Published in final edited form as:

Exp Eye Res. 2014 March ; 120: 118–126. doi:10.1016/j.exer.2014.01.014.

A Potential link between Bacterial Pathogens and Allergic Conjunctivitis by Dendritic Cells

Ruzhi Deng, M.D.^{1,2,*}, Zhitao Su, M.D.^{1,2,*}, Fan Lu, M.D., O.D.¹, Lili Zhang, M.D.^{2,3}, Jing Lin, M.D.^{2,3}, Xiaobo Zhang, M.D., Ph.D.², Cintia S de Paiva, M.D.², Stephen C. Pflugfelder, M.D.², and De-Quan Li, M.D., Ph.D.²

¹School of Optometry and Ophthalmology, Wenzhou Medical College, Wenzhou, China

²Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, Houston, Texas, USA

³Department of Ophthalmology, the Affiliated Hospital of Qingdao University Medical College, Qingdao, China.

Abstract

The association and mechanism of bacteria linking to the allergic inflammation have not been well elucidated. This study was to explore a potential link between bacterial pathogens and allergic conjunctivitis by dendritic cells (DCs). Bone marrow-derived DCs from BALB/c and MyD88 knockout mice were treated with or without bacterial pathogens or thymic stromal lymphopoietin (TSLP). Two murine models of the topical challenge with LPS or flagellin and experimental allergic conjunctivitis (EAC) were used for in vivo study. The mRNA expression was determined by reverse transcription and real time PCR, and protein production was evaluated by ELISA, Western blotting, immunofluorescent staining and flow cytometry. TSLP mRNA and protein were found to be largely induced by DCs challenged with microbial pathogens, highly by lipopolysaccharide (LPS) and flagellin. The expression of MyD88, NFκB1, NFκB2 and RelA accompanied by NFκB p65 nuclear translocation and TSLP induction were significantly stimulated by flagellin, but blocked by TLR5 antibody or NFκB inhibitor in DCs from *MyD88*^{+/+} but not *MyD88*^{-/-} mice. TSLP promoted the expression of CD40, CD80, OX40 ligand (OX40L), IL-13 and CCL17 by DCs. TSLP-producing DCs were identified in vivo in ocular surface conjunctiva and draining cervical lymph nodes from two murine models of topical challenge with LPS or flagellin, and EAC in BALB/c mice. TSLP/TSLPR/OX40L signaling was observed in DCs of EAC mice. Our findings demonstrate that DCs not only respond to TSLP, but also produce

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding Authors: Fan Lu, M.D., O.D., School of Optometry and Ophthalmology, Wenzhou Medical College, Wenzhou, China. lufan@mail.eye.ac.cn De-Quan Li, M.D., Ph.D. Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, 6565 Fannin Street, NC-205, Houston, TX 77030, USA. Tel: (713) 798-1123, Fax: (713) 798-1457, dequanl@bcm.tmc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Competing Interests: All authors have no financial and commercial conflicts of interests

TSLP via TLR/MyD88/NF κ B pathways in response to bacterial pathogens, suggesting a potential link between bacteria and allergic disease.

Keywords

Mucosal immunology; thymic stromal lymphopoietin; Toll-like receptor; dendritic cells; innate immunity; allergy; allergic conjunctivitis

INTRODUCTION

Allergic inflammatory diseases are well known to be induced by a Th2-dominant immune response. However, the molecular trigger that initiates Th2 allergic inflammation was not clear until studies discovered a pro-allergic cytokine thymic stromal lymphopoietin (TSLP), which has been identified to be capable of activating myeloid dendritic cells (DCs) to produce OX40 ligand (OX40L) that triggers a Th2 response (Demehri et al., 2009; Liu, 2007; Liu et al., 2007; Ying et al., 2008). 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, asthma, rhinitis and other allergic diseases (Bunyavanich et al., 2011; Holgate, 2007; Lee and Ziegler, 2007; Liu et al., 2007; Ying et al., 2008; Zhang et al., 2009; Ziegler, 2010). On ocular surface, our studies have revealed that epithelium-derived TSLP activated DCs to prime Th2 differentiation and triggered allergic inflammatory conjunctivitis through TSLP-TSLPR and OX40-OX40L signaling pathway (Li et al., 2011; Zheng et al., 2010b). Others found that conjunctival epithelial derived TSLP played an important role in ocular allergy through activation of DCs and mast cells (Matsuda et al., 2010). However, the association and mechanism of bacterial pathogens linking to the allergic inflammation have not been well elucidated.

TSLP is mainly produced by epithelial cells, and also by fibroblasts, smooth muscle cells and mast cells (Reche et al., 2001; Soumelis et al., 2002). Recently, a few reports have identified that DCs not only respond to TSLP but also are a source of TSLP (Kashyap et al., 2011; Spadoni et al., 2012). However, the role of DC-produced TSLP in allergic inflammation remains to be elucidated. DCs are the most potent antigen-presenting cells of the immune system. DCs not only instruct T- and B-lymphocytes, but also play an important role in the innate immune responses (Adema, 2009; Iwasaki, 2007). Mucosal surfaces, including the ocular surface, contain localized DCs capable of sensing the external stimuli and mounting local responses upon recognition of invading microorganisms (Duez et al., 2006; Ohbayashi et al., 2007; Rescigno et al., 2001). Inflammatory-mediators and especially the Toll like receptor (TLR) family of proteins have been shown to play a pivotal role in inducing the immune activation program in DCs. Based on the observation that TSLP is mainly induced via TLR-mediated innate response in epithelium exposed to microbial products (Kinoshita et al., 2009; Le et al., 2010; Ma et al., 2009), and in consideration of the important role of DCs in innate response, we hypothesize that DCs may potentially link bacterial pathogens to allergic inflammation in ocular surface by producing TSLP.

