**Functional Role of Thymic Stromal Lymphopoietin in Chronic Allergic Keratoconjunctivitis**

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**PURPOSE.** Previous reports have shown that thymic stromal lymphopoietin (TSLP) plays a role in atopic diseases. This study was undertaken to investigate the expression of TSLP in the giant papillae obtained from patients with vernal keratoconjunctivitis (VKC) or atopic keratoconjunctivitis (AKC), and its functional roles were analyzed.

**METHODS.** TSLP mRNA expression was examined in resected conjunctival samples obtained from four patients with VKC/AKC and three control subjects by reverse transcription-polymerase chain reaction. Anti-TSLP, anti-dendritic cell-limbic system-associated membrane protein (anti-DC-LAMP), and anti-trypsin immunohistochemical staining was performed with 10 resected giant papillae. Human conjunctival epithelial (HCJE) cells were stimulated with poly I:C, with and without endosomal inhibitor, to examine TSLP mRNA expression. Cultured human mast cells were stimulated with recombinant (r)TSLP to analyze the downstream effect of TSLP.

**RESULTS.** All four VKC/AKC samples showed TSLP mRNA expression; however, no TSLP mRNA expression was found in the control conjunctiva. Anti-TSLP immunohistochemical staining showed preferential expression in the epithelial cells and some infiltrated cells of the giant papillae, but not in the control conjunctiva. Double immunohistochemical staining with TSLP and DC-LAMP or trypsin showed the existence of activated dendritic cells and mast cells near TSLP-positive cells in the giant papillae. Real-time PCR analysis showed that poly I:C induced TSLP mRNA expression in HCJE cells in an endosomal-function-dependent manner and that rTSLP could induce IL-13 mRNA expression in the mast cells synergistically with IL-33.

**CONCLUSIONS.** The TSLP protein produced in conjunctival epithelial cells plays a role in severe ocular allergy through the activation of dendritic cells and mast cells in synergy with other cytokines. *Invest Ophthalmol Vis Sci. 2010;51:151–155* DOI:10.1167/iovs.09-4183

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**Material and Methods**

**Giant Papillae and Control Conjunctiva Samples**

Giant papillae were resected for therapeutic purposes from four patients, three with AKC and one with VKC, and control conjunctival tissue was biopsied from patients with cataract, pterygium, or meli-noma during surgery after written informed consent was obtained for TSLP mRNA analysis (Table 1). Additional giant papillae samples were obtained from six patients with AKC and four with VKC for TSLP immunostaining analysis (Table 2). All procedures were approved by the ethics committees of Juntendo University School of Medicine and Kyoto Prefectural University of Medicine, and the study was conducted in accordance with the tenets of the Declaration of Helsinki. AKC was defined as a bilateral chronic inflammation of the conjunctiva and lids associated with atopic dermatitis, and VKC was defined as a chronic, bilateral, conjunctival inflammatory condition found in individuals predisposed by their atopic background; detailed information about pa-
tient selection was described elsewhere.13 Upper bulbar conjunctivae resected from six patients with conjunctivochalasis were used as control samples, as previously described,13 after informed consent was obtained (Table 3).

Antibodies, Reagents, and Cell Lines

We purchased mouse anti-DC-LAMP (CD208) monoclonal antibody from Beckman Coulter Japan (Tokyo, Japan), mouse anti-human trypstatin antibody from Dako Japan (Kyoto, Japan), and Alexa-488-conjugated donkey anti-rat IgG and Alexa-594-conjugated donkey anti-mouse IgG antibodies from Invitrogen Japan (Tokyo, Japan). Rat anti-human TSLP monoclonal antibody was prepared as previously described.4 HCJE was kindly provided by Ilene K. Gipson (Schepens Eye Research Institute, Philadelphia, PA) and maintained with defined keratinocyte serum-free medium (KSFM; Invitrogen Japan). The human mast cell line LAD2 was kindly provided by Arnold Kirshenbaum (National Institutes of Health, Bethesda, MD), and maintained as previously described.11 Recombinant human (r)TSLP and recombinant human (r)IL-33 were obtained from Peprotech (London, UK), poly I:C (5 mg/mL) was obtained from InvivoGen (San Diego, CA), and bafilomycin A1 was obtained from Sigma-Aldrich (St. Louis, MO).

