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Human Corneal Epithelium-Derived Thymic Stromal Lymphopoietin Links the Innate and Adaptive Immune Responses via TLRs and Th2 Cytokines

Ping Ma^{1,2,3}, Fang Bian^{1,3,4}, Zhichong Wang², Xiaofen Zheng^{1,5}, Suksri Chotikavanich¹, Stephen C. Pflugfelder¹, and De-Quan Li¹

¹Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas

²State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun-Yat Sen University, Guangzhou, China

⁴Department of Ophthalmology, Union Hospital of Tongji Medical College, Huazhong Science and Technology University, Wuhan, Hubei Province, China

⁵Shanxi Eye Hospital, Taiyuan, Shanxi Province, China

Abstract

Purpose—To explore the crucial role of the human corneal epithelium-derived proallergic cytokine thymic stromal lymphopoietin (TSLP) in initiation and regulation of immune responses.

Methods—Primary corneal epithelial cells, established from donor limbal explants, were treated with 11 microbial ligands and proinflammatory and Th2 cytokines, alone or in combination. TSLP mRNA and protein were determined by real-time PCR and ELISA, respectively. NF- κ B activation was detected by immunostaining and Western blot.

Results—TSLP was found to be expressed by human corneal epithelium and its cultures. TSLP in corneal epithelial cells were largely induced in a concentration-dependent fashion by polyI:C, flagellin, and FSL-1, the ligands for toll-like receptor (TLR)-3, -5, and -6, respectively. Compared with the control, TSLP mRNA was increased by 60-, 12- and 8-fold and TSLP protein increased by 67-, 19- and 7-fold by these three ligands, respectively. The proinflammatory (TNF- α and IL-1 β) and Th2 (IL-4 and IL-13) cytokines moderately induced TSLP expression and production. IL-4 and -13 strongly synergized with PolyI:C, flagellin, and TNF- α to promote TSLP production in ex vivo tissues and in vitro cultures of corneal epithelium. PolyI:C, flagellin, or TNF- α also induced NF- κ B p65 protein nuclear translocation. The NF- κ B inhibitor quinazoline blocked p65

Corresponding author: De-Quan Li, Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, 6565 Fannin Street, NC-205, Houston, TX 77030; dequanl@bcm.tmc.edu.

³Contributed equally to the work and therefore should be considered equivalent authors.

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nuclear translocation and further suppressed TSLP expression and production induced by these stimuli.

Conclusions—These findings provide the first evidence of TSLP induction in the human eye and suggest the novel phenomenon that human corneal epithelium- derived TSLP may serve as a link between the innate and adaptive immune responses. TSLP may become a novel therapeutic target for allergic and inflammatory ocular surface diseases.

A long-recognized property of epithelial cells is their physical barrier function. Recent discoveries indicate that epithelial cells, as the first line of defense, play important roles, not only in host defense and inflammation, but also in regulation of immune responses. The mammalian immune system is composed of two branches that work in tandem to provide resistance to infection: the innate immune system and the adaptive immune system. The innate immune cells, represented primarily by monocytes, macrophages, dendritic cells, and granulocytes, are the first line of host defense and are responsible for immediate recognition and control of microbial invasion. In contrast, the adaptive immune system, represented by B and T lymphocytes, has a delayed response that is characterized by clonal expansion of cells that bind to a highly specific antigen and have immunologic memory.¹ The innate immune response relies on evolutionarily ancient germline-encoded receptors, the pattern-recognition receptors (PRRs)² that recognize highly conserved microbial structures. PRRs recognize microbial components known as pathogen-associated molecular patterns (PAMPs). A breakthrough in the understanding of the ability of the innate immune system to rapidly recognize pathogens occurred with the discovery of the toll-like receptors (TLRs), the most important family among the PRRs. Recently, expression of a variety of TLRs has been identified in the human corneal epithelium.^{3,4} Stimulation of TLR3 can induce the expression of proinflammatory cytokines, chemokines, and antiviral genes that help defend the cornea against viral infection.^{5,6} The role of ligand-stimulated TLR signaling in the corneal epithelium on regulation of innate and adaptive immunity remains to be elucidated.

