efficacy (11), suggesting that bronchial asthma develops by diverse immunological mechanisms. Respiratory infections caused by bacteria frequently activate the T_h1-cell response through activation of Toll-like receptors (12, 13) and are associated with the initiation and/or exacerbation of bronchial asthma in humans (14, 15). These clinical studies strongly suggest that some types of bronchial asthma may be explained by the activation of T_h1-cell responses. However, intranasal challenge with ovalbumin (OVA) alone cannot evoke asthma in mice carrying OVA-specific T_h1 cells (16, 17), indicating that the T_h1-cell response alone is not sufficient enough to induce these pathological alterations. We have demonstrated that intranasal challenge with exogenous IL-18 or bacterial LPS induces IL-18 production in mice. In conjunction with OVA, this can induce robust asthma in mice immunized with OVA and the T_h1 adjuvant, CFA (16–18). OVA initiates OVA-specific T_h1 cells to produce IFN-γ but not IL-13, whereas OVA with IL-18 is capable of activating these T_h1 cells to produce larger amounts of IFN-γ, as well as IL-13, IL-9 and various chemokines that recruit eosinophils and other leukocytes (16). Persistent stimulation with IL-18 and the antigen alters the T_h1 cells, resulting in them producing both IFN-γ and IL-13 (19). With respect to their potential to produce both pro-inflammatory and pro-atopic cytokines/chemokines, we designated T_h1 cells that were re-stimulated with antigen and IL-18 as super T_h1 cells (16, 18, 19). In asthmatic mice possessing super T_h1 cells, IL-13 is responsible for the eosinophilic airway inflammation and remodeling. AHR is caused by IFN-γ, but not IL-13 (16, 17), which is in contrast to T_h2 cell-initiated asthmatic alterations where IL-13 plays a common and critical role (20, 21). Thus, IL-18 is likely to be involved in the development of bacterial infection-associated asthma. However, it is entirely unknown whether bacteria or their products by themselves can trigger super T_h1 cell type bronchial asthma.

The site of a pathogenic infection often determines the phenotype of infection-associated atopic diseases presumably by recruiting and activating pathogen-specific effector T cells and by inducing IL-18 release from the site. Infection with bacteria such as *Staphylococcus aureus* sometimes exacerbates atopic dermatitis in humans (22). We recently observed that consecutive and topical application of *S. aureus* protein A (SpA) (23) induces atopic dermatitis-like skin alterations in naive NC/Nga mice that have a genetically impaired skin barrier (19). The $CD4^+$ T cells prepared from the DLNs of mice with SpA-induced dermatitis express a cytokine profile characteristic of super T_h1 cells. Administration of neutralizing anti-IL-18 antibodies protects against dermatitis as well as super T_h1-cell development (19). Based on these observations, we assumed that the mice carrying SpA-specific T_h1 cells were highly vulnerable to asthma upon intranasal challenge with SpA. To test this hypothesis, we generated a novel asthmatic inflammation mouse model to determine the requirement of IL-18 in the development of SpA-induced asthma. Severely immunodeficient mice that had been inoculated with SpA-stimulated human PBMCs exhibited airway inflammation following intranasal challenge with SpA. Treatment with neutralizing anti-human IL-18 antibodies prevented this airway inflammation. Thus, IL-18 could be a potential target for the treatment of asthmatic inflammation associated with bacterial infection.

Methods

Animals and reagents

Female BALB/c mice and BALB/c *nu/nu* mice were purchased from CLEA Japan (Osaka, Japan). C57BL/6 background *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice were from Taconic Farms (Hudson, NY, USA). All animals were bred and/or maintained in specific pathogen-free conditions at the animal facilities of Hyogo College of Medicine and were used at 6–10 weeks of age. Animal experiments were performed in accordance with the guidelines of the National Institutes of Health, as specified by the animal care policy of Hyogo College of Medicine. SpA from *S. aureus* Cowan I was purchased from CalbioChem (La Jolla, CA, USA). Recombinant murine IL-18 was purchased from MBL (Nagoya, Japan). Anti-mouse CD3ε mAb (2C11), anti-mouse CD4 mAb (GK1), anti-human CD4 mAb (RPA-T4) and anti-human CD45 mAb (HI30) were from BD Biosciences Pharmingen (San Diego, CA, USA). Neutralizing anti-IFN-γ mAb was partly purified from the ascites fluid collected from BALB/c *nu/nu* mice inoculated intraperitoneally with hybridoma 6A2 purchased from the American Type Culture Collection (Manassas, VA, USA) (17, 19). Soluble IL-13Rα2-Fc was purchased from R&D Systems (San Diego, CA, USA) (17, 19). Rabbit polyclonal anti-mouse IL-18 antibodies were prepared in our laboratory (19). We generated a neutralizing anti-human IL-18 mAb as described previously (24).

Induction of asthma

The experimental protocol for asthma induction was the same as described in our previous report except we used SpA instead of OVA (17) (Supplementary Figure 1 is available at *International Immunology* Online). Briefly, BALB/c mice were immunized with SpA (500 μg) in CFA, followed by a boost with SpA in incomplete Freund's adjuvant (IFA) at day 14. For the adoptive cell transfer study, $CD4^+$ T cells isolated from DLNs of the immunized and boosted mice were labeled with 5-carbozyfluorescein diacetate succinimidyl ester (CFSE) and 6-CFSE. The CFSE-labeled cells (1×10^7) were administered intravenously into naive BALB/c mice (17). Two weeks following the SpA boost or after $CD4^+$ T cell transfer, mice were exposed intranasally to 50 μl of SpA (250 μg) in PBS for three consecutive days. In some experiments, neutralizing anti-mouse IL-18 antibodies (500 μg) were injected intraperitoneally into the mice at 1 day before and 1 day after intranasal exposure to SpA (17, 19). Anti-IFN-γ mAb (100 μg) or IL-13R2α-Fc (20 μg) was intranasally administered as outlined previously (17). Mice were sacrificed at 24 h after the final intranasal exposure of SpA.

Invasive measurement of AHR

Invasive measurement of AHR was assessed as an increase in pulmonary resistance (RLung) in response to aerosolized β-methacholine as described previously (17). RLung was measured by Pulmos-II (MIPS, Osaka, Japan) hardware and software (MIPS).

Preparation of $CD4^+$ lymph node cells and lung homogenate

$CD4^+$ T cells from the DLN were purified by magnetic-activated cell sorting (17). Lungs were homogenized with

1 ml of lysis buffer according to the method described previously (19, 25).

Cytoplasmic staining for IFN-γ and IL-13

Cells were isolated from mediastinal lymph nodes of SpA-immunized mice after consecutive 3-day challenge with SpA and were incubated with immobilized anti-CD3 mAb and 100 U ml^{-1} of IL-2 in the presence or absence of rmIL-18 (100 ng ml^{-1}) for 48 h. Cytoplasmic staining of the cells for IFN-γ and IL-13 were performed using antigen-presenting cells (APC)-anti-CD4 mAb (RM4-5), FITC-anti-IFN-γ mAb (XMG1.2) and PE-anti-IL-13 mAb (eBio13A).

Preparation of human PBMCs

PBMCs from healthy volunteers (26) were cultured with 100 μg ml^{-1} of SpA for 4 days. Experimental protocols for the use of human PBMCs were approved by the College Review Board of Hyogo College of Medicine.

Establishment of mice implanted with human PBMCs

SpA-stimulated human PBMCs (1×10^7) were transplanted intravenously into *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice (27). One-week post-transplantation, we isolated lymphocytes from the peripheral blood and spleen of the recipient mice and analyzed proportions of human $CD45^+$ cells in each preparation by flow cytometry. We used mice that contained >5% human $CD45^+$ cells in their peripheral blood because they also contained > 10% dual $CD45^+/CD4^+$ cells in their spleen (described below). The mice that received human SpA-stimulated PBMCs were then exposed intranasally to SpA for three consecutive days. In order to block the action of human IL-18, anti-human IL-18 mAb (300 μg) was intranasally administered 1 h before SpA exposure. Twenty-four hours following the final administration of SpA, lungs were sampled for histological and confocal microscopic studies.

Preparation of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected (17) and total cell number was determined in each sample. Cytospin preparations of BALF were stained with Dif-Quik (Baxter Healthcare Corp., Miami, FL, USA). Eosinophils and neutrophils were distinguished from each other by their difference in staining.

Histology

Lung specimens were fixed in 10% buffered formalin and sections were stained with hematoxylin and eosin (17). Fields of view on a microscope were selected at random and printed in large scale to distinguish eosinophils from other cell types. Eosinophils and the total number of nucleated cells in each field of view were counted. The mean ± SD of 10 fields of view per sample were calculated.

