

The Role of Interleukin-33 in Chronic Allergic Conjunctivitis

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PURPOSE. The authors discovered a genetic association between the *ST2L* gene and atopy. The *ST2L* gene encodes a membrane-bound functional marker for Th2 cells. Recently, a novel Th2 cytokine, interleukin-33 (IL-33), was discovered to be a specific ligand for *ST2L*. The authors investigated the role of IL-33 in chronic allergic conjunctivitis.

METHODS. Immunohistochemical analysis was carried out using giant papillae samples obtained from patients with atopic keratoconjunctivitis. The authors used proinflammatory stimuli to clarify IL-33 mRNA/protein-inducing signals with cultured human conjunctival epithelial cells, fibroblasts, human umbilical vascular endothelial cells, and mast cells. These cells were also used to examine the expression of ST2L (IL-33R). Finally, cultured mast cells were stimulated with recombinant IL-33 (rIL-33) to examine the downstream signals.

RESULTS. The authors found IL-33 protein expression in human vascular endothelial cells in the giant papillae and in the control conjunctivae. IL-33 expression was also observed in conjunctival epithelium of the giant papillae but not in the control conjunctivae. IL-1 β stimulation upregulated IL-33 mRNA expression in conjunctival fibroblasts. The authors also confirmed mature IL-33 protein expression in ocular resident cells by Western blot analysis. Preferential ST2L expression was observed in human mast cells, and phosphorylation of p38 MAPK and IL-13 mRNA induction was observed in human cultured mast cells after rIL-33 stimulation. Phosphorylation of p38 MAPK was inhibited by soluble ST2 protein.

CONCLUSIONS. The IL-33-ST2 signaling cascade plays some roles in the pathophysiology of chronic allergic conjunctivitis through the activation of mast cells. (*Invest Ophthalmol Vis Sci.* 2009;50:4646–4652) DOI:10.1167/iops.08-3365

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Our previous study showed a strong genetic association between atopic dermatitis/high serum IgE status and a functional single nucleotide polymorphism (SNP) in the distal promoter region of *ST2*.¹ The *ST2* gene encodes two molecules by alternative splicing. One molecule is ST2L, which is controlled by the distal promoter and encodes a membrane receptor for a recently discovered Th2 cytokine, interleukin-33 (IL-33).² The other molecule is soluble ST2, which works as a decoy receptor for IL-33. The biological activities of IL-33, including IgE induction and eosinophil infiltration to the target tissues, can be explained by the signaling cascade through membrane-bound ST2L (IL-33R/IL-33 receptor), which had been extensively investigated as a marker for Th2 cells.^{3,4} Other lines of study showed that the IL-33 precursor is identical to a nuclear factor for high endothelial venule (NF-HEV), which is a gene cloned from high endothelial venule (HEV) tissue,⁵ and is identical to *DVS-27*, a gene cloned from rat subarachnoid hemorrhage model.⁶

In this study, we investigated the role of the IL-33/IL-33R signaling cascade in chronic allergic conjunctivitis because IL-33 shares homologous sequences with IL-1, a known proinflammatory cytokine, expressed by ocular surface epithelial cells and plays some roles in allergic conjunctivitis.⁷ In addition, IL-33 shares the receptor beta chain (IL-1RAP) with IL-1,⁸ indicating that both cytokines may share its downstream signaling cascades.

To elucidate the role of IL-33 in ocular surface inflammation, we examined the *in vivo* expression of IL-33 protein using biopsy samples obtained from atopic keratoconjunctivitis (AKC),⁹ a typical Th2-biased disease; superior limbic keratoconjunctivitis (SLK)^{10,11} and Mooren ulcer,¹² models for non-Th2-biased inflammation; and conjunctivochalasis, a noninflammatory control conjunctiva.¹³ We found IL-33 protein expression at human vascular endothelial cells and HEV in the conjunctival tissues. In chronic allergic conjunctivitis, IL-33 expression was also observed in the conjunctival epithelium. We also used proinflammatory stimuli to clarify the IL-33-inducing mechanism using cultured cells. IL-1 β could upregulate IL-33 mRNA expression in ocular surface epithelial cells and fibroblasts.

MATERIALS AND METHODS

Reagents, Antibodies, and Plasmids

Rabbit anti-human IL-33 polyclonal antibody was purchased from MBL (Nagoya, Japan), anti-human CD4 and CD31 antibodies were purchased from Dako Japan (Kyoto, Japan), and rat anti-PNAd IgM antibody (clone MECA-79) was purchased from BD Biosciences (Franklin Lakes, NJ). Phospho-p38 MAPK antibody was purchased from Promega (Madison, WI), and total p38 MAPK monoclonal antibody and normal rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant human interferon-gamma (rIFN- γ), recombinant human IL-1 β (rIL-1 β), and recombinant human IL-33 (rIL-33) were purchased from PeproTech (London, UK).

Immunohistochemistry

Immunofluorescence staining was performed to examine the expression of IL-33, CD4, CD31 (endothelial cell marker), and PNAd (HEV marker) in the giant papillae obtained from six patients with AKC and the control conjunctival tissues (obtained from five patients with conjunctivochalasis, four with SLK, and two with Mooren ulcer). Detailed information for the patients (with AKC, SLK, and conjunctivochalasis) has been described elsewhere.¹⁴ Cryosections (7 μ m) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and then stained with rabbit anti-IL-33, mouse anti-CD31, and rat anti-PNAd antibodies (BD Bioscience). We also immunostained sections from four skin biopsy samples obtained from patients with atopic eczema. The sections were then scanned with a confocal microscope (FV-1000; Olympus Corporation, Tokyo, Japan). Negative control specimens were immunostained with normal rabbit IgG antibody instead of rabbit anti-IL-33 antibody. Double immunostaining was carried out on pairs of the anti-IL-33 antibody with the mouse anti-CD31 antibody or with the rat anti-PNAd antibody. Donkey Alexa 488-conjugated anti-rabbit IgG antibody, donkey Alexa 594-conjugated anti-mouse IgG antibody, and donkey Alexa 594-conjugated anti-rat IgG antibody (all from Invitrogen Japan, Tokyo, Japan) were used as secondary antibodies. All procedures were approved by the ethics committee of Kyoto Prefectural University of Medicine, and the study was conducted in accordance with the tenets of the Declaration of Helsinki.