Thymic stromal lymphopoietin (TSLP) is a recently identified novel proallergic cytokine that strongly activates dendritic cells to induce an inflammatory Th2 response and initiate allergic and inflammatory diseases.^{7,8} TSLP is produced primarily by epithelial cells in the lungs, gut, and skin, although fibroblasts, smooth muscle cells, and mast cells all have the potential to produce TSLP.^{9,10} Recent work has shown that TSLP levels increase at sites of inflammation. For example, airway epithelium from asthmatics has shown increased TSLP expression that supports a role of TSLP in promoting T helper 2-type allergic inflammation. CD4⁺ T cells, primed by TSLP-treated dendritic cells, produce the proallergic cytokines IL-4, IL-13, IL-5, and TNF- α , but not IL-10 and IFN γ , on restimulation.¹⁰ Recent studies have demonstrated that TSLP plays an important role in the initiation and maintenance of the allergic immune response in atopic dermatitis and asthma.^{7,8,11,12} TSLP may become an important target for intervention in the initiation of allergic inflammatory responses.

The ocular surface epithelium may share properties with the airway and skin epithelia, despite belonging to a different mucosal surface. We hypothesize that corneal epithelial cells play a central role in innate and adaptive immune responses by producing a novel proallergic

cytokine TSLP, in response to microbial and inflammatory stimuli through TLR and NF- κ B signaling pathways that regulate dendritic cell activation for priming the Th2 response.

Methods

Materials and Reagents

Cell culture dishes, plates, centrifuge tubes, and other plastic ware were purchased from BD Biosciences (Lincoln Park, NJ); polyvinylidene difluoride (PVDF) membranes from Millipore (Bedford, MA); polyacrylamide ready gels (4%–15% Tris-HCl), sodium dodecyl sulfate (SDS), prestained SDS-PAGE low range standards, precision plus protein standards, and precision protein strep tacin-HRP conjugate from Bio-Rad (Hercules, CA); Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, amphotericin B, and gentamicin from Invitrogen (Grand Island, NY); and fetal bovine serum (FBS) from Hyclone (Logan, UT). The TLR1 ligand Pam3CSK4; the TLR2 ligands peptidoglycan from *Bacillus subtilis* (PGN-BS), lipoteichoic acid from *Staphylococcus aureus* (LTA-SA), and yeast zymosan; and the respective TLR5 to -9 ligands—flagellin from *S. typhimurium*, synthetic diacylated lipoprotein (FSL-1), imiquimod (R837), single-stranded GU-rich oligonucleotide complexed with LyoVec (ssRNA40/LyoVec), and type C CpG oligonucleotide (ODN 2395)—were purchased from InvivoGen (San Diego, CA). The TLR3 ligand polyinosinic-polycytidylic acid (polyI:C), and the TLR4 ligand, LPS from *Escherichia coli*, were from Sigma-Aldrich (St. Louis, MO). The human recombinant cytokines, TNF- α , IL-1 β , IFN γ , IL-4, and IL-13; a TSLP ELISA kit; and a TNF- α mouse monoclonal antibody (mAb) were from R&D Systems (Minneapolis, MN). Affinity-purified rabbit polyclonal antibodies (Ab) against TLR3 (M-300), TLR5 (H-127), and p65 were from Santa Cruz Biotechnology (Santa Cruz, CA), and TSLP Rabbit antibody was from ProSci Incorporated (Poway, CA). An RNA extraction kit (RNeasy Mini; Qiagen, Valencia, CA), enhanced chemiluminescence (ECL) reagents, and lyophilized beads (Ready-To-Go-Primer First-Strand Beads) were obtained from GE Healthcare, Inc. (Piscataway, NJ); gene expression assays and real-time PCR master mix (*TaqMan*) from Applied Biosystems (Applied Biosystems, Inc. [ABI], Foster City, CA); and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, and the BCA protein assay kit from Pierce Chemical (Rockford, IL).

Human Corneal Epithelial Cell Culture Models for TSLP Induction

Fresh human corneoscleral tissues (<72 hours after death) unsuitable for clinical use, from donors aged 19 to 67 years, were obtained from the Lions Eye Bank of Texas (Houston, TX). Human corneal epithelial cells (HCECs) were cultured in 12-well plates with explants from corneal limbal rims in a supplemented hormonal epidermal medium (SHEM) containing 5% FBS according to our published methods.^{13–15} Corneal epithelial cell growth was carefully monitored, and only the epithelial cultures without visible fibroblast contamination were used for this study. Confluent corneal epithelial cultures were switched to serum-free SHEM and treated for different periods (1, 4, 8, 16, 24, or 48 hours) with (1) a series of microbial components with multiple concentrations (the 11 ligands for TLR1 to -9 listed in Table 1); (2) the proinflammatory cytokines TNF- α (1–20 ng/mL) and IL-1 β (1–20 ng/mL); and (3) the Th2 cytokines, IL-4 and -13 (1–100 ng/mL), alone or in combination with either (1) or (2). Each experiment was repeated at least three times. The cells treated for

1 to 24 hours were lysed for total RNA extraction and mRNA expression. The supernatants of the conditioned medium in the cultures treated for 48 hours were collected and stored at -80°C for TSLP ELISA, and the cells were lysed in RIPA buffer for protein assay.

