

Thymic Stromal Lymphopoietin-Related Allergic Pathway in Patients With Vernal Keratoconjunctivitis

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Purpose: To investigate the expression of thymic stromal lymphopoietin (TSLP) and the relevant signaling pathways in the giant papillae obtained from patients with vernal keratoconjunctivitis (VKC) and to study the potential functional role and molecular mechanism of TSLP.

Methods: Giant papillae from VKC patients and control samples were used to perform immunohistochemical staining and analyze the mRNA expression of TSLP and related pathway by real-time polymerase chain reaction.

Results: TSLP was markedly expressed in the epithelial cells and some inflammatory cells of giant papillae, but not in the control conjunctival tissue. TSLP mRNA expression in the giant papillae of VKC was increased by 9.63 ± 0.99 (mean $\pm$ SD) fold compared with controls ($P < 0.01$). CD11c⁺ and OX40L⁺ immunoreactive cells largely infiltrated the giant papillae as observed by immunohistochemical staining. CD4⁺Th2 cell infiltration was observed through high immunoreactivity of CD4. Th2 cytokines (IL-4, IL-5 and IL-13) and OX40 in the VKC specimens showed increased expression. Augmented gene expression levels of CD4 (6.88 ± 1.84), OX40L (7.60 ± 1.79), OX40 (7.25 ± 1.38), IL-4 (6.89 ± 1.46), IL-5 (8.42 ± 1.55), and IL-13 (9.69 ± 1.94) were significantly different from controls ($P < 0.05$).

Conclusions: Our observations provide strong evidence that TSLP may be a crucial factor that contributes to the development and progression of allergic conjunctivitis. The results also demonstrated that TSLP activates dendritic cells to prime CD4⁺T cells to differentiate into Th2 type and triggers Th2-dominant allergic inflammation through the TSLP/OX40L/OX40 signaling as part of immunopathogenesis of VKC.

Key Words: vernal keratoconjunctivitis, thymic stromal lymphopoietin, immunopathogenesis, immunohistochemical staining, quantitative real-time PCR

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Vernal keratoconjunctivitis (VKC) is a severe bilateral chronic allergic conjunctivitis (AC), which is accompanied with superior or inferior tarsal giant papillae and results from a combination of IgE-induced and T-lymphocyte-induced immune responses.^{1,2} Thymic stromal lymphopoietin (TSLP), produced by epithelial and mast cells, is a novel allergy-related cytokine and can activate dendritic cells (DCs) by binding to the TSLP receptor (TSLPR) to induce inflammatory Th2-type cell differentiation, which is a basic initiator of allergic response.^{3,4}

Immunopathogenesis of AC has been theorized as follows: TSLP-activated DCs express OX40 ligand (OX40L), which binds to OX40 to differentiate CD4⁺T cells into inflammatory Th2-type cells that produce specific Th2-related cytokines IL-4, IL-5, and IL-13.^{4–7} Because of its important role in initiation and progression of allergic immune reaction in atopic disease,^{8,9} TSLP has the potential to become a determinant biomarker and therapeutic target for prevention of allergic response. Therefore, meticulous work is needed to specifically demonstrate immunopathogenesis signaling of TSLP-related AC. Short ragweed (SRW)-induced AC mice were used to demonstrate that pollen prominently downregulates the expression of miR-146a, a type of miRNA involved in various inflammation, and upregulates the expression of TSLP and its downstream signaling pathway, such as TSLP-TSLPR and OX40/OX40L. However, these studies do not present sufficient evidence to demonstrate the entire signaling pathway in patients with VKC.^{10–12}

TSLP mRNA and protein are highly expressed in the resected giant papillae obtained from patients with AC. DCs expressing OX40L have also been detected with immunohistochemical (IHC) methods.¹³ However, there has been no direct evidence of the TSLP-mediated signaling pathway and its corresponding expression in vivo. In the present study, we examined the presence of TSLP and systematically studied the TSLP-related pathway that induces an allergic inflammatory response using giant papillae resected from patients with VKC.

