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Recent Advances in Mucosal Immunology and Ocular Surface Diseases

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1. Introduction

The mucosa, a mucus-secreting membrane, is linings of surface and cavities that are exposed to the external environment and internal organs. The mucosal immune system has a unique anatomy and physiology, which provides protection to an organism's various mucous membranes from invasion by potentially pathogenic microbes. It provides three main functions: protecting the mucus membrane against infection, preventing the uptake of antigens, microorganisms, and other foreign materials, and moderating the organism's immune response to that material. The mucosal epithelium forms the initial interface between the environment and host, and functions not only as a barrier but also as a sensor providing bidirectional communication with other resident mucosal lymphoid cells with the capacity to respond to pathogenic microbes and other injurious agents.

It has become increasingly clear that epithelial cells play important roles not only in host defense and inflammation, but also in regulation of immune responses. The mammalian immune system is comprised of two branches, the innate immune system and the adaptive immune system, that work in tandem to provide resistance to infection. The innate immune cells, represented primarily by monocytes, macrophages, dendritic cells and granulocytes, are the first line of host defense and are responsible for immediate recognition and control of microbial invasion. In contrast, the adaptive immune system, represented by B and T lymphocytes, has a delayed response, which is characterized by clonal expansion of cells that bind to a highly specific antigen and have immunological memory [1]. Substantial new evidence now indicates that epithelial cells are central participants, as initiators, mediators and regulators, in the innate and adaptive immune responses, as well as in the transition from innate immunity to adaptive immunity.

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This new concept has been recently generated from studies mainly in skin and airway epithelial cells. In addition to its function as a physical barrier, human skin has been shown to be an important immune organ displaying various defense mechanisms, which can be divided into three major functional compartments: natural epithelial defense, innate immunity and antigen-elicited adaptive immunity [2-4]. Airway epithelial cells have been recognized to be at the interface of innate and adaptive immunity [5-7]. Airway epithelial cells produce antimicrobial host defense molecules and proinflammatory cytokines and chemokines in response to microbial pathogens. Recruitment of immune cells, including dendritic cells, T cells and B cells into the proximity of epithelium results in the enhancement of adaptive immunity through interactions with epithelial cells. Epithelial cells are also responsible for mucus production in both protective immune responses and allergic airway inflammatory diseases. The crucial roles of epithelial cells in the innate and adaptive immune responses for host defense have also been recognized in other epithelia, including gastrointestinal mucosa [8-10] and ocular surface [11-13]. This chapter is focused on recent advances of this new concept that mucosal epithelium plays a central role in initiating and regulating innate and adaptive immune responses in ocular inflammatory diseases, based on literature review and our new findings. These novel breakthroughs in mucosal immunology would facilitate new therapeutic strategies for treating ocular inflammatory diseases.

2. Toll-like receptors and ocular mucosal innate immunity

The innate immune response relies on evolutionarily ancient germline-encoded receptors, the pattern recognition receptors (PRRs) [14], which recognize highly conserved microbial structures (Table 1). PRRs recognize microbial components, known as pathogen-associated molecular patterns. A breakthrough in the understanding of the ability of innate immune system to rapidly recognize pathogens occurred with the discovery of the Toll-like receptors (TLRs), which is the most important family among the PRRs. At least 10 human TLRs have been identified to date. Each TLR has unique ligand specificity. In general, TLRs 1, 2, 4, 5 and 6 present on the cell plasma membrane and respond to a variety of components of bacteria and fungi (Table 1); and TLRs 3, 7, 8 and 9 mainly present on endosomal membranes inside cells and recognize viral nucleic acids [12]. Among 4 types of transmembrane proteins structurally, TLRs are single-pass type I transmembrane proteins with leucine rich repeats in the extracellular domain for ligand recognition, and a Toll/IL-1 receptor (TIR) domain in the cytoplasmic portion for intracellular signaling [12, 15-17]. TLRs are expressed on immune cells that are most likely to first encounter microbes, such as neutrophils, monocytes, macrophages, and dendritic cells [15]. Ligand recognition by TLRs facilitates the dimerisation of TLRs that triggers the activation of signaling pathways, which originates from the cytoplasmic TIR domain, and culminates in the activation of the nuclear transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which leads to the expression of pro-inflammatory molecules, such as TNF-α, IL-1β, IL-6 [16, 18-20]. In addition to innate immune cells, an array of TLRs is expressed by epithelial cells at interfaces between host and environment including that of the skin [2, 21], respiratory tract [5, 6], gastrointestinal tract [9], and ocular surface [12, 13]. Strategic expression of TLRs at such host/environment interfaces appears to play an important role in the first line of defense against microbial invasion at these sites.

TLR	Microbial components	Ligands used for study
TLR1/TLR2	Triacylated lipopeptide (LP)	Pam3CSK4 (1-100 μg/ml)
TLR2*	Bacterial lipoprotein	
	Peptidoglycan	peptidoglycan-BS (1-50 μg/ml)
	lipoteichoic acid	LTA-SA (0.1-10 μg/ml)
	Zymosan (fungi)	Zymosan (1-100 μg/ml)
TLR3	Double stranded RNA (viruses dsRNA)	Poly I:C (5-50 μg/ml)
TLR4	LPS (Gram negative bacteria)	LPS (1-50 μg/ml)
	Bacterial HSP60	
	Respiratory syncytial virus coat protein	
TLR5	Flagellin (flagellated bacteria)	Flagellin-ST (1-25 μg/ml)
TLR6/TLR2	Diacylated lipopeptides	FSL-1 (0.1-10 μg/ml)
TLR7	Imidazoquinolone antiviral drug	Imiquimod (R837, 1-50 μg/ml)
TLR8	Single stranded RNA (viruses ssRNA)	ssRNA40 (0.1-10 μg/ml)
	Imidazoquinolone antiviral drug	
TLR9	Unmethylated CpG motifs of bacterial DNA	C-CpG-ODN (1-50 μg/ml)
TLR10	Unknown	

* TLR2 forms heterodimers with TLR1 and TLR6: TLR1 associates with TLR2 to recognize tri-acyl lipopeptides; TLR6/TLR2 heterodimer recognizes diacyl lipopeptides.

Table 1. Human Toll-like receptors (TLRs) and their microbial ligands

Ocular mucosal epithelial cells have been identified to express an array of functional TLRs [12, 13]. The production of pro-inflammatory cytokines, chemokines and antimicrobial peptides is stimulated via TLR2 by corneal epithelial cells exposed to yeast zymosan [22] and peptidoglycan of Staphylococcus aureus [23]. This pathway may have a role in the pathogenesis of Gram positive bacterial keratitis. Signaling through TLR9 appears important in P. aeruginosa keratitis, and silencing TLR9 signaling reduces inflammation, but likely contributes to decreased bacterial killing in the cornea [24]. Stimulation of TLR3 can induce the expression of proinflammatory cytokines, chemokines and antiviral genes that help to defend the cornea against viral infection [25, 26]. However, the distinctive role of ligand-stimulated TLR signaling in epithelium on regulation of innate and adaptive immunity remains to be elucidated.

3. Ocular epithelial cytokine TSLP links innate and adaptive immunity via Th2 inflammatory responses

Compelling evidence has been recently provided that thymic stromal lymphopoietin (TSLP) represents a key initiator of allergic inflammation at the interface of epithelial and dendritic cells, and TSLP may have a determinant role in the initiation and maintenance of the allergic immune response in atopic dermatitis and asthma [27-31]. Skin-derived TSLP was found to trigger progression from epidermal-barrier defects to asthma, the atopic march, in mice [31, 32].

TSLP is a 140-amino acid IL-7-like 4-helix bundle cytokine that was first isolated from a murine thymic stromal cell line and shown to support B-cell development in the absence of IL-7 [33]. Mouse and human TSLP share a poor homology of 43% amino acid identity. The human TSLP gene is localized in chromosome 5q22, not far from the gene cluster encoding for all the Th2 cytokines, IL-4, IL-5 and IL-13 [34]. The TSLP receptor (TSLPR) complex consists of a TSLP binding chain and the IL-7 receptor α chain (IL-7Rα). Like TSLP, human and mouse TSLPR share approximately 40% amino acid identity. By interacting with the heterodimeric receptor TSLPR/IL-7Rα, TSLP appears to initiate phosphorylation of signal transducer and activator of transcription (STAT) 3 and STAT-5 [27, 35].