DCs induced from mouse bone marrow have been widely used for study (Rank et al., 2009; Zheng et al., 2010a). Using both in vitro cultured bone marrow-derived DCs and in vivo ocular surface of BALB/c mice treated with microbial pathogens, as well as an experimental allergic conjunctivitis murine model (Li et al., 2011; Zheng et al., 2010b), the present study explored a novel phenomenon that DCs produce TSLP via TLR-mediated innate signaling pathway, which potentially links bacterial pathogens to allergic inflammation.

MATERIALS AND METHODS

Animals

The animal research protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine. All animal use in this study were 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. The 6-8 weeks old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Me). 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). The genotyping was performed using a previously described method (Song et al., 2003). The *MyD88*^{-/-} mice grown to 6 to 8 weeks were used for experiments with age- and gender-matched *MyD88*^{+/+} mice as controls.

Generation of murine bone marrow-derived dendritic cells

Bone marrow-derived DCs were generated as previously described method (Zheng et al., 2010a) with minor modifications. Briefly, bone marrow was collected from femurs and tibiae from BALB/c, *MyD88*^{+/+} or *MyD88*^{-/-} mice. Cells were cultured at 10×10^6 per 100mm dish in culture medium (RPMI-1640 containing 10% FBS, 15ng/ml of rmGM-CSF and 5ng/ml of rmIL-4). On day 3, 10 ml of the culture medium was added to each dish. On days 6 and 8, 10 ml of supernatant were collected and centrifuged at 300g for 10 min; the cells were resuspended in 10 ml culture medium, and given back into original dish. On day 9, the non-adherent and loosely adherent DCs were harvested for experiments.

Treatment of murine bone marrow-derived dendritic cells

DCs at the density of 1.5×10^6 were incubated for 4-24 h with RPMI-FBS medium alone, TLR ligands (Pam3CSK4, PGN, polyI:C, LPS, flagellin, FSL-1, R837, ssRNA or C-CpG-ODN, ligands to TLRs 1-9 respectively, 10 μ g/ml each, except for 50 μ g/ml polyI:C and 1 μ g/ml LPS) or 1ng/ml of recombinant mouse TSLP (R&D Systems, purity >95%, endotoxin <1.0 EU per 1 μ g protein) for mRNA expression. DCs at the density of $1.0-5.0 \times 10^6$ were treated with LPS, flagellin or TSLP for protein assays of TSLP and DCs markers. All TLR ligands and TSLP were prepared in endotoxin-free water.

Murine model of ocular surface challenge with LPS and flagellin for TSLP induction

BALB/c mice were topically challenged with 1 μ g LPS or flagellin in 5 μ l PBS per eye three times a day for 2 days, 5 μ l PBS alone was used as a control. After 24h from final challenge, the whole globes and draining cervical lymph nodes (CLN) were excised, embedded in OCT

compound and frozen in liquid nitrogen. Cryosections were cut by a cryostat (HM 500; Micron, Waldorf, Germany) and stored at -80°C .

Murine model of experimental allergic conjunctivitis (EAC) induced by short ragweed (SRW) pollen

The murine EAC model was induced using the previously reported methods (Li et al., 2011; Zheng et al., 2010b). In brief, mice were immunized with 50 μg of SRW pollen (Greer Laboratories) in 5 mg of Imject Alum (Pierce Biotechnology) by footpad injection on day 0. Allergic conjunctivitis was induced by repeated topical challenges of 1.5 mg of SRW pollen suspended in 10 ml of PBS (pH 7.2) into each eye once a day from days 10 to 12. PBS eyedrop treated SRW-sensitized and untreated mice were used as controls. On day 13, 24 hours after the last SRW challenge, the whole eyeballs and CLNs were harvested for immunofluorescent staining.

RNA extraction, reverse transcription (RT) and quantitative real-time PCR (qPCR)

Total RNA was extracted with a RNeasy Micro Kit (Qiagen, Valencia, CA), quantified with a spectrophotometer (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE), and stored at -80°C . The first strand cDNA was synthesized from 1 μg total RNA using Ready-To-Go You-Prime First-Strand Beads, and qPCR was performed in Mx3005P QPCR System (Stratagene, La Jolla, CA) as previously described (Luo et al., 2004; Yoon et al., 2007). The results were analyzed by the comparative threshold cycle (Ct) method and normalized by GAPDH as an internal control (Ma et al., 2009).

Enzyme-linked immunosorbent assay (ELISA)

Double-sandwich ELISA for mouse TSLP was performed, according to the manufacturer's protocol and our previous publication (Li et al., 2011). Absorbance was read at a reference wavelength of 450 nm by a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA).

Western blot analysis

Western blot analysis was performed with a previous method (Ma et al., 2009). Briefly, the cell lysate proteins (50 $\mu\text{g}/\text{lane}$) were separated on SDS polyacrylamide gel and electronically transferred to PVDF membranes. The membranes were incubated with primary antibodies against TSLP (1:500, 2 $\mu\text{g}/\text{ml}$) or β -actin (1:500, 1 $\mu\text{g}/\text{ml}$) overnight at 4°C , then with horseradish peroxidase-conjugated secondary antibody for 1 h. The signals were detected with ECL Plus reagent using a Kodak image station 2000R (Eastman Kodak, New Haven, CT).