Reverse-Transcription–Polymerase Chain Reaction

Total RNA was extracted from the giant papillae tissue (NucleoSpin II RNA isolation kit; Macherey-Nagel GmbH & Co., Duren, Germany), and cDNAs were prepared from 1 μg of total RNA by using random primers and reverse transcriptase (Superscript II; Invitrogen) according to the manufacturer’s protocol. PCR primers for TSLP amplification were 5’-aacaaggtgctatacaag-3’ (forward) and 5’-aatgtcccttagaaaagtatg-3’ (reverse), which are designed for amplifying the common region of TSLP transcript variants 1 and 2 (GenBank accession numbers: NM_033035.4 and NM_138551.3, respectively; 849-bp length; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD).PCR reaction was performed as follows: initial denaturation at 94°C for 5 minutes and at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute (35 cycles). IL-415 and IL-1316 amplification was performed according to previously published methods, with the following pairs of primers: IL-4: 5’-ctcagacagaagctctggcagcacgagcagctgg-3’ (forward), and 5’-cagacagcaagactagccgccgacgccc-3’ (reverse); and IL-13: 5’-ccagggctcgtcttctcaaaagc-3’ (forward), 5’-ccgtgtgagcggagataaagctca-3’ (reverse).

Immunohistochemistry

Giant papillae were frozen in OCT compound, and cryostat sections were then cut, mounted on slides, and fixed in 4% paraformaldehyde in PBS. Nonspecific staining was blocked (30 minutes) with blocking buffer (10% normal donkey serum, 1% bovine serum albumin [BSA]) in PBS. Anti-TSLP monoclonal antibody (10 μg/mL) was then applied and reacted overnight at 4°C. After they were washed with PBS, the slides were incubated for 30 minutes with Alexa 488-conjugated anti-rat IgG. Double immunohistochemical staining was performed with pairs of anti-TSLP and anti-trypstatin antibodies and anti-TSLP and anti-CD208 antibodies. The pair of primary antibodies was applied to the samples simultaneously, and the secondary antibodies (Alexa 488 anti-rat IgG and Alexa 594 anti-mouse IgG antibodies) were then applied after the samples were washed with PBS.

HCJE Stimulation with Poly I:C and the Effect of Bafilomycin A for Poly I:C Stimulation

HCJE cells were grown in 12-well dishes and used in the subconfluent state. Poly I:C (5 μg/mL) was added to HCJE cells and incubated for 1, 3, and 8 hours in a CO2 incubator. Simultaneously, 10 nM bafilomycin A was added to some wells to inhibit endosomal functions in the HCJE cells.

Mast Cell Stimulation with rTSLP/rIL33 and Downstream Signal Analysis

LAD2 cells (2 × 10⁴ cells per well in a 24-well dish) were stimulated with rTSLP (10 ng/mL) for 1, 3, and 16 hours. rIL-33 (10 ng/mL) was was added to LAD2 cells and incubated for 1, 3, and 16 hours. rIL-33 (10 ng/mL) was then applied and reacted overnight at 4°C. After they were washed with PBS, the slides were incubated for 30 minutes with Alexa 488-conjugated anti-rat IgG. Double immunohistochemical staining was performed with pairs of anti-TSLP and anti-trypstatin antibodies and anti-TSLP and anti-CD208 antibodies. The pair of primary antibodies was applied to the samples simultaneously, and the secondary antibodies (Alexa 488 anti-rat IgG and Alexa 594 anti-mouse IgG antibodies) were then applied after the samples were washed with PBS.
added alone or simultaneously to LAD2 cells. IL-13 mRNA expression in LAD2 cells was quantified by real-time PCR.

**Real-Time PCR Analysis of TSLP and IL-33 mRNA Expression**

Total RNA was extracted from HCJE and LAD2 cells and cDNAs were prepared from 1 μg of total RNA by using random primers as just described. We used real-time PCR probes (TaqMan; Applied Biosystems [ABI], Foster City, CA) and primers specific for human TSLP (Hs01572934_g1), human IL-13 (Hs00174379_m1), and 18SrRNA (Assay-on-Demand gene expression products; ABI). Real-time PCR analysis was performed on a sequence-detection system (Prism 7300; ABI). The expression of TSLP in the HCJE cells was quantified by the standard curve method, by using 18SrRNA expression in the same cDNA as a control. We calculated a standard curve with full-length human TSLP cDNA obtained by PCR reaction and subcloned into pCRII dual promoter plasmid (Invitrogen). For IL-13 mRNA expression, the comparative Ct method was used, which utilizes 18SrRNA expression in the same cDNA as a control.