Cell Culture and Stimulation with rIL-1 β

The immortalized human conjunctival epithelial (HCJE) cell line was kindly provided by Ilene K. Gipson.¹⁵ Cultured human primary conjunctival fibroblasts were prepared and maintained as previously described.¹⁶ Conjunctival epithelial cells/fibroblasts were stimulated with rIL-1 β (10 ng/mL) and rIL-1 β + rIFN (10 ng/mL) for 24 hours. The HCJE cell line was cultured with defined keratinocyte serum-free growth medium (Invitrogen Japan). Conjunctival fibroblasts were cultured with 10% fetal calf serum in Dulbecco's minimum essential medium (Invitrogen Japan). Human umbilical vascular endothelial cells (HUVECs) were obtained from DS Pharma Biomedical (Osaka, Japan) and were cultured with human vascular endothelial culture medium according to the manufacturer's protocol. The human mast-cell line LAD2 was kindly provided by Arnold Kirshenbaum (National Institute of Allergy and Infectious Diseases, National Institutes of Health) and was maintained as previously described.¹⁷ HUVECs and LAD2 were also stimulated with rIL-1 β (10 ng/mL) for 24 hours.

Reverse Transcription and Real-Time PCR Analysis

With the use of an RNA purification kit (RNeasy; Qiagen Japan, Tokyo, Japan), total RNA was isolated from cultured cells (conjunctival epithelial cells and fibroblasts). cDNA was made with a reverse transcriptase (ReverTra-Ace; Toyobo, Osaka, Japan) using random hexamers according to manufacturer's protocol. Real-time PCR probes and primers specific for human IL-1 β (Hs00174097_m1), human IL-33 (Hs00369211_m1), human IL-13 (Hs00174379_m1), human IL-1 receptor alpha (IL-1R1; Hs00991010_m1), human ST2L (Hs00249389_m1), and human 18S-rRNA were purchased from Applied Biosystems (Assay on Demand Products; Applied Biosystems Inc., Foster City, CA). Real-time PCR analysis was performed with a detection system (ABI PRISM 7300 HT Sequence; Applied Biosystems). Relative expression of IL-33 in the cultured cells was quantified by the standard curve method, and relative expression of IL-1 β , IL-1R, IL-13, and ST2L was quantified by comparative Ct methods using 18S-rRNA expression in the same cDNA as the controls. The standard curve was made with pCRII-IL-33 plasmid prepared by PCR-based subcloning.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

HCJE cells and fibroblasts were collected and washed twice with phosphate-buffered saline (PBS), and the number of cells was counted.

Cells (2×10^4) were then solubilized in SDS-sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, and 0.04% bromophenol blue). Next, 50 mM dithiothreitol was added to the samples and incubated for 15 minutes at 65°C. Of each sample, 15 μ L was loaded onto 12% Tris-glycine gel with prestained protein standards (Precision Blue; Bio-Rad Japan, Tokyo, Japan). The electrophoresed protein was transferred to polyvinylidene fluoride (PVDF) membrane (Pall Japan, Tokyo, Japan). The membrane was then incubated with rabbit anti-human IL-33 polyclonal antibody (a 1:500 dilution in 1% nonfat skim milk) overnight at 4°C. After washing with Tris-buffered saline (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) containing 0.05% Tween 20 (TBS-T), the membrane was incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (GE Healthcare, Uppsala, Sweden) for 1 hour and was visualized with Western blotting reagents (ECL Plus; GE Healthcare).

Reporter Gene Assay

ST2L (the membrane-bound form of ST2) or soluble ST2 (sST2) were overexpressed in HCJE cells with the pBOS-human ST2L expression vector or the pEF6-human sST2 vector, respectively, as previously described.¹⁸ Mock transfection was carried out with empty pBOS vectors. Forty-eight hours after transfection, pNF κ B promoter plasmids (Stratagene Japan, Tokyo, Japan) and the pRL-TK vector (Promega) were cotransfected into the HCJE cells. The pRL-TK vectors served as an internal control for transfection efficiency into the epithelial cells. All transfections were carried out with reagent (Lipofectamine LTX; Invitrogen Japan). Twenty-four hours after reporter gene transfection, each sample was stimulated with rIL-33 (50 ng/mL), rIL-1 (10 ng/mL), or PBS only. Twenty-four hours after cytokine stimulation, luciferase activity was measured (Dual Luciferase Reporter Assay Kit, Promega).

Quantification of Relative IL-1R and ST2L (IL-33R) Expression

Real-time PCR analysis was carried out using cDNA obtained from unstimulated HCJE cells, conjunctival fibroblasts, HUVECs, and mast cells.