Immunofluorescent staining

Immunofluorescent staining was performed as our previous methods (Chen et al., 2004; Kim et al., 2004). In brief, DCs were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS, and cryosections were fixed in acetone. Rabbit anti-mouse antibodies against TSLP (4 $\mu\text{g}/\text{ml}$), TLR4 (2 $\mu\text{g}/\text{ml}$), TLR5 (2 $\mu\text{g}/\text{ml}$) or NF κ B-p65 (2 $\mu\text{g}/\text{ml}$), goat anti-mouse TSLPR (2 $\mu\text{g}/\text{ml}$) or OX40L (2 $\mu\text{g}/\text{ml}$) and hamster anti-mouse CD11c (10 $\mu\text{g}/\text{ml}$)

were applied for 2 h. Secondary antibodies conjugated AlexaFluor 488 or 594 were incubated for 1 h, with propidium iodide (PI) or DAPI used for nuclear counterstaining. Isotype IgG was used for negative control. The images were photographed using an epifluorescence microscope (Eclipse 400; Nikon).

Flow cytometry analysis

Flow cytometry was performed as previously described (Zheng et al., 2010a). Briefly, DCs were incubated with anti-CD16/32 Ab (50µg/ml) for 10min to block Fc receptors, and stained with FITC-conjugated anti-CD11c (20µg/ml), APC-conjugated anti-CD40 (20µg/ml) or anti-CD80 (10µg/ml), PE-conjugated anti-CD86, anti-I-A/I-E or anti-OX40L (10µg/ml each) for 30 min at 4°C. Dead cells were excluded by PI staining. The analysis was performed by the BD LSRII Benchtop cytometer by gating on a CD11c-positive forward scatter-high cell population, and data were analyzed using BD Diva Software (BD Pharmingen).

Statistical analysis

Student's t-test was used to compare differences between two groups. One-way ANOVA test was used to make comparisons among three or more groups, followed by Dunnett's post-hoc test. *P* values <0.05 were considered statistically significant.

RESULTS

Microbial pathogens stimulate TSLP expression and production by DCs

Mouse DCs were induced from bone marrow cells in culture for 8-9 days, and the purity of DCs was greater than 92% (92.9-98.3%), as evaluated by flow cytometry with DC surface marker CD11c (Fig. 1A). These DCs were found to express TLRs 1-9, as determined by RT-qPCR (data not show). The presence of TLR4 and TLR5 was confirmed by immunofluorescent staining showing their localization at membrane and cytoplasm of mouse DCs (Fig. 1B).

The DCs were cultured in the media with or without extracted or synthetic microbial products that are ligands to TLRs 1-9 (10µg/ml of Pam3CSK4, PGN, flagellin, FSL-1, R837, ssRNA, C-CpG-ODN, 1µg/ml of LPS or 50µg/ml of polyI:C) for 4-24 h. TSLP mRNA was expressed at very low level in untreated DCs, but it was largely induced by specific TLR ligands (Fig. 1C). The mRNA expression of TSLP by DCs reached the peak level at 4 h (Fig. 1D). As shown in Fig. 1 A&B, LPS and flagellin significantly induced TSLP mRNA expression to 10-14 fold ($P<0.01$) in dose-dependent fashion; pam3CSK4, polyI:C, FSL-1 and R837 also upregulated TSLP mRNA by 3-5 fold ($P<0.05$); while PGN, ssRNA and C-CpG-ODN did not significantly induce TSLP expression.

To further identify the TSLP production at protein level, DCs were treated for 24 h with LPS and flagellin, the ligands to TLRs 4 and 5 respectively, which strongly stimulated TSLP mRNA expression by DCs. TSLP protein was barely detected in the culture medium from untreated DCs, but was largely stimulated to 2-4 fold by LPS and flagellin ($P<0.05$), as determined by ELISA (Fig. 1E). Correspondingly, TSLP protein concentration in the cell

lysates was also significantly increased by LPS and flagellin ($P<0.05$), 2-4 fold higher than the untreated controls, as determined by Western blotting (Fig. 1F). The immunofluorescent staining further showed that TSLP was mainly immunolocalized in the cytoplasm, and the TSLP positive cells were largely increased in DCs treated with LPS or flagellin (Fig. 1G), when compared with untreated DCs.

Flagellin induces TSLP production via TLR5/MyD88/NF κ B signaling pathways

Myeloid differentiation primary-response protein 88 (MyD88) is a universal adapter protein necessary for response to most TLRs except TLR3 (Johnson et al., 2005; Piggott et al., 2005); and TLR signaling typically induces activation of the NF κ B. We hypothesized that flagellin promoted TSLP production via TLR-mediated signaling pathways. We incubated DCs from BALB/c mice with flagellin and observed the significantly increased mRNA expression of MyD88, NF κ B1 and NF κ B2 (all $P<0.01$), as well as RelA ($P<0.05$) that encodes NF κ B-p65 (Fig. 2A). Immunofluorescent staining revealed that NF κ B-p65 protein was mainly located in cytoplasm in untreated control DCs, but markedly translocated from cytoplasm to nucleus in response to flagellin (Fig. 2B), indicating NF κ B signaling activation. Interestingly, the stimulated mRNA expression of MyD88, NF κ B1 and NF κ B2, the nuclear translocation of NF κ B p65, and the increased TSLP production by flagellin were significantly blocked by TLR5 antibody or quinazoline, a NF κ B activation inhibitor (NF κ B-I) with an exception that NF κ B-I did not blocked the stimulated MyD88 mRNA, a upstream molecule of NF κ B (Fig. 2).