Human Corneal Epithelial Tissue Ex Vivo Model for TSLP Induction

A fresh corneoscleral tissue was cut into four equal-sized pieces. Each quarter of corneal tissue was placed into a well of eight-chamber slides with epithelial side up in $150\text{ }\mu\text{L}$ of serum-free SHEM medium, without or with polyI:C ($50\text{ }\mu\text{g/mL}$) or TNF- α (20 ng/mL) in the presence of IL-4 or -13 (100 ng/mL) for 24 hours in a 37°C incubator. The supernatants of the conditioned media were collected and stored at -80°C for TSLP ELISA. The corneal epithelial tissues were prepared for frozen sections for TSLP immunohistochemical staining.

TLR and NF κ B Signaling Pathway Assay

HCECs were preincubated with specific TLR antibodies ($5\text{ }\mu\text{g/mL}$ of TLR3 or TLR5 rabbit antibody) or NF κ B activation inhibitor (quinazoline $10\text{ }\mu\text{M}$) for 1 hour before polyI:C, flagellin, or TNF- α , respectively, was added for 4 hours. The cells in six-well plates were lysed for extraction of cytoplasmic and nuclear proteins with a nuclear extraction kit (Active Motif, Carlsbad CA), and stored at -80°C for Western blot analysis. The cells in eight-chamber slides were fixed for NF κ B p65 immunofluorescent staining. The cells in 12-well plates were subjected to total RNA extraction for TSLP expression by real-time PCR. The medium supernatants from the cultures treated for 48 hours were used for TSLP ELISA.

Total RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was isolated from cells (RNeasy Mini kit; Qiagen) according to the manufacturer's protocol, and quantified by spectrophotometry (ND-1000; NanoDrop Technologies, Wilmington, DE) and stored at -80°C . The first-strand cDNA was synthesized by RT from $1\text{ }\mu\text{g}$ of total RNA using lyophilized beads (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare) as previously described.^{16,17} The realtime PCR was performed (Mx3005P system; Stratagene) with a $20\text{-}\mu\text{L}$ reaction volume containing $5\text{ }\mu\text{L}$ of cDNA, $1\text{ }\mu\text{L}$ of gene expression assay primers and probe for TSLP (Hs00263639_m1) or GAPDH (Hs99999905_m1) and $10\text{ }\mu\text{L}$ gene expression master mix (*TaqMan*; ABI). The thermocycler parameters were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A nontemplate control was included to evaluate DNA contamination. The results were analyzed by the comparative threshold cycle (C_T) method and normalized by GAPDH.^{17,18}

Enzyme-Linked Immunosorbent Assay

Double-sandwich ELISA (R&D Systems) for human TSLP was performed, according to the manufacturer's protocol, to determine the TSLP protein levels in the supernatants of the conditioned media from cultures and tissues after different treatments.

Immunohistochemical and Immunofluorescent Staining

The human corneal frozen sections or corneal epithelial cells on eight-chamber slides were fixed in acetone at -30°C for 5 minutes. Cell cultures were permeabilized with 0.2% Triton

X-100 in PBS at RT for 10 minutes. Indirect immunostaining was performed according to our published methods,^{15,19,20} with primary rabbit antibodies against human TSLP (1:1000, 1 $\mu\text{g}/\text{mL}$) or p65 (1:100, 2 $\mu\text{g}/\text{mL}$). For histochemical staining, a goat anti-rabbit biotinylated second antibody (R&D Systems) and an ABC peroxidase system (Vectastain; Vector Laboratories, Burlingame, CA) were used. For fluorescent staining, AlexaFluor 488-conjugated secondary antibodies were applied, and propidium iodide (PI) was used for nuclear counterstaining. Secondary antibody alone without primary antibodies applied, or isotype IgG were used as the negative control. The staining was photographed with a microscope (Eclipse 400; Nikon, Garden City, NY) with a digital camera (DMX 1200; Nikon).

