

Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4–dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways

De-Quan Li, MD, PhD,^{a,*} Lili Zhang, MD,^{a,b,*} Stephen C. Pflugfelder, MD,^a Cintia S. De Paiva, MD,^a Xiaobo Zhang, MD,^a Guiqiu Zhao, MD, PhD,^b Xiaofen Zheng, MD,^a Zhitao Su, MD,^a and Yangluowa Qu, MS^a *Houston, Tex, and Qingdao, China*

Background: Allergic diseases affect a large population. Pollen, an ubiquitous allergen, is the trigger of seasonal rhinitis, conjunctivitis, and asthma, as well as an exacerbating factor of atopic dermatitis. However, the underlying mechanism by which pollen induces thymic stromal lymphopoietin (TSLP)–triggered allergic inflammation through epithelial innate immunity is largely unknown.

Objective: We sought to explore whether short ragweed (SRW) pollen induces TSLP/OX40 ligand (OX40L)/OX40 signaling through Toll-like receptor (TLR) 4–dependent pathways in patients with allergic disease.

Methods: Three models were used for this study, a well-characterized murine model of allergic conjunctivitis induced by SRW pollen, a topical challenge model on the murine ocular surface, and a culture model of primary human corneal epithelium exposed to aqueous extract of defatted SRW pollen (SRWe).

Results: The topical challenges with SRW pollen generated typical allergic conjunctivitis in BALB/c mice. Clinical signs, stimulated TSLP/OX40L/OX40 signaling, and T_H2 cytokine levels in the ocular mucosa and draining cervical lymph nodes were significantly reduced or eliminated in TLR4-deficient (*Tlr4-d*) or myeloid differentiation primary response gene 88 (*MyD88*) knockout (*MyD88*^{−/−}) mice compared with those seen in their wild-type littermates. SRWe stimulated TSLP production by ocular epithelia in wild-type but not *Tlr4-d* or *MyD88*^{−/−} mice. SRWe-stimulated TSLP was blocked by TLR4 antibody and nuclear factor κB inhibitor in murine and human corneal epithelia.

Conclusion: For the first time, we have shown that SRW pollen, acting as a functional TLR4 agonist, initiates TLR4-dependent TSLP/OX40L/OX40 signaling, which triggers T_H2-dominant allergic inflammation. These findings shed light on the understanding of mucosal epithelial innate immunity and create new therapeutic targets to cure allergic diseases. (*J Allergy Clin Immunol* 2011;■■■■:■■■-■■■.)

Key words: Allergy, pollen, conjunctivitis, Toll-like receptor, thymic stromal lymphopoietin, innate immunity

Allergic diseases, such as seasonal allergy, asthma, and atopic dermatitis, affect up to 20% to 30% of the population in industrialized countries, and up to 50% of these subjects report ocular allergic manifestations.^{1–3} The incidence of allergies has increased steadily over the past 30 years. T_H2-dominant hypersensitivity is a major contributor to allergic inflammatory disease, but the underlying mechanism for initiation of this adaptive immune disorder by mucosal epithelia remains a relative mystery. The molecular triggers for T_H2-induced allergic inflammation were not clear until studies identified a novel epithelium-derived proallergic cytokine, thymic stromal lymphopoietin (TSLP), which activates myeloid dendritic cells (DCs) to produce OX40 ligand (OX40L), which triggers a T_H2 inflammatory response. Compelling evidence demonstrates that TSLP represents one of the master switches initiating allergic inflammation at the interface between epithelial cells and DCs and has a determinant role in atopic dermatitis and asthma.^{4–6} The role of TSLP in ocular allergic inflammation has also been reported,^{7,8} including in our study. However, it is not clear whether TSLP is induced by environmental allergens, such as pollen, in patients with allergic diseases.

Pollen, an ubiquitous allergen, is the trigger of seasonal rhinitis, conjunctivitis, and asthma, as well as an exacerbating factor of atopic dermatitis. However, the underlying molecular mechanism by which pollen induces T_H2-dominant allergic inflammation through epithelial innate immunity pathways is largely unknown. Substantial evidence now indicates that epithelial cells are central participants in innate and adaptive immune responses.^{9–11} The innate immune response relies on evolutionarily ancient germline-encoded receptors, the pattern-recognition receptors,¹² which recognize highly conserved microbial components. A breakthrough in understanding the ability of the innate immune system to rapidly recognize pathogens is the discovery of Toll-like receptors (TLRs), the most important family among the pattern-recognition receptors. A variety of TLRs have been identified in skin and mucosal epithelia, including ocular surface

From ^athe Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, Houston, and ^bthe Department of Ophthalmology, Affiliated Hospital of Qingdao University Medical College, Qingdao.

*These authors contributed equally to this work.

Supported by Department of Defense CDMRP PRMRP grant FY06 PR064719 (DQL), National Institutes of Health grant EY11915 (SCP), an unrestricted grant from Research to Prevent Blindness, the Oshman Foundation, and the William Stamps Farish Fund.

Disclosure of potential conflict of interest: D.-Q. Li receives research support from the Department of Defense. S. C. Pflugfelder receives research support from the National Institutes of Health. The rest of the authors have declared that they have no conflict of interest.

Received for publication May 9, 2011; revised June 21, 2011; accepted for publication June 28, 2011.

Reprint requests: De-Quan Li, MD, PhD, Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, 6565 Fannin St, NC-205, Houston, TX 77030. E-mail: dequanl@bcm.tmc.edu.