Furthermore, we cultured bone marrow-derived DCs from *MyD88*^{-/-} knockout mice and their age- and gender-matched wild type *MyD88*^{+/+} littermates to investigate if MyD88 signaling is essential for TLR activation and TSLP induction. As shown in Fig. 3A, the mRNA expression of NF κ B1, NF κ B2, RelA and TSLP ($P<0.01$, 0.01, 0.05 and 0.01 respectively) was strongly stimulated in DCs from *MyD88*^{+/+} mice by flagellin when compared with the untreated control. But these stimulatory effects of flagellin were largely abolished in DCs of *MyD88*^{-/-} mice. TSLP induction, evaluated by its immunoreactivity (Fig. 3B), was also significantly increased by flagellin in DCs of MyD88 wild type, but not knockout mice.

TSLP activated DCs to produce OX40L and Th2 cytokines

DCs have been known to be activated by TSLP-primed condition to produce OX40L, which targets Th2 cells to trigger allergic inflammatory response (Ito et al., 2005; Zheng et al., 2010b). Mouse DCs were incubated with 1ng/ml rmTSLP for 4-24 h to evaluate the mRNA and protein expression. We observed that TSLP activated DCs with increased CD40 and CD80 for maturation as evaluated by RT-qPCR and flow cytometry (Fig. 4 A&B). TSLP significantly stimulated the OX40L mRNA levels to 2.6 fold ($P<0.05$, Fig. 4A). Flow cytometry analysis confirmed that TSLP treatment not only greatly increased number of OX40L positive (OX40L⁺) cells, but also increased the mean fluorescence intensity (MFI) of OX40L ($P<0.05$) in DCs (Fig. 4B). Immunofluorescent staining showed that OX40L was weakly expressed in the cytoplasm by a few untreated DCs, but its immunoreactivity was strongly increased in DCs exposed to TSLP (Fig. 4B). TSLP also stimulated expression of Th2 cytokine IL-13, and chemokine CCL17 by DCs (Fig. 4A).

TSLP was produced by infiltrated CD11c⁺ DCs in ocular surface and draining lymph nodes of BALB/c mice topically challenged by LPS or flagellin

To further identify whether DCs produce TSLP *in vivo*, LPS (1 µg/5 µl PBS per eye), flagellin (1 µg/5 µl PBS per eye), or 5 µl PBS alone was topically instilled in the conjunctival sac of BALB/c mice three times a day for 2 days. Double immunofluorescent staining revealed that murine corneal and conjunctival epithelia weakly expressed TSLP in normal control mice, but produced strong TSLP immunoreactivity when stimulated by LPS or flagellin, indicating TSLP is mainly produced by epithelial cells, a similar pattern to human ocular surface epithelia (Ma et al., 2009). Interestingly, TSLP immunoreactivity was also observed in CD11c⁺ DCs that infiltrated into the conjunctival stroma near epithelial area and draining CLN in the mice challenged with LPS or flagellin (Fig. 5). This finding further identified that DCs produce TSLP *in vivo* in murine mucosal ocular surface and draining cervical lymph nodes in response to microbial products.

TSLP-producing DCs in murine experimental allergic conjunctivitis

To confirm the role of TSLP-producing DCs in allergic disease, EAC model was induced in BALB/c mice sensitized and topically challenged by SRW pollen (EAC mice), with PBS-treated (PBS mice) and untreated mice as control groups. Repeated topical challenges with SRW allergen generated typical signs mimicking to human allergic conjunctivitis, including lid edema, conjunctival redness, chemosis, tearing, and frequent scratching of the eye lids. Consistent to previous reports (Li et al., 2011; Zheng et al., 2010b), the infiltration of CD11c⁺ DCs on the ocular surface was detected in this EAC model by immunostaining. As shown in Fig. 6A, CD11c⁺ DCs were accumulated in the SRW-challenged ocular surface, primarily in the stroma subjacent to conjunctival epithelia. Interestingly, double staining showed some DCs producing TSLP although its immunoreactivity in DCs was overlapped by strong staining of conjunctival epithelium, a major producer of TSLP. In draining CLN, we also observed the dramatic increase of positively immunoreactive cells to CD11c, TSLP, TSLPR and OX40L in EAC mice (Fig. 6B).

DISCUSSION

TSLP has been identified as a key initiator that strongly activates DCs to induce a Th2-dominant inflammatory response and trigger allergic diseases, including asthma, atopic dermatitis, allergic rhinitis and conjunctivitis (Demehri et al., 2009; Kay, 2001; Liu, 2007; Liu et al., 2007; Matsuda et al., 2010; Ying et al., 2008). It has been documented that epithelial cells are major producer of TSLP in both mice and humans, although other cells have the potential to produce TSLP (Reche et al., 2001; Soumelis et al., 2002). DCs express abundant TSLP receptor to respond to TSLP activation. Until very recently, TSLP was found to be expressed by DCs (Kashyap et al., 2011; Spadoni et al., 2012). However, the association and mechanism of bacterial pathogens linking to the allergic inflammation have not been well elucidated. In this study, we demonstrated that bacterial pathogens markedly induce TSLP production by mouse dendritic cells via TLR/MyD88/NFκB signaling pathways, and the DC-produced TSLP played a significant role in ocular surface and draining CLN in an EAC mouse model, suggesting a potentially link between bacterial pathogens to allergic inflammation.