Western Blot Analysis

Western blot analysis was performed with a previous method.¹⁶ In brief, the cytoplasm and nuclear protein samples (30 μg per lane), measured by a BCA protein assay kit, were mixed with 6 $\times$ SDS reducing sample buffer and boiled for 5 minutes before loading. The proteins were separated on an SDS polyacrylamide gel and transferred electronically to PVDF membranes. The membranes were blocked with 5% nonfat milk in TTBS (50 mM Tris [pH 7.5], 0.9% NaCl, and 0.1% Tween-20) for 1 hour at room temperature and incubated, first with primary antibodies against NF κ B p65 overnight at 4°C, and then with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The signals were detected with a chemiluminescence reagent (ECL; GE Healthcare), and the images were acquired by an imaging station (model 2000R; Eastman Kodak, Rochester, NY).

Statistical Analysis

TSLP production was compared between the untreated control and each treated condition of corneal epithelial cells with a two-tailed Student's *t*-test. Statistical significance was retained for $P < 0.05$.

Results

Large Induction of TSLP in Response to Microbes in HCECs

We hypothesized that HCECs could be stimulated to produce TSLP in response to TLR ligands. This proposition was tested in primary HCECs challenged by extracted or synthetic microbial components, representing the ligands respective to TLR1 to -9 in different concentrations (Table 1) for different periods (1–48 hours). Induction of TSLP expression at the mRNA and protein levels was evaluated by RT and real-time PCR and ELISA, respectively. TSLP mRNA was found to be weakly expressed by normal HCECs. TSLP mRNA levels were largely induced in the corneal epithelial cells challenged by certain viral or bacterial components, including polyI:C, flagellin, and FSL-1, which are the ligands respective to TLR3, -5, and -6 (Fig. 1A). The TSLP mRNA levels were slightly but not significantly induced by Pam3CSK4 and imiquimod (R837), which are the ligands respective to TLR1 and -7. TSLP expression was not induced by other bacterial components, such as the TLR2 ligands PGN-BS (1–50 $\mu\text{g}/\text{mL}$), LTA-SA (0.1–10 $\mu\text{g}/\text{mL}$), and zymosan (1–100 $\mu\text{g}/\text{mL}$); the TLR4 ligand LPS (1–50 $\mu\text{g}/\text{mL}$); the TLR9 ligand bacterial DNA unmethylated CpG motifs (C-CpG-ODN, 1–50 $\mu\text{g}/\text{mL}$); or the TLR8 ligand virus single

stranded RNA (ssRNA40, 0.1–10 $\mu\text{g/mL}$). The Expression of TSLP mRNA was induced to peak levels at 4 hours (Fig. 1B) by the major inducers, including the TLR3 ligand polyI:C (1 to 50 $\mu\text{g/mL}$; up to 60-fold), representing viral double-stranded RNA (dsRNA); the TLR5 ligand flagellin (0.1 to 10 $\mu\text{g/mL}$; up to 12-fold), the major component of the bacterial flagellar filament; and the TLR6 ligand FSL-1 (0.1 to 10 $\mu\text{g/mL}$; up to 8-fold), the bacterial diacylated lipopeptide (Fig. 1A). The expression of TSLP mRNA was induced in a concentration-dependent fashion by these major microbial ligands (Fig. 1C). A corresponding increase in TSLP protein was detected by ELISA in supernatants of cultured corneal epithelial cells treated with these TLR ligands for 48 hours. The TSLP protein levels were very low, at 5.1 ± 2.9 pg/mL in the normal control, the untreated HCECs cultured in SHEM medium (Figs. 1A, 1C). PolyI:C, flagellin, and FSL-1 stimulated TSLP production to 340.2 ± 103.6 , 99.1 ± 10.6 , and 34.1 ± 6.7 pg/mL ($P < 0.001$, 0.01, 0.05, $n = 4$, respectively, representing 67.2-, 19.4-, and 6.7-fold increases over the control, respectively).

Moderate Induction of TSLP in Response to Proinflammatory Cytokines in HCECs

The expression of TSLP mRNA and protein by primary HCECs was moderately induced by proinflammatory cytokines in a concentration-dependent manner. As shown in Figure 2, TNF- α and IL-1 β increased the expression of TSLP mRNA by 4.0 ± 0.7 - or 2.4 ± 0.3 -fold, respectively, and increased TSLP protein production up to 22.9 ± 6.5 and 10.4 ± 3.6 pg/mL, respectively (both $P < 0.05$, $n = 4$).