Confocal laser microscopic analysis

Frozen sections were fixed and incubated with FITC- or PE-conjugated mAb, followed by evaluation using a laser confocal microscope (model IX81; Olympus, Tokyo, Japan) (19).

Detection of cytokines and chemokines

Concentrations of IL-4, IL-13, tumor necrosis factor-α and IFN-γ in culture supernatants were determined with appropriate ELISA kits (Genzyme, Cambridge, MA, USA). Mouse IL-18 was measured by an ELISA kit from MBL. The concentrations of various mouse chemokines were measured with a Bio-Plex Cytokine assay kit (Bio-Rad, Hercules, CA, USA).

Statistics

Three to five mice were used for each experimental group. Data are expressed as the mean ± SD of triplicate samples. Significance between experimental and control groups was determined via an unpaired Student's *t*-test. A P value <0.05 was considered significant. Two to three experiments were performed per assay, and the representative data were shown.

Results

SpA-induced asthmatic inflammation

We examined whether intranasal challenge with SpA induces asthma-like airway inflammation in SpA-immunized mice. We immunized BALB/c mice subcutaneously with SpA in the T_h1 adjuvant, CFA, followed by a booster with SpA in IFA 2 weeks later. Twenty-eight days after the initial immunization, we administered SpA through a nasal tract for three consecutive days and examined the severity of asthmatic inflammation by measuring AHR, analyzing BALF preparations and lung histology (Supplemental Figure 1 is available at *International Immunology* Online). Invasive measurement of AHR revealed that SpA-immunized mice exhibited substantial AHR upon intranasal SpA challenge (Fig. 1A). None of the mice exhibited AHR after treatment with PBS (Fig. 1A) and naive mice were free from AHR even after SpA challenge (Fig. 1A). Thus, SpA immunization and SpA challenge are both required for the development of AHR. Following intranasal challenge with SpA, SpA-immunized mice demonstrated an increase in the number of eosinophils and neutrophils in BALF (Fig. 1B). These increases were not observed after treatment with PBS (Fig. 1B). The severity of AHR, consistent with our previous observations (16, 17), coincided with the cell numbers of eosinophils in BALF. This was also the case for the density of eosinophilic inflammation around the airway. Histological analysis revealed that only SpA-immunized mice developed severe inflammation around the airway following challenge with SpA, but not with PBS (Fig. 1C, E and F). Furthermore, intranasal challenge with SpA, but not PBS or OVA, induced eosinophilia (Supplementary Figure 2 is available at *International Immunology* Online), suggesting that SpA works in an antigen-specific manner. Naive mice exhibited only modest lung inflammation, if any, after intranasal SpA challenge (Fig. 1D). Eosinophils accumulated around the airway of the SpA-immunized and -challenged mice but not in mice treated with the other combinations of immunogens (Fig. 1J). Taken together, these results indicate that SpA-immunized and -challenged mice fulfill the clinical signs of asthmatic inflammation and are a suitable mouse model for bacterial infection-associated asthma-like inflammatory illnesses.

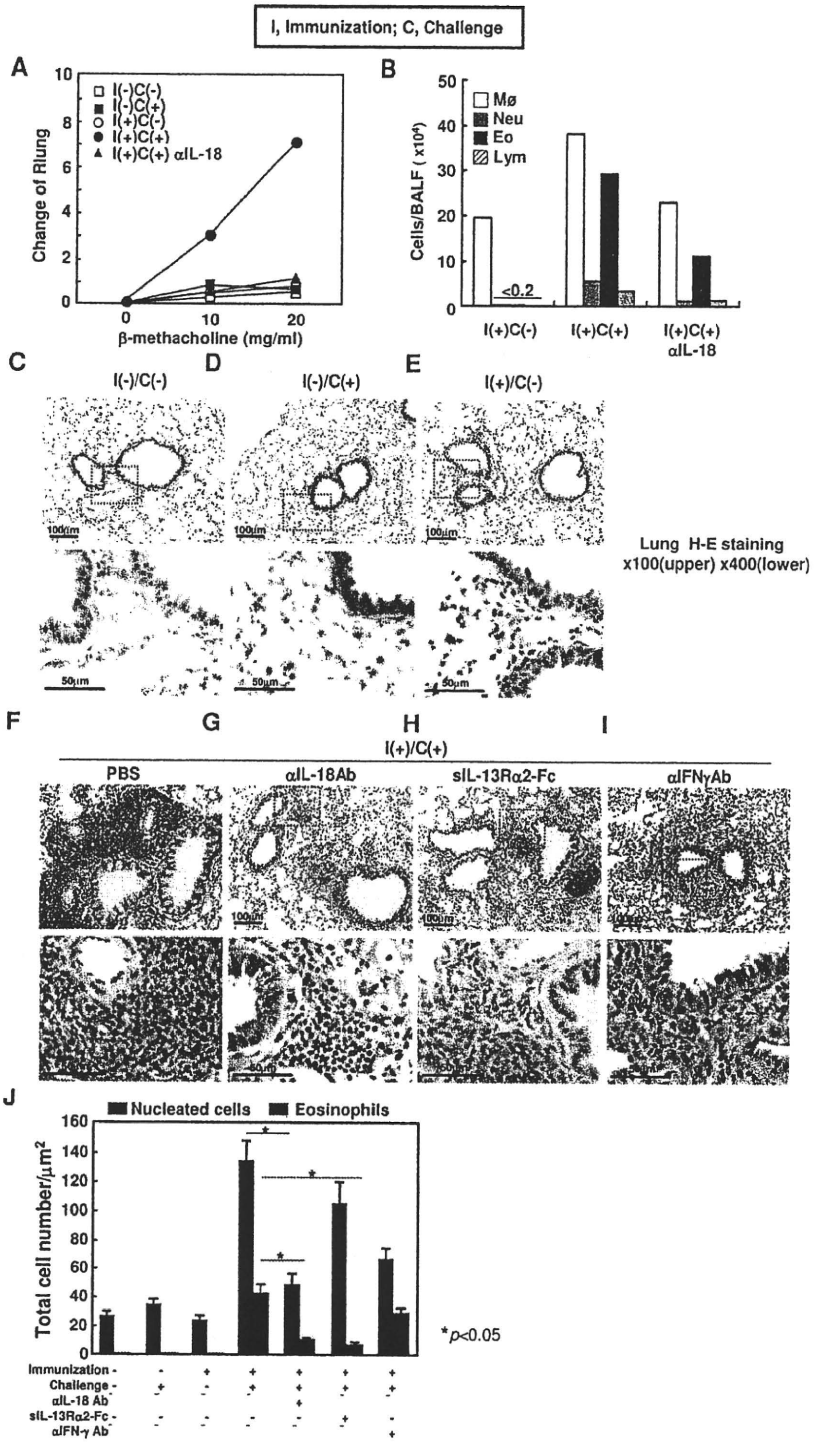

Fig. 1. Requirement of endogenous IL-18 for SpA-induced bronchial asthma. SpA-immunized mice 'I(+)' or naive mice 'I(−)' were intranasally challenged with SpA (250 μg/50 μl) 'C(+)' or PBS (50 μl) 'C(−)'. Neutralizing anti-IL-18 antibodies (500 μg) (A, B, G and J), soluble IL-13R2α-Fc (sIL-13Rα-Fc) (20 μg) (H) or neutralizing anti-IFN-γ (αIFNγ antibody) (100 μg) was administered twice into SpA-immunized mice intravenously, (I) 1 day before or after 3-day intranasal challenge with SpA (αIL-18 antibody). Twenty-four hours after the last SpA challenge, invasive measurement of AHR (A), cellular analysis of BALF (B) and histological analysis of lung specimens (C–I) were performed. (C–I) Upper panels are at low magnifications, and the lower panels are high magnification images of the areas indicated by a red-dotted square in the corresponding

Requirement of IL-18 for SpA-induced asthma

As previously reported, SpA-induced atopic dermatitis develops in an IL-18-dependent manner (19). We investigated whether IL-18 plays a pivotal role in the development of SpA-induced asthmatic inflammation. To test this, we administered neutralizing anti-IL-18 antibodies into the SpA-immunized mice 1 day before and 1 day after the initial intranasal SpA challenge (Supplementary Figure 1 is available at *International Immunology* Online). Administration of neutralizing anti-IL-18 antibodies profoundly reduced AHR (Fig. 1A) and significantly hampered respiratory inflammation and eosinophilia (Fig. 1B, G and J). Consistently (2, 9, 16), blocking the action of IL-13, but not IFN-γ, protected against eosinophilia in the airway (Fig. 1H–J). Conversely and consistently, blockade of IFN-γ, but not of IL-13, prevented AHR (16) (Supplementary Figure 3 is available at *International Immunology* Online). It would appear that endogenous IL-18 seems to be important in the development of SpA-induced asthmatic airway inflammation due to IL-13 production.