Mast Cell Stimulation with rIL-33 and Downstream Signal Analysis

To analyze the rIL-33-induced phosphorylation of p38 MAPK, LAD2 cells (2×10^4 cells/well in a 24-well dish) were stimulated with rIL-33 (50 ng/mL) for 15 minutes. As control experiments, neutralization of rIL-33 was carried out by the addition of anti-ST2 antibody (clone 2A5; MBL) or recombinant soluble ST2 (R&D Systems, Minneapolis, MN). Western blot analysis was carried out using phosphospecific p38 mitogen-activated protein kinase (MAPK) and total p38 MAPK antibodies. IL-13 mRNA expression in LAD2 cells, with rIL-33 (50 ng/mL) or rIL-1 β (10 ng/mL) stimulation for 6 hours and 24 hours, was also quantified by real-time PCR, as described.

RESULTS

Immunohistochemistry of Giant Papillae

IL-33 immunohistochemical staining with rabbit anti-human IL-33 polyclonal antibody was carried out on giant papillae samples obtained from patients with AKC (Figs. 1, 2A–D). IL-33 immunostaining was observed in PNAd⁺ HEV cells (Fig. 1C, arrowheads), though not all the IL-33⁺ cells were PNAd⁺ (Fig. 1C, arrows). Negative control staining using control rabbit IgG showed no definite staining (Fig. 1D). IL-33⁺ immunostaining could be observed in vascular endothelial cells (Fig. 2) and in some part of the epithelial cells (Figs. 2A, 2C) and fibroblasts (Fig. 2D, asterisks) of giant papillae. Double immunostaining with anti-CD4 antibody showed that the IL-33⁺ vascular endothelial cells were surrounded by CD4⁺ T cells (Fig. 2B, arrow). Double immunostaining with anti-CD31 antibody con-

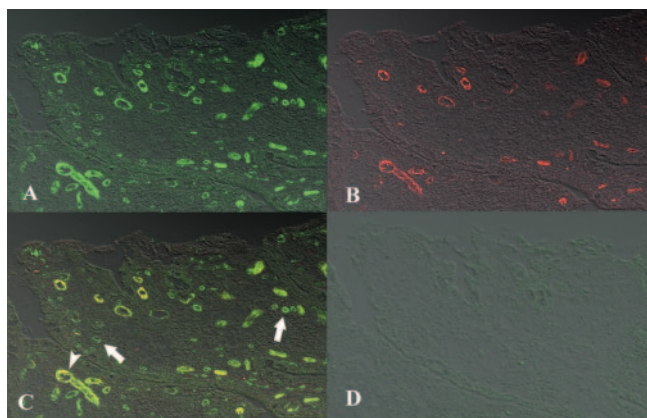


FIGURE 1. Immunohistochemical analysis of giant papillae using rabbit anti-human IL-33 antibody. Giant papillae obtained from a patient with AKC were immunostained with the anti-IL-33 antibody (A). The same slides were also immunostained with anti-PNAd (HEV) monoclonal antibody (B). The merged image is shown (C). *Arrows:* PNAd⁺ IL-33 staining. *Arrowhead:* IL-33⁺ HEV. Negative control staining of the adjacent section with normal rabbit IgG is shown (D). Original magnification, 200 $\times$.

firmed positive IL-33 staining in vascular endothelium (Fig. 2C). Nuclear IL-33⁺ staining was observed in some vascular endothelial cells (Fig. 2C, arrowhead). On the other specimens, cytoplasmic IL-33 staining was observed in vascular endothelial cells (Fig. 2D, arrows), and fibroblasts were also positive for IL-33 staining (Fig. 2D, asterisks). A skin biopsy section obtained from a patient with atopic dermatitis showed positive IL-33 immunostaining in epithelium and vascular endothelium (Fig. 2F).

IL-33 immunostaining was also observed in vascular endothelium of conjunctivae obtained from a patient with conjunctivochalasis (Fig. 2E), a patient with SLK (Fig. 3A), and the nuclei of HEV in conjunctival tissue obtained from a patient with Mooren ulcer (Figs. 3B, 3C). However, no definitive IL-33⁺ staining was observed in the epithelial cells or in the fibroblasts of the conjunctivae obtained from those patients.

IL-1 β Stimulation Induced IL-33 mRNA Expression in HCJE/Conjunctival Fibroblasts

IL-33 mRNA expression in HCJE cells and fibroblasts was quantified with real-time PCR. In human HCJE cells, the induction of IL-33 mRNA expression was 1.2-fold by IL-1 β stimulation and 1.4-fold by IL-1 β + IFN- γ stimulation. The overall magnitude of IL-33 mRNA expression was greater in conjunctival fibroblasts than in conjunctival epithelial cells, and 16-fold IL-33 mRNA induction was observed by IL-1 β stimulation. No IL-33 mRNA expression was observed in cultured human mast cells. On the other hand, IL-1 β treatment reduced constitutive IL-33 mRNA expression in HUVECs (Fig. 4A). For the purpose of comparison, relative IL-1 β mRNA expression was also quantified by real-time PCR. HCJE cells showed the most abundant IL-1 β mRNA expression. IL-1 β mRNA induction was observed in conjunctival fibroblasts and HUVECs. Only a negligible amount of IL-1 β mRNA expression was detected in mast cells (Fig. 4B).

IL-33 Western Blot Analysis

Western blot analysis was carried out with rabbit anti-human IL-33 polyclonal antibody using HCJE lysate and conjunctival fibroblast lysate, both stimulated with rIL-1 β or rIL-1 β + IFN- γ for 24 hours. In the case of fibroblasts, a marked increase of pro-IL-33 was observed after stimulation (Fig. 5, arrow). No definitive bands corresponding to pro-IL-33 molecular weight

(30 kDa, arrow) were observed in HCJE cells. A modest amount of mature IL-33 protein was observed in HCJE cells and conjunctival fibroblasts, but no increase of mature IL-33 protein was observed with rIL-1 β treatment (Fig. 5, asterisk). No additive effect for rIL-1 β + IFN- γ stimulation was observed for IL-33 expression.