PATIENTS AND METHODS

Patient Selection

All procedures were approved by the ethics committees of the Eye Hospital of Wenzhou Medical University, and the study was conducted in accordance with the tenets of the Declaration of Helsinki. VKC was defined as a chronic allergic recurrent conjunctival inflammation correlated with

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ethnic and environmental predisposition, characterized by giant papillae on the upper tarsal conjunctiva. Other typical symptoms include ocular redness, itching, and discharge. Inclusion criteria for patients with severe VKC included 3 or more giant papillae of >1 mm on the upper tarsal conjunctiva. Eighteen patients with VKC were enrolled into our study according to inclusion criteria and were treated with preservative-free artificial tears and dexamethasone eye drops for at least 1 week before surgical intervention. Nine patients with VKC recovered after pharmacologic treatment, and the remaining patients with no improvement were referred for resection of the giant papillae. The age of the remaining patients ranged from 8 to 28 years, and the symptoms lasted for 6 months to 4 years. Pterygium was defined as a fleshy, wing-shaped growth from the conjunctiva, crossing over the limbus onto the cornea. Conjunctivochalasis was defined as an accumulation of loose bulbar conjunctiva between the lower lid and the globe.¹⁴ All operations were undertaken after informed consent was obtained.

Sample Collection and Preparation

Fifteen giant papillae samples were collected from patients with severe VKC for surgical intervention. Fifteen control conjunctiva tissues were obtained from 3 instances: 8 samples were from 8 donors in the eye bank, 3 were from 3 patients after pterygium excision, and 4 from 1 patient after bilateral conjunctivochalasis surgery. The patient demographics are listed in Supplemental Digital Content 1 (see Supplemental Table, <http://links.lww.com/ICO/A732>). Both in VKC and control group, 9 samples from 9 eyes were used to perform immunohistochemical staining and 6 samples from 6 eyes were used to analyze the mRNA expression. Sections of the resected giant papillae were frozen during surgery and treated with hematoxylin–eosin staining. The remaining were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 6 hours, dehydrated in a 30% sucrose-PBS solution for 30 minutes, rapidly frozen in OCT compound, and stored at -80°C before use.

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

Giant papillae tissues were trimmed and dissected under a microscope and were dissociated with 1 mL TRIzol (Invitrogen, Grand Island, NY). Dried RNA was dissolved with 40 μL RNase-free water and stored at -80°C after the supernatant was separated and centrifuged at 13,000 rpm at 4°C with 200 μL chlorophenol (Sigma-Aldrich, St. Louis, MO), an equal volume of isopropyl alcohol, and 1 mL of 75% ethanol. The RNA solution was added to 350 μL buffer RLT (containing β -mercaptoethanol) and 250 μL 100% ethanol and transferred to a purification column. The purified RNA solution was collected and stored at -80°C after 35 μL RNase-free water was added and centrifuged at 13,000 rpm. A total of 5 μL RNA solution and 1- μL sample buffer was added to agarose gel to perform electrophoresis at 140 V for 20 minutes. Photographs of the gel were taken by Gel Doc XR when electrophoresis was completed.

We used RNase-free Dnasel (Invitrogen, Carlsbad, CA) to react with RNA samples at 37°C for 30 minutes and then EDTA

at 65°C for 10 minutes to prevent DNA contamination. Reverse transcription (RT) was performed as follows: 8 μL RNA, 1 μL random primer, and 1 μL 10 mM dNTPs were mixed, incubated at 70°C for 5 minutes, and then added to 2 μL 10 $\times$ RT Buffer, 4 μL 25 mM MgCl_2 , 2 μL 0.1 mM DTT, 1 μL RNase out, and 1 μL Superscript III reverse transcriptase, followed by incubation at 25°C for 10 minutes, 42°C for 50 minutes, and 85°C for 5 minutes to synthesize single-strand cDNA.

Real-time polymerase chain reaction (PCR) was analyzed with ABI7500 (Applied Biosystems, Foster City, CA) equipped with a 25- μL reaction system. The reaction system was prepared and plated with 23 μL of sample per well in 96-well plates and mixed with 2 μL cDNA. Three copies were made for every sample corresponding to 3 wells. The plate was placed on ABI7500 quantitative PCR. Real-time PCR was performed as follows: initial denaturation at 95°C for 5 minutes and at 9°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 76°C for 1 minute (40 cycles).

Results were analyzed by the comparative threshold cycle (C_t) method and normalized by GAPDH.

Antibodies, IHC Staining of Giant Papillae, and Cell Counting

We purchased antihuman TSLP antibody from ProSci (Poway, CA), antihuman IL-4, IL-5, and IL-13 antibody from BioLegend (San Diego, CA), antihuman CD11c⁺ and CD4 antibody from BD Pharmingen (San Diego, CA), goat anti-mouse IgG-FITC, goat anti-rabbit IgG, goat anti-rat IgG, goat anti-mouse IgG, OX40L antibody, and OX40 antibody from Santa Cruz Biotechnology (Santa Cruz, CA).