Super T_h1-cell differentiation

As IL-13 was profoundly involved in inflammation of the airways (Fig. 1H and J), we examined whether this experimental immunization/challenge protocol induces the development of $CD4^+$ DLN cells into super T_h1 cells or into IL-13-secreting T_h2 cells. We stimulated $CD4^+$ DLN cells with immobilized anti-CD3 mAb and measured the concentrations of T_h2 and super T_h1 cytokines. $CD4^+$ DLN cells from SpA-immunized and -challenged mice produced larger amounts of IFN-γ and IL-13, but little IL-4, compared with SpA-immunized mice without SpA challenge (Fig. 2A), indicating their development into super T_h1 cells, but not T_h2 cells, during intranasal SpA challenge. Furthermore, we examined whether both IL-13 and IFN-γ are produced by a single $CD4^+$ cell isolated from SpA-challenged and SpA-immunized mice. We isolated cells from mediastinal lymph nodes of SpA-challenged and SpA-immunized mice and incubated the cells with plate-bound anti-CD3 in the presence or absence of exogenous IL-18. We found a very small proportion of $IL\text{-}13^+IFN\text{-}\gamma^+$ $CD4^+$ T cells after TCR stimulation alone (Fig. 2B). However, upon TCR and IL-18 stimulation, the proportion of $IL\text{-}13^+IFN\text{-}\gamma^+$ $CD4^+$ T cells was significantly elevated (Fig. 2B), suggesting that super T_h1-cell differentiation occurs in SpA-immunized mice after intranasal challenge with SpA. At the same, this stimulation induced an increase in two other populations: IL-13-produicng cells and IFN-γ-producing cells (Fig. 2B). Thus, three populations, consisting of IL-13-produicng cells, IFN-γ-producing cells and IL-13 plus IFN-γ-producing cells, contribute to induction of SpA-induced bronchial asthma.

Next, we investigated the roles of endogenous IL-18 in super T_h1-cell development. $CD4^+$ DLN cells prepared from the mice additionally treated with anti-IL-18 antibodies produced much less IFN-γ and IL-13 than those from SpA-induced asthmatic mice (Fig. 2A), suggesting the possibility that IL-18 release during SpA challenge participates in super T_h1-cell differentiation. To test this possibility, we examined whether IL-18 is produced in the asthmatic lung. SpA immunization alone failed to induce significant increase in IL-18 concentration within the lung tissue (Fig. 2C). SpA immunization and challenge seemed to increase IL-18 levels significantly in the lung (Fig. 2C). These results suggest that airway constituents such as respiratory epithelial cells and/or alveolar macrophages might release IL-18 in response to SpA.

Induction of chemokines attracting eosinophils and neutrophils in the lungs

As IL-18 is capable of inducing chemokine production from epithelial cells, T_h1 cells and super T_h1 cells (16, 18), we examined whether IL-18 could induce expression of chemokines in the lung, particularly chemokines recruiting eosinophils and neutrophils, during intranasal SpA challenge. Lung homogenates from mice immunized with SpA only contained almost basal amounts of chemokines attracting eosinophils, including CCL5 (RANTES) and CCL11 (Eotaxin), and neutrophils, such as CXCL1 (KC) and CCL2 (MCP-1), when compared with naive mice (Fig. 3). However, it was only after SpA challenge that pulmonary levels of CCL5, CCL11, CCL2 and CXCL1 were significantly elevated (Fig. 3). This was also the case for the chemokines attracting diverse types of leukocytes, such as, CCL3 (MIP-1α) and CCL4 (MIP-1β), as well as the pro-inflammatory cytokines, IL-1β and IL-6 (Fig. 3). In contrast, T_h2 cytokines, IL-4 and IL-5 were not induced after SpA challenge (Fig. 3). As expected, treatment with neutralizing anti-IL-18 antibodies during intranasal exposure to SpA significantly reduced chemokine expression levels (Fig. 3). Thus, the expression of these chemokines could be induced by IL-18.

Importance of SpA-activated super T_h1 cells in the development of airway inflammation

We examined whether super T_h1 cells are effector cells of SpA-induced asthmatic inflammation. To test this, we transferred $CD4^+$ DLN cells from SpA-immunized mice into naive mice, followed by intranasal administration of SpA for three consecutive days. Upon daily exposure to PBS, mice receiving the $CD4^+$ DLN cells demonstrated an intact response to methacholine treatment and evaded airway inflammation (Figs 1A and C and 4A and C). Upon exposure to SpA, these mice exhibited obvious AHR (Fig. 4A) and airway inflammation (Fig. 4C), prompting us to investigate whether donor $CD4^+$ DLN cells migrated into the airway as a response to SpA challenge in order to exert their effector functions. We labeled the donor cells with CFSE, injected them into naive recipient mice and analyzed their localization in the recipient lung after SpA challenge. Many CFSE-labeled cells had migrated into the lung (Fig. 4B). Most of the

upper panels. Upper scale bars indicate 100 μm, while the lower ones represent 50 μm. Lung sections were stained with hematoxylin and eosin (H&E). The mean ± SD of total nucleated cells per square micrometer (black bars) and eosinophils per square micrometer (red bars) in 10 fields of view selected at random are shown (J). Mø, macrophages; Neu, neutrophils; Eo, eosinophils; Lym, lymphocytes. Data are representative of three independent experiments with five mice per group.

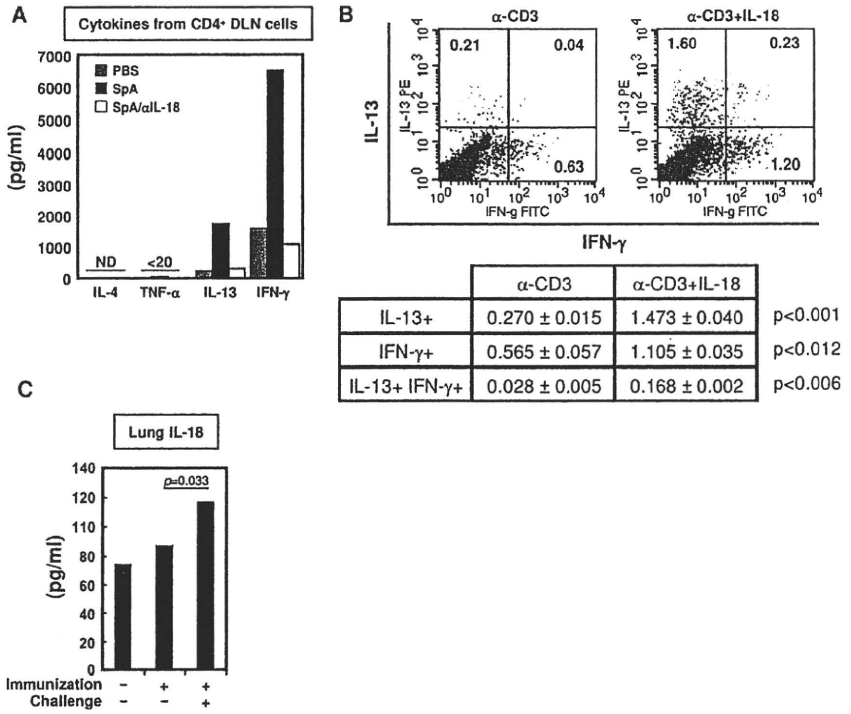

Fig. 2. IL-18-dependent differentiation toward super T_h1 cells. (A) DLN cells were prepared from variously treated mice as shown in the legend to Fig. 1A, and $CD4^+$ T cells (1×10^5) were incubated on immobilized anti-CD3ε mAb. Various cytokine concentrations in each supernatant were measured by ELISA. (B) Mediastinal lymph node cells were prepared from SpA-challenged SpA-immunized mice and were incubated with immobilized anti-CD3ε mAb alone (α-CD3) or anti-CD3ε mAb plus IL-18 for 48 h, followed by cytoplasmic staining for IFN-γ and IL-13. (C) Lung homogenates were prepared from naive mice, SpA-immunized mice and SpA-immunized and -challenged mice, for the measurement of IL-18 by ELISA. Data are representative of three independent experiments with five mice in each group.

pulmonary $CD4^+$ T cells co-expressed CFSE (Fig. 4B), indicating that the donor $CD4^+$ T cells but few of the recipient cells, accumulated in the lung. Upon exposure to PBS, few CFSE-labeled cells or $CD4^+$ T cells were observed in the recipient lung (data not shown). These results demonstrate that SpA-specific $CD4^+$ DLN cells migrate and are fully activated after being exposed to SpA, eventually leading to the development of asthmatic inflammation. Blocking the action of IL-18 protected the mice from AHR and airway inflammation (Fig. 4A and C), suggesting that SpA-induced IL-18 in the airway in combination with administered SpA might differentiate the donor cells toward super T_h1 cells, thereby becoming highly pathogenic effector cells.