**RESULTS**

**RT-PCR Analysis of Giant Papillae Obtained from Patients with VKC/AKC**

Total RNA was extracted from the giant papillae and control conjunctiva tissue, then RT-PCR was performed. TSLP mRNA expression was detected for all the giant papillae samples (Fig. 1, lanes 1–4); however, no TSLP mRNA expression was detected for control samples (Fig. 1, lanes 5–7).

**Immunohistochemical Localization of TSLP**

Anti-TSLP immunohistochemical staining was performed using giant papillae obtained from AKC/VKC patients as well as control conjunctiva. The epithelium of giant papillae showed cytoplasmic-positive immunostaining for TSLP protein (Fig. 2A). There was a clear boundary between the TSLP-positive and -negative epithelium (Fig. 2A). Additional positive TSLP staining was observed in some infiltrating cells in the giant papillae samples (Fig. 2A and 2B, asterisks). DC-LAMP-positive, activated dendritic cells were observed beneath the TSLP-positive conjunctival epithelium and near the TSLP-positive cells in the substantia propria (Fig. 2A, arrowheads). No TSLP immunostaining was observed in the control conjunctiva (Fig. 2C). Double-immunostaining with anti-TSLP and anti-tryptase antibodies revealed that tryptase-positive mast cells were located beneath the TSLP-positive epithelium (Fig. 3), and some of the mast cells were found within and under the epithelium.
formed in triplicate. The immunostaining results are representative of results in three independent experiments performed in triplicate.

(Fig. 3B). A few mast cells were also positive for TSLP immunostaining (Fig. 3A, asterisks). The immunostaining results are summarized in Tables 2 and 3.

### Poly I:C-Induced TSLP Expression in HCJE Cells

cDNAs were synthesized from total RNA isolated from poly I:C-stimulated HCJE cells. TSLP mRNA induction was observed in HCJE cells stimulated with poly I:C (5 μg/mL) at 3 hours after stimulation (Fig. 4). Simultaneous addition of 10 nM bafilomycin A showed inhibition of poly I:C-induced TSLP mRNA expression (Fig. 4).

### TSLP-Induced IL-33 mRNA Expression in Synergy with IL-33

The human mast cell line LAD2 was stimulated with rTSLP (10 ng/mL), rIL-33 (10 ng/mL), and rTSLP+rIL-33 for 1, 3, and 16 hours. Relative IL-13 mRNA expression was quantified with real-time PCR. rTSLP alone did not induce IL-13 mRNA expression, and rIL-33 induced modest IL-13 expression (30-fold, compared with the unstimulated LAD2 cells). Co-stimulation of LAD2 cells with rTSLP and rIL-33 synergistically induced IL-13 mRNA expression (150-fold) and peaked at 1 hour after co-stimulation (Fig. 5).

### DISCUSSION

In this study, we detected in vivo expression of TSLP mRNA/protein in the giant papillae tissues obtained from patients with VKC/AKC, and no TSLP expression was observed in the control samples (Figs. 1, 2A, 2C). To the best of our knowledge, this is the first report of TSLP expression in an ocular surface disorder. As has been reported about other allergic diseases such as atopic dermatitis and bronchial asthma, preferential TSLP expression was observed in the epithelial cells of chronic allergic conjunctivitis. Restricted TSLP protein expression was clearly observed at the surface epithelial cells (Fig. 2A). This finding was also consistent with those in a previous report on atopic dermatitis.

We also observed some TSLP-positive cells in the substantia propria of the giant papillae (Fig. 2, arrow). Corrigan et al. recently reported that TSLP-positive neutrophils, mast cells, and macrophages were observed in the antigen-challenged dermis obtained from atopic patients. Therefore, we theorize that the TSLP-positive cells in the giant papillae may be these inflammatory cells. We found that at least some mast cells express TSLP protein (Fig. 3A, asterisks), which is consistent with previous studies that showed that the mast cells activated through the IgE receptor express TSLP mRNA. Our double-immunohistochemistry results also showed that tryptase-positive mast cells localize in the vicinity of TSLP-positive epithelial cells in the giant papillae (Fig. 3B, arrow), and our result adds support to the proposal of Miyata et al. that mast cells are essential for TSLP expression in the epithelium of the mouse allergic rhinitis model.