Six- μm sections were made with a cryostat (HM 505E; Micron, Waldorf, Germany). The sections were fixed with acetone in PBS at 4°C for 10 minutes and treated with 3% H_2O_2 for 10 minutes. After the reaction was blocked with 10% normal goat serum, primary antibody was applied and incubated at 4°C overnight. The primary antibodies applied for this study included antihuman TSLP, CD11c, CD4, OX40, OX40L, IL-4, IL-5, and IL-13 antibody. After washing with PBS, the sections were incubated with secondary antibodies (all from Santa Cruz Biotechnology) and horseradish peroxidase. The stained slides were examined and photographed with an inverted microscope (Olympus, Tokyo, Japan) with a digital camera (SPOT Insight 2; SPOT Imaging, Sterling Heights, MI).

We categorized the results according to the average of positive cells in 5 non-overlapping fields counted at $\times 400$ magnifications to express the levels of IHC staining. The criteria of the staining results and quantitative statistics were formulated as follows: brown staining in cells is a positive result. The score 0 (no positive cell) implies negative; 1 (less than 5 positive cells) implies sporadic; 2 (positive cells range 6–25) implies positive; and 3 (more than 25 positive cells) implies markedly positive.

Statistical Analysis

The chi-square test was used to compare the statistical significance of cell count between the 2 groups (VKC vs. controls). The Student *t* test was used to compare the statistical

significance of the mRNA expression between the 2 groups. P values < 0.05 were considered statistically significant; all data were analyzed with SPSS software (version 18.0; SPSS Inc, Chicago, IL).

RESULTS

Patients' Information

Information of VKC and control patients is provided in Supplemental Digital Content 1 (see Supplemental Table, <http://links.lww.com/ICO/A732>).

Overexpression of TSLP in Giant Papillae

IHC staining confirmed increased TSLP expression mainly in the conjunctival epithelium when compared with that in controls observed at a magnification of $\times 400$ (Fig. 1B). The average score of cell counting was 2.622 ± 0.510 in the VKC group, and 0.033 ± 0.181 in the control group ($P < 0.05$). As evaluated by RT and real-time PCR, TSLP mRNA expression was found to be upregulated in giant papillae from patients with VKC compared with that in controls (Fig. 1A). This finding shows that TSLP mRNA expression and protein production in the conjunctival tissue are increased in the experimental group, suggesting possible involvement of TSLP in human AC. Positive TSLP staining (arrowheads) was mainly accumulated in the conjunctival epithelia of patients with VKC. A few cells (arrow) in the substantia propria were also positive for TSLP.

Expression of OX40L Increased in the DCs of Giant Papillae

DCs accumulate in conjunctival tissue from the experimental group examined by quantitative real-time PCR and immunostaining for the CD11c, a biomarker of DCs. IHC staining showed that CD11c-positive DCs accumulate in the allergic conjunctival tissue, mostly in the substantia propria under the conjunctival epithelium (Fig. 2B). The mean cell counting score was 0.078 ± 0.269 in the control group, and

2.344 ± 0.737 in the VKC group ($P < 0.05$). RT and real-time PCR further showed higher levels of CD11c mRNA expression in the conjunctival tissue from patients with VKC than controls (Fig. 2A). We also detected the presence of OX40L, a ligand expressed by DCs, in the conjunctival tissue. More OX40L-immunopositive cells were observed in the substantia propria, which were localized similar to CD11c cells of patients with VKC, than in controls (Fig. 2B). The VKC group had 2.578 ± 0.618 cell counting score in average, and the control had 0.056 ± 0.230 ($P < 0.05$). To confirm the immunostaining results, OX40L mRNA transcripts were investigated in the conjunctival tissue of eye sections obtained from patients with VKC and controls (Fig. 2A). These results suggest that TSLP activates DCs, which then express OX40L in human conjunctiva.

Expression of OX40 Increased in CD4⁺ T Cells of Giant Papillae

We also found a significant increase in the number of CD4-immunopositive cells in the conjunctival tissue, especially in the substantia propria (Figs. 3A, B). The mean score of cell counting was 0.1 ± 0.302 in the control group, and 2.567 ± 0.654 in the VKC group ($P < 0.05$). Infiltration of T-lymphocytes in the allergic conjunctival tissue was demonstrated by increased CD4 mRNA expression compared with that in controls (Fig. 3A). To determine the factors related to Th2 differentiation, the expression level of OX40 was detected. The number of OX40-immunoreactive cells and the level of OX40 mRNA (Figs. 3A, B) were significantly increased, primarily in the giant papillae group compared with that in the control group. The average score was 2.667 ± 0.600 in the VKC group, and 0.067 ± 0.251 in the control ($P < 0.05$).