DCs produce TSLP both in vitro and in vivo in response to bacterial pathogens

Although it has not been identified that bacteria could directly cause allergic disease, the involvement of bacterial agents like LPS and flagellin in initiation of allergic inflammation has been recently documented (Hammad et al., 2009; Le et al., 2010; Ortiz-Stern et al., 2011; Prescott et al., 2008; Reginald et al., 2011; Yang et al., 2009). Based on the important role of DCs in immune response and the observation that TSLP is mainly produced by epithelial cells via TLR-mediated innate response (Kinoshita et al., 2009; Le et al., 2010; Ma et al., 2009), we hypothesized that DCs are capable of producing TSLP in response to microbial pathogens. We incubated the murine bone marrow-derived DCs with TLR ligands 1-9, and found that several TLR ligands, especially bacterial products LPS and flagellin, the ligands to TLR4 and TLR5 respectively, significantly stimulated TSLP expression by mouse DCs at both mRNA and protein levels, as determined by RT-qPCR, ELISA, Western blotting and immunofluorescent staining (Fig. 1).

DCs are highly mobile and are present in the right place at the right time for the regulation of immunity. They are positioned as sentinels in the periphery, where they frequently encounter foreign antigens and penetrate epithelium to sample antigens (Rescigno et al., 2001), then they readily relocate to secondary lymphoid organs, particularly lymph nodes, to position themselves optimally for encounter with naive or central memory T cells (Allan et al., 2006; Randolph et al., 2005). To further identify TSLP produced by DCs in vivo, we performed double immunofluorescent staining of DC marker CD11c and TSLP with 2 mouse models. As shown in Figures 5 and 6, TSLP-producing DCs (CD11c⁺/TSLP⁺ cells) were infiltrated in the ocular surface, primarily in the stroma subjacent to conjunctival epithelia, and accumulated in the draining cervical lymph nodes of BALB/c mice with LPS or flagellin topical challenge, or experimental allergic conjunctivitis (EAC), respectively. The response was more dramatic in mice with EAC than topical challenge. It may result from the different treatment type (systemic plus topical vs. topical only) and time period (14 vs. 2 days) between EAC and topical challenge models. In draining CLN, we also observed the dramatic increase of positively immunoreactive cells to CD11c, TSLP, TSLPR and OX40L in EAC mice (Fig. 6B). These results suggest the possible role of TSLP-producing DCs that express TSLPR and produce OX40L in ocular surface and local lymph nodes of EAC mice.

DCs produce TSLP via TLR/MyD88/NFκB signaling pathways

TLR, which recognize conserved microbial components, are important pattern-recognition receptors. TLR consist of a family of at least 11 mammalian receptors that bind a restricted repertoire of ligands and recruit common adaptor molecules to induce cell signaling (Medzhitov and Janeway, Jr., 2000). The functional TLRs 1-7 and TLR9 have been identified to be expressed in human ocular surface (Kumar and Yu, 2006), and TLRs 2, 4, 5, 7 and 9 were reported to be expressed in mouse BM- DCs (Sallusto and Lanzavecchia, 2002).

MyD88 is an adapter protein necessary for response to TLR4 and TLR5 (Johnson et al., 2005; Piggott et al., 2005). TLR signaling typically induces activation of NFκB. NFκB1 or NFκB2 is bound to RelA to form the NFκB complex. Nuclear factor p65 is encoded by the

RelA gene (Nolan et al., 1991). Activated NF κ B complex translocates into the nucleus and binds DNA at kappa-B-binding motifs. We observed that flagellin significantly increased the mRNA expression of MyD88, NF κ B1, NF κ B2, RelA and TSLP, induced NF κ B-p65 nuclear translocation, and stimulated TSLP production, which were markedly blocked by TLR5 antibody and NF κ B inhibitor (Fig. 2). Furthermore, the stimulatory responses of TLR/MyD88/NF κ B signaling and TSLP induction by flagellin were seen in DCs of MyD88 wild-type littermate, but not seen in DCs of *MyD88*^{-/-} knockout mice (Fig. 3). All these results demonstrated that TSLP production in DCs was via TLR/MyD88/NF κ B signaling pathways in response to microbial pathogens.

TSLP-producing DCs may amplify local inflammatory response through a potential autocrine mechanism

TSLP has been identified to prime or activate myeloid DCs to produce OX40L that triggers a Th2 response (Liu, 2007; Soumelis et al., 2002). The present study revealed that DCs not only respond to TSLP, but also produce TSLP in response to microbial pathogens. The production of TSLP by DCs might potentially create an autocrine mechanism, by which DCs would respond to TSLP that is produced by themselves. Upon stimulation by TSLP, DCs became mature and produce OX40L and Th2 cytokines (Fig. 4). The role of TSLP-producing DCs and stimulated TSLP/OX40L signaling were further observed in an EAC mouse model (Fig. 6).

In conclusion, our findings demonstrate that bacterial ligands, LPS and flagellin, induce expression and production of pro-allergic cytokine TSLP by DCs via TLR/MyD88/NF κ B signaling pathways. DCs not only respond to TSLP by producing OX40L that trigger Th2-dominant allergic response, but also produce TSLP in response to bacterial pathogens. These findings suggest a potential link between bacterial pathogens and allergic conjunctivitis by DCs, as well as a potential autocrine mechanism by which local allergic inflammatory response may be amplified by DC-produced TSLP.

Acknowledgments

This study was supported by National Eye Institute, National Institutes of Health grant EY11915 (SCP) and Core Grant for Vision Research EY002520, Alkek Foundation (DQL), an unrestricted grant from Research to Prevent Blindness, the Oshman Foundation and the William Stamps Farish Fund.

