 mRNA and protein levels in the conjunctival tissue of naive or eye drop-challenged mice (1 mg 5 μl^{-1} PBS per eye, every 2 h, three times). At 24 h post-challenge, the conjunctiva was isolated from each group of mice. Relative IL-33 mRNA expression was measured by real-time PCR (B). IL-33 protein from homogenized conjunctiva was measured by ELISA (C). Results show the mean ± SD of five animals per group and are representative of two independent experiments. Statistical differences between samples were determined using the Student's *t*-test (B and C). (D) Bone marrow-derived basophils were cultured with rmIL-33 (0–100 ng ml^{-1}) or the soluble fraction from homogenized conjunctiva (0–320 mg ml^{-1}) from naive or RW-challenged mice, as described above for part B and C, in the presence or absence of anti-IL-33 polyclonal antibody (10 μg ml^{-1}) for 24 h. Supernatants were harvested and tested for IL-6 production by ELISA. Representative results from two independent experiments are shown.

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 T_h2 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 T_h2 cells but also by eosinophils. Thus, our data strongly indicated that T_h2 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_h2 cells to produce T_h2 cytokines in the conjunctiva and IL-5 from T_h2 cells in combination with IL-33 stimulate these IL-33Rα^+ 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_h2 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 contribution 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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Basophils are potent antigen-presenting cells that selectively induce Th2 cells

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Basophils and mast cells are important effector cells in helminth-infected host and IgE-mediated allergic inflammation. Although they have the same progenitors, basophils and mast cells complete their terminal differentiation in the bone marrow and peripheral tissues, respectively, and only basophils circulate in the blood. Although it is recognized that basophils are important for Th2 responses, and it is also well established that IL-4 is required for Th2 differentiation from naïve CD4$^+$ T cells, the nature of the cells that produce "early" IL-4, remained elusive until recently. Three groups independently demonstrated that basophils are the predominant APC in inducing Th2 response against helminth parasites and allergens. Basophils express MHC class II and CD80/86, have the potential to take-up and process protein Ag (particularly Ag–IgE complex) and to present peptide in the context of MHC class II, and to produce IL-4. These Ag-pulsed basophils induce the development of Th2 cells both *in vitro* and *in vivo*. Thus, basophils contribute to Th2/IgE response by the production of IL-4 and presentation of MHC class II/peptide complex to naïve CD4$^+$ T cells, in contrast to the Th1-inducing action of DC. In this review, we summarize what is known regarding basophil function in allergy and parasite infection, examine the novel Ag-presenting function of basophils and discuss potential clinical implications of this finding.

Key words: Ag–IgE complex · Basophils · Helminth infection · Th2 response

Introduction

Mast cells, basophils and eosinophils are key effector cells in response to parasite infection and allergic inflammation [1–5]. Basophils and eosinophils are granulocytes, which mature in the bone marrow, circulate in the blood and are recruited to allergic inflammatory sites [3–5]. In contrast, progenitors of mast cells migrate from the bone marrow to the peripheral tissues and undergo their terminal differentiation *in situ*; mast cells that complete their differentiation in the skin or intestine develop into connective tissue mast cells and mucosal mast cells, respectively [1, 2]. Mast cells and basophils express the high-affinity receptor for IgE and, upon crosslinking of FcεR1-bound IgE with multivalent Ag, rapidly produce diverse preformed mediators, cytokines (*e.g.* IL-4 and IL-13) and lipid mediators, leading to the induction of immediate-type hypersensitivity [1–5]. Here, the author reviews the major functions of basophils as effector cells in the development of allergic inflammation and their novel function as Th2-inducing APC in helminth infection and allergy.

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Basophil development and its role in allergy

As mentioned, mast cells, basophils and eosinophils are the key innate effector cells involved in parasite-induced immune responses and allergic inflammation. Basophils are short-lived cells that account for less than 1% of circulating granulocytes in the blood. In contrast, mast cells are located in the tissue and mast cell progenitors have the potential to proliferate locally in the tissue in response to IL-3, IL-4 and IL-9, resulting in local mastocytosis.