Involvement of human IL-18 in SpA-induced airway inflammation in transiently humanized mice

Finally, we investigated whether IL-18 is a therapeutic target for the treatment of airway inflammation in humans, associated with bacterial infection. First, we tried to generate mice transiently carrying human immune competent cells. We incubated PBMCs from healthy donors with SpA *in vitro*, transferred them into immunodeficient mice without T cells, B cells and NK cells and investigated whether human PBMCs settled in the recipient mice by calculating proportions of human $CD45^+$ hematopoietic cells (27) in peripheral immune tissues. Human $CD45^+$ cells were robustly observed in the spleen and peripheral blood of the recipient mice at day 7 after PBMC transfer (Fig. 5). About one-third of human $CD45^+$ cells co-expressed the human CD4 marker (Fig. 5).

Like the $CD4^+$ DLN cell-transplanted mice (Fig. 4), the SpA-stimulated humanized mice developed airway inflammation upon intranasal challenge with SpA, concomitant with dense accumulation of human $CD4^+$ T cells around the airway (Fig. 6C and D). This result indicated that SpA-stimulated human $CD4^+$ T cells migrated into the airway and presumably evoked pulmonary inflammation in response to exogenous SpA and endogenous IL-18. Upon PBS exposure, however, the host mice showed weak airway inflammation with modest but apparent accumulation of human $CD4^+$ cells (Fig. 6A and B). We investigated the role of human IL-18 and confirmed that anti-human IL-18 mAb (24) potently neutralized human IL-18 (Supplementary Figure 4 is available at *International Immunology* Online). This mAb prevented SpA-induced airway inflammation in these mice by attenuating airway accumulation of human $CD4^+$ T cells (Fig. 6E and F). Therefore, human cell-derived IL-18 might fully activate human SpA-specific T_h1 cells to become pathogenic effector cells. Indeed, human PBMCs could release super T_h1 cell-inducing cytokines, such as IL-12 and IL-18, and super T_h1 cytokines, such as IL-13 and IFN-γ, in response to SpA *in vitro* (Supplementary Figure 5 is

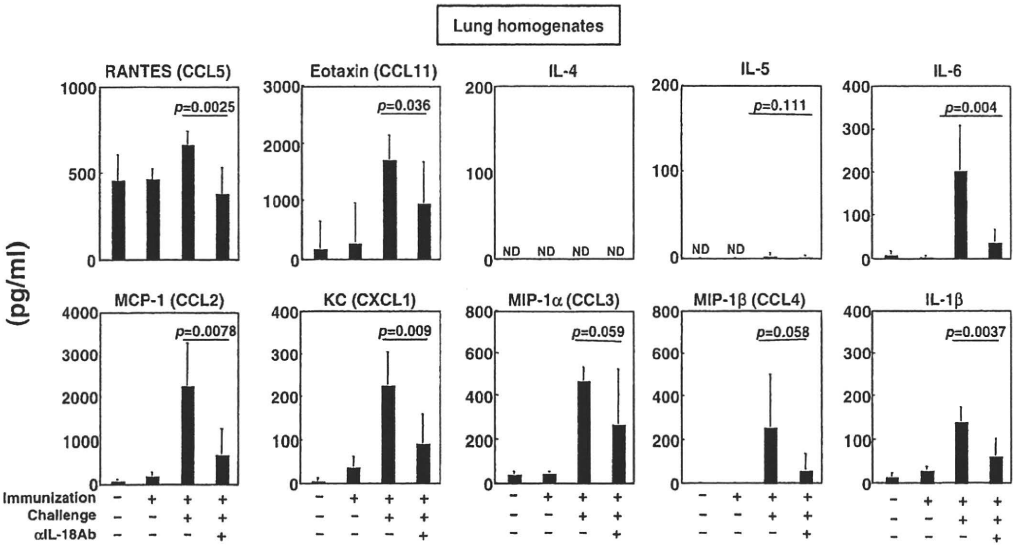

Fig. 3. IL-18 involvement in the induction of chemokines for neutrophils and eosinophils. Lung homogenates were prepared from the mice of three experimental groups (five mice per group) as shown in the legend to Fig. 1A and naive mice (five mice per group). The concentration of CCL2 (MCP-2), CCL3 (MIP1α), 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*$^{-/-}$*Cγ*$^{-/-}$ 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*$^{-/-}$*Cγ*$^{-/-}$ 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.

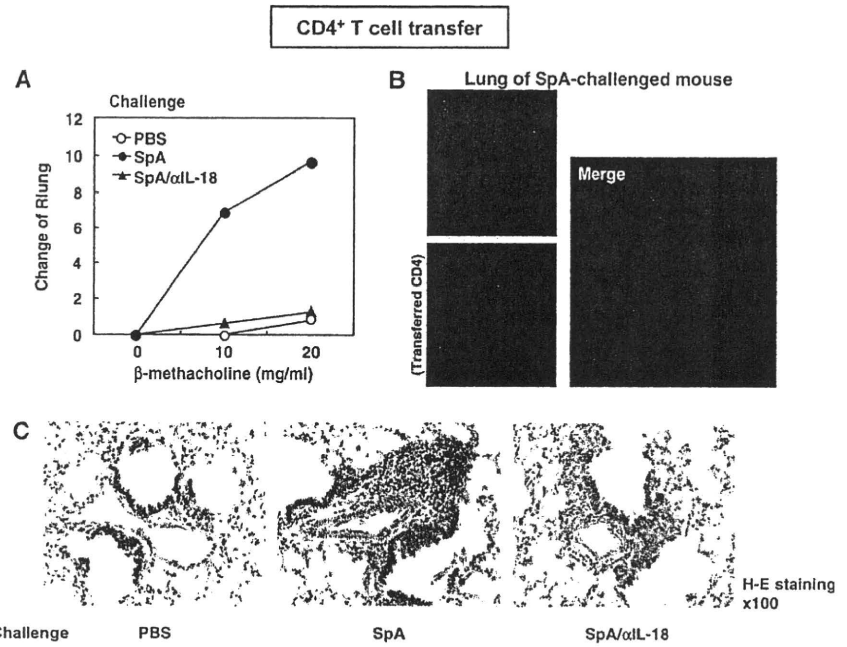

Fig. 4. SpA-sensitized CD4+ T cells can transfer disease susceptibility. CD4+ DLN cells isolated from mice immunized and boosted with SpA were labeled with CFSE and transferred into naive mice (1×10^7 per mouse). Recipient mice received intranasal challenge with SpA (250 μg/50 μl) (SpA), PBS or SpA and 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_h2 type and super T_h1 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_h1 cell-bearing mice, suggesting that OVA plus LPS activates T_h1 cells via endogenous IL-18. In contrast, the role of endogenous IL-18 in allergic T_h2/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_h1 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_h2 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_h2-cell response develops only under the limited condition of immunization with the protein in combination with T_h2-cell adjuvant. Beyond this dogma, it was shown that multiple intra-tracheal challenges with house dust mite alone could trigger a T_h2-cell response without prior immunization with antigen/T_h2 adjuvant complex. Mice lacking TLR4 expression on their respiratory epithelial cells, but not hematopoietic cells, were able to evade T_h2 type asthma. Because house dust mites possess intrinsic TLR4 agonists, TLR4 on respiratory

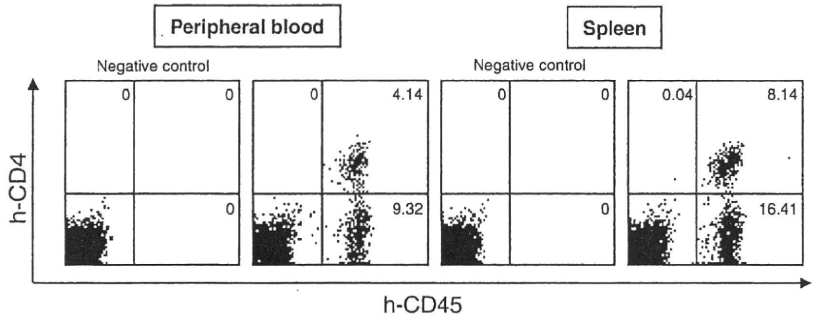

Fig. 5. Mice injected with human PBMCs. Seven days after SpA-stimulated PBMC (1×10^7) were injected into mice, the *Rag2*$^{-/-}$*Cγ*$^{-/-}$ mice were analyzed for repopulation with human lymphocytes in their peripheral blood and spleen. Cells were incubated with PE-conjugated anti-human CD4 and FITC-labeled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