0091-6749/\$36.00

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doi:10.1016/j.jaci.2011.06.041

Abbreviations used

CLN:	Cervical lymph node
DC:	Dendritic cell
EAC:	Experimental allergic conjunctivitis
HCEC:	Human corneal epithelial cell
<i>MyD88</i> :	Myeloid differentiation primary response gene 88
NF- κ B:	Nuclear factor κ B
OX40L:	OX40 ligand
RT:	Reverse transcription
SRW:	Short ragweed
SRWe:	Aqueous extract of defatted short ragweed pollen
TLR:	Toll-like receptor
<i>Tlr4-d</i> :	TLR4-deficient
TSLP:	Thymic stromal lymphopoietin
TSLPR:	Thymic stromal lymphopoietin receptor

epithelium,^{13,14} whereas the role of ligand-stimulated epithelial TLR signaling in regulating innate and adaptive immunity remains to be elucidated.

On the basis of the observation that we and other groups have made¹⁵⁻¹⁷ that TSLP is mainly induced through the TLR-mediated innate response in epithelia exposed to microbial products, we hypothesized that pollen, such as *Ambrosia* species short ragweed (SRW), might serve as a functional TLR4 agonist that induces production of TSLP, an epithelium-derived proallergic cytokine, through innate immune signaling to trigger T_H2-dominant allergic inflammation. The ocular surface is the most common site for the development of allergic inflammation because allergens can easily deposit directly onto the exposed mucosal surface of the eye. The present study was conducted using a well-characterized murine model of allergic conjunctivitis induced by SRW pollen in BALB/c, *Tlr4-d*, and myeloid differentiation primary response gene 88 (*MyD88*) knockout mice, as well as a murine topical ocular surface challenge model and a culture model of primary human corneal epithelial cells (HCECs) exposed to an aqueous extract of defatted SRW pollen (SRWe), to uncover the novel molecular signaling pathways involved in pollen-induced allergic inflammation.

METHODS**Reagents**

Ambrosia artemisiifolia SRW pollen and lyophilized SRWe were purchased from Greer Laboratories (Lenoir, NC). The amount of endotoxin in these SRW preparations was 0.00038 ± 0.00026 EU/ μ g of protein, as measured by using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ). Imject Alum was from Pierce Biotechnology (Rockford, Ill). LPS from *Escherichia coli* was from Sigma-Aldrich (St Louis, Mo).

Animals

The animal research protocol was approved by the Center for Comparative Medicine at Baylor College of Medicine. All animals used in this study were maintained in specific pathogen-free conditions in microisolator cages and were treated in accordance with the guidelines provided in the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. BALB/c and BALB/c-based homozygous *Tlr4-d* (C.C3-*Tlr4*^{Lps-d/J}) mice aged 6 to 8 weeks of both sexes were purchased from the Jackson Laboratory (Bar Harbor, Me). Age- and sex-matched mice were used for each experiment. Heterozygous *MyD88*^{+/-} knockout mice on a C57BL/6 background were kindly provided by Dr Shizuo Akira (Research

Institute for Microbial Disease, Osaka University, Japan) through Dr Eric Pearlman (Department of Ophthalmology and Visual Sciences, Case Western Reserve University, Cleveland, Ohio).¹⁸ The genotyping was performed by means of PCR of the tail DNA with 3 specific primers (MyD88F, TGGCATGCCTCCATCATAGTTAACC; MyD88R, GTCAGAAACAAC-CACCACCATGC; MyD88R-Neo, ATCGCCTTCTATCGCCTTCTTGACG) by using a previously described method.¹⁹ The age- and sex-matched *MyD88*^{+/-} littermates of the *MyD88*^{-/-} mice grown to 8 to 10 weeks were used for experiments.

Murine model of experimental allergic conjunctivitis induced by SRW pollen

The murine experimental allergic conjunctivitis (EAC) model was induced by using previously reported methods,^{20,21} with modifications.⁸ In brief, mice were immunized with 50 μ g of SRW pollen (Greer Laboratories) in 5 mg of Imject Alum (Pierce Biotechnology) by means of footpad injection on day 0. Allergic conjunctivitis was induced by means of topical application of 1.5 mg of SRW pollen suspended in 10 μ L of PBS (pH 7.2) into each eye once a day from days 10 to 12. PBS eye drop–treated SRW-sensitized and untreated mice were used as control animals. Animals were examined clinically for signs of an immediate hypersensitivity responses 20 minutes after each topical challenge with SRW pollen. A clinical scoring scheme described by Magone et al²⁰ was used to evaluate chemosis, conjunctival redness, lid edema, and tearing. On day 13, 24 hours after the last SRW challenge, the corneal epithelium, conjunctiva, cervical lymph nodes (CLNs), and whole eyes were harvested for gene expression assays and histopathological studies. Four mice per group were used in each experiment, and the same experiments were repeated 4 times.

Murine model for TSLP induction by means of ocular surface challenge with SRWe

SRWe (Greer Laboratories) was topically instilled at 150 μ g/5 μ L per eye or 5 μ L of PBS as a control for 4 to 24 hours. SRWe was applied without or with preinstilled rat anti-mouse TLR4 antibody (1 μ g/5 μ L per eye; eBioscience, San Diego, Calif) or its isotype, rat IgG_{2a}. In *Tlr4-d*, *MyD88*^{+/-}, and *MyD88*^{-/-} mice, LPS from *E coli* (Sigma-Aldrich) at 5 μ g/5 μ L per eye was used as a positive control for SRWe treatment. Tissue specimens, including corneal epithelium and conjunctival tissues, were collected at different time points and stored at -80°C before use for TSLP mRNA and protein assays. Four mice per group were used in each experiment, which was repeated 4 times.

In vitro culture models for TSLP induction by primary HCECs exposed to SRWe

Fresh human corneal tissue (<72 hours after death) from donors aged 19 to 67 years without allergic conditions was obtained from the Lions Eye Bank of Texas (Houston, Tex). HCECs were cultured in 12-well plates by using explants from corneal limbal rims in a supplemented hormonal epidermal medium containing 5% FBS by using our previous methods.^{22,23} Confluent corneal epithelial cultures were switched to serum-free supplemented hormonal epidermal medium and treated for different time periods (4–48 hours) with different concentration of SRWe (0.1–50 μ g/mL). SRWe at 10 μ g/mL was applied without or with preincubated mouse anti-human TLR4 antibody (10 μ g/mL, eBioscience); its isotype, murine IgG_{2a} κ ; or quinoxaline, a nuclear factor κ B (NF- κ B) activation inhibitor (10 μ mol/L, EMD Biosciences, San Diego, Calif). Each experiment was repeated 4 times.