Production of Th2-Related Allergic Inflammatory Cytokines IL-4, IL-5, and IL-13

These CD4⁺T cells seem to be of Th2-type because Th2-related cytokines—IL-4 and IL-13—were found to be expressed at higher levels in the conjunctival tissue of the VKC group than in the controls (Fig. 4A). Immunostaining results

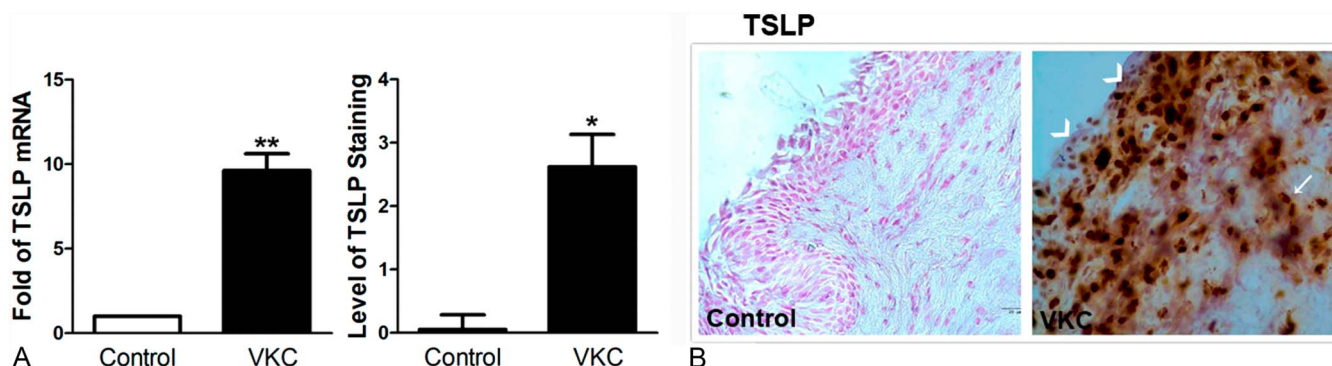


FIGURE 1. mRNA expression and immunoreactive level of TSLP in the conjunctival epithelia of patients with VKC, with nonatopic tissue as controls. A, TSLP mRNA expression levels are indicated as a relative fold in the conjunctival epithelia of patients with VKC over the controls, which were detected by RT and real-time PCR, with GAPDH as an internal control. (* $P < 0.05$; ** $P < 0.01$, $n = 6$). B, Positive TSLP staining (arrowheads) mainly accumulated in the conjunctival epithelium of patients with VKC. A few cells (arrow) in the substantia propria are also positive for TSLP (original magnification, $400\times$) with nonallergic tissue as a negative control (original magnification, $400\times$).