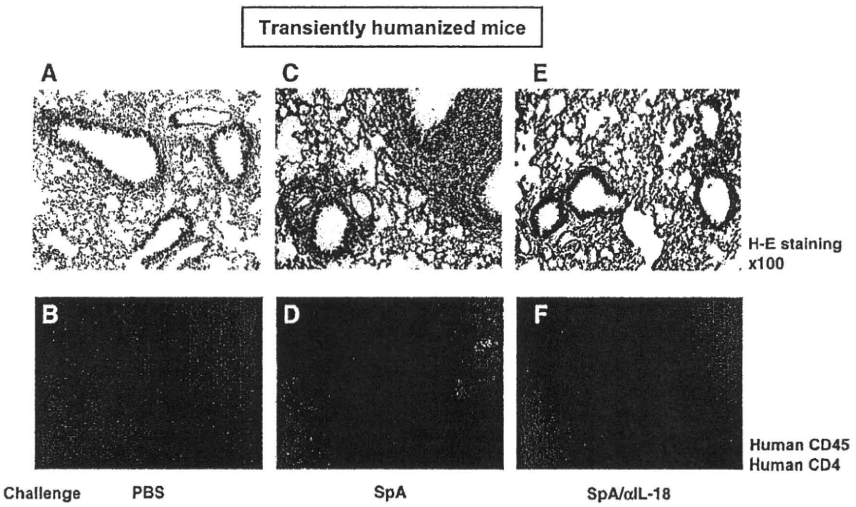

Fig. 6. Blocking the action of human IL-18 prevents SpA-induced airway inflammation in transiently humanized mice. Mice having received SpA-stimulated PBMC (1×10^7) were intranasally treated with PBS (A and B) or challenged with SpA (250 μg/50 μl) (SpA; C and D) or with SpA and neutralizing anti-human 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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References

1 Busse, W. W. and Lemanske, R. F. Jr. 2001. Asthma. *N. Engl. J. Med.* 344:350.
2 Wills-Karp, M. 2004. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* 202:175.
3 Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17:255.
4 Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. and DeKruyff, R. H. 2002. Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 3:715.
5 Anderson, B. 2008. Endotyping asthma: new insights into key pathogenic mechanisms in a complex, heterogenous disease. *Lancet.* 372:1107.
6 Frey, U. and Suki, B. 2008. Complexity of chronic asthma and chronic obstructive pulmonary disease: implications for risk assessment, and disease progression and control. *Lancet.* 372:1088.
7 Kubo, M., Ozaki, A., Tanaka, S., Okamoto, M. and Fukushima, A. 2006. Role of suppressor of cytokine signaling in ocular allergy. *Curr. Opin. Allergy Clin. Immunol.* 6:361.
8 Hoshino, A., Tsuji, T., Maatsuzaki, J. *et al.* 2004. STAT6-mediated signaling in Th2-dependent allergic asthma: critical role for the development of eosinophilia, airway ahyper-responsiveness and mucus hypersecretion, distinct from its role in Th2 differentiation. *Int. Immunol.* 16:1497.
9 Cohn, L., Elias, J. A. and Chupp, G. L. 2004. Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* 22:789.
10 Medoff, B. D., Thomas, S. Y. and Luster, A. D. 2008. T cell trafficking in allergic asthma: the ins and outs. *Annu. Rev. Immunol.* 26:205.
11 Bisgaard, H., Hermansen, M. N., Buchvald, F. *et al.* 2007. Childhood asthma after bacterial colonization of the airway in neonates. *N. Engl. J. Med.* 357:1487.
12 Kaisho, T. and Akira, S. 2006. Toll-like receptor function and signaling. *J. Allergy Clin. Immunol.* 117:979.
13 Akira, S., Uematsu, S. and Takeuchi, O. 2006. Pathogen recognition and innate immunity. *Cell* 124:783.
14 Singh, A. M. and Busse, W. W. 2006. Asthma exacerbations 2: aetiology. *Thorax* 61:809.
15 Cosentini, R., Tarsia, P., Canetta, C. *et al.* 2008. Severe asthma exacerbation: role of acute *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* infection. *Respir. Res.* 9:48.
16 Sugimoto, T., Ishikawa, Y., Yoshimoto, T., Hayashi, N., Fujimoto, J. and Nakanishi, K. 2004. Interleukin 18 acts on memory T helper cells type 1 to induce airway inflammation and hyperresponsiveness in a naive host mouse. *J. Exp. Med.* 199:535.
17 Hayashi, N., Yoshimoto, T., Izuhara, K., Matsui, K., Tanaka, T. and Nakanishi, K. 2007. T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyperresponsiveness and lung fibrosis by IFN-γ and IL-13 production. *Proc. Natl Acad. Sci. USA* 104:14765.
18 Tsutsui, H., Yoshimoto, T., Hayashi, N., Mizutani, H. and Nakanishi, K. 2004. Induction of allergic inflammation by interleukin-18 in experimental animal models. *Immunol. Rev.* 202:115.
19 Terada, M., Tsutsui, H., Imai, Y. *et al.* 2006. Contribution of IL-18 to atopic-dermatitis-like skin inflammation induced by *Staphylococcus aureus* product in mice. *Proc. Natl Acad. Sci. USA* 103:8816.
20 Yamagata, S., Tomita, K., Sato, R., Niwa, A., Higashino, H. and Tohda, Y. 2008. Interleukin-18-deficient mice exhibit diminished chronic inflammation and airway remodelling in ovalbumin-induced asthma model. *Clin. Exp. Immunol.* 154:295.
21 Hartwig, C., Tschernig, T., Mazzega, M., Braun, A. and Neumann, D. 2008. Endogenous IL-18 in experimentally induced asthma affects cytokine serum levels but is irrelevant for clinical symptoms. *Cytokine* 42:298.
22 Leung, D. Y. and Bieber, T. 2003. Atopic dermatitis. *Lancet* 361:151.
23 Gomez, M. I., Lee, A., Reddy, B. *et al.* 2004. *Staphylococcus aureus* protein A induces airway epithelial inflammatory responses by activating TNFR1. *Nat. Med.* 10:842.
24 Hamasaki, T., Hashiguchi, S., Ito, Y. *et al.* 2005. Human anti-human IL-18 antibody recognizing the IL-18-binding site 3 with IL-18 signaling blocking activity. *J. Biochem.* 138:433.
25 Ishikawa, Y., Yoshimoto, T. and Nakanishi, K. 2006. Contribution of IL-18-induced innate T cell activation to airway inflammation with mucus hypersecretion and airway hyperresponsiveness. *Int. Immunol.* 18:847.
26 Hata, H., Yoshimoto, T., Hayashi, N., Hada, T. and Nakanishi, K. 2004. IL-18 together with anti-CD3 antibody induces human Th1 cells to produce Th1- and Th2-cytokines and IL-8. *Int. Immunol.* 16:1733.
27 Goldman, J. P., Blundell, M. P., Lopes, L., Kinnon, C., DiSanto, J. P. and Thrasher, A. J. 1998. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor g chain. *Br. J. Haematol.* 103:335.
28 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19:423.
29 Kato, A. and Schleimer, R. P. 2007. Beyond inflammation: airway epithelial cells are at the interface of innate and adaptive immunity. *Curr. Opin. Immunol.* 19:711.
30 Hammad, H., Chieppa, M., Perros, F., Willart, M. A., Germain, R. N. and Lambrecht, B. N. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 15:410.
31 Trompette, A., Divanovic, S., Visintin, A. *et al.* 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457:585.
32 Cayrol, C. and Girard, J.-P. 2009. The IL-1-like cytokine IL-33 is activated after maturation by caspase-1. *Proc. Natl Acad. Sci. USA* 106:9021.
33 Saenz, S. A., Taylor, B. C. and Artis, D. 2008. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunol. Rev.* 226:172.
34 Nakano, H., Tsutsui, H., Terada, M. *et al.* 2003. Persistent secretion of IL-18 in the skin contributes to IgE response in mice. *Int. Immunol.* 15:611.
35 Tanaka, T., Tsutsui, H., Yoshimoto, T. *et al.* 2001. Interleukin-18 is elevated in the sera from patients with atopic dermatitis and from atopic dermatitis model mice, NC/Nga. *Int. Arch. Allergy Immunol.* 125:236.
36 Tanaka, H., Miyazaki, N., Oashi, K. *et al.* 2001. IL-18 might reflect disease activity in mild and moderate asthma exacerbation. *J. Allergy Clin. Immunol.* 107:331.
37 Higa, S., Hirano, T., Mayumi, M. *et al.* 2003. Association between interleukin-18 gene polymorphism 105A/C and asthma. *Clin. Exp. Allergy* 33:1097.
38 Novak, N., Kruse, S., Potreck, J. *et al.* 2005. Single nucleotide polymorphisms of the IL18 gene are associated with atopic eczema. *J. Allergy Clin. Immunol.* 115:828.
39 Arimitsu, J., Hirano, T., Higa, S. *et al.* 2006. IL-18 gene polymorphisms affect IL-18 production capability by monocytes. *Biochem. Biophys. Res. Comm.* 342:1413.