It was demonstrated that epithelial cell-derived TSLP could strongly activate human myeloid dendritic cells to mature dendritic cells that produce OX40 ligand (OX40L) in the absence of IL-12 to induce an inflammatory Th2 response characterized by high level of pro-inflammatory cytokine TNF-α with low level of anti-inflammatory cytokine IL-10, distinct from the regulatory Th2 responses characterized by low TNF-α and high IL-10 production [27, 28]. This suggests that TSLP represents a key initiator of allergic inflammation at the interface of epithelial cells and dendritic cells. TSLP was also demonstrated to direct the innate phase of allergic immune responses through activating mast cells. Therefore, TSLP and OX40L may represent important targets for intervention in the initiation of allergic inflammatory responses.

Epithelial cells appear to be the major potential producer of TSLP in both mice and humans, although fibroblasts, smooth muscle cells, and mast cells all have the potential to produce TSLP [35, 36]. The expression of TSLP by epithelial cells has been recently shown to be stimulated by microbial ligands, inflammatory and Th2 cytokines [37-39]. Using ocular mucosal epithelium as a model, Li and associates [40] have evaluated the expression and production of TSLP by primary human corneal epithelial cells in response to 11 extracted or synthetic microbial components that are ligands of TLRs 1-9 (Table 1). As shown in Figure 1A, TSLP expression and production were found to be largely induced by the ligands to TLRs 3, 5 and 6, which were polyinosinic-polycytidylic acid (polyI:C), flagellin and FSL-1, respectively, representing viral dsRNA and the bacterial components flagellin and lipopeptides. PolyI:C and flagellin, the major TSLP inducers, stimulated TSLP production to 67- and 19-fold, respectively. The TSLP mRNA reached the peak levels rapidly in 4 hours in response to these ligands. The specificity of this response was confirmed when respective antibody against TLR3 or TLR5 significantly blocked TSLP expression induced by polyI:C and flagellin, respectively. The pattern of TLR-dependant TSLP induction indicates that human corneal epithelial cells are able to rapidly initiate an innate immune response to virus or bacteria through TLR-mediated pathways. TSLP was also moderately induced by pro-inflammatory cytokines at both mRNA and protein levels. TNF-α or IL-1β induced a concentration dependent increase in the TSLP mRNA and protein, but their stimulatory effects were much weaker than that of polyI:C and flagellin, which stimulated TSLP protein production over 15- and 4-fold higher, respectively, than TNF-α. These data suggest that TSLP induction is mainly through a TLR-dependant innate immune response to microbes in human ocular epithelium.

IL-4 and IL-13, the major cytokines secreted by Th2 cells, not only moderately induced TSLP mRNA and protein, but also strongly synergized with microbial ligands, such as polyI:C, or pro-inflammatory cytokine TNF-α to promote TSLP expression and production (Figure 1B). This synergized induction of TSLP was further confirmed in an ex vivo experiment model

using fresh human corneal epithelial tissues (Figure 1C). These findings demonstrate that adaptive immunity-derived Th2 cytokines are capable of amplifying the TSLP expression and production by the corneal epithelium, which in turn has the capability of priming Th2 cell differentiation through dendritic cell activation [27, 28]. These findings suggest that blocking TSLP could be a novel strategy for treatment of allergic diseases or other TSLP-driven conditions.

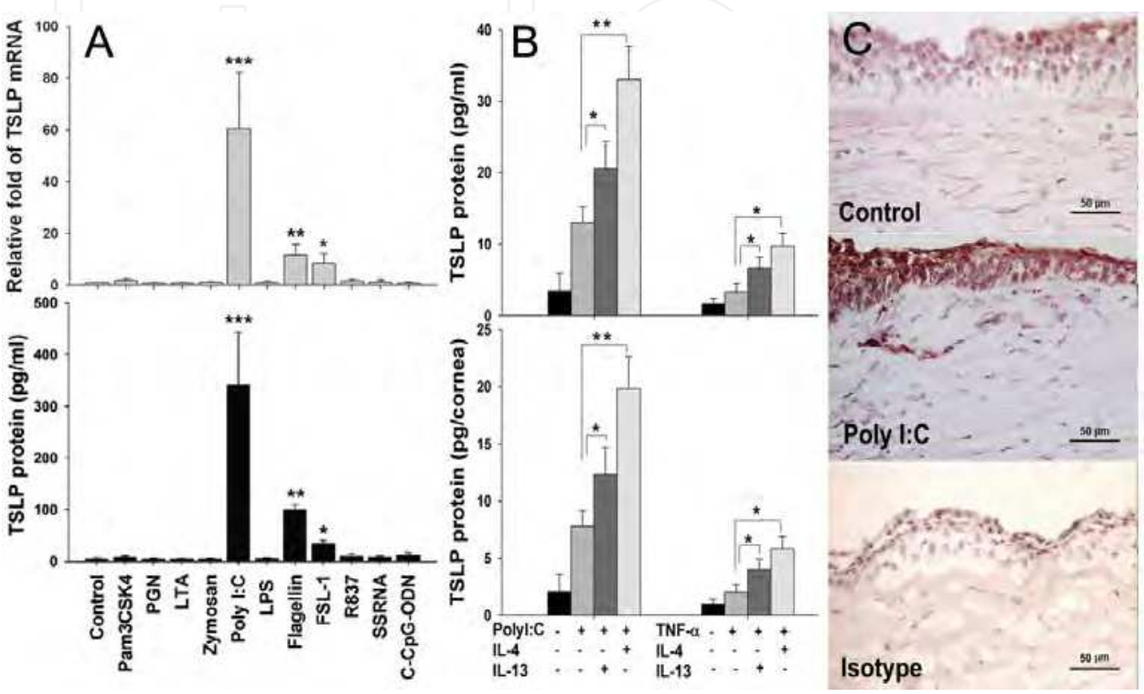

Fig. 1. TLR-mediated induction of TSLP by microbial ligands, TNF-α and Th2 cytokines. **A**. TSLP induction in HCECs. The confluent primary HCECs were incubated with 50μg/ml polyI:C or 10μg/ml of Pam_3CSK_4, peptidoglycan (PGN), LTA, Zymosan, LPS, flagellin, FSL-1, R837, single stranded RNA (ssRNA40) or C-CpG-ODN for 4 hours for TSLP mRNA expression by RT-qPCR, or for 48 hours for TSLP protein in the culture supernatants by ELISA; **B.** TSLP induction in an ex vivo model of human corneal tissues. A fresh corneoscleral tissue was cut into 4 equal size pieces. Each quarter of corneal tissue was placed into a well of 8-chamber slides in 150 μl of serum-free SHEM medium without or with polyI:C (50 μg/ml) or TNF-α (20 ng/ml) in the presence of IL-4 or IL-13 (100 ng/ml) for 24 hours. The culture supernatants were used for TSLP ELISA. Results shown are mean ± SD of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $P < 0.001$. **C.** The corneal tissues were prepared for cryosections for TSLP immunohistochemical staining with isotype IgG as negative control.

Although the recognition of different ligands by specific TLRs leads to activation of an intracellular signaling cascade in a myeloid differentiation primary response gene 88 (MyD88)-dependent or independent fashion, all TLRs share NF-κB signal transduction pathways for activation of the transcription factors [20]. TNF-α is also well known to promote activation of the NF-κB signaling pathway [41]. TSLP induction has been observed through NF-κB activation in airway epithelial cells [30] and synovial fibroblasts [42]. As evaluated by Western blot analysis and immunofluorescent staining, NF-κB was found to be

dramatically activated in corneal epithelial cells exposed to polyI:C, flagellin or TNF-α for 4 hours. This activation was evidenced by nuclear translocation of p65 protein, one of the two proteins in NF-κB heterodimer. This p65 nuclear translocation and TSLP induction, stimulated by polyI:C, flagellin or TNF-α were markedly blocked by TLR3, TLR5 or TNF-α specific antibody, respectively, and also by quinazoline, a NF-κB activation inhibitor [40]. These findings confirm that TSLP is mainly induced by microbial components, proinflammatory cytokines and Th2 cytokines in human corneal epithelial cells via TLR and NF-κB signaling pathways, suggesting that epithelium-derived TSLP links the innate and adaptive immune responses,