Moderate Induction of TSLP by Th2 Cytokines and Their Synergistic Effects with TLR Ligands or Proinflammatory Cytokines on HCECs

Of interest, production of TSLP by corneal epithelia was also moderately induced by Th2 cell-secreted cytokines, but not by Th1 cytokine IFN- γ . Addition of exogenous IL-4 and -13 increased the TSLP mRNA expression 5.5 ± 1.9 - and 4.2 ± 2.7 fold, respectively (Fig. 3A) and TSLP protein production to 26.4 ± 10.6 and 21.8 ± 7.3 pg/mL (Fig. 3B) in HCEC culture supernatants. The stimulatory effects of IL-4 and -13 on TSLP induction were slightly higher, but not significantly, than those of the proinflammatory cytokines TNF- α and IL-1 β .

Of further interest, we discovered that Th2 cytokines strongly synergized with TLR ligands and proinflammatory cytokines to promote the expression and production of TSLP by HCECs. IL-4 in combination with TLR3 ligand polyI:C stimulated the TSLP mRNA by 106.2 ± 10.5 -fold and protein levels to 484.7 ± 112.6 pg/mL, respectively, significantly higher than the effect of polyI:C alone, $P < 0.01$, $n = 3$; Fig. 3). IL-13 also synergized with polyI:C to induce the TSLP mRNA 90.0 ± 12.3 -fold and protein levels to 401.0 ± 108.4 pg/mL, respectively, $P < 0.05$ ($n = 3$) compared with polyI:C alone. IL-4 and IL-13 also synergized with the TLR5 ligand flagellin to promote TSLP mRNA expression by 22.2 ± 9.8 - and 19.8 ± 7.3 -fold respectively, and protein production to 271.1 ± 89.1 and 221.4 ± 82.1 pg/mL, respectively. Furthermore, IL-4 and IL-13 showed a synergistic effect with TNF- α on TSLP mRNA expression (a 192 ± 11.8 - and 11.5 ± 7.3 -fold increase, respectively) and protein production (to 131.7 ± 59.8 and 70.9 ± 37.6 pg/mL, respectively).

TSLP Induction in an Ex Vivo Model of Human Corneal Tissues

To further confirm the TSLP induction in whole corneal tissue, fresh human corneal tissues were incubated ex vivo with polyI:C or TNF- α in combination with IL-4 or -13 for 24 hours. The TSLP protein levels secreted from the corneal epithelial tissue were markedly induced from 0 to 3.4 pg/mL in the control cultures to 33.1 ± 4.6 (with polyI:C+IL-4), 20.6 ± 3.9 (polyI:C+IL-13), 97 ± 1.8 (TNF- α +IL-4), or 6.6 ± 1.5 (with TNF- α +IL-13) pg/mL, respectively, resulted in three separate experiments (Fig. 4A). Adjusted for ex vivo production per cornea, polyI:C plus IL-4 or -13 induced TSLP production to 198 ± 2.8 or 12.3 ± 2.3 pg/cornea (Fig. 4A). TSLP immunohistochemical staining further confirmed this stimulation in corneal tissues. The corneal epithelium had stronger staining with TSLP antibody after incubation with polyI:C for 24 hours, compared with untreated control (Fig. 4B).

Mediation of TSLP Induction in HCECs by TLR and NF κ B Signaling Pathways

To study the pathways involved in TSLP induction, we preincubated the HCECs with TLR3 Ab for polyI:C, TLR5 Ab for flagellin, or the NF κ B activation inhibitor quinazoline (NF κ B-I). The polyI:C-stimulated TSLP mRNA and protein production were dramatically blocked by TLR3 antibody or NF κ B-I (Fig. 5A). When evaluated by Western blot analysis (Fig. 5B) and immunofluorescent staining (Fig. 5C), the NF- κ B p65 protein was markedly activated through translocation from cytoplasm to nucleus in corneal epithelial cells after treated with polyI:C for 4 hours. This p65 nuclear translocation was largely blocked by preincubation with rabbit anti-TLR3 antibody (5 μ g/mL) or NF κ B-I (10 μ M). Similar levels of inhibition were observed when the cells were treated with flagellin in the absence or presence of TLR5 Ab or NF κ B-I (data not shown). TNF- α -stimulated TSLP production (Fig. 6A) and p65 nuclear translocation (Figs. 6B, 6C) were also dramatically inhibited by its monoclonal antibody or NF κ B-I.