Total RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA was isolated from murine tissue specimens and human cells by using a Qiagen RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol and quantified by using a NanoDrop ND-1000

Spectrophotometer (Thermo Fisher Scientific, Inc, Wilmington, Del) and stored at -80°C . The first-strand cDNA was synthesized by means of reverse transcription (RT) from 1 μg of total RNA by using Ready-To-Go You-Prime First-Strand Beads, as previously described.^{24,25} Real-time PCR was performed with specific primers and probes by using TaqMan gene expression assays and TaqMan master mix (Applied Biosystems, Foster City, Calif) in the Mx3005P QPCR System (Stratagene, La Jolla, Calif). TaqMan Gene Expression Assay IDs for murine glyceraldehyde-3-phosphate dehydrogenase, TSLP, thymic stromal lymphopoietin receptor (TSLPR), OX40L, CD11c, OX40, CD4, IL-4, IL-5, IL-13, and IFN- γ were Mm99999915, Mm00498739, Mm00497362, Mm00442039, Mm00498698, Mm00445259, Mm00437214, Mm00442754, Mm0099999063, Mm00434204, and Mm00801778_m1, respectively. The results were analyzed by using the comparative threshold cycle method²⁶ and normalized by glyceraldehyde-3-phosphate dehydrogenase, as previously reported.^{25,27}

ELISA

ELISA MAX Deluxe kits for human and murine TSLP were from BioLegend (San Diego, Calif). The TSLP protein levels in the cell lysates from murine ocular specimens and in the supernatants from HCECs were measured according to the manufacturer's protocols, similar to our previous report.¹⁵

Immunohistochemical and immunofluorescent staining

The eyes and lids of the mice in each group were excised, embedded in OCT compound (VWR, Suwanee, Ga), and flash-frozen in liquid nitrogen. Sagittal 8- μm cryosections from murine globes were cut with a cryostat (HM 500; Micron, Waldorf, Germany) and stored at -80°C before use. Immunohistochemical and immunofluorescent staining was performed as previously described.^{28,29} The primary antibodies used for this study included the following: rat anti-mouse CD4, hamster anti-mouse CD11c, and rat anti-mouse IL-4 from BD PharMingen (San Jose, Calif); rat anti-mouse OX40L and rat anti-mouse/human IL-5 were from BioLegend; rabbit anti-human TSLP from ProSci, Inc (Poway, Calif); goat anti-mouse TSLPR and OX40 from Santa Cruz Biotechnology (Santa Cruz, Calif); and rabbit anti-human IL-13 from AbD Serotec (Raleigh, NC).

Statistical analysis

The Student *t* test was used to compare differences between 2 groups. One-way ANOVA was used to make comparisons among 3 or more groups, followed by the Dunnett *post hoc* test. *P* values of less than .05 were considered statistically significant.

RESULTS

TSLP/OX40L/OX40 signaling in a $\text{T}_\text{H}2$ -dominant EAC murine model induced by SRW pollen

The EAC model was induced in BALB/c mice sensitized and topically challenged with SRW pollen (SRW mice), with PBS-treated (PBS mice) and untreated mice as control groups, to explore the role of TSLP and its signaling molecules in patients with pollen-triggered allergic inflammation. Repeated topical challenges with SRW allergen generated typical signs that mimic human allergic conjunctivitis, including lid edema, conjunctival redness, chemosis, tearing, and frequent scratching of the eye lids. These signs and scratching behavior were detected within 20 minutes and persisted for at least 8 hours after SRW challenge. Some mice had signs that persisted for 24 hours.

Evaluated by means of RT-quantitative real-time PCR (qPCR), TSLP mRNA expression was significantly upregulated in the corneal (1.95 ± 0.22 -fold) and conjunctival (2.53 ± 0.58 -fold)

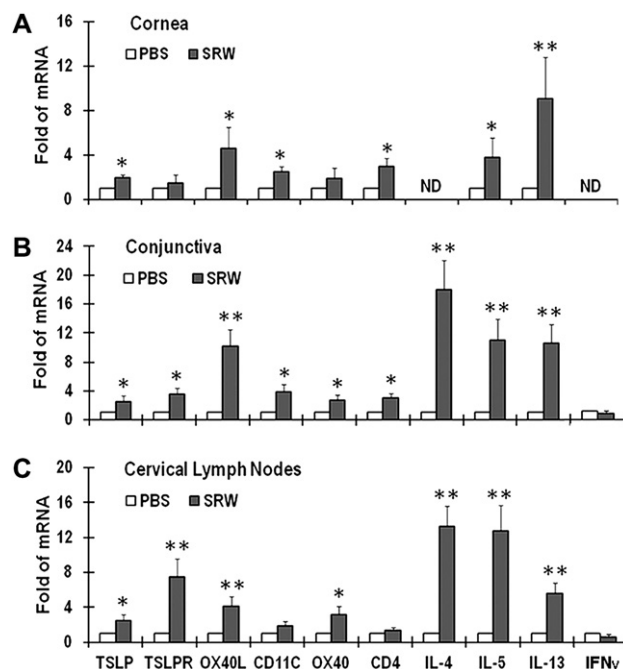


FIG 1. Stimulated expression of TSLP signaling molecules and $\text{T}_\text{H}2$ cytokines in BALB/c mice with SRW pollen-induced EAC. The mRNA expression of TSLP and its downstream signals in DCs (TSLPR, OX40L, and CD11c) and T cells (OX40 and CD4) and the expression of $\text{T}_\text{H}1$ (IFN- γ) and $\text{T}_\text{H}2$ cytokines (IL-4, IL-5, and IL-13) by corneal epithelium (A), conjunctiva (B), and CLNs (C) were evaluated by using RT-qPCR in BALB/c mice with EAC, with PBS-treated mice as controls. Results shown are means $\pm$ SDs of 4 independent experiments. **P* < .05 and ***P* < .01 (*n* = 4). ND, Not detectable.