4. TSLP/OX40L/OX40 signaling initiates Th2-dominant allergic conjunctivitis

Allergic diseases like seasonal allergy, asthma, atopic dermatitis, affect up to 20-30% of the population in industrialized countries, and up to 50% of these individuals reporting ocular allergic manifestations [43-45]. The incidence of allergies has increased steadily over the past 30 years. Th2-dominant hypersensitivity is a major contributor to allergic inflammatory diseases, but the underlining mechanism for initiation of this adaptive immune disorder by mucosal epithelia remains a relative mystery. The molecular triggers for Th2 allergic inflammation were not clear until studies identified a novel epithelium-derived pro-allergic cytokine TSLP, which activates myeloid dendritic cells (DCs) to produce OX40 ligand (OX40L) that triggers a Th2 inflammatory response. TSLP has been identified as a key initiator in the development of human allergic disease [31, 46, 47], including asthma, atopic dermatitis and allergic conjunctivitis, a triad of common atopic IgE-dependent allergic diseases [48]. The direct link between TSLP expression and the pathogenesis of atopic dermatitis and asthma in vivo has been demonstrated [29]. TSLP was found to be highly expressed by keratinocytes in skin lesions of atopic dermatitis and was associated with dendritic cell activation in situ [49]. Evidence associating TSLP with human asthma has also been reported [29, 32]. Patients suffering from one member of the triad often show symptoms of one or both of the other members, suggesting a common genetic or initiating element in these diseases [31].

Using a well characterized murine model of experimental allergic conjunctivitis (EAC) induced by short ragweed (SRW) pollen [50, 51], Li and colleagues observed that the repeated topical challenges with ragweed pollen allergen generated typical signs of allergic conjunctivitis in the pollen-sensitized BALB/c mice, which developed lid edema, conjunctival redness, chemosis, and tearing, as well as frequent scratching of the eye lids [52]. They found that TSLP mRNA expression was significantly upregulated in the corneal and conjunctival epithelia from mice sensitized and challenged with pollen when compared with phosphate buffered saline (PBS) alone and untreated normal controls. Immunohistochemical staining confirmed an increase in TSLP production in the eyes challenged with SRW pollen. As shown in Figure 2A, the corneal and conjunctival epithelia in EAC BALB/c mice displayed much stronger TSLP staining throughout the entire epithelium, especially the superficial epithelial layers of the conjunctiva, than the PBS-treated control. These data indicate the stimulated TSLP mRNA expression and protein production by ocular surface epithelia in the SRW-induced EAC murine model.

The accumulation of CD11c positive ($CD11c^+$) dendritic cells on the ocular surface was detected in this EAC model by reverse transcription and quantitative real-time polymerase

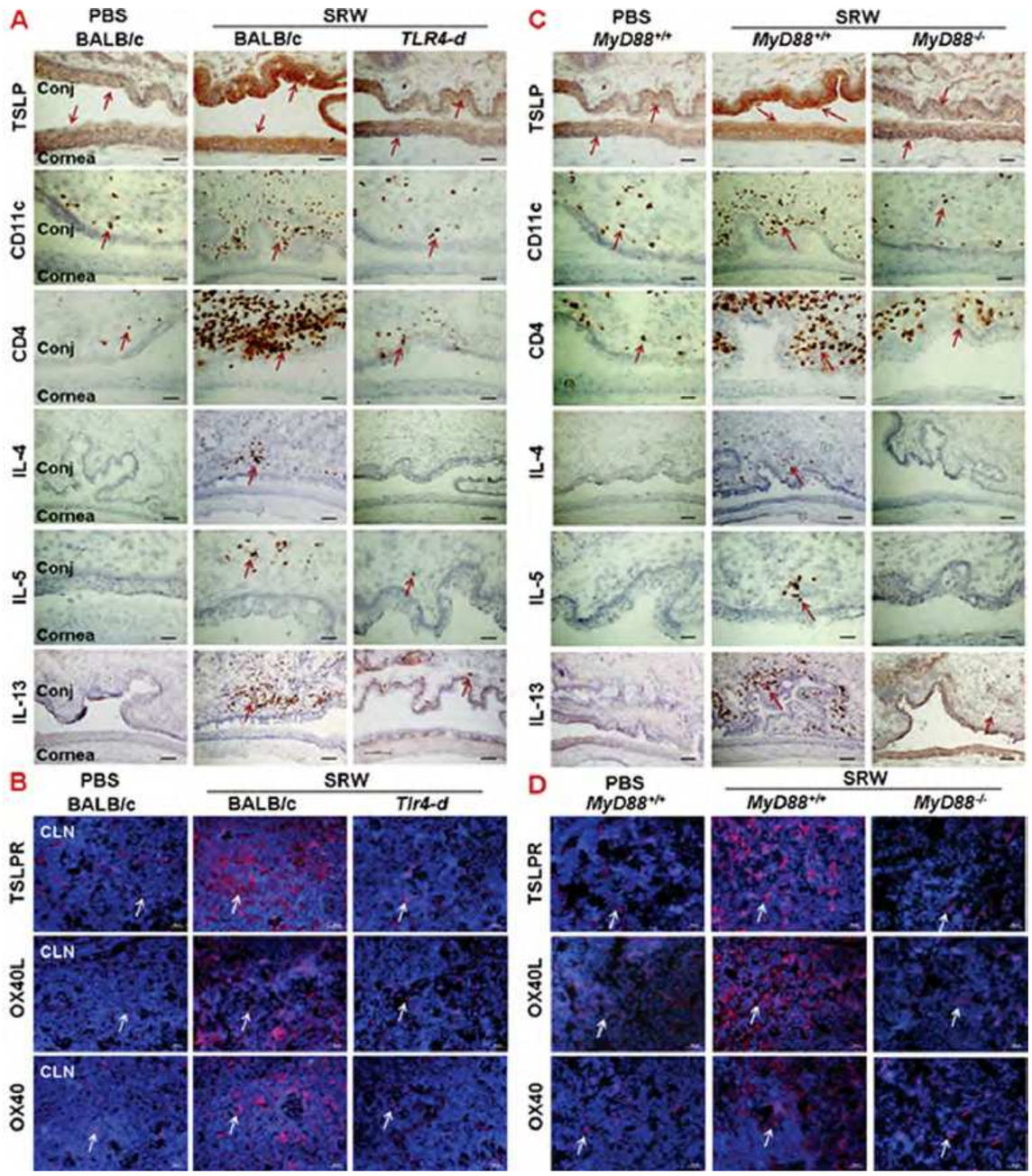

Fig. 2. The stimulated production of TSLP signaling proteins and Th2-dominant inflammation in SRW-induced EAC model requires TLR4 and MyD88. **Top panel**: The representative images showing immunohistochemical staining of epithelial TSLP, markers for dendritic (CD11c) and T cells (CD4), and Th2 cytokines (IL-4, IL-5 and IL-13) on cornea and conjunctiva (Conj) of wild type and *Tlr4* deficient BALB/c mice challenged by SRW pollen, with PBS-treated mice as controls (**A**), and of C57BL/6 based wild type *MyD88*$^{+/+}$ and knockout *MyD88*$^{-/-}$ mice challenged by SRW pollen, with PBS-treated mice as controls (**C**). **Bottom panel**: The representative images showing immunofluorescent staining of TSLP activated signals, TSLPR, OX40L and OX40 on cervical lymph nodes (CLN) of wild type and

Tlr4 deficient BALB/c mice challenged by SRW pollen with PBS-treated mice as controls (**B**), and of C57BL/6 based wild type *MyD88*$^{+/+}$ and knockout *MyD88*$^{-/-}$ mice challenged by SRW pollen, with PBS-treated mice as controls (**D**). Bar: 20μm; Arrows: red or red brown positive staining signals.

chain reaction (RT-qPCR) and immunostaining. The increased mRNA levels of CD11c, TSLPR and OX40L were observed in ocular surface, especially in conjunctival tissues, where their transcripts increased to 4-10 fold, respectively ($P< 0.05$ or <0.01), from SRW pollen-challenged mice, compared with PBS controls. The large amount of CD11c$^+$ dendritic cells was accumulated in the ocular surface of the pollen challenged eyes, primarily in the stroma subjacent to the conjunctival epithelia, in EAC mice, but not in the control mice treated with PBS (Figure 2A). These results suggest that ocular surface was infiltrated with TSLP activated dendritic cells that express TSLPR and produce OX40L in the EAC mice.