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A study by Arinobu *et al.* [6] has identified a common progenitor of basophils and mast cell precursor (BMCP), which arise from the granulocyte/monocyte progenitor (GMP). Eosinophil precursor also arises from GMP. The development from GMP to eosinophil precursor and BMCP, and from BMCP to basophil precursor or MCP, are regulated by the level and order of expression of transcription factors, C/EBPα and GATA-2 [6]. Morphologically, basophils and eosinophils have lobulated nucleus and secretory granules in the cytoplasm. Mast cells are round cells with a non-segmented nucleus and intracellular granules. Although basophils and mast cells are heterogeneous in their development and morphology, they are regarded to share a pathological role in allergic responses, as demonstrated by their potential to produce cytokines, vasoactive histamine and lipid mediators after FcεR1 crosslinkage [1–5]. Thus, individuals with atopy, after repeated exposure to a particular Ag such as pollen, exhibit immediate-type hypersensitivity. Furthermore, there is tight correlation between FcεR1 expression on basophils and IgE level in human peripheral blood [7], suggesting a positive feedback mechanism for the IgE-mediated immediate-type hypersensitivity reaction [8, 9]. Thus, once individuals with atopy start to produce IgE, they develop progressive allergic inflammation by increasing production of IgE and expression of FcεR1 on effector cells.

Basophil activation in parasitic infection and allergic inflammation: Role of IL-3

There appears to be at least two major pathways of basophil activation during allergic inflammation, one involving Ag/IgE signaling and the other that is mediated by PAMP and soluble mediators such as IL-18 and IL-33.

An important cytokine involved in both pathways of basophil activation is IL-3. IL-3 is not only an important growth factor for mast cells and basophils, IL-3 stimulation also induces basophil production of IL-4. Furthermore, basophils, when stimulated with a combination of IL-3 and crosslinking of FcεR1 by Ag, strongly produce IL-4 and IL-13, suggesting the importance of crosstalk between IL-3-mediated signaling and FcεR1-mediated signaling for IL-4 and IL-13 production. Furthermore, FcR common γ-chain (FcγR) may also be important in basophil activation. Recently, Hida *et al.* [10] demonstrated that basophils lacking FcγR could proliferate normally but failed to produce IL-4 in response to IL-3, suggesting that FcγR-mediated IL-3 signal is crucial in IL-4 production by basophils.

The effect of IL-3 can also be observed in IgE-independent basophil IL-4 production. We previously demonstrated that basophils express IL-18R and IL-33R and produce IL-4, IL-6, IL-13 and chemical mediators when stimulated with IL-3 plus IL-18/IL-33 *in vitro* (Fig. 1, left panel) [11, 12]. These results suggest the potential of basophils to induce allergic inflammation in an IgE-independent manner (innate-type allergic inflammation). Mouse basophils also express TLR1, TLR2, TLR4 and TLR6 and produce Th2 cytokines including IL-4 and IL-13 in response to stimulation with TLR ligands plus IL-3 [13].

Thus, as stated, there are at least two major basophils activation pathways during allergic inflammation. One is an Ag/IgE-dependent pathway responsible for "acquired-type allergic inflammation" and the other is an IL-18, IL-33 or PAMP-dependent pathway responsible for "innate-type allergic inflammation."

IL-3 is also important for generation and peripheral accumulation of basophils during parasitic infections [14]. Infection of wild-type mice with *Strongyloides venezuelensis* or *Nippostrongylus brasiliensis* causes accumulation of basophils in the liver and spleen of the host [15]; however, this accumulation is not observed in IL-3-deficient mice [14]. Thus, IL-3 produced by Th2 cells is critically involved in generation, accumulation and activation of basophils.

In terms of the interactions between allergic inflammation and parasitic infection, we showed previously that nasal administration of IL-18 or IL-33 induces bronchial asthma entirely independently of allergen and IgE [12, 16]. As these cytokines are stored in the epithelial cells, infection with pathogens, including helminth parasites, bacteria, fungi and viruses or exposure to allergens, can induce the release of IL-18 and IL-33 from epithelial cells, causing IL-18 and/or IL-33-mediated allergic inflammation (Fig. 2), which is dependent on IL-18R and/or IL-33R and the adapter protein MyD88 pathway (Fig. 1). Basophils and mast cells also produce Th2 cytokines in response to parasite Ag (*e.g.* IPSE-α-1, a soluble glycoprotein Ag from eggs of *Schistosoma mansoni* and has been shown to stimulate basophils in an IgE-specific but Ag-nonspecific manner [17]). Basophils and mast cells may also respond to other parasite Ag, suggesting their role in defense against intestinal nematode such as *S. venezuelensis*, *N. brasiliensis* or *Trichuris muris*. Eosinophils are also effector cells of parasite infection – they defend against the tissue stage of helminth that is too large to be phagocytosed. IgE antibodies that bind to the surface of helminths activate eosinophils to produce granule content such as the major basic protein, which is highly toxic to helminths. Recruitment of eosinophils is also a well-known late hallmark of allergic inflammation and contributes to pathological processes in allergic diseases. Thus, basophils, mast cells and eosinophils are major effector granulocytes in parasitic infection and allergic inflammation.