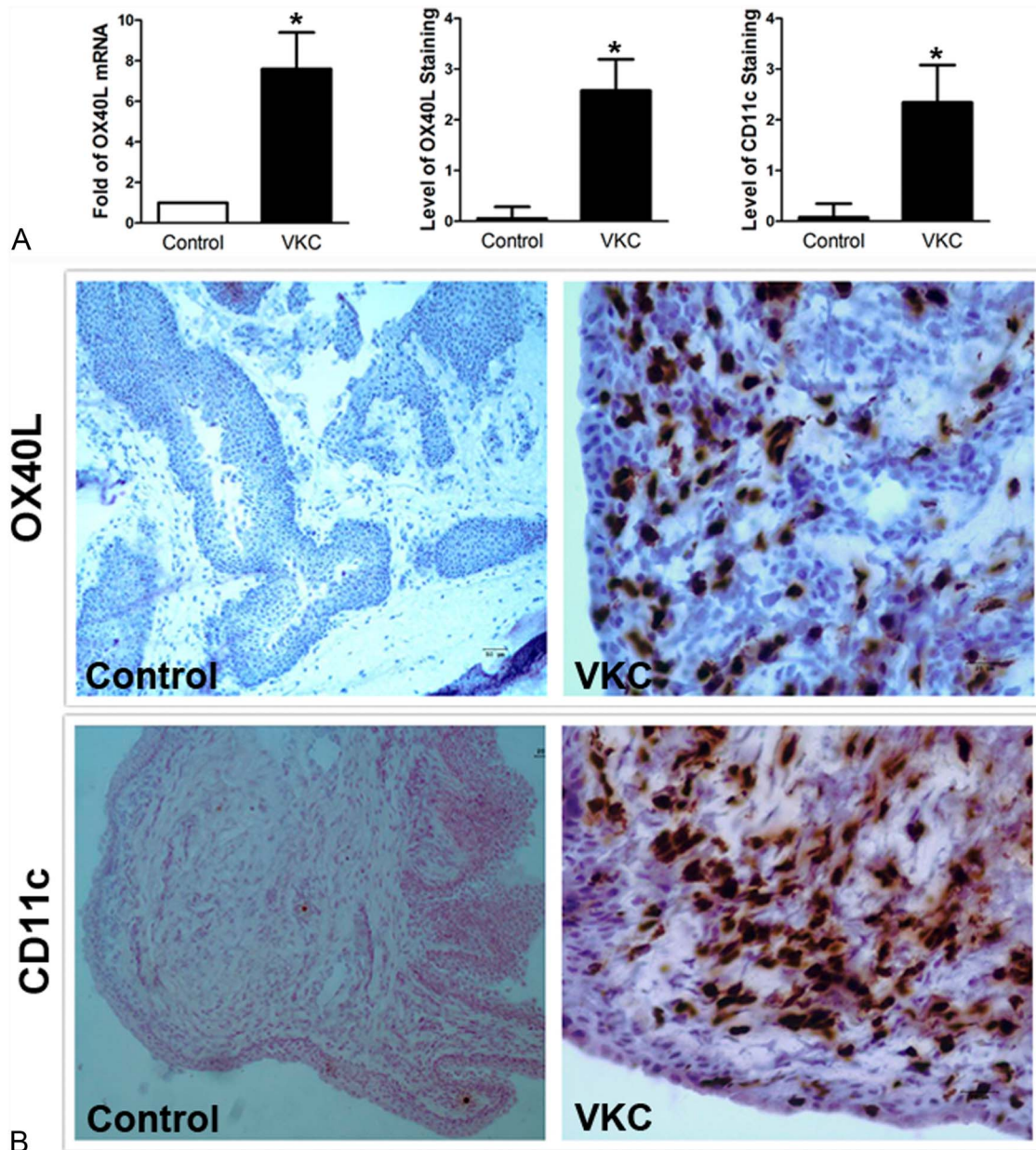


FIGURE 2. OX40L and CD11c mRNA expression and immunoreactive level in the conjunctival epithelia of patients with VKC, with nonatopic tissue as controls. A, mRNA expression levels of OX40L are indicated as a relative fold in the conjunctival epithelia of patients with VKC over the controls, which were determined by RT and real-time PCR, with GAPDH as an internal control. (* $P < 0.05$; $n = 6$). B, Representative images of CD11c and OX40L immunohistochemical staining of the conjunctival tissue of patients with VKC (original magnification, $\times 400$), with nonallergic tissue as a negative control (original magnification, $\times 100$).

confirmed that Th2 cells producing IL-4, IL-5, and IL-13 primarily infiltrated the conjunctival substantia propria (Fig. 4B). The average scores of cell counting in control groups were sequentially 0.044 ± 0.207 , 0.079 ± 0.286 , and 0.067 ± 0.251 , and 2.644 ± 0.708 , 2.478 ± 0.691 , 2.422 ± 0.749 in VKC groups ($P < 0.05$). These results suggest that activated DCs further express OX40L to interact with OX40 expressed by naive CD4⁺ T cells and primed the naive CD4⁺ T cells for differentiation into Th2 cells, which are involved in immune response to human allergic diseases

through the production of IL-4, IL-5, and IL-13. All data on mRNA expression are provided separately in Table 1.

DISCUSSION

We show the representative images of TSLP's IHC staining of the conjunctival tissue of patients with VKC and a control at a similar site and at a magnification of $\times 400$ to indicate that TSLP was involved as an epithelium-derived proallergic cytokine. We also found that some TSLP staining