ST2L (IL-33R)-Dependent NF- κ B Activation in HCJE

HCJE cells were transfected with the ST2L (IL-33R) expression vector or the sST2 (a decoy receptor for IL-33) expression vector. NF- κ B activation by IL-1 β stimulation was observed in all samples examined (Fig. 6A). ST2L-transfected HCJE cells showed a higher NF- κ B activation signal than did those of mock-transfected HCJE or sST2-transfected cells (Fig. 6A).

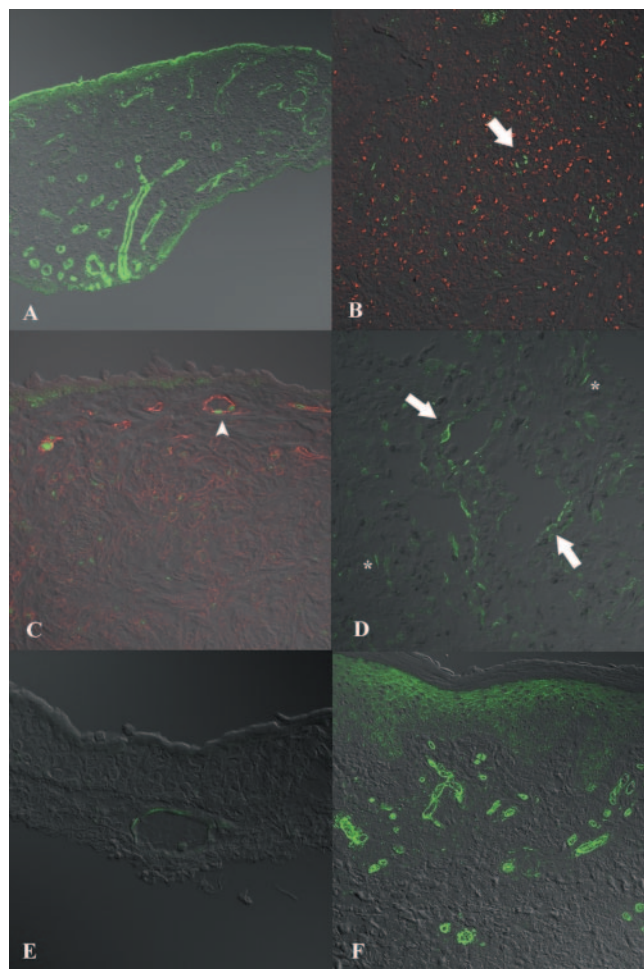
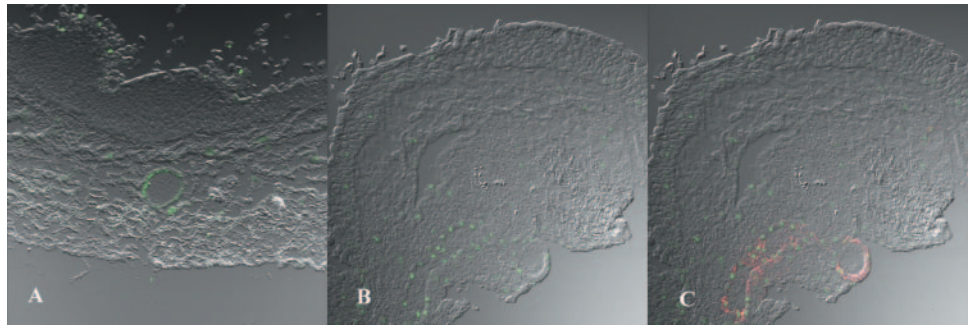


FIGURE 2. Immunohistochemical analysis of giant papillae using anti-human IL-33 antibody. Giant papillae obtained from patients with AKC were immunostained with anti-IL-33 polyclonal antibody (A) and double immunostained with anti-IL-33 and anti-CD4 monoclonal antibody (B). *Arrow:* IL-33⁺ vascular endothelium surrounded by CD4⁺ T cells. (C) Double-immunostained with anti-IL-33 and anti-CD31 monoclonal antibody. *Arrowhead:* nuclear IL-33 staining in the vascular endothelium. (D) Cytoplasmic IL-33 staining in the vascular endothelium (*arrows*); IL-33⁺ fibroblastic staining (*asterisks*). (E) Control conjunctiva obtained from a patient with conjunctivochalasis. Positive IL-33 immunostaining was observed in the vascular endothelium. (F) IL-33 immunostaining of a skin biopsy specimen of a patient with atopic eczema. Positive staining was observed in the epithelium and vascular endothelium. Original magnifications, 200 $\times$ (A, B, F); 400 $\times$ (C–E).

FIGURE 3. Immunohistochemical staining of conjunctival specimens obtained from patients with SLK or Mooren ulcer using anti-IL-33 antibody. Frozen sections made from conjunctival specimens obtained from a patient with SLK (A) and a patient with Mooren ulcer (B, C) were stained by polyclonal anti-IL-33 antibody. Nuclear IL-33 staining was observed in the vascular endothelium. Double immunostaining was carried out with anti-PNAd (HEV) antibody (C). Original magnification, 200 $\times$.



IL-1R and ST2L (IL-33R) Receptor Expression Levels Are Distinct among the Cell Types

The relative mRNA expression of IL-1R and ST2L was quantified by real-time PCR. Abundant IL-1R mRNA expression was observed in HCJE cells and conjunctival fibroblasts, whereas a negligible amount of IL-1R expression was observed in mast cells. On the other hand, abundant ST2L mRNA expression was observed in mast cells but not in HCJE cells or conjunctival fibroblasts. HUVECs showed modest IL-1R and ST2L mRNA expression (Fig. 6B).