Basophils in chronic allergic inflammation and systemic anaphylactic shock

Recent studies suggest that basophils also induce IgE-mediated chronic allergic inflammation and IgG1-mediated systemic anaphylactic shock [4, 18, 19]. Mukai *et al.* demonstrated that a single injection of multivalent Ag in the ear of mice passively sensitized with Ag-specific IgE induces immediate-phase, late-phase and delayed-onset of ear swelling characterized by infiltration with basophils and eosinophils [18]. Mast cell-deficient mice did not develop immediate- and late-phase ear swelling, suggesting mast cells are responsible for inducing these ear swellings. In contrast, depletion of basophils in wild-type mice diminished delayed-onset of ear swelling and eosinophilic infiltration. Moreover, transfer of basophils into FcεR1-deficient mouse showed that basophils are responsible for inducing delayed-onset ear swelling that is

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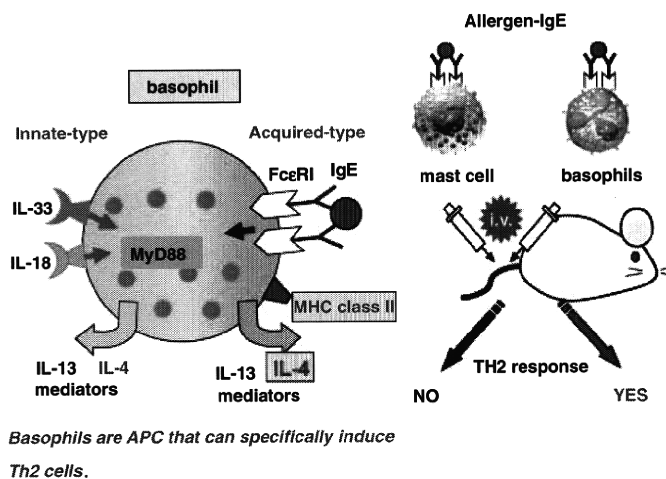

Figure 1. Basophils are effectors cells and also inducers of Th2 response *in vivo*. Basophils, which produce IL-4, IL-13, and other mediators when stimulated with IL-3 plus Ag/IgE complex (acquired-type activation) also produce these cytokines and mediators, when stimulated with IL-3 plus IL-18 or IL-3 plus IL-33 (innate-type allergy). Furthermore, intravenous administration of basophils (but not mast cells) pulsed with allergen induce the development of Th2 cells in the peripheral lymphoid organs.

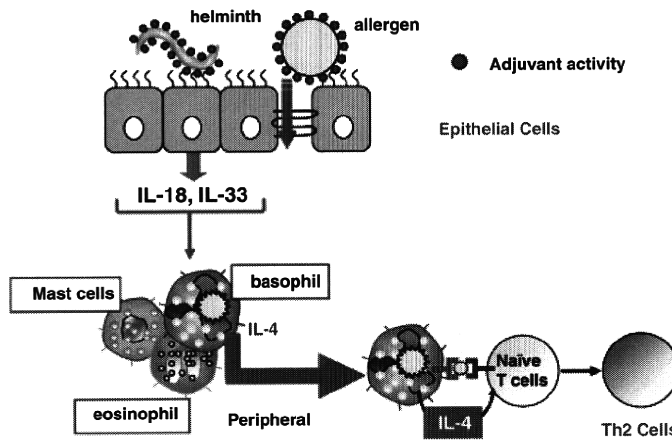

Figure 2. Interaction between epithelial cells and basophils. IL-18 or IL-33 derived from epithelial cells stimulated with helminth or allergen induces mast cells, basophils and eosinophils to produce Th2 cytokines, chemokines and chemical mediators. Among these granulocytes, basophils strongly produce IL-4 in response to IL-18 or IL-33. Basophils also uptake and process allergen and express allergen-derived peptide with MHC class II. These basophils prime Th2 responses in an MHC class II-dependent and IL-4-dependent manner.

associated with marked eosinophilic infiltration. Therefore, basophils seem to induce delayed-onset or chronic allergic inflammation by recruiting eosinophils [18].