It is known that one of the major functions of TSLP is activating dendritic cells, which prime naïve T cells to produce the proallergic cytokines, such as IL-4, -5, and -13. We therefore determined the existence of activated dendritic cells by using the dendritic cell activation marker DC-LAMP and found the existence of activated dendritic cells in the vicinity of the TSLP-positive cells (Fig. 2, asterisks). The DC-LAMP-positive dendritic cells were observed not only underneath the TSLP-positive epithelial cells but also near the TSLP-positive cells in the substantia propria (Fig. 2A, arrow). This result adds support to the recent results of Corrigan et al., who proposed a possible role of TSLP-positive inflammatory cells in the activation of dendritic cells in the dermis of atopic skin challenged with allergen.

As a next step, we examined the expression of TSLP mRNA in HCJE cells with added poly I:C. As reported previously in bronchial epithelial cells and corneal epithelial cells, poly I:C stimulation induces TSLP mRNA expression in the conjunctival epithelial cells. We analyzed the time course of TSLP mRNA expression and found that it peaked at 3 hours after poly I:C stimulation (Fig. 4). Our results are consistent with those of Ma et al., who showed peak TSLP mRNA expression at 3 hours after stimulation of human corneal epithelial cells. We then tried to inhibit poly I:C-induced TSLP mRNA expression by the endosomal inhibitor bafilomycin. Previous reports showed that poly I:C-induced IL-6 expression in a peripheral blood mononuclear cell (PBMC) requires an acidic pH (pH 5.7–6.5), and bafilomycin A could inhibit poly I:C-induced IL-6 expression by inhibiting the endosomal proton pump. Our results showed that a 10 nM bafilomycin treatment blocked TSLP mRNA expression and found that it peaked at 3 hours after poly I:C stimulation. We then tried to inhibit poly I:C-induced TSLP mRNA expression by the endosomal inhibitor bafilomycin. Previous reports showed that poly I:C-induced IL-6 expression in a peripheral blood mononuclear cell requires an acidic pH (pH 5.7–6.5), and bafilomycin A could inhibit poly I:C-induced IL-6 expression by inhibiting the endosomal proton pump.
pression induced by 3-hour poly I:C stimulation (Fig. 4). We theorize that drugs that raise endosomal pH may be useful for inhibiting poly I:C-mediated TSLP expression. It is known that bafilomycin A is too toxic for clinical use; therefore, omeprazole, a proton pump inhibitor, or chloroquine, a competing basic compound that raises endosomal pH, which are widely used in the treatment of gastric ulcer and malaria, respectively, may be useful for this purpose.22

Finally, we examined the possible effector cells for TSLP in the chronic allergic conjunctivitis. Mast cells have been reported as one of the effector cells in TSLP signaling.23 A previous report showed that the coculture of skin fragments from patients with atopic dermatitis (AD) with human mast cells induces TSLP protein expression and that anti-TSLP antibody treatment suppresses TSLP expression.24 However, the authors did not perform direct stimulation of human mast cells with recombinant (r)TSLP. Therefore, we stimulated human mast cells with rTSLP and found that rTSLP treatment alone induced a minimum of IL-13 mRNA induction. A surprising finding showed that co-stimulation with another epithelial cell–derived Th2 cytokine, IL-33, had a synergistic effect for IL-13 mRNA expression in LAD2 cells (Fig. 5). In their study, Allakhverdi et al.23 showed partial suppression of IL-13 expression using anti-TSLP antibody in a mast cell–lesional, AD–skin coculture model, so it is reasonable to consider that other skin-derived factor(s) also contribute to IL-13 mRNA expression. Very recently, we reported IL-33 protein expression in the epithelial cells of giant papillae, and we theorize that TSLP and IL-33 cooperate to induce Th2 cellular immunity in vivo.16 Therefore, we speculated that TSLP may contribute to Th2 polarization of Th2 cells expressing its receptor in allergen-induced late phase cutaneous responses in atopic subjects. Allergy. 2009;64(7):1014–22.