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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.

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

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

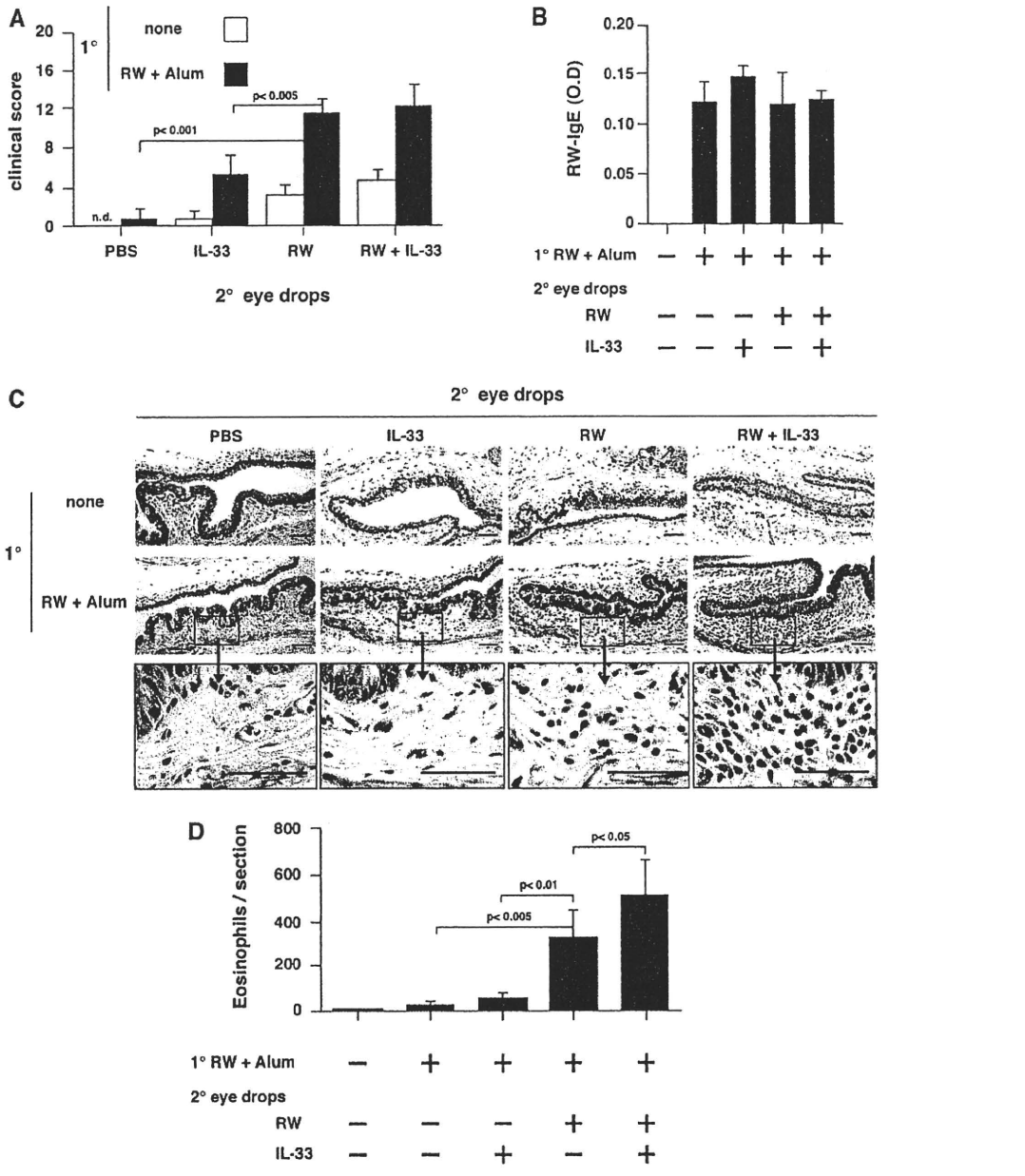

Fig. 1. Induction of AC and conjunctival eosinophilic inflammation by RW or RW plus IL-33 in RW-immunized mice. BALB/c mice were immunized with RW as described in Methods. Naive mice or RW-immunized mice were topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and/or IL-33 (1 μg 5 μl^{-1} PBS per 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,

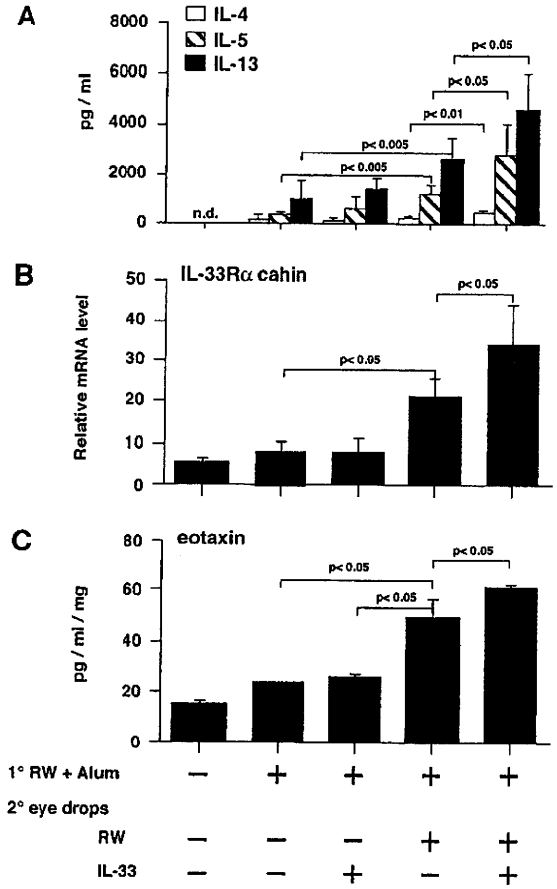

Fig. 2. Enhancement of T_h2 cytokine production and IL-33R expression by cervical lymph node cells after eye drop challenge with RW or RW plus IL-33. BALB/c mice were immunized with RW and topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and/or IL-33 (1 μg 5 μl^{-1} PBS per eye). Cervical lymph 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).

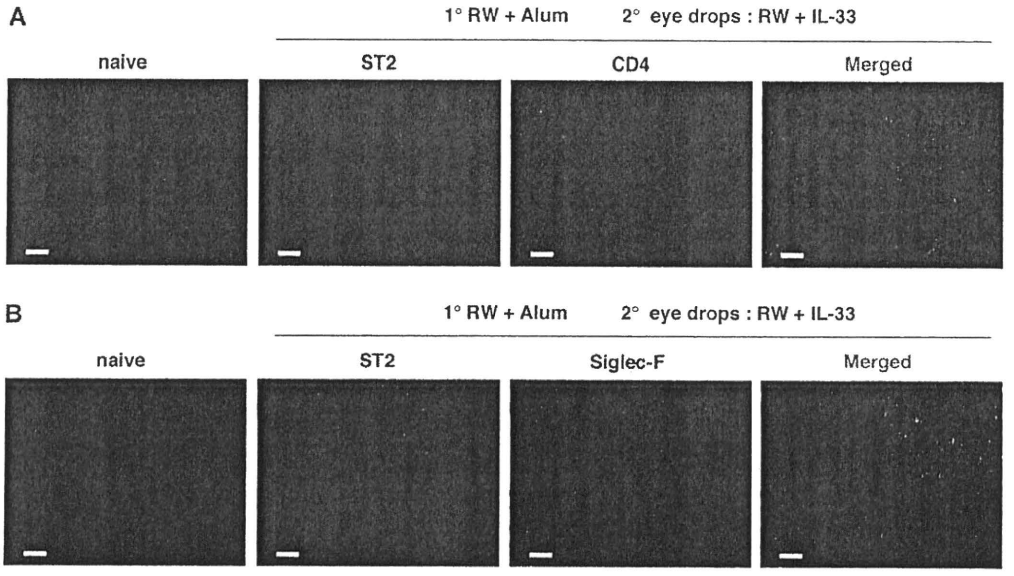

Fig. 3. Confocal microscopic examination of IL-33Rα chain$^+$ cells in the conjunctiva tissue of RW-immunized mice after challenge with RW and/or IL-33. BALB/c mice were immunized with RW and topically challenged with RW (1 mg 5 μl^{-1} PBS per eye) and IL-33 (1 μg 5 μl^{-1} PBS per eye). At 24 h post-challenge, 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,