Th2-dominant inflammatory response was clearly observed on ocular surface in the mice challenged by pollen. The infiltration of T lymphocytes was evidenced by the increased CD4 mRNA expression and a markedly increased number of CD4 immunopositive (CD4$^+$) cells in the ocular surface, especially in the conjunctiva, of EAC mice (Figure 2A), when compared with PBS control mice. These CD4$^+$ T cells appear to be Th2 lineage because the transcripts of three key Th2 cytokines, IL-4, IL-5 and IL-13, were all found to be expressed at significantly higher levels in corneal and conjunctival tissues from the EAC mice than the PBS controls. Immunostaining data confirmed that the IL-4, IL-5 and IL-13-producing Th2 cells were largely infiltrated in conjunctival stroma (Figure 2A).

To confirm the TSLP signaling in SRW pollen-induced EAC mice, the ocular surface draining cervical lymph nodes (CLN) were collected for evaluation (Figure 3A). Compared with PBS controls, the mRNA levels of TSLPR and OX40L were significantly stimulated to 7.5 and 4.1 fold respectively (both $P<0.01$) while CD11c expression only slightly increased in CLN from EAC mice, indicating CD11c$^+$ DCs were markedly activated by ocular surface epithelia-derived TSLP. The mRNA levels of OX40 (3.4 fold, $P<0.05$) and Th2 cytokines, IL-4, IL-5 and IL-13 (13.2, 12.8 and 5.5 fold, respectively, all $P<0.01$), significantly increased while CD4 expression was not changed in the CLN from EAC mice, indicating naive CD4$^+$ T cells were largely differentiated to Th2 cells that might be primed by OX40L produced by TSLP activated dendritic cells [28]. The increased TSLPR$^+$, OX40L$^+$ and OX40$^+$ cells in the draining CLN of the pollen challenged EAC mice were further confirmed by immunofluorescent staining that showed dramatically increased immunoreactivity of these 3 signaling proteins at cell membrane and cytoplasm in CLN (Figure 2B). All these results demonstrate that TSLP/OX40L/OX40 signaling plays a critical role in the development of Th2-dominant allergic inflammation in pollen induced EAC model in BALB/c mice, suggesting that TSLP signaling molecules could be novel therapeutic targets to treat allergic inflammatory disease.

5. Cutting edge breakthrough: Short ragweed pollen triggers ocular allergic inflammation through TLR4-dependent innate immune response

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 knowledge gap and potential challenge because

failure to resolve allergen driven inflammation potentially leads to recurrent or chronic allergic diseases. Pollen, a ubiquitous allergen, affects a large population of allergic patients. Pollen 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 Th2-dominant allergic inflammation via epithelial innate immunity pathways is largely unknown. Substantial evidence now indicates that epithelial cells are central participants in innate and adaptive immune responses [4, 5, 7]. Based on the observation that we and other groups have made [40, 53, 54] that TSLP is mainly induced via TLR mediated innate response in epithelia exposed to microbial products, we hypothesized that pollen, such as Ambrosia artemisiifolia short ragweed (SRW), the most widespread plant in North America, may serve as a functional TLR4 agonist that induces production of a proallergic cytokine TSLP via innate immune response to trigger Th2-dominant allergic inflammation. To uncover the novel phenomenon and molecular signaling pathways involved in pollen induced allergic inflammation, a comprehensive set of experiments has been conducted using a well-characterized murine model of allergic conjunctivitis induced by SRW pollen in BALB/c, TLR4 deficient and MyD88 knockout mice, as well as a murine topical ocular surface challenge model and a culture model of primary human corneal epithelial cells exposed to an aqueous extract of defatted SRW pollen.

TLR-mediated TSLP induction has been recognized [39, 53, 54]. We have demonstrated that TSLP was largely induced by specific TLR ligands in human corneal epithelial cells [40]. MyD88 is a universal adapter protein necessary for response to all TLRs except TLR3 [55, 56]. *Tlr4*-deficient (*Tlr4*-d, C.C3-Tlr4Lps-d/J) and MyD88 knockout ($MyD88^{-/-}$) mice have been used to identify TLR4 mediated signaling [57, 58]. To explore whether SRW pollen stimulates TSLP through TLR4-dependent innate response, we sensitized and topically challenged the wild-type BALB/c, *Tlr4*-d (Jackson Laboratory, Bar Harbor, ME) and $MyD88^{-/-}$ mice (gifts from Dr. Shizuo Akira, Research Institute for Microbial Disease, Osaka University, Japan) with SRW pollen.

Compared with wild-type BALB/c mice, the ocular allergic signs, stimulated TSLP/OX40L/OX40 signaling and Th2-dominant inflammatory response by ocular mucosa, especially conjunctival tissues, were dramatically reduced or eliminated in BALB/c based *Tlr4*-d mice. As shown in Figure 3A, the mRNA levels of TSLP, OX40L, OX40 and Th2 cytokines (IL-4, IL-5 and IL-13) were significantly stimulated in cornea, conjunctiva and draining CLN from wild-type BALB/c, 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 Th2 response in ocular mucosal tissues (Figure. 2A) and draining CLN (Figure. 2B) 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 less severe than BALB/c mice. The expression of TSLP and its signaling molecules, TSLPR, OX40L and OX40, as well as Th2 cytokines IL-4, IL-5 and IL-13 was significantly stimulated in the cornea, conjunctiva and CLN from SRW challenged wild type $MyD88^{+/+}$ mice at both mRNA (Figure. 3B) and protein levels (Figure 2C, 2D). Clinical allergic signs and stimulated production of TSLP signaling molecules (TSLPR, OX40L and OX40) and Th2 cytokines (IL-4, IL-5 and IL-13) were dramatically reduced or eliminated in SRW challenged $MyD88^{-/-}$ mice as evaluated by RT-qPCR (Figure

3B) and immunostaining (Figure 2C, 2D). These findings suggest that MyD88 pathway is involved in the TLR4-dependent TSLP signaling induced by SRW pollen.