REFERENCES

- Adema GJ. Dendritic cells from bench to bedside and back. *Immunol. Lett.* 2009; 122:128–130. [PubMed: 19121337]
- Allan RS, Waithman J, Bedoui S, Jones CM, Villadangos JA, Zhan Y, Lew AM, Shortman K, Heath WR, Carbone FR. Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming. *Immunity.* 2006; 25:153–162. [PubMed: 16860764]
- Bunyavanich S, Melen E, Wilk JB, Granada M, Soto-Quiros ME, Avila L, Lasky-Su J, Hunninghake GM, Wickman M, Pershagen G, O'Connor GT, Weiss ST, Celedomicronn JC. Thymic stromal lymphopoietin (TSLP) is associated with allergic rhinitis in children with asthma. *Clin. Mol. Allergy.* 2011; 9:1. [PubMed: 21244681]
- Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li D-Q. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells.* 2004; 22:355–366. [PubMed: 15153612]

- Demehri S, Morimoto M, Holtzman MJ, Kopan R. Skin-derived TSLP triggers progression from epidermal-barrier defects to asthma. *PLoS. Biol.* 2009; 7:e1000067. [PubMed: 19557146]
- Duez C, Gosset P, Tonnel AB. Dendritic cells and toll-like receptors in allergy and asthma. *Eur. J. Dermatol.* 2006; 16:12–16. [PubMed: 16436336]
- Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 2009; 15:410–416. [PubMed: 19330007]
- Holgate ST. The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol.* 2007; 28:248–251. [PubMed: 17466594]
- Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N, Qin FX, Yao Z, Cao W, Liu YJ. TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp. Med.* 2005; 202:1213–1223. [PubMed: 16275760]
- Iwasaki A. Mucosal dendritic cells. *Annu. Rev. Immunol.* 2007; 25:381–418. [PubMed: 17378762]
- Johnson AC, Heinzel FP, Diaconu E, Sun Y, Hise AG, Golenbock D, Lass JH, Pearlman E. Activation of toll-like receptor (TLR)2, TLR4, and TLR9 in the mammalian cornea induces MyD88-dependent corneal inflammation. *Invest Ophthalmol. Vis. Sci.* 2005; 46:589–595. [PubMed: 15671286]
- Kashyap M, Rochman Y, Spolski R, Samsel L, Leonard WJ. Thymic stromal lymphopoietin is produced by dendritic cells. *J. Immunol.* 2011; 187:1207–1211. [PubMed: 21690322]
- Kay AB. Allergy and allergic diseases. First of two parts. *N. Engl. J. Med.* 2001; 344:30–37. [PubMed: 11136958]
- Kim HS, Jun S, X, de Paiva CS, Chen Z, Pflugfelder SC, Li D-Q. Phenotypic characterization of human corneal epithelial cells expanded ex vivo from limbal explant and single cell cultures. *Exp. Eye Res.* 2004; 79:41–49. [PubMed: 15183099]
- Kinoshita H, Takai T, Le TA, Kamijo S, Wang XL, Ushio H, Hara M, Kawasaki J, Vu AT, Ogawa T, Gunawan H, Ikeda S, Okumura K, Ogawa H. Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA. *J. Allergy Clin. Immunol.* 2009; 123:179–186. [PubMed: 19056108]
- Kumar A, Yu FS. Toll-like receptors and corneal innate immunity. *Curr. Mol. Med.* 2006; 6:327–337. [PubMed: 16712478]
- Le TA, Takai T, Vu AT, Kinoshita H, Chen X, Ikeda S, Ogawa H, Okumura K. Flagellin Induces the Expression of Thymic Stromal Lymphopoietin in Human Keratinocytes via Toll-Like Receptor 5. *Int. Arch. Allergy Immunol.* 2010; 155:31–37. [PubMed: 21109746]
- Lee HC, Ziegler SF. Inducible expression of the proallergic cytokine thymic stromal lymphopoietin in airway epithelial cells is controlled by NFkappaB. *Proc. Natl. Acad. Sci. U. S. A.* 2007; 104:914–919. [PubMed: 17213320]
- Li DQ, Zhang L, Pflugfelder SC, de Paiva CS, Zhang X, Zhao G, Zheng X, Su Z, Qu Y. Short ragweed pollen triggers allergic inflammation through Toll-like receptor 4-dependent thymic stromal lymphopoietin/OX40 ligand/OX40 signaling pathways. *J. Allergy Clin. Immunol.* 2011; 128:1318–1325. [PubMed: 21820713]
- Liu YJ. Thymic stromal lymphopoietin and OX40 ligand pathway in the initiation of dendritic cell-mediated allergic inflammation. *J Allergy Clin. Immunol.* 2007; 120:238–244. [PubMed: 17666213]
- Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt RW, Omori M, Zhou B, Ziegler SF. TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu. Rev. Immunol.* 2007; 25:193–219. [PubMed: 17129180]
- Luo L, Li D-Q, Doshi A, Farley W, Corrales RM, Pflugfelder SC. Experimental dry eye stimulates production of inflammatory cytokines and MMP-9 and activates MAPK signaling pathways on the ocular surface. *Invest Ophthalmol Vis. Sci.* 2004; 45:4293–4301. [PubMed: 15557435]
- Ma P, Bian F, Wang Z, Zheng X, Chotikavanich S, Pflugfelder SC, Li DQ. Human corneal epithelium-derived thymic stromal lymphopoietin links the innate and adaptive immune responses via TLRs and Th2 cytokines. *Invest Ophthalmol. Vis. Sci.* 2009; 50:2702–2709. [PubMed: 19151401]