Mast Cell Stimulation with rIL-33-Induced p38 MAPK Activation and IL-13 Upregulation

Human mast cell line LAD2 was stimulated with rIL-33 for 5, 15, and 30 minutes, and phosphospecific p38 MAPK immunoblotting was then carried out. Prominent p38 phosphorylation was observed 15 minutes after rIL-33 stimulation (Fig. 7A). IL-13 mRNA expression in rIL-33-stimulated LAD2 cells was quantified with real-time PCR analysis. Relative magnitude of IL-13 mRNA expression was shown. Marked IL-13 induction was observed in rIL-33-stimulated LAD2 cells but not in rIL-1 β -stimulated mast cells (Fig. 7B).

DISCUSSION

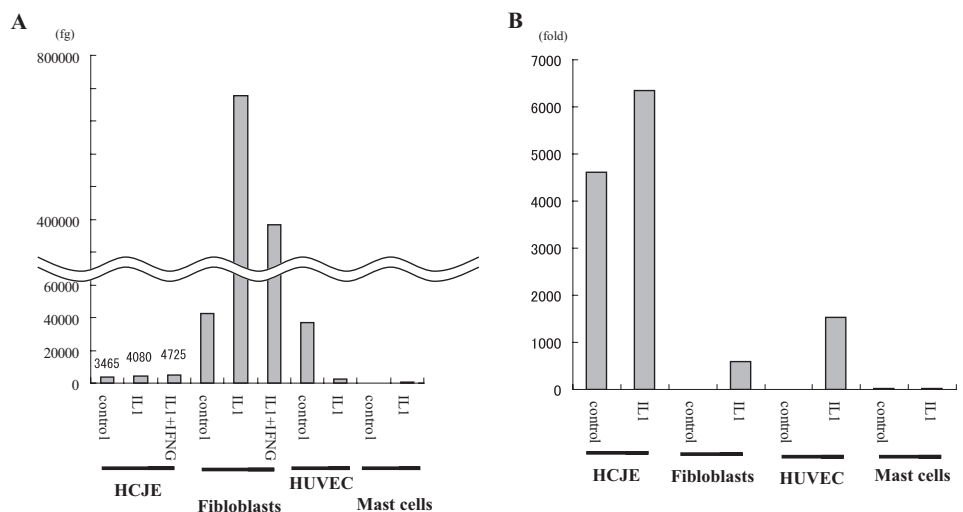
Immunohistologic analysis of ocular tissue showed constitutive IL-33 expression in vascular endothelial cells (Figs. 1–3). These results are consistent with previous reports that showed IL-33 expression at vascular endothelial cells in human colon, lung, liver, and skin tissue.¹⁹ We found IL-33⁺ HEV at the stromal region of the giant papillae (Fig. 1C, arrowhead). Given that the

IL-33 molecule was originally cloned from HEV by differential display,⁵ it is reasonable that we found IL-33⁺ HEV cells in our samples. It is reported that HEV cells are abundant in the T-cell zone of lymphatic tissue, and they play a major role in lymphocyte homing to lymphatic tissue.²⁰ Our results also showed that IL-33⁺ vascular cells were surrounded by CD4⁺ T cells (Fig. 2B, arrow). Because of the preferential ST2L (IL-33R) expression in Th2 cells compared with Th1 cells,^{3,4} IL-33⁺ HEV cells may play a role in the activation and infiltration of Th2 lymphocytes, as suggested by previous reports.^{21,22}

IL-33 protein expression is observed not only in the nucleus of endothelial cells (Fig. 2C, arrowhead), as reported in previous reports,^{19,23} but also in the cytoplasm in some vascular endothelial cells (Fig. 2D, arrows). Given that the IL-33 nuclear localization signal is located at the N-terminal of the pro-IL-33 protein,²⁴ it is reasonable that it is expressed in cytoplasm, at least in mature IL-33 protein, in which N-terminal amino acid sequences were cleaved by caspase-1.² Based on these results, we theorize that nuclear and cytoplasmic IL-33 expression is possible in conjunctival tissue, and nuclear IL-33 localization suggests the existence of the pro-IL-33 protein.

In the giant papillae of patients with AKC and in skin biopsy specimens obtained from patients with atopic eczema, positive IL-33 staining was observed not only in the vascular endothelium but also in epithelial cells (Figs. 2A, 2C, 2F). On the other hand, IL-33 expression is restricted to the vascular endothelium of conjunctivae of patients with conjunctivochalasis (Fig. 2E), SLK (Fig. 3A), and Mooren ulcer (Figs. 3B, 3C). We used conjunctivae samples obtained from patients with conjunctivochalasis as noninflammatory control conjunctivae, as shown previously.¹³ Although we analyzed only a limited number of samples, extravascular IL-33 protein expression might be one

FIGURE 4. Real-time PCR analysis of IL-33 mRNA expression using cultured cells with IL-1 β stimulation. HCJE cells, conjunctival fibroblasts, HUVECs, and mast cells were stimulated with rIL-1 β (10 ng/mL) for 24 hours (A). HCJE cells and fibroblasts were also stimulated with rIL-1 β + rIFN- γ (10 ng/mL). After total RNA extraction and reverse transcription, real-time PCR analysis was carried out. The standard curve was made with the IL-33-pCRII dual promoter plasmid (1000 = 1 pg plasmid). Relative IL-1 β mRNA expression was quantified using the same cDNA samples (B). The relative amount of IL-1 β mRNA was shown by fold changes using the IL-1 β expression in unstimulated HUVECs as standard. These results are representative of three independent experiments.