Discussion

Recent studies have provided compelling evidence that in addition to their physical barrier function, epithelial cells actively participate, as initiators, mediators, and regulators, in innate and adaptive immune responses for host defense.^{21,22} TSLP, a novel proallergic cytokine, has been found to be produced by several mucosal epithelia and it appears to play a determinant role in the initiation and maintenance of the allergic immune response in atopic dermatitis and asthma.^{7,8,11,12} The expression and regulation of TSLP by the ocular surface epithelia has not been investigated. Using fresh human corneoscleral tissue and primary cultured corneal epithelial cells, we have documented that TSLP is expressed by normal corneal epithelium and is largely induced by microbial components, inflammatory cytokines, and Th2 cytokines through TLR and NF κ B signaling pathways. Our findings provide new evidence that corneal epithelial cell-derived TSLP may serve as a link between the innate and adaptive immune responses on the ocular surface.

TLR-Dependent Induction of TSLP by HCECs in Response to Microbial Components

The discovery of the TLRs, the most important family among the PRRs, is a breakthrough in the understanding of the ability of the innate immune system to rapidly recognize pathogens.

At least 10 human TLRs have been identified to date. Each TLR has unique ligand specificity. In general, TLR1, -2, -4, -5, and -6 are present on the cell plasma membrane and respond to a variety of components of bacteria and fungi, whereas TLR3, -7, -8, and -9 are mainly present on endosomal membranes inside cells and recognize virus nucleic acids.³ Recently, TLR3 was also found to be present on cell surface in human endothelial²³ and corneal epithelial cells.⁶ TLRs are expressed on immune cells that are most likely to first encounter microbes, such as neutrophils, monocytes, macrophages, and dendritic cells.²⁴ In addition to innate immune cells, an array of TLRs are expressed by epithelial cells at host/environment interfaces, including the skin,^{25,26} respiratory tract,^{21,27} gastrointestinal tract,²⁸ and ocular surface.^{3,4} HCECs have been shown to express functional TLR1 to -9 except for TLR8,³ to be able to recognize the presence of Gram-positive bacteria and to initiate innate immune responses by producing the proinflammatory cytokines and β -defensin-2.²⁹ In this study, we have evaluated the expression and production of TSLP by HCECs in response to 11 extracted or synthetic microbial components that are ligands of TLR1 to -9 (Table 1). As shown in Figure 1, TSLP expression and production were largely induced by polyI:C, flagellin, and FSL-1, the ligands for TLR3, -5, and -6, representing viral dsRNA and the bacterial components flagellin and lipopeptides, respectively. PolyI:C and flagellin were major TSLP inducers, stimulating TSLP production by 67- and 19-fold, respectively. The TSLP mRNA reached peak levels rapidly in 4 hours in response to these ligands. The specificity of this response was confirmed when respective antibodies against TLR3 and -5 significantly inhibited TSLP expression. The pattern of TLR-dependent TSLP induction indicates that HCECs are able to rapidly initiate an innate immune response to virus or bacteria through certain TLR pathways.

TSLP was also moderately induced by proinflammatory cytokines at both mRNA and protein levels. TNF- α or IL-1 β induced a concentration dependent increase in the TSLP mRNA and protein (Fig. 2). Supported by a previous report that the proinflammatory and Th2 cytokines synergize to induce TSLP production by human skin keratinocytes,³⁰ our findings suggest a role for TSLP in ocular surface inflammation. But the stimulatory effects of TNF- α and IL-1 β on TSLP expression and production were much weaker than that of polyI:C and flagellin, which stimulated TSLP protein production over 15- and 4-fold higher, respectively, than did TNF- α . These data suggest that TSLP induction is mainly through a TLR-dependent innate immune response to microbes in the HCECs.

TSLP Induction in HCECs by the Adaptive Immunity–Derived Th2 Cytokines IL-4 and -13

It has been reported recently that epithelial cell-derived TSLP could strongly activate human myeloid dendritic cells to become mature dendritic cells that produce OX40 ligand (OX40L) to induce a Th2-based inflammatory response characterized by high TNF- α and low IL-10 production, distinct from a regulatory Th2 responses characterized by low TNF- α and high IL-10 production (for a review, see Refs.^{7, 8}). TSLP may act directly on naive, but not memory, CD4⁺ T cells, and promote their proliferation in response to antigen.³¹ These data indicate that epithelial cell-derived TSLP promotes Th2 differentiation of certain CD4⁺ T cells. Our results demonstrated that IL-4 and -13, the major cytokines secreted by Th2 cells, not only moderately induced TSLP mRNA and protein alone, but also strongly synergized with microbial ligands (polyI:C and flagellin) or the proinflammatory cytokine TNF- α to

promote TSLP expression and production (Fig. 3). This synergized induction of TSLP was further confirmed in an ex vivo experiment model using fresh human corneal epithelial tissues (Fig. 4). Our findings demonstrate that adaptive immunity-derived Th2 cytokines are capable of amplifying TSLP expression and production by the corneal epithelium.^{7,8} These findings suggest that blocking TSLP could be a novel strategy for treatment of allergic diseases or other TSLP-driven conditions. Further study is needed to investigate whether and how corneal epithelium-derived TSLP activates dendritic cells to promote Th2 differentiation of CD4⁺ T cells in the adaptive immune system.