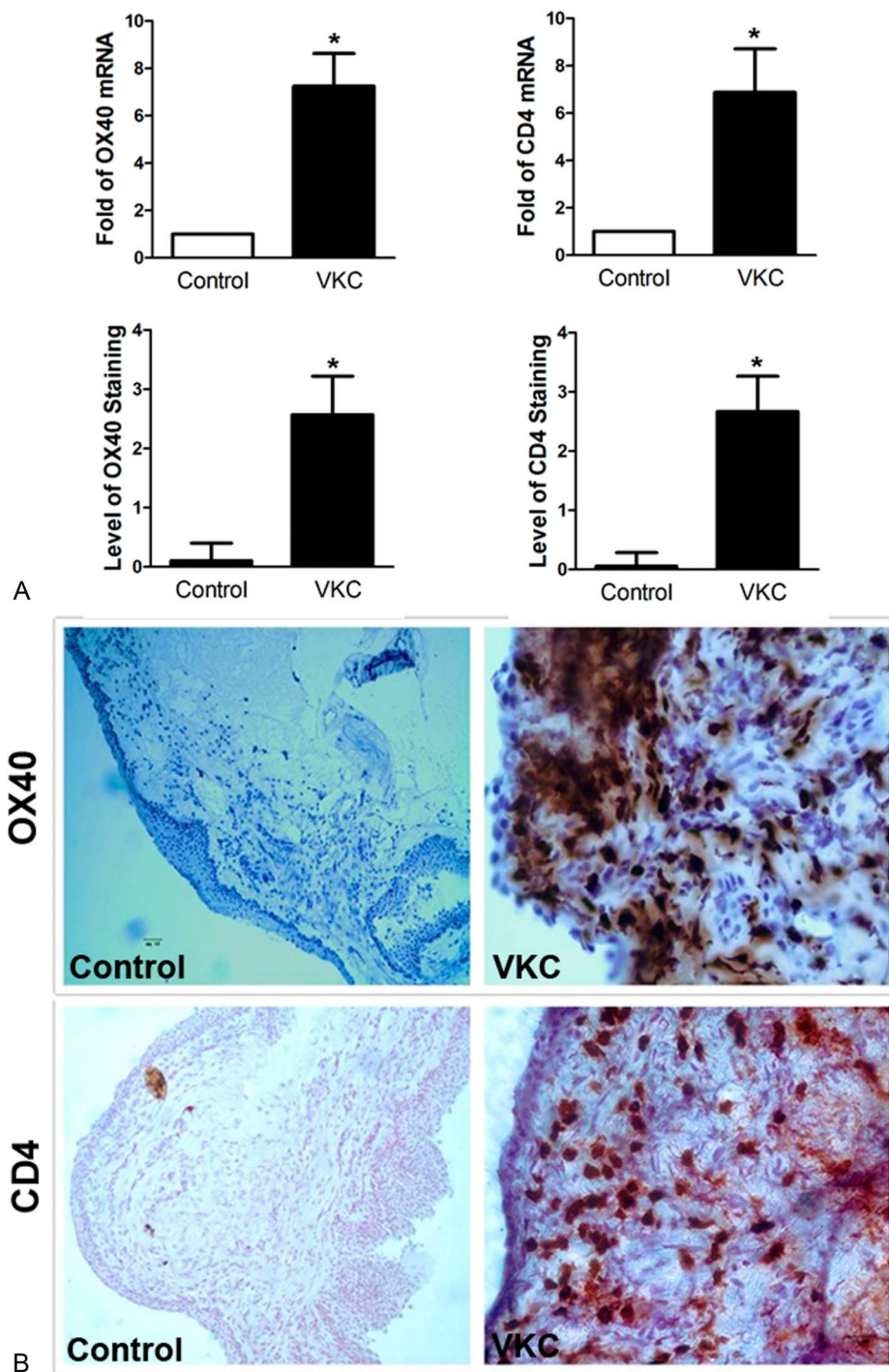


FIGURE 3. mRNA expression or immunoreactive level of OX40 and CD4 in the conjunctival epithelia of patients with VKC, with nonallergic tissue as controls. A, mRNA expression levels of OX40 and CD4 are indicated as a relative fold in the conjunctival epithelia of patients with VKC over the controls, which were detected by RT and real-time PCR, with GAPDH as an internal control. (* $P < 0.05$; $n = 6$). B, Representative images of OX40 and CD4 immunohistochemical staining of conjunctival tissue of patients with VKC (original magnification, $\times 400$), with nonallergic tissue as a negative control (original magnification, $\times 100$).

beneath the epithelium was accompanied with a high mRNA level of TSLP. It is reported that inflammatory cells such as macrophages, mast cells, and neutrophils were observed in TSLP-positive responses in the allergen-challenged dermis obtained from atopic subjects.¹⁵ The result of IHC staining except for TSLP at a magnification of $\times 100$ in controls and $\times 400$ in patients with VKC shows significant difference in their outcomes. We selected patients with pterygium or

conjunctivochalasis as controls because we could obtain the redundant conjunctival tissue after surgical treatment. To minimize the confounding variables from ages, we tried to choose the conjunctival samples from young donors in the eye bank. The conjunctival epithelium is responsible for natural barriers and inflammation in synergy with the lacrimal film through expression of adhesion factor, surface antigen, and cytokines.^{16,17} Recent studies have indicated that TSLP,