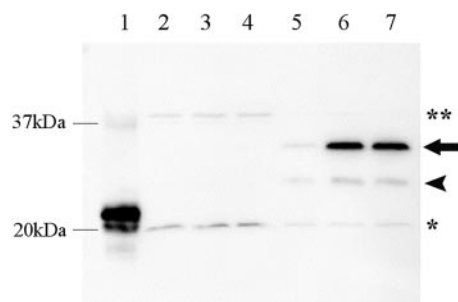


FIGURE 5. Immunoblotting analysis of IL-33 protein using HCJE cells and fibroblasts. Cultured conjunctival epithelial cells and fibroblasts were stimulated with rIL-1 β (10 ng/mL) and rIL-1 β + rIFN- γ (10 ng/mL) for 24 hours. Western blot analysis was carried out using rabbit anti-human IL-33 polyclonal antibody. The molecular weight of mature IL-33 protein was approximately 20 kDa (*asterisk*), whereas for the proform of IL-33 protein it was approximately 30 kDa (*arrow*). An additional band of unknown origin was observed around 27 kDa (*arrowhead*). A proposed dimeric form of IL-33 protein was observed around 40 kDa (*double asterisks*). *Lane 1*, rIL-33 (100 ng); *lanes 2–4*, conjunctival epithelial cells; *lanes 5–7*, conjunctival fibroblasts; *lanes 2 and 5*, no stimulation; *lanes 3 and 6*, rIL-1 β stimulation; *lanes 4 and 7*, rIL-1 β + rIFN- γ stimulation.

of the pathologic elements of chronic allergic disease. Because Leonardi et al.²⁵ reported that IL-1 β protein was expressed in 4 of 6 tear samples obtained from patients with vernal keratoconjunctivitis (VKC; average, 21.7 pg/mL) but not in any of the tear samples obtained from controls, it is possible that the tears of AKC and VKC patients induce extravascular IL-33 expression at the ocular surface.

We next tried to detect proinflammatory stimuli to induce IL-33 expression. A previous report showed that the IL-33 (DVS-27) gene is induced prominently by IL-1 β and moderately by IFN- γ in human artery smooth muscle cells.⁶ We then stimulated HCJE cells, corneal fibroblasts, HUVECs, and mast cells with IL-1 β for 24 hours and found that IL-33 mRNA was induced by rIL-1 β stimulation in HCJE cells and conjunctival fibroblasts. Our result was consistent with previous results for IL-33 induction in dermal fibroblasts by a combination of IL-1 β /TNF- α .² A weak additive effect for rIL-1 β + IFN- γ costimulation was observed in the mRNA level. On the other hand, IL-33 mRNA expression was suppressed in HUVECs by the same IL-1 β . This result is consistent with the previous results by Kuchler et al.²³ that showed IL-33 downregulation by IL-1 stimulation. We also examined IL-1 β mRNA expression using the same cDNA samples (Fig. 4B) and found that constitutive

IL-1 β mRNA expression was observed in HCJE cells and that IL-1 β mRNA expression was induced in conjunctival fibroblasts and in HUVECs with the stimulation of IL-1 β itself. Interestingly, the reverse kinetics of IL-33 mRNA and IL-1 β mRNA expression was observed in IL-1 β -stimulated HUVECs (Fig. 4A vs. 4B). We considered that the reverse kinetics was not contradictory because the loss of constitutive IL-33 expression in the nucleus may activate transcriptional machinery in HUVECs, as has been shown in other reports.²³ As a result of our *in vivo* and *in vitro* experiments, we agree with the theory proposed by Carriere et al.²⁴ that IL-33 may work as a dual-function protein (a nuclear protein with repressive properties and as a proinflammatory cytokine) and other types of dual-function proteins such as IL-1 α ²⁶ and HMGB1.²⁷

As a next step, we tried to detect IL-33 protein because it was reported that caspase-1-dependent cleavage is required for mature IL-33.² Immunoblotting experiments showed clear induction of the pro-IL-33 molecule in response to rIL-1 β stimulation for 24 hours in conjunctival fibroblasts (Fig. 5, lanes 5 and 6, arrow); however, no definitive increase in mature IL-33 was observed (Fig. 5, lanes 5 and 6, asterisk). No additive effect for IL-33 protein induction was observed with IL-1 β + IFN- γ stimulation compared with the effect of IL-1 β stimulation (Fig. 5, lanes 4 and 7).

In the case of HCJE cells, the induction of pro-IL-33 protein was not observed in contrast to that of conjunctival fibroblasts (Fig. 5, lanes 2 and 3, arrow); however, a comparable amount of mature IL-33 expression was observed (Fig. 5, lanes 2 and 3 vs. lanes 5 and 6, asterisk). A previous report showed caspase-1 protein expression in ocular surface epithelial cells.²⁸ These results might be attributed to the more efficient IL-33 maturation process by caspase-1 in conjunctival epithelial cells compared with fibroblasts. The origin of higher molecular weight bands around 40 kDa (Fig. 5, double asterisks) observed in HCJE cells and in rIL-33 is unknown, but they might be a dimeric form of IL-33. Our immunohistologic results showed that some of the epithelial cells (Figs. 2A, 2C) and some of the fibroblastic cells (Fig. 2D) in the giant papillae were IL-33⁺. Further studies are essential to elucidate IL-33 protein maturation and subcellular localization.