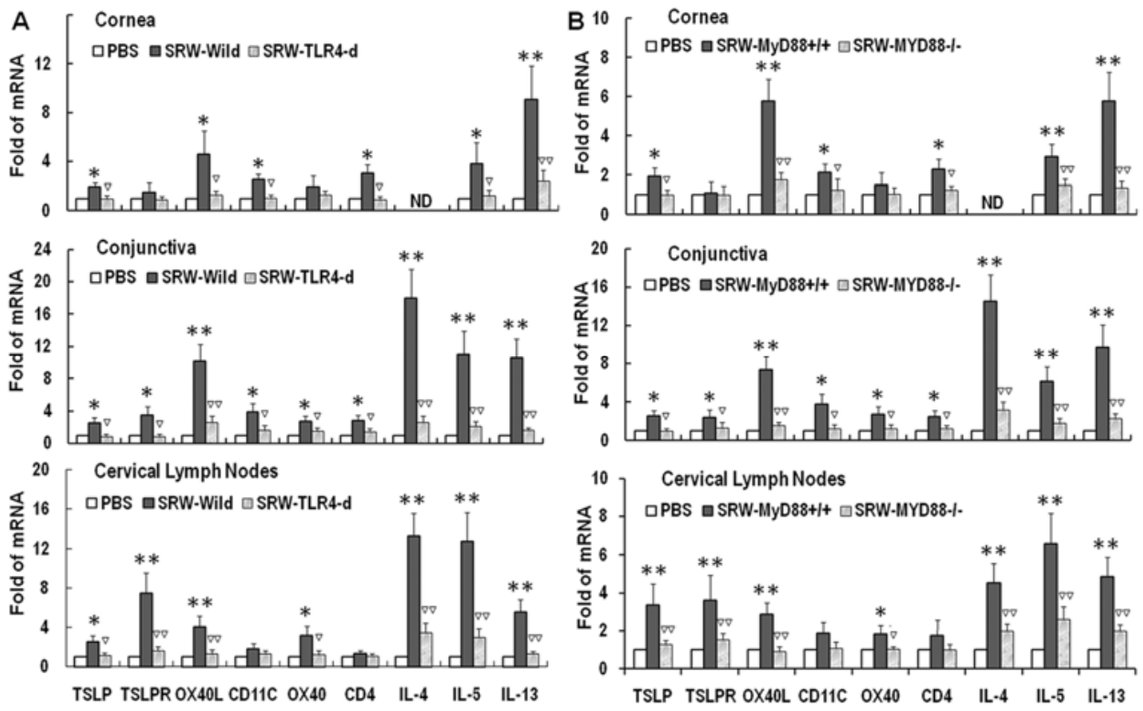

Fig. 3. The stimulated expression of TSLP signaling molecules and Th2 cytokines in SRW-induced EAC model requires TLR4 and MyD88. The mRNA expression of proallergic cytokine TSLP, its downstream signals in dendritic (TSLPR, OX40L, & CD11c) and T cells (OX40 & CD4), as well as Th2 cytokines (IL-4, IL-5 and IL-13) by corneal epithelium, conjunctiva and cervical lymph nodes in wild type and *Tlr4* deficient BALB/c mice sensitized and topically challenged by SRW with PBS-treated mice as controls (**A**), and in C57BL/6 based wild type *MyD88*$^{+/+}$ and knockout *MyD88*$^{-/-}$ mice, sensitized and topically challenged by SRW pollen, with PBS-treated mice as controls (**B**). The mRNA levels are presented as relative fold in EAC mice over the controls, which were evaluated by RT and real-time qPCR using TaqMan gene expression assay system with GAPDH as an internal control. Results shown are the Mean ± SD of four independent experiments. **P*<0.05, ***P*<0.01; *n*=4, compared with PBS controls; $^{\nabla}$P<0.05, $^{\nabla\nabla}$P<0.01, n=4, compared with wild type mice.

To confirm that SRW pollen directly stimulates TSLP production by ocular mucosal epithelia through TLR4-dependent innate immunity pathway, we created a topical challenge murine model using an aqueous extract of defatted SRW pollen (SRWe) at 150μg/5μl/eye for 4-24 hours. TSLP mRNA was 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 Figure 4 A & B, SRWe significantly stimulated TSLP mRNA by 2 fold in corneal and conjunctival epithelia (Both *P*<0.05), and its protein levels by 3.2 fold (from 150.2±37.6 pg/mg cellular protein to 480.00±89.6 pg/mg) in cornea epithelia and 2.2 fold (from 128.6±29.8 pg/mg to 281.6±19.3 pg/mg) in conjunctiva in BALB/c mice when

compared with untreated or PBS-treated controls. The SRWe-stimulated TSLP were significantly decreased at both mRNA and protein levels by TLR4 blocker, a rat anti-mouse TLR4 antibody, but not by its isotype rat IgG2a.

The SRWe topical challenge did not increase TSLP mRNA (Figure 4C) and protein levels (Figure 4D) in corneal and conjunctival epithelia of *Tlr4-d* mice. Similarly, a TLR4 agonist lipopolysaccharides (LPS, 5μg/5μl/eye) stimulated TSLP production by corneal and conjunctival epithelia in BALB/c mice, but not in *Tlr4-d* mice. Furthermore, we applied this topical challenge model to MyD88 knockout mice and their wild type controls. SRWe

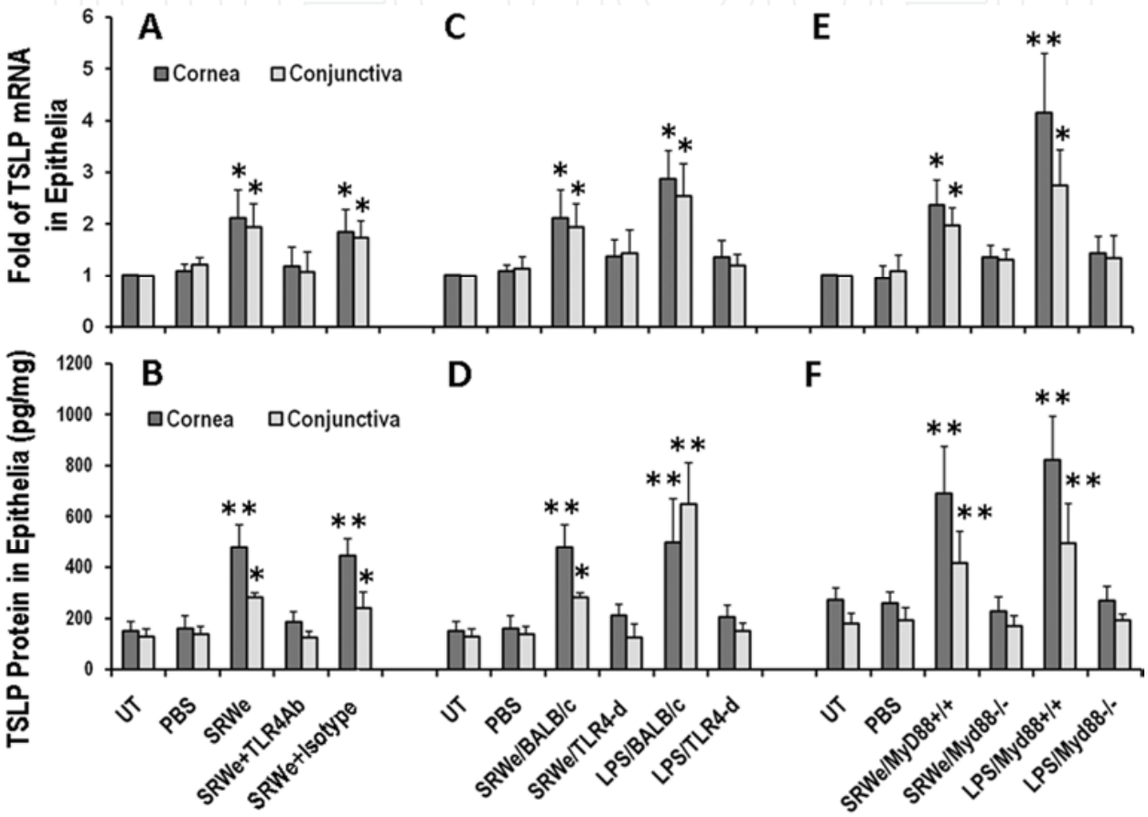

Fig. 4. Aqueous extract of defatted SRW pollen (SRWe) induces TSLP expression and production by murine ocular surface epithelia through TLR4- and MyD88-dependent innate immunity pathway. **A, B**. BALB/c mice were topically instilled with SRWe at 150μg/5μl/eye for 6 or 24 hours for TSLP mRNA (A) or protein (B) respectively, without or with pre-instilled rat anti-mouse TLR4 antibody (1μg/5μl/eye) or its isotype rat IgG2a. Untreated (UT) and PBS-treated mice were used as controls. Corneal epithelium and conjunctiva were harvested for TSLP mRNA and protein by RT-qPCR and ELISA respectively. **C, D**. TSLP mRNA (C) and protein (D) induction by topically challenged SRWe or LPS (5μg/5μl/eye) in wild type and *Tlr4-d* BALB/C mice, with untreated or PBS-treated mice as controls. **E, F.** TSLP mRNA (E) and protein (F) induction by topically challenged SRWe or LPS in wild type $MyD88^{+/+}$ and knockout $MyD88^{-/-}$ mice, with untreated or PBS-treated mice as controls. Results shown are the Mean ± SD of four independent experiments. **P*<0.05, ***P*<0.01; n=4, compared with PBS controls.

promoted TSLP production by ocular epithelia at both mRNA (Figure 4E) and protein levels (Figure 4F) only in *MyD88*$^{+/+}$ mice, but not in *MyD88*$^{-/-}$ mice, a similar pattern to that observed following LPS topical challenge. Taken together, these data demonstrated that SRWe directly stimulates TSLP production by ocular mucosal epithelia via a TLR4-dependent innate pathway.

To explore whether this phenomenon occurs in humans, we investigated TSLP expression in human corneal epithelium. TSLP mRNA was upregulated at 4 hours and its protein was detected at 24 hours in human corneal epithelial cells (HCECs) exposed to SRWe, which is consistent with our previous report [40]. TSLP induction at mRNA (Figure 5A) and protein levels (Figure 5B) was concentration-dependently stimulated by SRWe in primary HCECs. TSLP protein was barely detectable (4.83±1.60 pg/ml) in the supernatant of normal HCEC cultures. SRWe at 10μg/ml increased the TSLP protein to 48.92±4.23 pg/ml (P<0.01), the levels comparable to that stimulated by 10ng/ml of TNF-α in HCECs [40]. The SRWe (10μg/ml)-stimulated TSLP mRNA was significantly blocked by pre-incubation of cells with 10μg/ml of neutralizing monoclonal antibody against human TLR4, but not by its isotype mouse IgG2a k (Figure 5C). Furthermore, SRWe stimulated TSLP expression was also significantly inhibited by quinazoline, a NF-κB Activation Inhibitor (Figure 5C). These findings were confirmed by detection of increased TSLP protein levels as shown in Figure 5D. These data demonstrate that SRW induces TSLP production in human corneal epithelial cells through TLR4 and NF-κB innate signaling pathways.