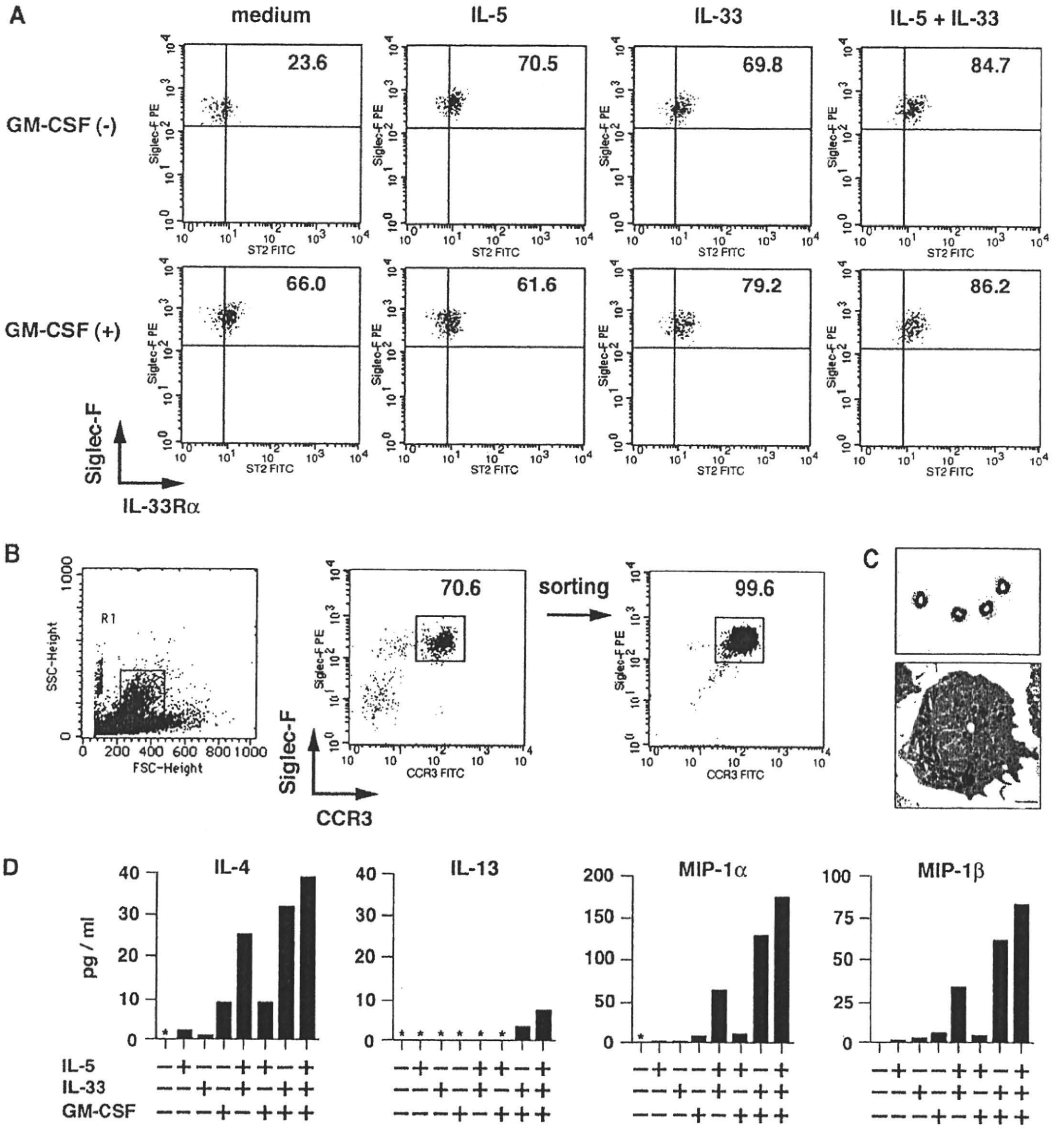

Fig. 4. Eosinophils stimulated with IL-5 increase IL-33Rα chain expression and become responsive to IL-33. (A) Spleen cells (2×10^6 ml^{-1}) from naive BALB/c mice were stimulated with medium alone, IL-5 (40 ng ml^{-1}) and/or IL-33 (100 ng ml^{-1}) in the presence or absence of GM-CSF (50 ng ml^{-1}) 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).

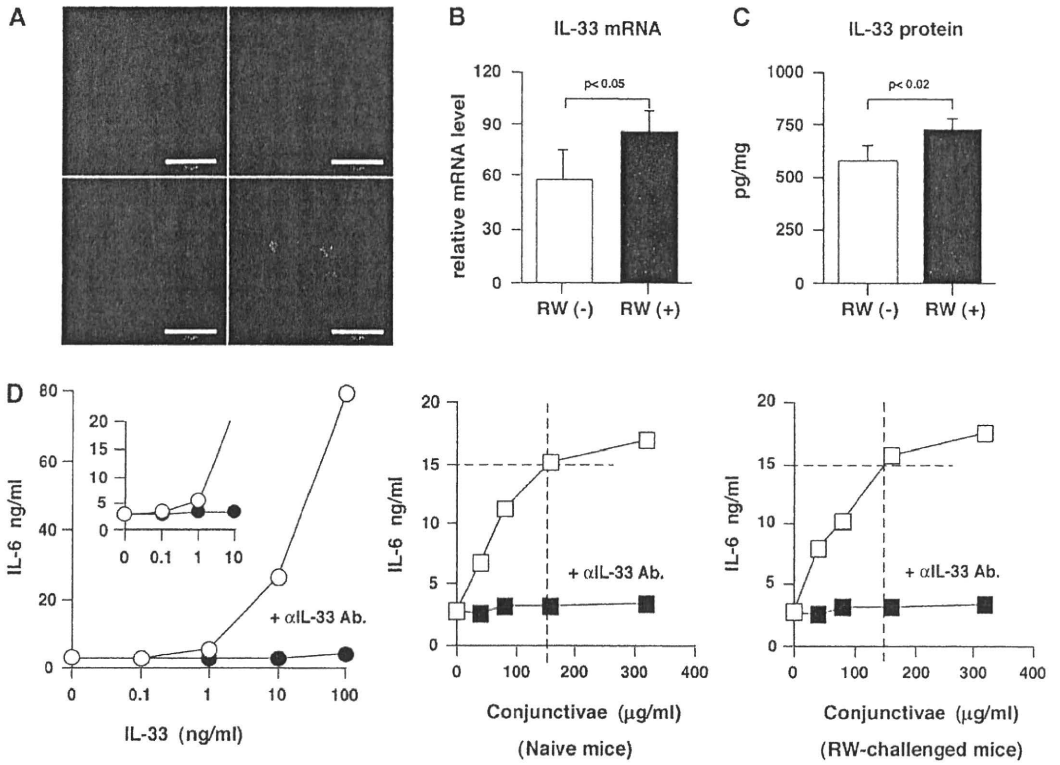

Fig. 5. Expression of IL-33 by the conjunctiva of mice before and after eye drop challenge with RW. (A) Eyes were enucleated from naive BALB/c mice, fixed in 4% PFA, embedded in paraffin and cut into 4-μm vertical plane sections including the optic nerve. Immunofluorescence staining was performed using 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

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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References