It is well documented that mast cells and IgE are crucially involved in the development of systemic anaphylaxis. Interestingly, mice deficient for mast cells or IgE nevertheless develop systemic anaphylaxis, suggesting that an alternative pathway may be involved. Tsujimura *et al.* [19] clearly demonstrated that basophils and IgG1 induce mast cell-independent systemic anaphylaxis.

Role of Th cells in allergy and infection

Allergen-activated Th2 cells produce cytokines that induce allergen-specific IgE production by B cells and recruitment of mast cells, eosinophils and basophils to the site of allergic inflammation. Helminth infection also induces Th2 responses, resulting in high levels of IgE and recruitment of mast cells, eosinophils and basophils to the infected organ. Naïve $CD4^+$ T cells develop into Th1, Th2, Th17 cells and Treg upon activation by appropriate combination of antigenic signal, costimulation and cytokine signals by APC and accessory cells [20]. IFN-γ and IL-12 induce the development of Th1 cells, which are characterized by a high-level production of IFN-γ and are indispensable for eradication of intracellular pathogens [21]. IL-4 triggers the differentiation of Th2 cells [22]. Th2 cells are critically involved in clearing extracellular multi-cellular parasites such as helminths and in helping B cells to produce antibodies. Th2 cells are also involved in the pathogenesis of allergic inflammation. Th17 cells play an important role for the defense against extracellular pathogens and fungi [23]. Differentiation of Th17 cells is induced by TGF-β and IL-6 in the mouse and by TGF-β and IL-6 or IL-21 in the human [23] (see also review on IL-6 in this issue [24]). Treg can be induced by TGF-β and are involved in maintaining immune tolerance [25].

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The initial source of the differentiation factors for both Th1 and Th2 cells are cells of the innate immune system responding to microbial Ag, parasitic Ag, or allergens. DC recognize bacteria through TLR and mature to express costimulatory molecules CD80/86 and to produce IL-12 and IL-18, favoring development of Th1 cells [26–28]. Thus, DC infected with intracellular bacteria induce Th1 cells.

Of particular importance to allergic inflammation and parasite infections is the nature of APC involved in polarizing Th2 responses. Ag-pulsed DC can also induce the development of Ag-specific naïve $CD4^+$ T cells into Th2 cells under the presence of IL-4 *in vitro* [20]; however, the APC involved in the development of Th2 response under physiological conditions remains uncertain. Several reports indicate that there are several pathways for the differentiation of naïve $CD4^+$ T cells into Th2 cells [29–33]. The Notch-ligand Jagged 1 and Jagged 2 on DC can trigger Th2 differentiation independently of IL-4 and STAT6 signaling [29]. Epithelial cells-derived cytokine, thymic stromal lymphopoetin (TSLP), activates DC to express OX40L, which induces the development of Th2 cells [30]. Aluminum adjuvant also induces Th2-cell differentiation, although exact mechanism remains uncertain [31]. In addition, M2 macrophages (also known as alternatively activated macrophages), eosinophils and mast cells are also important for the development of Th2 cells [32, 33].

Previous studies suggested that basophils may be critical in Th2 immunity. Min and colleagues [34] showed that naïve $CD4^+$ T cells stimulated with peptide-pulsed DC develop into Th2 cells when cultured with basophils from wild-type mice but not from IL-4-deficient mice. As both DC and basophils are added to the same culture, it was initially considered that DC deliver antigenic-specific signal and basophils promote the development of Th2 response by providing early IL-4 signal to Ag-activated $CD4^+$ T cells. It was also previously reported that helminth infection induces the development of Th2 cells and accumulation of basophils in the spleens and livers of host mice [15], suggesting that the relationship between Th2 cells and basophils may be more direct. *In vivo*, mice deficient in interferon-regulatory factor 2 show expansion of basophil and spontaneous Th2 differentiation [35], suggesting promotion of Th2 immune response by basophils. Furthermore, this Th2 differentiation is markedly reduced by the introduction of mutation in the gene-encoding c-Kit, because this mutation reduces the number of basophils [35]. Thus, although indirect evidence support the role of basophils in Th2 immunity, it is important to formally prove that basophils produce "early" IL-4, required for the development of naïve $CD4^+$ T cells into Th2 cells. Recently, three groups independently demonstrated that basophils and not DC, are the critical APC involved in Th2 differentiation *in vivo* [36–38].