- Matsuda A, Ebihara N, Yokoi N, Kawasaki S, Tanioka H, Inatomi T, de Waal MR, Hamuro J, Kinoshita S, Murakami A. Functional role of thymic stromal lymphopoietin in chronic allergic keratoconjunctivitis. *Invest Ophthalmol. Vis. Sci.* 2010; 51:151–155. [PubMed: 19741251]
- Medzhitov R, Janeway C Jr. Innate immune recognition: mechanisms and pathways. *Immunol. Rev.* 2000; 173:89–97. [PubMed: 10719670]
- Nolan GP, Ghosh S, Liou HC, Tempst P, Baltimore D. DNA binding and I kappa B inhibition of the cloned p65 subunit of NF-kappa B, a rel-related polypeptide. *Cell.* 1991; 64:961–969. [PubMed: 2001591]
- Ohbayashi M, Manzouri B, Flynn T, Toda M, Ikeda Y, Nakamura T, Ono SJ. Dynamic changes in conjunctival dendritic cell numbers, anatomical position and phenotype during experimental allergic conjunctivitis. *Exp. Mol. Pathol.* 2007; 83:216–223. [PubMed: 17560570]
- Ortiz-Stern A, Kanda A, Mionnet C, Cazareth J, Lazzari A, Fleury S, Dombrowicz D, Glaichenhaus N, Julia V. Langerin+ dendritic cells are responsible for LPS-induced reactivation of allergen-specific Th2 responses in postasthmatic mice. *Mucosal. Immunol.* 2011; 4:343–353. [PubMed: 21048704]
- Piggott DA, Eisenbarth SC, Xu L, Constant SL, Huleatt JW, Herrick CA, Bottomly K. MyD88-dependent induction of allergic Th2 responses to intranasal antigen. *J. Clin. Invest.* 2005; 115:459–467. [PubMed: 15650773]
- Prescott SL, Noakes P, Chow BW, Breckler L, Thornton CA, Hollams EM, Ali M, van den Biggelaar AH, Tulic MK. Presymptomatic differences in Toll-like receptor function in infants who have allergy. *J. Allergy Clin. Immunol.* 2008; 122:391–9. 399. [PubMed: 18571707]
- Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nat. Rev. Immunol.* 2005; 5:617–628. [PubMed: 16056255]
- Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL-33-activated dendritic cells induce an atypical TH2-type response. *J Allergy Clin. Immunol.* 2009; 123:1047–1054. [PubMed: 19361843]
- Reche PA, Soumelis V, Gorman DM, Clifford T, Liu M, Travis M, Zurawski SM, Johnston J, Liu YJ, Spits H, de Waal MR, Kastelein RA, Bazan JF. Human thymic stromal lymphopoietin preferentially stimulates myeloid cells. *J. Immunol.* 2001; 167:336–343. [PubMed: 11418668]
- Reginald K, Westritschnig K, Werfel T, Heratizadeh A, Novak N, Focke-Tejkl M, Hirschl AM, Leung DY, Elisyutina O, Fedenko E, Valenta R. Immunoglobulin E antibody reactivity to bacterial antigens in atopic dermatitis patients. *Clin. Exp. Allergy.* 2011; 41:357–369. [PubMed: 21155910]
- Rescigno M, Urbano M, Valzasina B, Francolini M, Rotta G, Bonasio R, Granucci F, Kraehenbuhl JP, Ricciardi-Castagnoli P. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* 2001; 2:361–367. [PubMed: 11276208]
- Sallusto F, Lanzavecchia A. The instructive role of dendritic cells on T-cell responses. *Arthritis Res.* 4 Suppl. 2002; 3:S127–S132.
- Song XJ, Li D-Q, Farley W, Luo LH, Heuckeroth RO, Milbrandt J, Pflugfelder SC. Neurturin-deficient mice develop dry eye and keratoconjunctivitis Sicca. *Invest Ophthalmol Vis Sci.* 2003; 44:4223–4229. [PubMed: 14507865]
- Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, Gilliet M, Ho S, Antonenko S, Lauerma A, Smith K, Gorman D, Zurawski S, Abrams J, Menon S, McClanahan T, de Waal-Malefyt RR, Bazan F, Kastelein RA, Liu YJ. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat. Immunol.* 2002; 3:673–680. [PubMed: 12055625]
- Spadoni I, Iliev ID, Rossi G, Rescigno M. Dendritic cells produce TSLP that limits the differentiation of Th17 cells, fosters Treg development, and protects against colitis. *Mucosal. Immunol.* 2012; 5:184–193. [PubMed: 22236997]
- Yang M, Kumar RK, Foster PS. Pathogenesis of steroid-resistant airway hyperresponsiveness: interaction between IFN-gamma and TLR4/MyD88 pathways. *J. Immunol.* 2009; 182:5107–5115. [PubMed: 19342691]
- Ying S, O'Connor B, Ratoff J, Meng Q, Fang C, Cousins D, Zhang G, Gu S, Gao Z, Shamji B, Edwards MJ, Lee TH, Corrigan CJ. Expression and cellular provenance of thymic stromal lymphopoietin and chemokines in patients with severe asthma and chronic obstructive pulmonary disease. *J. Immunol.* 2008; 181:2790–2798. [PubMed: 18684970]