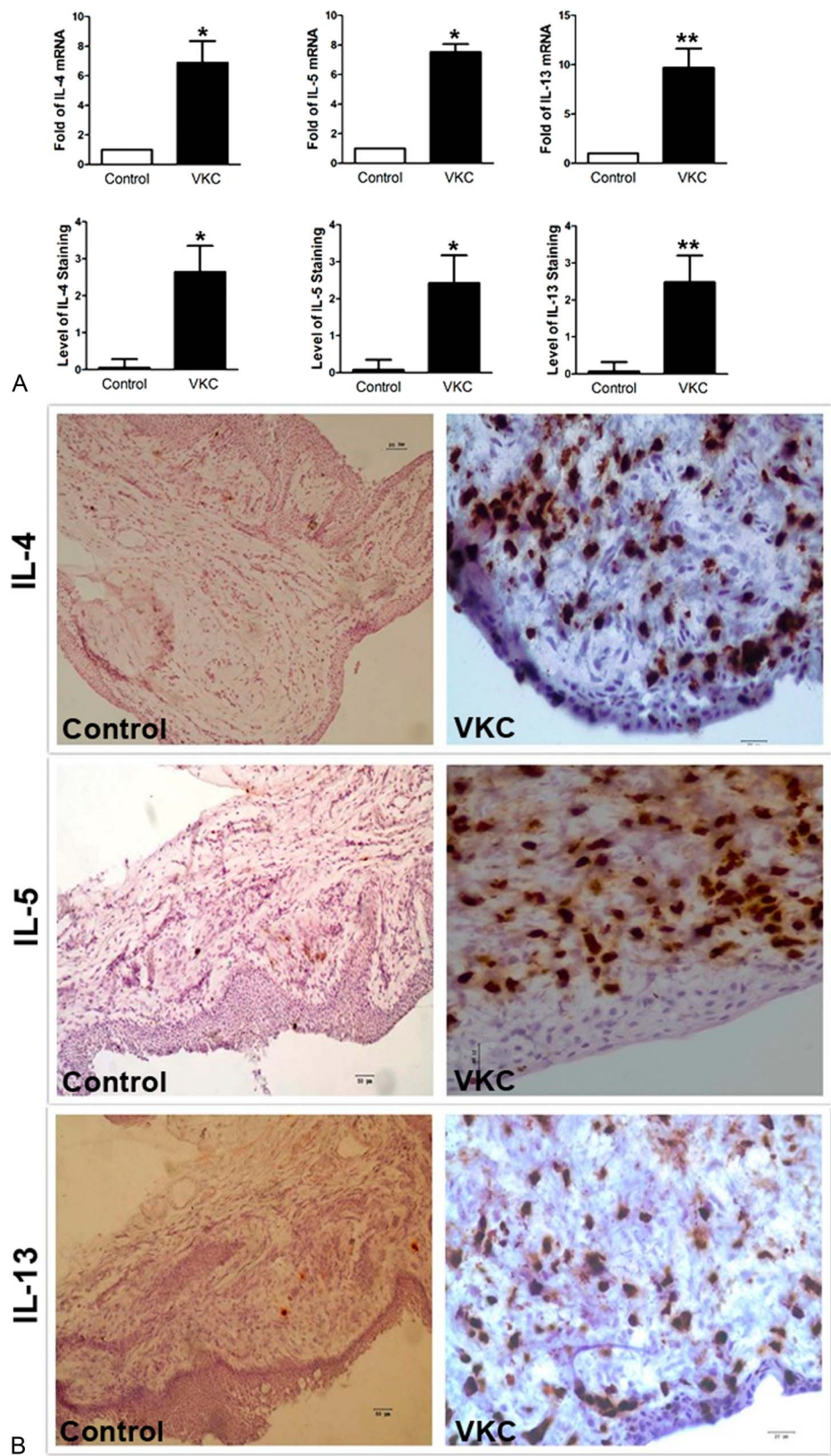


FIGURE 4. mRNA expression and immunoreactive levels of IL-4, IL-5, and IL-13 in the conjunctival epithelia of patients with VKC, with nonatopic tissue as controls. A, mRNA expression levels of IL-4, IL-5, and IL-13 are indicated as a relative fold in the conjunctival epithelia of patients with VKC patients over the controls, which were determined by RT and real-time PCR, with GAPDH as an internal control. (* $P < 0.05$; ** $P < 0.01$, $n = 6$). B, Representative images of immunohistochemical staining for IL-4, IL-5, and IL-13 of the conjunctival tissue of patients with VKC (original magnification, $\times 400$), with nonallergic tissue as a negative control, (original magnification, $\times 100$).