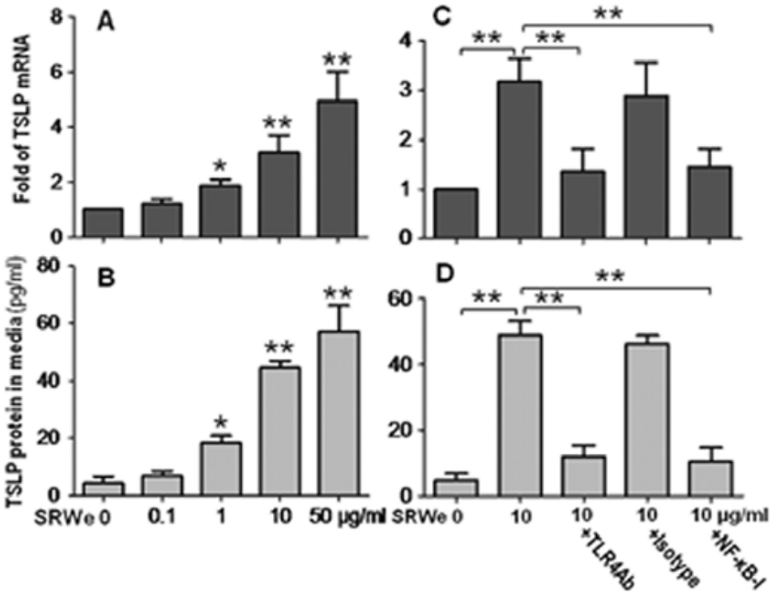

Fig. 5. SRWe induces TSLP expression and production by human corneal epithelial cells (HCECs) through TLR4 and NF-κB signaling pathways. **A, B**. Confluent cultures of primary HCECs were treated with 0.1 to 50 μg/ml of SRWe for 4 hours for TSLP mRNA or 48 hours for TSLP protein in the supernatants. **C, D.** HCECs were pre-incubated with mouse TLR4 antibody (10μg/ml), isotype mouse IgG2a k, or NF-κB activation inhibitor quinazoline (NFkB-I, 10μM) for 1 hour before adding 10μg/ml SRWe for 4 hours for TSLP mRNA or 48 hours for TSLP protein in the supernatants. Results shown are the Mean ± SD of four independent experiments. *P<0.05, **P<0.01; n=4.

Traditionally, TLRs recognize conserved microbial components as ligands or agonists. Recent studies have revealed that TLR4 recognizes a wider variety of ligands than previous 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 (PAMP), TLR4 responds to human endogenous structural proteins derived from tissue injury or during inflammation, the damage-associated molecular patterns (DAMP), such as type III repeat extra domain A of fibronectin, oligosaccharides of hyaluronic acid, human heat-shock protein Hsp60 and Hsp70 (see review [59]). 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 [60], and aqueous extract of Rhodiola imbricata rhizome, a medicinal plant [61].

In conclusion, we have for the first time uncovered a novel phenomenon and a unknown mechanism that short ragweed pollen, serving as a functional TLR4 agonist, induces TSLP/OX40L/OX40 signaling to trigger Th2-dominant allergic inflammation via TLR4-dependent innate immunity pathways [62]. These novel findings shed light on the understanding of innate mucosal epithelial immunity involved in allergic inflammation, and may create new therapeutic targets to cure allergic disease.

6. Epithelium-derived interleukin 33 initiates allergic inflammation

The IL-1 receptor family has several members, including the classical IL-1 receptor (IL-1R) and the IL-18 receptor (IL-18R). In 1989, one member of the family, ST2, a protein encoded by IL-1 receptor-like 1 (IL-1RL1) gene, was identified as an orphan receptor [63]. Investigation into the function of ST2 revealed its participation in inflammatory processes, particularly regarding mast cells, type 2 $CD4^+$ T helper cells and the production of Th2-associated cytokines. In fact, ST2 was characterized as a specific cellular marker that differentiated Th2 from Th1 T cells. Clinical and experimental observations led to the association of ST2 with disease entities such as asthma, pulmonary fibrosis, rheumatoid arthritis, collagen vascular diseases and septic shock [64]. In 2005, the discovery of IL-33 as a ST2 ligand provided new insights into ST2 signaling [65]. By binding to ST2 receptor, IL-33 can activate Th2 cells and mast cells to secrete the proinflammatory and Th2 cytokines and chemokines that lead to severe pathological changes in mucosal organs [66].

IL-33 is produced mainly by epithelial and endothelial cells, fibroblast, and others [66-68]. IL-33 expression has been found to be up-regulated by stimulation with inflammatory cytokines, TNF-α and IL-1β [69]. However, the expression and regulation of IL-33 by mucosal surface epithelia has not been well elucidated. Using fresh donor corneal tissues and primary HCECs, we recently observed that IL-33 is mainly expressed by epithelium and largely induced by microbial products through TLR and NF-κB signaling pathways [70]. The findings suggest that the mucosal epithelial cell-derived cytokine IL-33 may play an important role in allergic inflammatory diseases through innate immune responses.

As shown in Figure 6A, IL-33 expression and production were largely induced by polyI:C, lipopolysaccharides (LPS), flagellin, FSL-1 and R837, the ligands for TLR3, -4, -5, -6 and -7, representing viral dsRNA and the bacterial components flagellin and lipopeptides, respectively. PolyI:C and flagellin were major IL-33 inducers, stimulating IL-33 production by 7- and 4-fold, respectively, with the peak mRNA levels at 8 hours by HCECs. The IL-33 induction by these 2 ligands was further confirmed using an ex vivo donor corneal tissue

model (Figure 6B). The specificity of TLR-dependent response by HCECs was also confirmed when an antibody against TLR3 or TLR5 significantly inhibited IL-33 expression by polyI:C or flagellin respectively (Figure 6C). The pattern of TLR-dependent IL-33 induction indicates that HCECs are able to rapidly initiate an innate immune response to virus or bacteria, and play an important role in allergic inflammatory disease.