- Yoon KC, de Paiva CS, Qi H, Chen Z, Farley WJ, Li DQ, Pflugfelder SC. Expression of Th-1 chemokines and chemokine receptors on the ocular surface of C57BL/6 mice: effects of desiccating stress. *Invest Ophthalmol. Vis. Sci.* 2007; 48:2561–2569. [PubMed: 17525185]
- Zhang Z, Hener P, Frossard N, Kato S, Metzger D, Li M, Chambon P. Thymic stromal lymphopoietin overproduced by keratinocytes in mouse skin aggravates experimental asthma. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:1536–1541. [PubMed: 19188585]
- Zheng X, de Paiva CS, Li DQ, Farley WJ, Pflugfelder SC. Desiccating stress promotion of Th17 differentiation by ocular surface tissues through a dendritic cell-mediated pathway. *Invest Ophthalmol. Vis. Sci.* 2010a; 51:3083–3091. [PubMed: 20130281]
- Zheng X, Ma P, de Paiva CS, Cunningham MA, Hwang CS, Pflugfelder SC, Li DQ. TSLP and downstream molecules in experimental mouse allergic conjunctivitis. *Invest Ophthalmol Vis Sci.* 2010b; 51:3076–3082. [PubMed: 20107175]
- Ziegler SF. The role of thymic stromal lymphopoietin (TSLP) in allergic disorders. *Curr. Opin. Immunol.* 2010; 22:795–799. [PubMed: 21109412]

Highlight

- Dendritic cells not only respond to but also produce pro-allergic cytokine TSLP.
- LPS and flagellin induce TSLP expression via TLR/MyD88/NF κ B pathways.
- There is a potential link between bacteria and allergic disease by dendritic cells.

Fig. 1.

Dendritic cells (DCs) from BALB/c mice produce TSLP in response to microbial pathogens.

A. The purity of DCs, which were induced from bone marrow cells in RPMI-1640 containing 10% FBS, 15ng/ml rmGM-CSF and 5ng/ml rmIL-4 for 9 days, was evaluated by flow cytometry with DC surface marker CD11c. **B.** Representative images showed TLR4 and TLR5 localization (Red) in DCs by immunofluorescent staining with DAPI (Blue) as nuclear counterstaining. **C.** TSLP mRNA expression was determined by RT-qPCR in murine DCs exposed to microbial products, ligands to TLRs 1-9 (10μg/ml of Pam3CSK4, PGN, flagellin, FSL-1, R837, ssRNA, C-CpG-ODN, 1μg/ml of LPS or 50μg/ml of polyI:C,) for 4 h. **D.** The time course and dose response of TSLP mRNA by DCs exposed to LPS or flagellin. **E** and **F**, TSLP protein production in culture medium or cell lysates of DCs treated with LPS (1μg/ml) or flagellin (10μg/ml) for 24 h was determined by ELISA and western blotting respectively. **G.** Representative images showing TSLP localization (green) in DCs exposed to LPS (1μg/ml) or flagellin (10μg/ml) for 24 h by immunofluorescent staining with PI (Red) as nuclear counterstaining. Each bar in the diagrams represents mean $\pm$ SD of three to five independent experiments. *P<0.05; **P<0.01.

Fig. 2.

TSLP production by DCs from BALB/c mice is induced via TLR/NFκB signaling pathways. **A**, The mRNA expression of *MyD88*, NFκB1, NFκB2, RelA and TSLP was evaluated by RT-qPCR in DCs treated with flagellin (10 μg/ml) for 4 h with or without 1 h prior incubation of TLR5 antibody or NFκB-I. **B**, NFκB-p65 with nuclear translocation and TSLP production by immunofluorescent staining (Green) with PI counterstaining (Red) were observed in DCs treated with flagellin (10 μg/ml) for 24 h. *P<0.05; **P<0.01, compared with control, ^<0.05; ^^<0.01, compared with flagellin.

Fig. 3.

TSLP production by DCs is induced via *MyD88* signaling pathways. **A**, The mRNA expression of NFκB1, NFκB2, RelA, and TSLP was evaluated by RT-qPCR in DCs from *MyD88*^{+/+} and *MyD88*^{-/-} mice exposed to flagellin (10 g/ml) for 4 h. **B**, TSLP production was evaluated by immunofluorescent staining (Green) with PI counterstaining (Red) in DCs from *MyD88*^{+/+} and *MyD88*^{-/-} mice treated with flagellin (10 g/ml) for 24 h. Each bar in the diagrams represents mean ± SD of three to five independent experiments. *P<0.05; **P<0.01, compared with *MyD88*^{+/+} control, ^<0.05; ^^<0.01, compared with *MyD88*^{+/+} flagellin.

Fig. 4.

Effects of TSLP on DC activation. The DCs were treated with rmTSLP (1ng/ml) for 4-24 h to evaluate: **A.** the mRNA expression of CD40, CD80, OX40L, IL-13 and CCL17 by RT-qPCR, **B.** the protein production of CD40, CD80 and OX40L by flow cytometry analysis and **C.** OX40L protein production by immunofluorescent staining (Red) with DAPI (Blue) nuclear counterstaining.

Fig. 5.

Mucosal DCs produce TSLP in murine conjunctiva and cervical lymph nodes in vivo. BALB/c mice were topically challenged in the conjunctival sac with 1μg LPS or 1μg flagellin in 5 μl PBS per eye, three times a day for 2 days, 5 μl PBS was used as a control. Frozen sections of eyeballs and cervical lymph nodes were used for double immunofluorescent staining with CD11c (Red) and TSLP (Green) using DAPI counterstaining (Blue). Arrows indicate positive staining signals.

Fig. 6.

TSLP-producing DCs in murine experimental allergic conjunctivitis. The EAC model was induced in BALB/c mice sensitized and topically challenged by SRW pollen (EAC), with PBS-treated mice (PBS) as controls. **A.** Double immunofluorescent staining showed the infiltrated CD11c⁺ DCs (red) that produce TSLP (green) in conjunctiva of EAC mice; **B.** the dramatic increase of positively immunoreactive cells to CD11c, TSLP, TSLPR and OX40L in draining cervical lymph nodes (CLN) of EAC mice.