epithelia (all *P* < .05) from SRW mice compared with the PBS-treated and untreated control groups (Fig 1, A and B). Immunohistochemical staining confirmed the increase of TSLP production in the SRW-challenged eyes. TSLP immunoreactivity was absent or weak in the ocular surfaces of the PBS-treated and untreated groups. The corneal and conjunctival epithelia in SRW-challenged mice displayed much stronger TSLP staining throughout the entire epithelium, especially in the superficial epithelial layers of the conjunctiva (Fig 2, A). These data indicate that TSLP mRNA expression and protein production by ocular surface epithelia increased in this SRW-induced EAC model.

The accumulation of CD11c⁺ DCs on the ocular surface was detected in this EAC model by means of RT-qPCR and immunostaining. As shown in Fig 1, A and B, the increased mRNA levels of CD11c, TSLPR, and OX40L were observed in the ocular surface, especially in conjunctival tissues, where their transcripts increased to 4.16-, 3.67-, or 10.19-fold, respectively (*P* < .05, *P* = .05, or *P* < .01), from SRW-challenged mice compared with PBS mice. The large number of CD11c⁺ DCs accumulated in the SRW-challenged ocular surface, primarily in the stroma subjacent to the conjunctival epithelium (Fig 2, A). These results suggest that in SRW-induced EAC, the ocular surface was infiltrated with TSLP-activated DCs that express TSLPR and produce OX40L.

A $\text{T}_\text{H}2$ -dominant inflammatory response was clearly observed on the ocular surface in SRW-challenged mice. T-lymphocyte infiltration was evidenced by the markedly increased CD4 mRNA level (Fig 1, A and B) and immunopositive (CD4⁺) cells in the ocular surface, especially in the conjunctiva from SRW-challenged mice (Fig 2, A) compared with PBS-treated

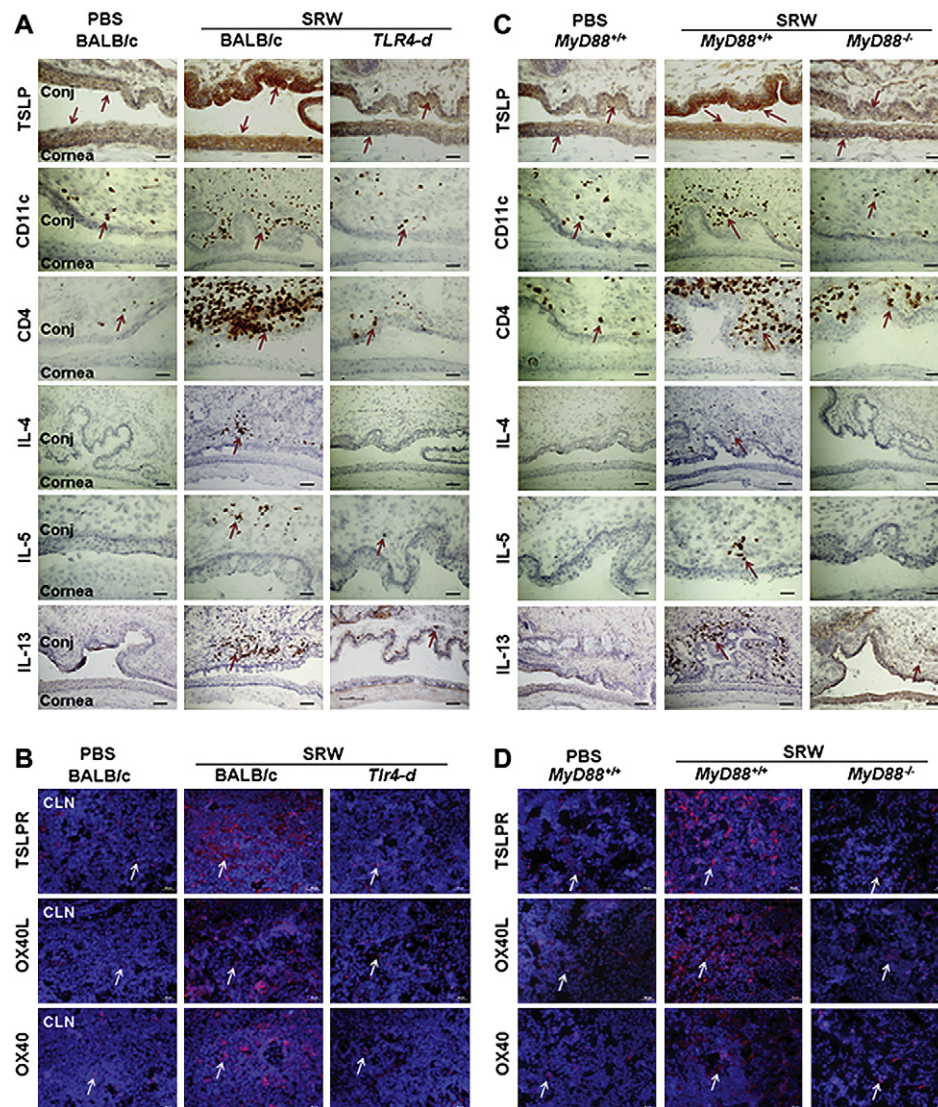


FIG 2. Stimulated production of TSLP signaling proteins and T_H2 -dominant inflammation in an SRW-induced EAC model requires TLR4 and MyD88. **A** and **C**, Immunohistochemical staining of TSLP signaling molecules and T_H2 cytokines on the cornea and conjunctiva (Conj) of wild-type and *Tlr4-d* BALB/c mice (Fig 3, A) and C57BL/6-based wild-type *MyD88*^{+/+} and *MyD88*^{-/-} mice (Fig 3, C) challenged with SRW pollen, with PBS-treated mice as controls. **B** and **D**, Immunofluorescent staining of TSLP-activated signals, TSLPR, OX40L, and OX40 in CLNs of different strains, as described above. Bar = 20 μ m. Arrows, Red or red-brown positive staining signals.