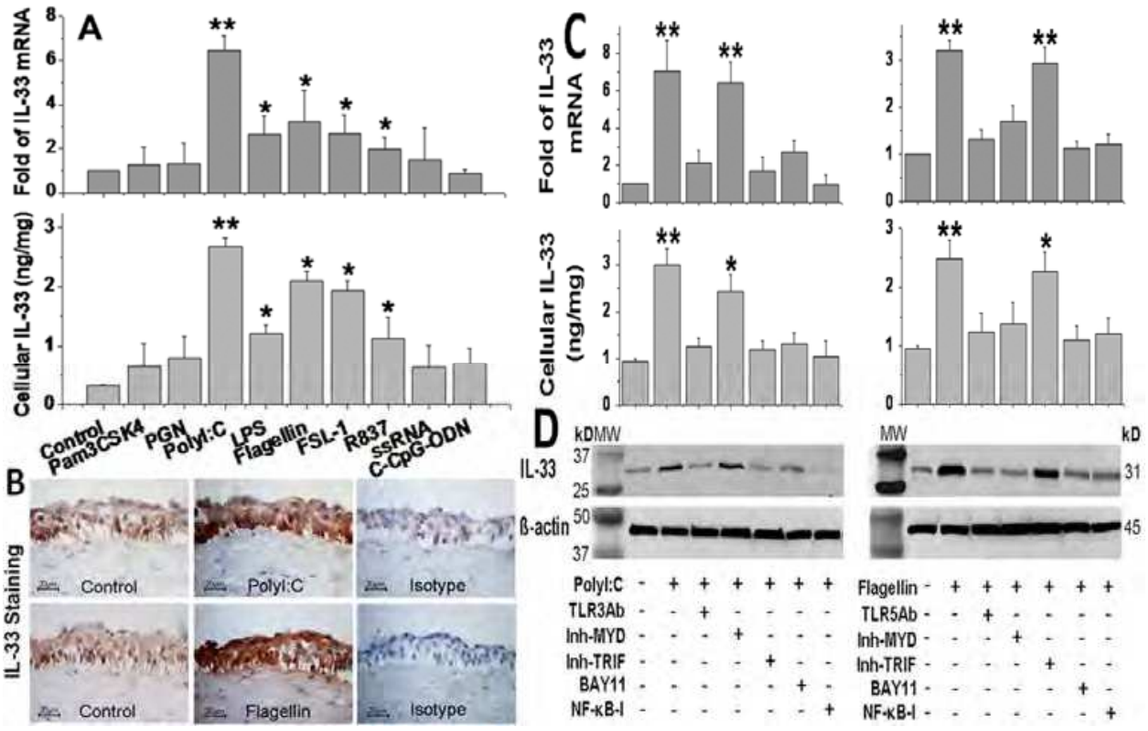

Fig. 6. TLR-dependent induction of IL-33 by microbial ligands in human corneal epithelium. **A.** IL-33 mRNA and protein levels induced by primary human corneal epithelial cells (HCECs) exposed to 50μg/ml polyI:C or 10μg/ml of Pam3CSK4, peptidoglycan (PGN), polyI:C, LPS, flagellin, FSL-1, R-837, ssRNA40 or C-CpG-ODN for 8 or 48 hours, evaluated by quantitative real-time PCR or ELISA, respectively. Results shown are the mean ± SD of four independent experiments. *P< 0.05; **P < 0.01. **B.** The immunohistochemical staining showing IL-33 induction in an ex vivo human corneal tissues by polyI:C (50μg/ml) or flagellin (10μg/ml) for 24 hours with isotype IgG as a negative control. **C.** TLR and NF-κB signaling pathways involved in IL-33 induction by polyI:C or flagellin in HCECs exposed to polyI:C (50μg/mL) or flagellin (10μg/mL) in the absence or presence of preincubated rabbit TLR3Ab (10μg/mL), TLR5Ab (10μg/mL), BAY11-7082 (10μM) or quinazoline (10μM) for 1 hour, and Pepinh-MYD (40μM) or Pepinh-TRIF (40μM) for 6 hours. The cultures treated by ligands for 8 hours for IL-33 mRNA, or for 48 hours for IL-33 protein by ELISA and by Western blot with β-actin as control (**D**). Results shown are the mean ± SD of four independent experiments. *P< 0.05; **P < 0.01.

IL-33 is an extracellular inflammatory cytokine while it also acts as a nuclear transcription factor [71]. It has been shown that IL-33 is mainly localized to the nucleus of endothelial cells and bound to chromatin. IL-33 mRNA is primarily translated and synthesized in vivo as a 30-kDa precursor, a pro-IL-33 protein. As a member of IL-1 super family and like IL-1 and

IL-18, pro-IL-33 protein sequence does not have a signal peptide that directs it for secretion via the ER–Golgi pathway [66, 72]. Like pro-IL-1β, human pro-IL-33 was reported to be cleaved by caspase-1 to generate active form, an 18-kDa fragment (mature IL-33), which is sufficient to activate signaling via the IL-33 receptor ST2. Recombinant mature IL-33 has been known to induce Th2-associated cytokines and inflammatory cytokines via its receptor, ST2 [72, 73]. However, processing of pro-IL-33 in vivo has not been clarified yet. It is not clear whether caspase-1 cleavage of pro-IL-33 occurs in vivo and whether, as for IL-1β, this cleavage is a prerequisite for IL-33 secretion and bioactivity.

Our data have showed that no significantly changes of IL-33 protein levels can be detected by ELISA in the culture supernatants, other than in cell lysate, of the HCECs. IL-33 protein levels significantly increased in the cell lysate, but not in the culture supernatants, of HCECs exposed to polyI:C or flagellin for 24-48 hours, when compared with the untreated control. However, the stimulated cellular IL-33 protein was released outside the cells into culture supernatant after co-incubation with ATP for additional 30 minutes, as evaluated by ELISA and Western blot analysis [70]. The finding supports a notion that caspase 1-dependant activation may involved in the release and secretion of IL-33 protein since ATP has been known to activate caspase-1 through triggering the P2X7 receptor [74]. Further studies are necessary to clarify the underlining mechanism.

As shown in Figure 6C evaluated by RT-qPCR and ELISA, as well as in Figure 6D by Western blotting, synthetic dsRNA polyI:C-induced IL-33 mRNA expression and protein production were markedly blocked by TLR3 antibody and TIR-domain-containing adaptor inducing interferon (TRIF) inhibitory peptide (Pepinh-TRIF), but not by TLR5 antibody or MyD88 inhibitory peptide (Pepinh-MYD), while extracted bacterial component flagellin-induced IL-33 production was dramatically suppressed by TLR5 antibody and Pepinh-MYD, but not by TLR3 antibody or Pepinh-TRIF, in corneal epithelial cells. The stimulated IL-33 induction by polyI:C or flagellin were also significantly blocked by IκB-α inhibitor BAY11-7082 or NF-κB activation inhibitor quinazoline. Further study has shown that NF-κB was dramatically activated with p65 protein nuclear translocation in corneal epithelial cells exposed to polyI:C or flagellin for 4 hours, as evaluated by Western blot analysis and immunofluorescent staining. BAY 11 selectively inhibits the phosphorylation and degradation of IκB-α, blocked the nuclear translocation of NF-κB p65 protein. NF-κB activation inhibitor quinazoline also blocked the p65 nuclear translocation. These findings demonstrate a novel phenomenon that a newly defined pro-allergic cytokine IL-33 is largely induced by microbial components through TLR and NF-κB signaling pathways in human corneal epithelium. This suggests that human ocular mucosal epithelium plays an important role in initiating Th2-dominant allergic inflammation via innate immune responses.

In a clinical study, we have tested conjunctival impression cytology specimens obtained from 8 patients with active atopic conjunctivitis and 8 normal subjects by RT-qPCR (Figure 7). The mRNA levels of TSLP, TSLPR, OX40L, OX40, IL-33 and ST2 were found to be significantly elevated in the atopic group compared with the normal control subjects, suggesting a potential role of TSLP/TSLPR/OX40L/OX40 and IL-33/ST2 signaling pathways in allergic conjunctivitis (Figure 7A). In SRW pollen induced EAC mice, the transcripts and proteins of IL-33, ST2, and IL-1 receptor accessory protein (IL1RAP) were also found to be significantly increased in the corneal epithelium, conjunctiva and CLN, as evaluated by RT-qPCR (Figure 7B) and immunostaining (Figure 7C). IL-33, ST2 and TLRs could become novel biomarkers and molecular targets for the intervention to treat allergic inflammatory diseases.