TABLE 1. mRNA Expression of TSLP Signaling Molecules and Th2 Cytokines in Giant Papillae

	TSLP (mean ± SD)	OX40L (mean ± SD)	OX40 (mean ± SD)	CD4 (mean ± SD)	IL-4 (mean ± SD)	IL-5 (mean ± SD)	IL-13 (mean ± SD)
Giant papillae (n = 6)	9.63 ± 0.99	7.60 ± 1.79	7.25 ± 1.38	6.88 ± 1.84	6.89 ± 1.46	8.42 ± 1.55	9.69 ± 1.94
Control (n = 6)	1	1	1	1	1	1	1
P	0.002	0.004	0.01	0.02	0.018	0.011	0.0007

an IL-7-like cytokine expressed by the epithelium and mast cells, plays an important role in initiation of allergic diseases such as atopic dermatitis and airway inflammation.^{9,18,19} Recent basic reviews have reported the mechanism of TSLP involved in allergic ocular diseases in human biopsies and cultured cells.^{20,21} We observed that TSLP-immunopositive cells primarily infiltrated the epithelium layer (Fig. 3A); this finding was consistent with a previous report.^{4,12}

The expression of TSLPR is observed in several types of cells such as DCs.^{22,23} Activated DCs migrate as antigen presenting cells (APCs) to secondary lymphoid organs, such as draining lymph nodes, to regulate immune function through interaction with T lymphocytes.²⁴ TSLP-activated human CD11c⁺ DCs express OX40L and produce Th2 chemokines: thymus and activation-regulated chemokine and macrophage-derived chemokine, but neither IL-12 nor proinflammatory cytokines.^{5,25} Furthermore, we detected the apparent immunopositive staining of CD11c and OX40L in the substantia propria. This finding supports the reports of Zheng et al and Li et al,^{10,11} who proposed TSLP/OX40L/OX40 response pathways in a murine model of AC induced by an SRW pollen. Activation of naive CD4⁺T cells requires both interaction of the T-cell receptor with the MHC-peptide complex on the APCs as the first signal and essential costimulatory interactions as the second signal provided by the APC.²⁶

The specificity of T-cell responses is managed by the antigen-specific T-cell receptor. However, complete T-cell activation generally exists only in the presence of additional interactions between costimulators such as OX40/OX40L.²⁷ As a member of the tumor necrosis factor receptor super family, OX40 is an essential costimulatory receptor that is not constitutively expressed in naive T cells but is induced after antigen recognition.²⁸ CD4⁺ T cells triggered by OX40 can proliferate and differentiate into effector T cells and memory T cells.²⁹ OX40 costimulation is demonstrated to prime Th2 differentiation or produce Th2-related cytokines in vitro, and OX40-OX40L interactions also play an important role in Th2-related signaling in vivo as shown in the experimental models of asthma.^{28,30–33} In the present study, we found infiltration of CD4⁺Th2 cells according to a marked immunoreactivity or mRNA expression of OX40, CD4, IL-4, IL-5, and IL-13 in the giant papillae. These findings suggest that in human AC, TSLP-activated DCs seem to be involved in differentiation of CD4⁺ T cells into Th2 cells through OX40L–OX40 interaction that produces IL-4, IL-5, and IL-13.

It has been reported that the proinflammatory (TNF-α and IL-1α) and Th2-related (IL-4 and IL-13) cytokines slightly activate expression and production of TSLP. IL-4 and IL-13 have synergistic effects with PolyI:C to promote TSLP expression in human skin keratinocytes.³⁴ Poly I:C,

flagellin, or TNF-α also induce nuclear translocation of the NF-κβ p65 protein; hence, Ma et al²¹ used NF-β inhibitor quinazoline to block NF-κβ p65 nuclear translocation and further decrease TSLP expression and production induced by Poly I:C in human corneal tissue. Previous reports show that poly I:C-induced IL-6 production in mononuclear cells requires an acidic environment at pH 5.7 to 6.5, and bafilomycin A was used to block poly I:C-induced IL-6 production by inhibiting the endosomal proton pump.³⁵ It is well known that bafilomycin A is quite toxic and hence has limited clinical use; therefore, we hypothesize that chloroquine, a competing basic compound that increases endosomal pH, or omeprazole, a proton pump inhibitor, which are widely used to treat malaria and gastric diseases, respectively, may be useful for inhibiting poly I:C-mediated TSLP expression in human tissue.

Concluding Remarks

We found that immunopathogenesis of TSLP is associated with Th2 signaling at protein and mRNA levels in human conjunctival tissue. This study may provide critical targets based on which immunological therapies could be used to treat AC. However, this study does not provide effective evidence to confirm the Th2 differentiation pathway from CD4⁺-naive T cells in human cervical lymph nodes. Further studies are essential to investigate the dynamic expression of TSLP in acute and chronic AC and the direct effect of DCs on Th2 differentiation in lymph nodes.

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