mice. These $CD4^+$ T cells appear to be in the T_H2 lineage because the transcripts of 3 key T_H2 cytokines, IL-4, IL-5, and IL-13, were expressed at significantly higher levels, whereas mRNA expression of the T_H1 cytokine IFN- γ was at lower levels in corneal ($P < .05$ or $P < .01$) and conjunctival (all $P < .01$) tissues of SRW-challenged mice than in the PBS-treated control group (Fig 1, A and B). Immunostaining data confirmed that the conjunctival stroma was largely infiltrated by IL-4-, IL-5-, and IL-13-producing T_H2 cells (Fig 2, A).

The ocular surface draining CLNs were collected for evaluation to confirm the presence of TSLP signaling in mice with SRW-induced EAC. Compared with PBS-treated mice, mRNA levels of TSLPR and OX40L were significantly stimulated to 7.47- and 4.07-fold, respectively (both $P < .01$), whereas CD11c expression slightly increased in CLNs from SRW-challenged mice,

indicating that $CD11c^+$ DCs were markedly activated by ocular epithelium-derived TSLP. Interestingly, the mRNA levels of OX40 (3.39-fold, $P < .05$) and the T_H2 cytokines IL-4, IL-5, and IL-13 (13.24-, 12.78-, and 5.54-fold, respectively, all $P < .01$) were significantly increased, whereas IFN- γ mRNA expression was lower and CD4 expression did not change in the CLNs from SRW-challenged mice, indicating naive $CD4^+$ T cells were largely differentiated to T_H2 cells primed by OX40L produced by TSLP-activated DCs.⁴ The increased numbers of TSLPR⁺, OX40L⁺, or OX40⁺ cells in the draining CLNs of SRW-challenged mice was confirmed by dramatically increased cell membrane and cytoplasmic immunoreactivity of these 3 signaling proteins in CLNs (Fig 2, B). All these results suggest a critical role of TSLP/OX40L/OX40 signaling in T_H2 -dominant EAC induced by SRW pollen.

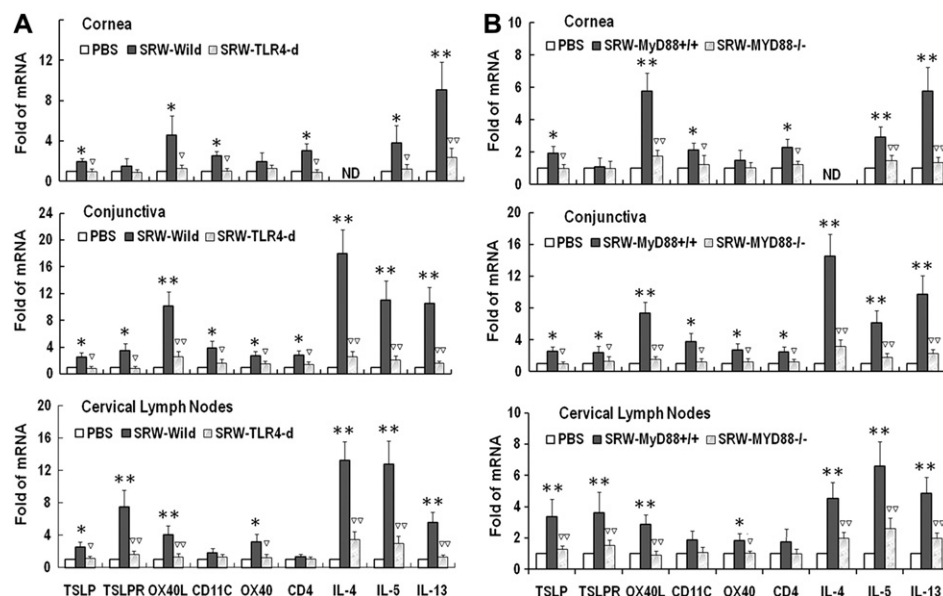


FIG 3. Stimulated expression of TSLP signaling molecules and T_H2 cytokines in an SRW-induced EAC model requires TLR4 and MyD88. The mRNA expression of TSLP signaling molecules and T_H2 cytokines by corneal epithelium, conjunctiva, and CLNs in an EAC model with wild-type and *Tlr4-d* BALB/c mice (**A**) and with C57BL/6-based *MyD88*^{+/+} and *MyD88*^{-/-} mice (**B**) sensitized and topically challenged by SRW pollen is shown, with PBS-treated mice as controls. Results shown are means $\pm$ SDs. * $P < .05$ and ** $P < .01$ ($n = 4$) compared with PBS-treated control animals; $\nabla P < .05$ and $\nabla\nabla P < .01$ ($n = 4$) compared with wild-type mice.

SRW pollen-induced TSLP-triggered allergic inflammation through a TLR4-dependent innate immune response

TLR-mediated TSLP induction has been recognized.^{16,17,30} We have demonstrated that TSLP was largely induced by specific TLR ligands in HCECs.¹⁵ MyD88 is a universal adapter protein necessary for response to all TLRs except TLR3.^{31,32} *Tlr4-d* (C.C3-Tlr4^{Lps-d/J}) and *MyD88*^{-/-} mice have been used to identify TLR4-mediated signaling.^{33,34} To explore whether SRW pollen stimulates TSLP through a TLR4-dependent innate response, we sensitized and topically challenged the *Tlr4-d* and *MyD88*^{-/-} mice with SRW pollen.