1 Bielory, L. 2000. Allergic and immunologic disorders of the eye. Part II: ocular allergy. *J. Allergy Clin. Immunol.* 106:1019.
2 Stahl, J. L. and Barney, N. P. 2004. Ocular allergic disease. *Curr. Opin. Allergy Clin. Immunol.* 4:455.
3 Calonge, M. 1999. Classification of ocular atopic/allergic disorders and conditions: an unsolved problem. *Acta Ophthalmol. Scand. Suppl.* 228:10.
4 Niederkorn, J. Y. 2008. Immune regulatory mechanisms in allergic conjunctivitis: insights from mouse models. *Curr. Opin. Allergy Clin. Immunol.* 8:472.
5 Miyazaki, D., Tominaga, T., Yakura, K. *et al.* 2008. Conjunctival mast cell as a mediator of eosinophilic response in ocular allergy. *Mol. Vis.* 14:1525.
6 Carreras, I., Carreras, B., McGrath, L., Rice, A. and Easty, D. L. 1993. Activated T cells in an animal model of allergic conjunctivitis. *Br. J. Ophthalmol.* 77:509.
7 Magone, M. T., Chan, C. C., Rizzo, L. V., Kozhich, A. T. and Whitcup, S. M. 1998. A novel murine model of allergic conjunctivitis. *Clin. Immunol. Immunopathol.* 87:75.
8 Ozaki, A., Seki, Y., Fukushima, A. and Kubo, M. 2005. The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model. *J. Immunol.* 175:5489.
9 Schmitz, J., Owyang, A., Oldham, E. *et al.* 2005. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 23:479.
10 Arend, W. P., Palmer, G. and Gabay, C. 2008. IL-1, IL-18, and IL-33 families of cytokines. *Immunol. Rev.* 223:20.
11 Cayrol, C. and Girard, J. P. 2009. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. *Proc. Natl Acad. Sci. USA* 106:9021.
12 Kondo, Y., Yoshimoto, T., Yasuda, K. *et al.* 2008. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. *Int. Immunol.* 20:791.
13 Xu, D., Chan, W. L., Leung, B. P. *et al.* 1998. Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells. *J. Exp. Med.* 188:1485.
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: synergism with IL-18 for IFN-gamma production. *J. Immunol.* 161:3400.
15 Born, T. L., Thomassen, E., Bird, T. A. and Sims, J. E. 1998. Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. *J. Biol. Chem.* 273:29445.
16 Nakanishi, K., Yoshimoto, T., Tsutsui, H. and Okamura, H. 2001. Interleukin-18 regulates both Th1 and Th2 responses. *Annu. Rev. Immunol.* 19:423.
17 Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U. 2007. IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl Acad. Sci. USA* 104:18660.
18 Chackerian, A. A., Oldham, E. R., Murphy, E. E., Schmitz, J., Pflanz, S. and Kastelein, R. A. 2007. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* 179:2551.
19 Hoshino, K., Kashiwamura, S., Kuribayashi, K. *et al.* 1999. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. *J. Exp. Med.* 190:1541.
20 Ho, L. H., Ohno, T., Oboki, K. *et al.* 2007. IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. *J. Leukoc. Biol.* 82:1481.
21 Yoshimoto, T., Bendelac, A., Hu-Li, J. and Paul, W. E. 1995. Defective IgE production by SJL mice is linked to the absence of CD4+, NK1.1+ T cells that promptly produce interleukin 4. *Proc. Natl Acad. Sci. USA* 92:11931.
22 Andrews, A. L., Bucchieri, F., Arima, K. *et al.* 2007. Effect of IL-13 receptor alpha2 levels on the biological activity of IL-13 variant R110Q. *J. Allergy Clin. Immunol.* 120:91.
23 Fujisawa, T., Kato, Y., Atsuta, J. *et al.* 2000. Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. *J. Allergy Clin. Immunol.* 105:126.

24 Ishikawa, Y., Yoshimoto, T. and Nakanishi, K. 2006. Contribution of IL-18-induced innate T cell activation to airway inflammation with mucus hypersecretion and airway hyperresponsiveness. *Int. Immunol.* 18:847.

25 Lohning, M., Stroehmann, A., Coyle, A. J. *et al.* 1998. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl Acad. Sci. USA* 95:6930.

26 Zhang, M., Angata, T., Cho, J. Y., Miller, M., Broide, D. H. and Varki, A. 2007. Defining the *in vivo* function of Siglec-F, a CD33-related Siglec expressed on mouse eosinophils. *Blood* 109:4280.

27 Kitaura, M., Nakajima, T., Imai, T. *et al.* 1996. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. *J. Biol. Chem.* 271:7725.

28 Lukacs, N. W., Standiford, T. J., Chensue, S. W., Kunkel, R. G., Strieter, R. M. and Kunkel, S. L. 1996. C-C chemokine-induced eosinophil chemotaxis during allergic airway inflammation. *J. Leukoc. Biol.* 60:573.

29 Lukacs, N. W., Strieter, R. M., Shaklee, C. L., Chensue, S. W. and Kunkel, S. L. 1995. Macrophage inflammatory protein-1 alpha influences eosinophil recruitment in antigen-specific airway inflammation. *Eur. J. Immunol.* 25:245.

30 Lukacs, N. W., Strieter, R. M., Warmington, K., Lincoln, P., Chensue, S. W. and Kunkel, S. L. 1997. Differential recruitment of leukocyte populations and alteration of airway hyperreactivity by C-C family chemokines in allergic airway inflammation. *J. Immunol.* 158:4398.

31 Carriere, V., Roussel, L., Ortega, N. *et al.* 2007. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor *in vivo*. *Proc. Natl Acad. Sci. USA* 104:282.

32 Moussion, C., Ortega, N. and Girard, J. P. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*: a novel 'alarmin'? *PLoS One* 3:e3331.

33 Hayakawa, H., Hayakawa, M., Kume, A. and Tominaga, S. 2007. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J. Biol. Chem.* 282:26369.

34 Pushparaj, P. N., Tay, H. K., H'Ng, S. *et al.* 2009. The cytokine interleukin-33 mediates anaphylactic shock. *Proc. Natl Acad. Sci. USA* 106:9773.

35 Sakashita, M., Yoshimoto, T., Hirota, T. *et al.* 2008. Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin. Exp. Allergy* 38:1875.

36 Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A. 2009. Human basophils and eosinophils are the direct target leukocytes of the novel IL-1 family member IL-33. *Blood* 113:1526.

37 Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R. and Smith, D. E. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 20:1019.

38 Gudbjartsson, D. F., Bjornsdottir, U. S., Halapi, E. *et al.* 2009. Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat. Genet.* 41:342.

39 Guo, L., Wei, G., Zhu, J. *et al.* 2009. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc. Natl Acad. Sci. USA* 106:13463.

40 Mui, A. L., Wakao, H., O'Farrell, A. M., Harada, N. and Miyajima, A. 1995. Interleukin-3, granulocyte-macrophage colony stimulating factor and interleukin-5 transduce signals through two STAT5 homologs. *EMBO J.* 14:1166.

41 Cherry, W. B., Yoon, J., Bartemes, K. R., Iijima, K. and Kita, H. 2008. A novel IL-1 family cytokine, IL-33, potently activates human eosinophils. *J. Allergy Clin. Immunol.* 121:1484.

42 Suzukawa, M., Koketsu, R., Iikura, M. *et al.* 2008. Interleukin-33 enhances adhesion, CD11b expression and survival in human eosinophils. *Lab. Invest.* 88:1245.

43 Perrigoue, J. G., Saenz, S. A., Siracusa, M. C. *et al.* 2009. MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat. Immunol.* 10:697.

44 Sokol, C. L., Chu, N. Q., Yu, S., Nish, S. A., Laufer, T. M. and Medzhitov, R. 2009. Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nat. Immunol.* 10:713.

45 Yoshimoto, T., Yasuda, K., Tanaka, H. *et al.* 2009. Basophils contribute to T(H)2-IgE responses *in vivo* via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 10:706.

46 Dinarello, C. A. 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annu. Rev. Immunol.* 27:519.

47 Bianchi, M. E. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukoc. Biol.* 81:1.

48 Haraldsen, G., Balogh, J., Pollheimer, J., Sponheim, J. and Kuchler, A. M. 2009. Interleukin-33-cytokine of dual function or novel alarmin? *Trends Immunol.* 30:227.

49 Bagarozzi, D. A. Jr and Travis, J. 1998. Ragweed pollen proteolytic enzymes: possible roles in allergies and asthma. *Phytochemistry* 47:593.

50 Traidl-Hoffmann, C., Kasche, A., Jakob, T. *et al.* 2002. Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. *J. Allergy Clin. Immunol.* 109:831.

51 Gunawan, H., Takai, T., Ikeda, S., Okumura, K. and Ogawa, H. 2008. Protease activity of allergenic pollen of cedar, cypress, juniper, birch and ragweed. *Allergol. Int.* 57:83.

52 Gunawan, H., Takai, T., Kamijo, S. *et al.* 2008. Characterization of proteases, proteins, and eicosanoid-like substances in soluble extracts from allergenic pollen grains. *Int. Arch. Allergy Immunol.* 147:276.

53 Bacsi, A., Dharajiya, N., Choudhury, B. K., Sur, S. and Boldogh, I. 2005. Effect of pollen-mediated oxidative stress on immediate hypersensitivity reactions and late-phase inflammation in allergic conjunctivitis. *J. Allergy Clin. Immunol.* 116:836.