We then attempted to find the effector cells for IL-33 among ocular surface resident cells. At first we examined HCJE cells because it is known that IL-1 stimulation may activate these cells through the IL-1R signaling cascade.^{29,30} Our result showed that ST2L transfection is required for NF- κ B activation in response to IL-33 in HCJE cells (Fig. 6A), which means that the amount of ST2L expression is a determinant factor for the responsiveness against IL-33. Therefore, we examined ST2L

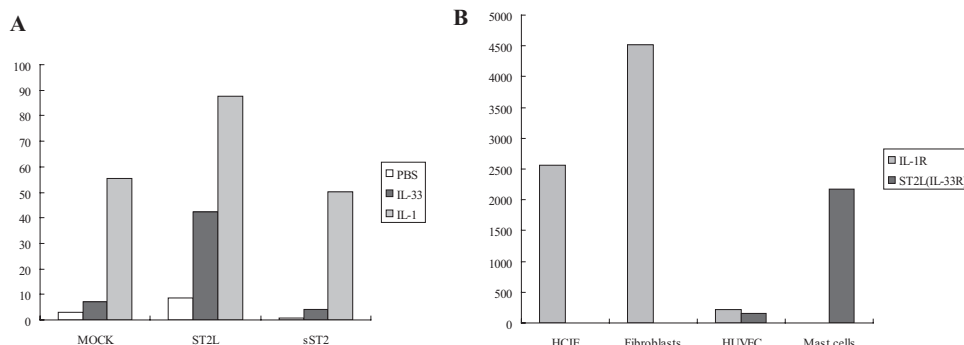
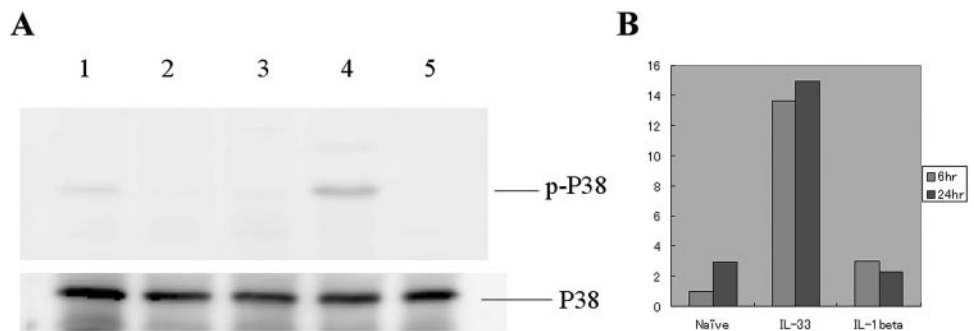


FIGURE 6. Receptor amount-dependent responses against rIL-1 β or rIL-33. The reporter gene assay ST2L (IL-33R) or soluble ST2 (sST2; a decoy receptor for IL-33) were overexpressed in conjunctival epithelial cells using the pBOS-ST2L expression vector or the pEF6-sST2 vector, respectively (A). Forty-eight hours after transfection, pNF- κ B and pRL-TK (internal control) reporter plasmids were cotransfected into the HCJE cells. Twenty-four hours after reporter gene transfection, each sample was stimulated with rIL-33 (50 ng/mL), rIL-1 (10 ng/mL), or PBS only. Twenty-four hours after stimulation, luciferase activity was measured. Results show the representative data of five independent experiments. (B) IL-1R and IL-33R expression. IL-1R and ST2L (IL-33R) mRNA was quantified by real-time PCR analysis using cDNA made from HCJE cells, conjunctival fibroblasts, HUVECs, and human mast cells. Relative mRNA expression was shown by fold changes using mast cells (for IL-1R) and HCJE (for ST2L) as standards. Results show the representative data of three independent experiments.

FIGURE 7. Mast cell stimulation with rIL-33-induced phosphorylation of p38 MAPK and IL-13 mRNA expression. **(A)** Human mast cell line LAD2 (2×10^4 cells/well in a 24-well dish) was stimulated with rIL-33 (50 ng/mL) for 15 minutes. Control experiments include anti-ST2 antibody + rIL-33 (lane 1), recombinant soluble ST2 + rIL-33 (lane 2), recombinant soluble ST2 + rIL-33 (preincubated in tube; lane 3), rIL-33 only (lane 4), and PBS (lane 5). Western blot analysis was carried out using phosphospecific p38 MAPK and total p38 MAPK antibodies. **(B)** IL-13 mRNA expression in LAD2 cells after rIL-33 (50 ng/mL) or rIL-1 β (10 ng/mL) stimulation for 16 hours. After total RNA extraction, real-time PCR analysis was carried out. This result is representative of three independent experiments.



mRNA expression level by real-time PCR and found that ST2L (IL-33R) mRNA is abundantly expressed in human mast cells but not in HCJE or fibroblasts (Fig. 6B). Based on these results, we considered that the human mast cell is a good candidate to be an effector cell of IL-33.

Consistent with the results of previous studies,^{31–33} we found that rIL-33 could activate the p38 MAPK signaling cascade (Fig. 7A) and could induce IL-13 mRNA expression in human mast cells (Fig. 7B). Neutralizing experiments showed that the sST2 molecule could completely inhibit rIL-33-mediated p38 MAPK activation and that anti-ST2 antibody could partially inhibit p38 MAPK activation (Fig. 7A). This result suggests that the sST2 molecule may have a therapeutic value for allergic diseases, as suggested by the mouse asthma model.¹⁸ It should also be noted that the ability of rIL-33 to induce IL-13 mRNA expression in mast cells was far stronger than that of rIL-1 β (Fig. 7B). Based on these results we considered that the ratio of IL-1R1/IL-33R expression depends on the cell types (Fig. 6B) and that it may have some functional significance.

In summary, we found in vivo IL-33 protein expression in epithelium and vascular endothelium of the giant papillae in patients with severe chronic allergic conjunctivitis. We also found IL-1 β -induced IL-33 mRNA expression and pro-IL-33 protein expression, particularly in the cultured conjunctival fibroblasts. The IL-33-ST2L (IL-33R) signaling cascade may play some role in the pathophysiology of chronic allergic conjunctivitis through the activation of mast cells. Further investigations are essential to elucidate the molecular mechanism of IL-33 expression control and its role in atopic disease.