Compared with wild-type BALB/c mice, the ocular allergic signs, stimulated TSLP/OX40L/OX40 signaling, and T_H2 -dominant inflammatory response by ocular mucosa, especially conjunctival tissues, were dramatically reduced or eliminated in BALB/c-based *Tlr4-d* mice. As shown in Fig 3, A, the mRNA levels of TSLP, OX40L, and OX40 and the T_H2 cytokines (IL-4, IL-5, and IL-13) were significantly stimulated in the cornea, conjunctiva, and draining CLNs from wild-type BALB/c mice but not in those from *Tlr4-d* mice. The immunostaining results confirmed that SRW pollen did not stimulate TSLP and its downstream molecules or a T_H2 response in ocular mucosal tissues (Fig 2, A) and draining CLNs (Fig 2, B) of *Tlr4-d* mice. These findings suggest that TLR4-dependent TSLP signaling was involved in the SRW pollen-induced allergic inflammation.

The SRW topical challenges triggered the typical allergic signs and scratching behavior in wild-type *MyD88*^{+/+} mice, although these were less severe than those seen in BALB/c mice. The expression of TSLP and its signaling molecules, TSLPR, OX40L, and OX40, as well as the T_H2 cytokines IL-4, IL-5, and IL-13, was significantly stimulated in the cornea, conjunctiva, and

CLNs from SRW-challenged wild-type *MyD88*^{+/+} mice at both the mRNA (Fig 3, B) and protein (Fig 2, C and D) levels. Clinical allergic signs and stimulated production of TSLP signaling molecules and T_H2 cytokines were dramatically reduced or eliminated in SRW-challenged *MyD88*^{-/-} mice, as evaluated by means of RT-qPCR (Fig 3, B) and immunostaining (Fig 2, C and D). These findings suggest that the MyD88 pathway is involved in the TLR4-dependent TSLP signaling induced by SRW pollen.

SRWe-induced TSLP expression and production by murine ocular surface epithelia through TLR4-dependent innate pathway

To confirm that SRW pollen directly stimulates TSLP production by ocular mucosal epithelia through the TLR4-dependent innate immunity pathway, we created a topical challenge murine model by using SRWe at 150 μ g/5 μ L per eye for 4 to 24 hours. TSLP mRNA levels were induced as early as 4 hours and reached peak levels at 8 hours, and TSLP protein levels increased in 24 hours in ocular epithelia exposed to SRWe. As shown in Fig 4, A and B, SRWe significantly stimulated TSLP mRNA by 2-fold in corneal and conjunctival epithelia (both $P < .05$) and its protein levels by 3.2-fold (from 150.2 $\pm$ 37.6 pg/mg cellular protein to 480.00 $\pm$ 89.6 pg/mg) in corneal epithelia and 2.2-fold (from 128.6 $\pm$ 29.8 pg/mg to 281.6 $\pm$ 19.3 pg/mg) in the conjunctiva in BALB/c mice when compared with untreated or PBS-treated control animals. The SRWe-stimulated TSLP was significantly blocked at both the mRNA and protein levels by a rat anti-mouse TLR4 antibody but not by its isotype, rat IgG_{2a}.

Interestingly, the SRWe topical challenge did not increase TSLP mRNA (Fig 4, C) and protein (Fig 4, D) levels in corneal

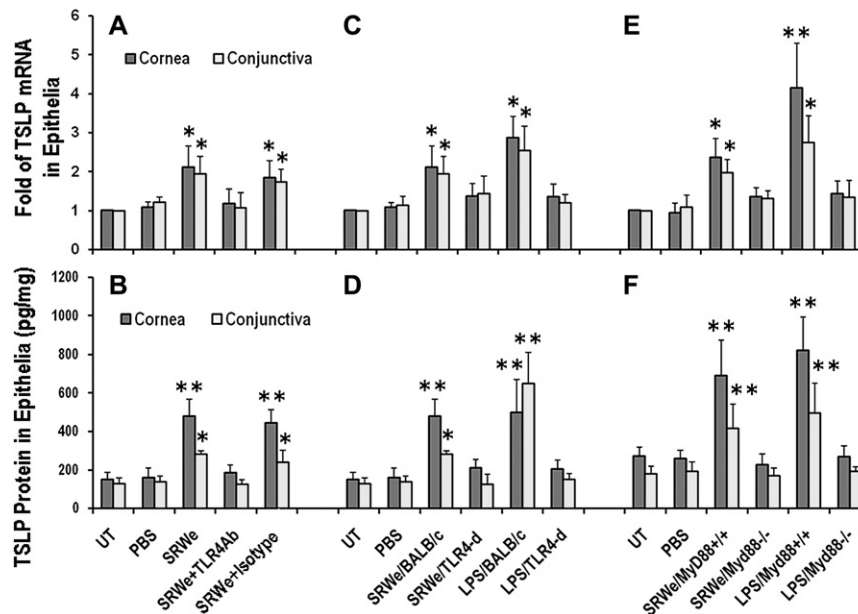


FIG 4. SRWe induces TSLP mRNA (A, C, and E) and protein (B, D, and F) by murine corneal and conjunctival epithelia through a TLR4- and MyD88-dependent pathway. Fig 4, A and B, BALB/c mice were topically administered SRWe at 150 μ g/5 μ L per eye without or with preinstilled rat anti-mouse TLR4 antibody (1 μ g/5 μ L per eye) or its isotype rat IgG_{2a}. Fig 4, C and D, TSLP induction by topically challenged SRWe or LPS (5 μ g/5 μ L per eye) in wild-type and *Tlr4-d* BALB/C mice. Fig 4, E and F, TSLP induction by means of topical challenge in wild-type *MyD88*^{+/+} and *MyD88*^{-/-} mice. Untreated or PBS-treated mice were used as controls. Results shown are means $\pm$ SDs (n = 4). *P < .05 and **P < .01 compared with PBS-treated controls.