TLR and NF κ B Signaling Pathways Involved in TSLP Induction

Although the recognition of different ligands by specific TLRs leads to activation of an intracellular signaling cascade in a MyD88-dependent or -independent fashion, all TLRs share NF κ B signal transduction pathways for activation of the transcription factors.³² TNF- α is also well known to promote activation of the NF κ B signaling pathway.³³ TSLP induction was recently observed through NF κ B activation in airway epithelial cells¹² and synovial fibroblasts.³⁴ As shown in Figures 5 and 6, when evaluated by Western blot analysis and immunofluorescent staining, NF κ B was dramatically activated with p65 protein nuclear translocation in corneal epithelial cells exposed to polyI:C, flagellin, or TNF- α for 4 hours. This p65 nuclear translocation and TSLP induction, stimulated by polyI:C, flagellin, or TNF- α were markedly blocked by TLR3, TLR5, or TNF- α specific antibody, respectively, and also by the NF κ B activation inhibitor quinazoline. These findings confirmed that TSLP induction in HCECs was via the TLR and NF κ B signaling pathways. Further study is necessary to investigate whether this NF κ B signaling is activated through MyD88-dependent or -independent pathways in corneal epithelial cells.

In summary, this study provides the first evidence of TSLP induction by the corneal epithelium in the human eye. We identified that TSLP production is largely induced by microbial components, proinflammatory cytokines, and Th2 cytokines through TLR and NF κ B signaling pathways. These findings suggest a novel phenomenon that human corneal epithelium-derived TSLP may serve as a potential link between the innate and adaptive immune responses and that TSLP could be a novel molecular target for the treatment of TSLP-mediated allergic and inflammatory conditions.

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Figure 1.

TLR-dependent induction of TSLP by microbial ligands in HCECs. (A) The confluent primary HCECs were incubated with 50 $\mu\text{g/mL}$ polyI:C or 10 $\mu\text{g/mL}$ of Pam₃CSK₄, PGN, LTA, zymosan, LPS, flagellin, FSL-1, R-837, ssRNA40, or C-CpG-ODN for 4 hours for TSLP mRNA expression by RT and real-time PCR, or for 48 hours for TSLP protein in the culture supernatants by ELISA. (B) The time course of TSLP mRNA induction by RT-QR-PCR in HCECs exposed to 50 $\mu\text{g/mL}$ of polyI:C, or 10 $\mu\text{g/mL}$ of flagellin or FSL-1 for 1 to 24 hours. (C) Concentration-dependent induction of TSLP by HCECs exposed to 5 to 50

$\mu\text{g/mL}$ polyI:C or 0.1 to 10 $\mu\text{g/mL}$ flagellin or FSL-1 for 4 hours for TSLP mRNA or 48 hours for TSLP protein in the supernatants. Results shown are the mean $\pm$ SD of three to five independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

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Figure 2.

TSLP induction by proinflammatory cytokines in HCECs. The confluent primary HCECs were incubated with 1 to 20 ng/mL of TNF- α or IL-1 β , as indicated for 4 hours for TSLP mRNA expression by RT and real-time PCR, or for 48 hours for TSLP protein production in the culture supernatants by ELISA. Results shown are the mean $\pm$ SD of three independent experiments. * P < 0.05.

Figure 3.

TSLP induction by Th2 cytokines alone and in combination with microbial ligands or proinflammatory cytokines in HCECs. The confluent primary HCECs were incubated with 100 ng/mL of IFN- γ , IL-4, or IL-13 in the presence or absence of 50 μ g/mL polyI:C, 10 μ g/mL flagellin, or 20 ng/mL TNF- α , as indicated for 4 hours for TSLP mRNA expression by RT and real-time PCR (A), or for 48 hours for TSLP production in the culture supernatants by ELISA (B). Results shown are mean $\pm$ SD of three independent experiments. * P < 0.05; ** P < 0.01.