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References

- Shimizu M, Matsuda A, Yanagisawa K, et al. Functional SNPs in the distal promoter of the ST2 gene are associated with atopic dermatitis. *Hum Mol Genet.* 2005;14:2919–2927.
- Schmitz J, Owyang A, Oldham E, et al. 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.* 2005;23:479–490.
- Yanagisawa K, Naito Y, Kuroiwa K, et al. The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. *J Biochem.* 1997;121:95–103.
- Xu D, Chan WL, Leung BP, et al. Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. *J Exp Med.* 1998;187:787–794.
- Baekkevold ES, Roussigne M, Yamanaka T, et al. Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. *Am J Pathol.* 2003;163:69–79.
- Onda H, Kasuya H, Takakura K, et al. Identification of genes differentially expressed in canine vasospastic cerebral arteries after subarachnoid hemorrhage. *J Cereb Blood Flow Metab.* 1999;19:1279–1288.
- Keane-Myers AM, Miyazaki D, Liu G, Dekaris I, Ono S, Dana MR. Prevention of allergic eye disease by treatment with IL-1 receptor antagonist. *Invest Ophthalmol Vis Sci.* 1999;40:3041–3046.
- Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S, Kastelein RA. IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J Immunol.* 2007;179:2551–2555.
- Foster CS, Rice BA, Dutt JE. Immunopathology of atopic keratoconjunctivitis. *Ophthalmology.* 1991;98:1190–1196.
- Corwin ME. Superior limbic keratoconjunctivitis. *Am J Ophthalmol.* 1968;66:338–340.
- Matsuda A, Tagawa Y, Matsuda H. Cytokeratin and proliferative cell nuclear antigen expression in superior limbic keratoconjunctivitis. *Curr Eye Res.* 1996;15:1033–1038.
- Brown SI. What is Mooren's ulcer? *Trans Ophthalmol Soc U K.* 1978;98:390–392.
- Yokoi N, Komuro A, Nishii M, et al. Clinical impact of conjunctivochalasis on the ocular surface. *Cornea.* 2005;24:S24–S31.
- Matsuda A, Okayama Y, Ebihara N, et al. Hyperexpression of the high-affinity IgE receptor- β chain in chronic allergic keratoconjunctivitis. *Invest Ophthalmol Vis Sci.* 2009;50:2871–2877.
- Gipson IK, Spurr-Michaud S, Argüeso P, Tisdale A, Ng TF, Russo CL. Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. *Invest Ophthalmol Vis Sci.* 2003;44:2496–2506.
- Kawasaki S, Nishida K, Sotozono C, Quantock AJ, Kinoshita S. Conjunctival inflammation in the chronic phase of Stevens-Johnson syndrome. *Br J Ophthalmol.* 2000;84:1191–1193.
- Kirshenbaum AS, Akin C, Wu Y, et al. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of Fc ϵ RI or Fc γ RI. *Leuk Res.* 2003;27:677–682.
- Hayakawa H, Hayakawa M, Kume A, Tominaga S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem.* 2007;282:26369–26380.
- Moussion C, Ortega N, Girard JP. 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.* 2008;3:e3331.
- Kraal G, Mebius RE. High endothelial venules: lymphocyte traffic control and controlled traffic. *Adv Immunol.* 1997;65:347–395.
- Smithgall MD, Comeau MR, Yoon BR, Kaufman D, Armitage R, Smith DE. 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.* 2008;20:1019–1030.
- Komai-Koma M, Xu D, Li Y, McKenzie AN, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. *Eur J Immunol.* 2007;37:2779–2786.
- Kuchler AM, Pollheimer J, Balogh J, et al. Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon

- angiogenic or proinflammatory activation. *Am J Pathol*. 2008;173:1229–1242.
24. Carriere V, Roussel L, Ortega N, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci U S A*. 2007;104:282–287.
 25. Leonardi A, Borghesan F, DePaoli M, Plebani M, Secchi AG. Pro-collagens and inflammatory cytokine concentrations in tarsal and limbal vernal keratoconjunctivitis. *Exp Eye Res*. 1998;67:105–112.
 26. Werman A, Werman-Venkert R, White R, et al. The precursor form of IL-1 α is an intracrine proinflammatory activator of transcription. *Proc Natl Acad Sci U S A*. 2004;101:2434–2439.
 27. Lotze MT, Tracey KJ. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.
 28. Solomon A, Rosenblatt M, Li DQ, et al. Doxycycline inhibition of interleukin-1 in the corneal epithelium. *Invest Ophthalmol Vis Sci*. 2000;41:2544–2557.
 29. Moore JE, McMullen TC, Campbell IL, et al. The inflammatory milieu associated with conjunctivalized cornea and its alteration with IL-1 RA gene therapy. *Invest Ophthalmol Vis Sci*. 2002;43:2905–2915.
 30. Narayanan S, Corrales RM, Farley W, McDermott AM, Pflugfelder SC. Interleukin-1 receptor-1-deficient mice show attenuated production of ocular surface inflammatory cytokines in experimental dry eye. *Cornea*. 2008;27:811–817.
 31. Iikura M, Suto H, Kajiwarra N, et al. IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest*. 2007;87:971–978.
 32. Allakhverdi Z, Smith DE, Comeau MR, Delespesse G. Cutting edge: the ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J Immunol*. 2007;179:2051–2054.
 33. Ho LH, Ohno T, Oboki K, et al. IL-33 induces IL-13 production by mouse mast cells independently of IgE-Fc ϵ RI signals. *J Leukoc Biol*. 2007;82:1481–1490.