and conjunctival epithelia of *Tlr4-d* mice. Similarly, LPS (5 μ g/5 μ L per eye) stimulated TSLP production in corneal and conjunctival epithelia of BALB/c mice but not in *Tlr4-d* mice. Furthermore, we applied this topical challenge model to *MyD88*^{-/-} mice and their wild-type controls. Interestingly, SRWe promoted TSLP production by ocular epithelia at both the mRNA (Fig 4, E) and protein (Fig 4, F) levels only in *MyD88*^{+/+} mice, but not in *MyD88*^{-/-} mice, a similar pattern to that observed after LPS topical challenge. Taken together, these data demonstrated that SRWe directly stimulates TSLP production in the ocular mucosal epithelia through a TLR4-dependent innate pathway.

SRWe induces TSLP expression and production by HCECs through TLR4 and NF- κ B signaling pathways

To explore whether this phenomenon occurs in human subjects, we investigated TSLP expression in human corneal epithelium. TSLP mRNA was upregulated at 4 hours, and its protein was detected at 24 hours in HCECs exposed to SRWe, which is consistent with our previous report.¹⁵ TSLP induction at the mRNA (Fig 5, A) and protein (Fig 5, B) levels was concentration-dependently stimulated by SRWe in primary HCECs. TSLP protein was barely detectable (4.83 ± 1.60 pg/ml) in the supernatants of normal HCEC cultures. SRWe at 10 μ g/mL increased the TSLP protein to 48.92 ± 4.23 pg/mL ($P < .01$), with the levels comparable with those after stimulation by 10 ng/mL TNF- α in HCECs.¹⁵ Interestingly, the SRWe (10 μ g/mL)-stimulated TSLP mRNA was significantly blocked by preincubation of cells with 10 μ g/mL neutralizing mAb against human TLR4 but not by its isotype, murine IgG_{2a} κ (Fig 5, C). Furthermore, SRWe-stimulated TSLP expression was also significantly

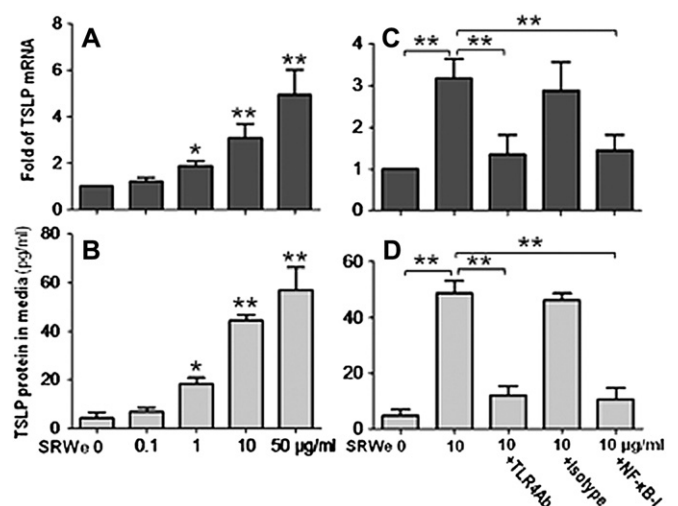


FIG 5. SRWe induces TSLP mRNA and protein by HCECs through TLR4 and NF- κ B signaling pathways. A and B, Primary HCECs were treated with 0.1 to 50 μ g/mL SRWe for 4 hours for TSLP mRNA or 48 hours for TSLP protein measurement in the supernatants. C and D, HCECs were preincubated with murine TLR4 antibody (10 μ g/mL), isotype murine IgG_{2a} κ , or the NF- κ B activation inhibitor quinazoline (NF- κ B-I, 10 μ mol/L) for 1 hour before adding 10 μ g/mL SRWe for 4 hours for TSLP mRNA or 48 hours for TSLP protein measurement in the supernatants. Results shown are means $\pm$ SDs (n = 4). *P < .05 and **P < .01.

inhibited by quinazoline, an NF- κ B activation inhibitor (Fig 5, C). These findings were confirmed by means of detection of increased TSLP protein levels, as shown in Fig 5, D. These data demonstrate that SRW induces TSLP production in HCECs through the TLR4 and NF- κ B innate signaling pathways.

DISCUSSION

Although there have been numerous studies on the development of allergen-induced inflammation, the mechanisms leading to resolution of allergic inflammation remain poorly understood. This represents an important challenge because failure to resolve allergen-driven inflammation potentially leads to recurrent or chronic allergic disease. Pollen is an ubiquitous allergen that affects a large population, and *A. artemisiifolia* SRW is the most widespread plant in North America. This study uncovered a previously unknown mechanism by which pollen induces TSLP-triggered T_H2 -dominant inflammation through a TLR4-dependent innate pathway by using multiple *in vivo* and *in vitro* models.

Traditionally, TLRs recognize conserved microbial components as ligands or agonists. Recent studies have revealed that TLR4 recognizes a wider variety of ligands than previously thought. In addition to its first identified ligand, bacterial LPS, TLR4 was found to recognize certain viral proteins, such as the F protein from respiratory syncytial virus and mouse mammary tumor virus. Not limited to pathogen-associated molecular patterns, TLR4 responds to human endogenous structural proteins derived from tissue injury or during inflammation: damage-associated molecular patterns, such as type III repeat extra domain A of fibronectin, oligosaccharides of hyaluronic acid, and human heat shock proteins 60 and 70.³⁵ Interestingly, a few reports have revealed the potential for protein extracts from plants and herbs to activate TLR4, such as taxol, an antitumor agent derived from the Yew plant,³⁶ and aqueous extract of *Rhodiola imbricata* rhizome, a medicinal plant.³⁷

To identify that SRW pollen serves as a functional TLR4 agonist capable of inducing TSLP-triggered T_H2 -dominant allergic inflammation, we demonstrated DRW pollen-stimulated TSLP/OX40L/OX40 signaling in a well-characterized murine EAC model.^{20,21,38} In addition to ocular allergy, SRW pollen and extracts have been widely used for allergic models, including asthma, rhinitis, peritonitis, and skin allergy, in mice, guinea pigs, dogs, and other animal species.^{1,39,40} However, there is no report showing that SRW pollen triggers TSLP signaling in these allergic models.