Figure 4. TSLP induction in an ex vivo model of human corneal tissues. A fresh corneoscleral tissue was cut into four equal-sized pieces. Each quarter of corneal tissue was placed into a well of an eight-chamber slide in 150 μ L of serum-free SDEM without or with polyI:C (50 μ g/mL) or TNF- α (20 ng/mL) in the presence of IL-4 or -13 (100 ng/mL) for 24 hours in a 37°C incubator. **(A)** The culture supernatants were used for TSLP ELISA. Results shown are the mean $\pm$ SD of three independent experiments. * P < 0.05; ** P < 0.01. **(B)** The corneal tissues were prepared for frozen sections for TSLP immunohistochemical staining with isotype IgG as the negative control.

Figure 5.

TLR and NF κ B signaling pathways in TSLP induction by polyI:C in HCECs. The HCECs were preincubated with 5 μ g/mL TLR3 Ab or 10 μ M. quinazoline, an NF κ B activation inhibitor (NF κ B-I) for 1 hour before adding 50 μ g/mL polyI:C. (A) The cells in 12-well plates treated for 4 or 48 hours were used for determination of TSLP mRNA by RT and real-time PCR or TSLP production in the supernatants by ELISA, respectively. Results shown are the mean $\pm$ SD of three independent experiments, $*P < 0.05$. (B) The cells treated for 4 hours in six-well plates were subjected to cytoplasm and nuclear protein extraction for

NF κ B p65 activation by Western blot analysis with 30 μ g total proteins per lane. C-p65, cytoplasm p65; N-p65, nuclear p65. (C) The cells seeded in chamber slides were fixed by acetone for immunofluorescent staining with rabbit anti-human p65 antibody and AlexaFluor 488-conjugated second antibodies. The images are representatives of those from three independent experiments.

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Figure 6.

NF κ B signaling pathways in TSLP induction by TNF- α in HCECs. The HCECs were preincubated with 5 μ g/mL TNF- α mAb or 10 μ M quinazoline, an NF κ B activation inhibitor (NF κ B-I) for 1 hour before adding 20 ng/mL TNF- α . (A) The cells in 12-well plates treated for 4 or 48 hours were used for TSLP mRNA or protein production, respectively. Results shown are the mean $\pm$ SD of three independent experiments. $*P < 0.05$. (B) The cells in six-well plates treated for 4 hours were subjected to cytoplasm and nuclear protein extraction for NF κ B p65 activation by Western blot analysis with 30 μ g total proteins per lane. C-p65,

cytoplasmic p65; N-p65, nuclear p65. (C) The cells seeded in chamber slides were fixed by acetone for immunofluorescent staining with rabbit anti-human p65 antibody and AlexaFluor 488 conjugated second antibodies. The images are representative of those from three independent experiments

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Table 1**Human TLRs and Their Ligands**

TLR	Principal Exogenous Ligands	Ligands Studied
TLR1/TLR2	Triacylated lipopeptide (LP)	Pam3CSK4 (1–100 $\mu\text{g}/\text{mL}$)
TLR2 *	Bacterial lipoprotein	
	Peptidoglycan	PGN-BS (1–50 $\mu\text{g}/\text{mL}$)
	Lipoteichoic acid	LTA-SA (0.1–10 $\mu\text{g}/\text{mL}$)
	Zymosan (fungi)	Zymosan (1–100 $\mu\text{g}/\text{mL}$)
TLR3	Double-stranded RNA (viruses dsRNA)	PolyI:C (5–50 $\mu\text{g}/\text{mL}$)
TLR4	LPS (Gram-negative bacteria)	LPS (1–50 $\mu\text{g}/\text{mL}$)
	Bacterial HSP60	
	Respiratory syncytial virus coat protein	
TLR5	Flagellin (flagellated bacteria)	Flagellin-ST (1–25 $\mu\text{g}/\text{mL}$)
TLR6/TLR2	Diacylated lipopeptides	FSL-1 (0.1–10 $\mu\text{g}/\text{mL}$)
TLR7	Imidazoquinolone antiviral drug	Imiquimod (R837, 1–50 $\mu\text{g}/\text{mL}$)
TLR8	Single stranded RNA (virus ssRNA)	ssRNA40 (0.1–10 $\mu\text{g}/\text{mL}$)
	Imidazoquinolone antiviral drug	
TLR9	Unmethylated CpG motifs of bacterial DNA	C-CpG-ODN (1–50 $\mu\text{g}/\text{mL}$)
TLR10	Unknown	

* TLR2 forms heterodimers with TLR1 and TLR6; TLR1 associates with TLR2 to recognize triacyl lipopeptides; the TLR6/TLR2 heterodimer recognizes diacyl lipopeptides.