Th2 development: Basophils as IL-4 provider

Medzhitov and colleagues [39] previously reported that basophils are important for the development of Th2 cells in response to papain. At day 3 after papain stimulation, basophils migrated into the T-cell zones of the draining lymph nodes, in which the basophils produce IL-4 and/or TSLP, which promote Th2 differentiation *in vivo*. Papain is a cysteine protease hydrolate enzyme from papaya that mimics the activity of proteases secreted by helminth parasites. Depletion of basophils with antibody against FcεR1 diminishes the development of Th2 cells, suggesting that basophils are involved in Th2 cell differentiation. This study [39] strongly indicates that basophils are critically involved in Th2 responses by their unique function to produce early IL-4 and TSLP in response to papain or bromelain. It remains uncertain, however, whether basophil-derived IL-4 is indeed involved in the development of Th2 cells in response to stimuli other than protease allergens.

Basophils as Th2-inducing APC

Data from our group also supported a role of basophils in Th2 responses – we reported that IL-18 and IL-33 synergize with IL-3 to strongly induce basophil, but not mast cell, production of IL-4 and IL-13 *in vitro*, respectively [11, 12] (Fig. 1, left panel), suggesting a role of basophils in promoting Th2 response by producing IL-4. Furthermore, basophils are shown to be an important regulator of Th2 responses *in vivo*, particularly in helminth-infected mice [3, 15]. As the size of helminths is too large to be phagocytosed directly by DC, it is more likely that DC take up Ag shed or secreted by parasites and present the Ag on MHC class II complex to naïves T cells in the context of IL-4 from parasite Ag-stimulated basophils. Although this is a persuasive hypothesis, the exact role of basophils in Th2 development remains to be formally demonstrated.

As noted, three groups independently demonstrated that contrary to our intuition, DC are not required for the development of Th2 responses to protease allergens, helminthic parasites or complexes of Ag and IgE [36–38]. All three groups demonstrated that basophils express MHC class II, CD80/86 and produce IL-4. Two groups showed that basophils induce Th2 cells in the absence of DC [36, 38]. Our group demonstrated that administration of Ag-pulsed basophils but not Ag-pulsed DC or mast cells selectively induces Th2 cells *in vivo* [37] (Fig. 1, right panel). Together, the three studies [36–38] suggest that basophils induce allergen or helminth-induced Th2 response by functioning as Th2-inducing APC. Artis and colleagues [36], using MHC II^{CD11c} transgenic mice, where MHC class II expression is restricted to $CD11c^+$ DC, demonstrated that these mice, when inoculated with *T. muris*, fail to develop Th2 response and to expel helminths. MHC II^{CD11c} transgenic mice do not secrete intestinal goblet-specific immune effector molecule resistin-like molecule β, which is induced by Th2 cells. Artis and colleagues [36] simultaneously demonstrated that this infection induced the development of Th1 cells, suggesting that $CD11c^+$ cells are required for the generation of Th1 cells; basophils, on the other hand, are dominant Th2-inducing APC that express IL-4 and MHC class II, as supported by depletion of basophils *in vivo*, which led to impaired protective Th2 immunity to *T. muris* in wild-type

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mice. Contrary to these findings, however, Min and colleagues [14] demonstrated that basophil depletion in *N. brasiliensis*-infected mice did not affect the development of Th2 cells, suggesting that *N. brasiliensis* infection induces Th2 immunity even in the absence of basophils. We therefore need further studies to reconcile this apparent discrepancy.

Medzhitov and colleagues [38] demonstrated that skin DC are dispensable for mounting Th2 responses to papain. This group previously reported that, as with injection of the soluble Ag of *S. mannsoni* eggs, papain rapidly induces recruitment of basophils to the lymph node [39]. In the lymph nodes, basophils secrete IL-4 and TSLP, which are critically involved in the development of Ag-specific Th2 cells. Given that this treatment simultaneously induced recruitment of DC, Medzhitov and colleagues [39] initially considered that basophils function as accessory cells and DC present Ag in the presence of IL-4 from basophils. In the follow-up study, Medzhitov and colleagues [38] very clearly demonstrated that skin DC are not required for the development of Th2 cells in the draining lymph nodes. In this study [38], papain was injected into the ear, where skin DC capture Ag and present Ag-derived peptides to naïve T cells in the draining lymph nodes. If skin DC capture Ag and present it at the lymph node, rapid removal of this Ag-pulsed DC by prompt excision of the injection site should inhibit the Th2 response; however, this treatment failed to inhibit development of Th2 cells, suggesting that Ag capture by skin DC is not required for induction of papain-specific Th2 development. Instead, soluble papain can directly enter lymph nodes from injection site. Furthermore, selective depletion of $CD11c^+$ DC did not inhibit Th2 development to papain, although mice failed to develop Th1 responses. Artis's [36] and Medzhitov's [38] groups used the same strategy to deplete DC, using the CD11c-restricted diphteria toxin receptor mice, in which CD11c-expressing DC are efficiently depleted upon delivery of diphteria toxin, the two groups demonstrated that DC depletion only inhibited the development of Th1 cells without affecting the development of Th2 response. These results strongly indicated that other type/s of APC might be required for Th2 cytokine-dependent immune response. Medzhitov's group [38] demonstrated that OVA-pulsed basophils induce the development of OVA-specific naïve $CD4^+$ T cells into Th2 cells *in vitro*. They also show basophils can uptake, process and present soluble Ag. They further demonstrated that adoptive transfer of OVA-pulsed basophils induced Th2 response in MHC class II-deficient mice.