Second, we demonstrated that TSLP signaling in T_H2 -dominant inflammation is stimulated by SRW pollen through a TLR4-dependent innate immune response. This novel phenomenon was revealed by strong evidence that all clinical signs of allergic conjunctivitis, TSLP production and its downstream signals and T_H2 -dominant inflammation, were significantly reduced or eliminated in *Tlr4-d* or *MyD88*^{-/-} mice when compared with their wild-type littermates. Furthermore, we measured the SRW-specific IgE levels in sera from SRW-induced EAC mice in different strains. Interestingly, the results showed that SRW-specific IgE levels increased significantly in wild-type BALB/c mice sensitized by means of SRW pollen footpad injection but did not change in *Tlr4-d* and *MyD88*^{-/-} mice with the same treatment (see additional text and Fig E1 in this article's Online Repository at www.jacionline.org). These results suggest a possible TLR4-dependent pathway in the sensitization stage, but this requires further studies for confirmation.

Third, the direct evidence that SRW stimulates TSLP expression and production by means of ocular surface epithelia through TLR4 innate immune pathways was identified in an *in vivo* murine model of SRWe topical challenge and an *in vitro* culture

model using primary HCECs. We showed that SRWe indeed stimulated TSLP production in a dose-dependent fashion, and its stimulation was largely blocked by exogenous TLR4 antibody or by deletion of *Tlr4* or *MyD88* genes. In the human culture model we observed that the SRWe-stimulated TSLP expression and production was also blocked by the NF- κ B activation inhibitor quinoxaline. This finding is consistent with previous reports,⁵ including ours,¹⁵ and further supports our hypothesis that SRW serves as a functional TLR4 agonist because NF- κ B is a well-known signaling pathway of TLRs,^{41,42} including TLR4.⁴³ Further studies are necessary to explore the signaling pathways involved in the NF- κ B-mediated TLR4 innate immune response induced by SRW.

In conclusion, we have shown, for the first time, that SRW pollen, serving as a functional TLR4 agonist, induces TSLP/OX40L/OX40 signaling to trigger T_H2 -dominant allergic inflammation through TLR4-dependent innate immunity pathways. These novel findings shed light on the understanding of innate mucosal epithelial immunity involved in allergic inflammation and might create new therapeutic targets to cure allergic disease.

We thank Dr Marshall Bowes Hamill for his kind support and the Lions Eye Bank of Texas for providing human corneal tissues.

Clinical implications: The discovery of SRW pollen as a functional TLR4 agonist that induces TSLP/OX40L/OX40 signaling to trigger T_H2 -dominant inflammation creates a new intervention strategy and potential for a cure for allergic diseases.

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METHOD FOR MEASURING SRW-SPECIFIC IGE LEVELS IN MURINE SERUM

Murine blood was collected for preparation of serum on day 11 after being immunized with SRW pollen through footpad injection and before ocular challenge. Serum SRW-specific IgE levels were measured by using a murine IgE ELISA MAX Deluxe kit (BioLegend), according to the manufacturer's protocol. Briefly, the immunoplates (Nalge Nunc International, Naperville, Ill) were coated with SRW (120 $\mu\text{g/mL}$) and IgE capture antibody overnight at 4°C. After blocking with 1% BSA in PBS for 1 hour at room temperature, serial dilutions (1:20–1:200) of serum samples and IgE standards were added and incubated for 2 hours at room temperature. The plates were then washed with PBS plus 0.05% Tween and incubated for 2 hours with horseradish peroxidase-conjugated rat anti-mouse IgE antibodies. After washing with PBS plus 0.05% Tween, a color reaction was developed

with 3, 3', 5, 5'-tetramethylbenzidine and stopped with 1 N H_2SO_4 . Absorbance was read at 450 nm with a microplate reader.

RESULTS OF SRW-SPECIFIC IGE LEVELS IN AN SRW-SENSITIZED EAC MODEL

Interestingly, the results showed that SRW-specific IgE levels increased significantly in wild-type BALB/c mice immunized with SRW pollen but did not increase in *Tlr4-d* and *MyD88*^{−/−} mice with the same treatment (see Fig E1). These results suggest a possible TLR4-dependent pathway in the sensitization stage, which is consistent with a new report,^{E1} but further study is required to confirm this conclusion.

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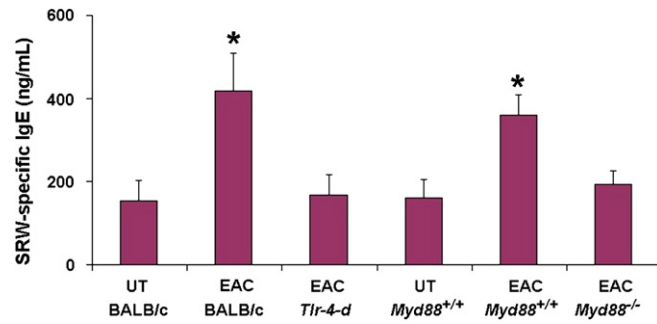


FIG E1. Serum SRW-specific IgE levels in an SRW-sensitized EAC model in different strains. Results shown are means $\pm$ SDs ($n = 3$). * $P < .05$ compared with untreated (UT) mice.