Basophils produce IL-4 and IL-13 upon stimulation with Ag/IgE complex. In addition, our *in vitro* studies demonstrated that, among mast cells and basophils, only basophils strongly produce IL-4 and IL-13 in response to IL-3 and IL-18 or IL-33 [11, 12]. These data suggest a role of basophils in the development of Th2 cells. These observations led us to examine the possibility whether basophils directly induce the development of Th2 cells, instead of functioning as accessory cells *in vitro* [37].

Splenic basophils from mice inoculated with *S. venezuelensis* produce large amounts of IL-4, IL-6 and IL-13 in the medium even in the absence of exogenous IL-3. In contrast, splenic basophils from naïve mice produce small amounts of IL-4, IL-6 and IL-13 only in IL-3-containing medium. Furthermore, basophils from infected mice express MHC class II and strongly induce the development of OVA-specific naïve $CD4^+$ T cells into Th2 cells *in vitro* in the presence of OVA peptide, IL-2 and IL-3 without IL-4 (neutral culture condition). Thus, we initially regarded only basophils from infected mice as potent APC; however, we soon found that splenic basophils from naïve mice also express comparable level of MHC class II and have the capacity to strongly induce the development of Th2 cells *in vitro* under neutral conditions [37]. We next examined bone marrow basophils and showed that these also have the potential to induce the development of Th2 cells. We purified basophils from bone marrow cells cultured with IL-3 for 10 days. Similar to splenic basophils, bone marrow basophils express MHC class II, CD80, CD86 and CD62L. Furthermore, bone marrow basophils can take-up and process protein Ag and express peptide in association with MHC class II. In particular, bone marrow basophils can efficiently uptake a low dose of Ag/IgE complex, and present Ag/MHC class II and produce IL-4, suggesting that they are potent Th2-inducing APC.

We also demonstrated that i.v. administration of OVA-pulsed basophils, which we prepared by culturing basophils with DNP–OVA and anti-DNP–IgE complexes, strongly induce OVA-specific Th2 cells in the spleen of naïve mouse (Fig. 1, right panel). We found that basophils' APC activity was enhanced when pulsed with DNP-OVA in the presence of anti-DNP IgE. In contrast, i.v. administration of OVA-pulsed DC failed to induce Th2 cells, although this treatment induced IFN-γ-producing Th1 cells. Thus, basophils are potent Th2-inducing APC *in vivo*. We transferred only $0.25–0.5 \times 10^6$ basophils and found dramatic induction of Th2 responses. We have also demonstrated that single i.v. administration of low-dose DNP–OVA/anti-DNP–IgE complex into naïve mice rapidly and preferentially induced OVA-specific Th2 cells in an endogenous basophil-dependent manner. Such sensitized mice promptly produced OVA-specific IgG1 antibody in response to i.v. administration of soluble OVA. Furthermore, IL-3 treatment prepares mice to be highly susceptible to Th2-inducing action of IgE complex by increasing the number of basophils.

Clinical implication: Basophils as a potential therapeutic target

Animals respond to allergen exposure by producing Ag-specific IgE. Such sensitized individuals, upon re-exposure to the same allergens, increase the production of IgE, which form allergen–IgE complexes by binding to allergens. These IgE complexes are captured by basophils that develop into Th2-inducing APC and present allergen-derived peptide with MHC class II and provide IL-4 to naïve $CD4^+$ T cells. Thus, basophils play a very important role in amplification of Th2-IgE responses, suggesting that they may be an important therapeutic target and depletion of basophils by antibody such as anti-FcεR1 might be an effective

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