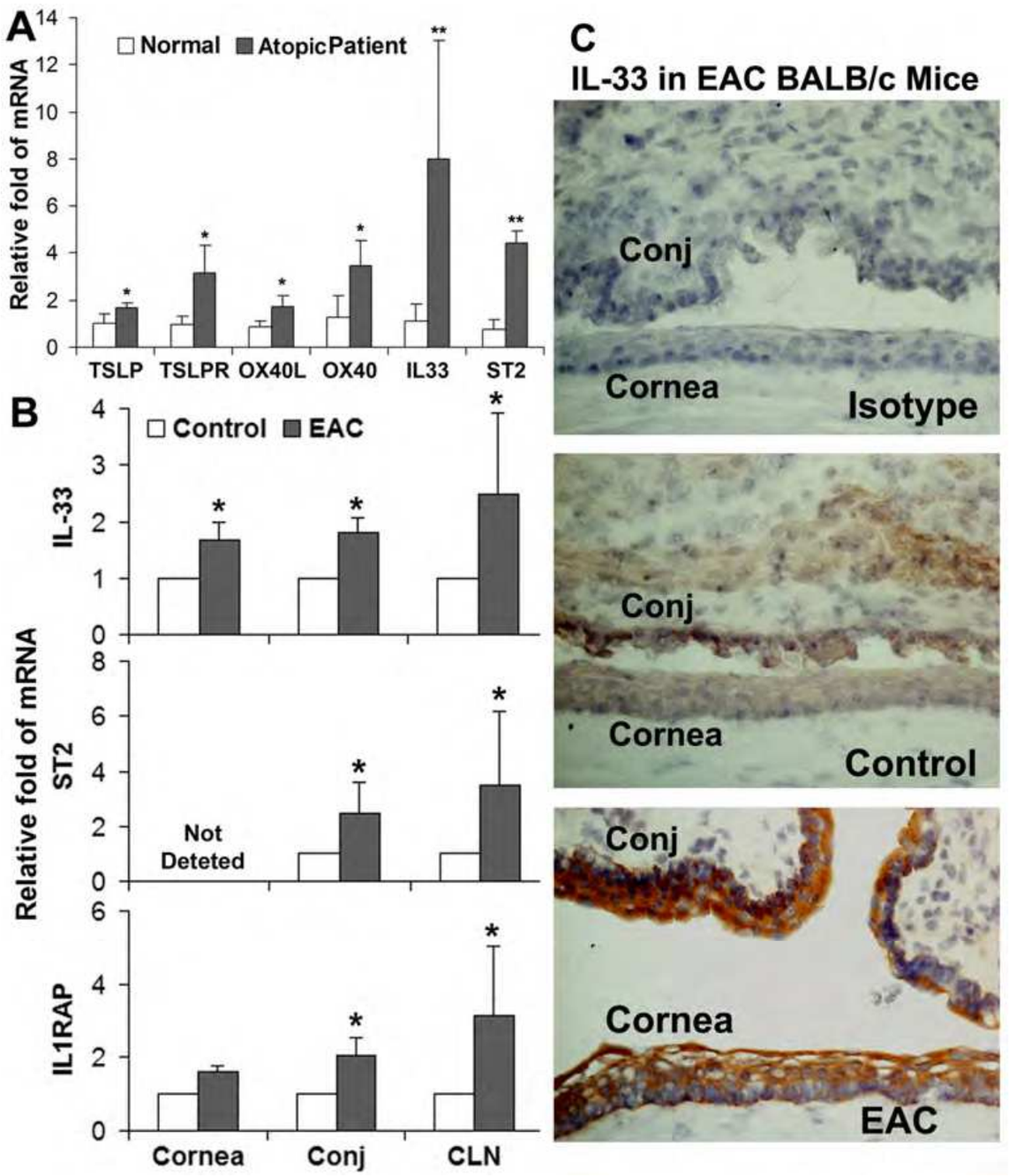

Fig. 7. The role of IL-33 in allergic disease. **A.** Elevated mRNA levels of TSLP and IL-33 signaling molecules in conjunctiva of atopic patients compared with normal subjects by RT-qPCR. * $P<0.05$, ** $P<0.01$, n=8. **B.** The mRNA levels of IL-33, ST2 and IL1RAP in corneal epithelium (Cornea), conjunctiva (Conj) and cervical lymph nodes (CLN) of in BALB/c mice with PBS-treated mice as controls. * $P<0.05$, n=3. **C.** Immunostaining images showing the stimulated IL-33 in ocular surface of EAC BALB/c mice with untreated mouse control and isotype IgG negative control.

7. Th17 pathway links innate and adaptive immunity

Th17 has been recently identified as a new T helper cell subset. $CD4^+$ T helper (Th) cells now include three different types based on their cytokine signatures: interferon-γ (IFN-γ)-secreting Th1, IL-4, -5, and -13-secreting Th2 [75], and IL-17-producing Th17 cells [76]. Th17 cells have been recognized to be key effector T cells in a variety of human inflammatory and autoimmune diseases as well as experimental animal models [77, 78]. The IL-17 family includes 6 members (IL17A-F). IL-17A (also known as IL-17) and IL-17F are the founding members of the IL-17 cytokine family. The genes encoding IL-17A and IL-17F are localized in the same chromosomal region in mice and in humans. But IL-17F has significantly weaker biological activity than IL-17A [79, 80]. IL-17E, also known as IL-25, is produced by Th2 cells and mast cells. In contrast, IL-17B, IL-17C and IL-17D have not been well investigated [81, 82]. The receptor for IL-17A (IL-17R or IL-17RA) is a single-pass transmembrane protein of approximately 130 kDa. Four additional receptors (IL-17RB-RE) have been identified, but are not well characterized. IL-17RC was recently identified to be a receptor for IL-17F [83]. It has been reported that Th17 cells also produce IL-22 [84, 85] and CC-chemokine attractant ligand 20 (CCL20) [86] in mice and humans. Therefore, distinct from Th1 and Th2 cells, Th17 cells produce a unique and expanding array of pro-inflammatory cytokines.

Compelling evidence has demonstrated that the differentiation of Th17 cells from naïve $CD4^+$ T cells is initiated by cytokines IL-6 or TGF-β, and expanded by cytokines IL-23, IL-1β and IL-21. IL-6 or TGF-β was proposed as a major initiator necessary for Th17 differentiation [87-89]. IL-23 was the first cytokine shown to selectively regulate IL-17 expression [90, 91], but it might not be required for the initial differentiation of Th17 cells in vivo [92]. Recently, IL-1β was found to promote Th17 cell development and proliferation in the presence of TGF-β and IL-6 [88]. IL-21, produced by activated T cells and natural killer (NK) cells [93], may be required for full commitment of Th17 cells [94, 95]. Hence IL-23, IL-1β and IL-21 may possibly maintain and expand the differentiated Th17 cells in the presence of IL-6 and TGF-β [88]. Furthermore, STAT3 has been found to mediate the initiation of Th17 cell differentiation by these inducing cytokines [87]. Activation of STAT3 induces the expression of retinoic-acid-receptor-related orphan receptor-α (RORα) and RORγt [96], two transcription factors that promote the Th17-cell-associated gene-expression program, leading to the production of IL-17, IL-17F, IL-22 and CCL20.

Using peripheral $CD4^+$ T cell isolated from mouse spleen and cervical lymph nodes, our team evaluated the differential effects of these inducing cytokines in promoting Th17 differentiation [97]. The results showed that IL-6 and TGF-β1, only minimally induced IL-17 production at both mRNA and protein levels. In the presence of IL-6 and TGF-β1, IL-23 was the strongest stimulator of the Th17 signature cytokines IL-17A and IL-17F, IL-22, and chemokine CCL20, as well as STAT3 among the 3 expanding cytokines IL-23, IL-1β and IL-21. In the 4 cytokine system, IL-1β stimulated much higher levels of IL-17 family cytokines, 1.5-2 fold greater than IL-21 in the presence of TGF-β1, IL-6 and IL-23. These findings suggest that TGF-β1 and IL-6 initiate low level differentiation of Th17 cells; and their maintenance and development need other expanding factors, among which IL-23 plays a potent role and IL-1β amplifies this expansion further in Th17 differentiation [97].

A variety of mucosal epithelia have been found to produce Th17 inducing cytokines, including TGF-β1, IL-6, IL-23, and IL-1β [98, 99]. IL-1β is a well recognized proinflammatory

cytokine produced by mucosal epithelia in response to stress, infection or wounding. In an attempt to mimic known stressors of the ocular surface, we measured production of Th17 inducing cytokines in cultured HCECs in response to hyperosmotic stress, microbial components and inflammatory cytokines.

The ocular surface epithelium is subjected to hyperosmotic stress in dry eye conditions. Exposure of human epithelial cells to hyperosmotic stress has been noted to activate mitogen-activated protein kinase pathways and stimulate production of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-8 [100]. We have also observed that TGF-β1, IL-6 and IL-23 were highly induced in the corneal epithelium in response to hyperosmotic stress.

We evaluated the expression and production of Th17-inducing cytokines by HCECs in response to 9 extracted or synthetic microbial components that are ligands of TLRs 1-9, respectively. TGF-β1, IL-6, IL-23, and IL-1β expression and production were found to be largely induced by polyI: C, flagellin, and R837, the respective ligands for TLRs3, 5 and 7, representing viral or bacterial infections. Among these TLR agonists, polyI:C was the strongest stimulator of Th17 inducing cytokines by HCECs.

Hyperosmotic stress and microbial components also promoted production of pro-inflammatory cytokines including TNF-α, which plays an important role in ocular surface disease [100, 101]. Consequently, TNF-α stimulus was found to markedly induce TGF-β1, IL-6, IL-23, and IL-1β.

Based on our findings that among TLR ligands, polyI:C is the potent stimulator of Th17 inducing cytokines, and that TNF-α is a representative pro-inflammatory factor, we evaluated the Th17 inducing capacity of conditioned media (CM) of HCECs treated with polyI:C and TNF-α [97]. It has been observed that Th17 cell differentiation was significantly stimulated in $CD4^+$ T cells exposed to the 50% conditioned media of HCECs challenged by polyI:C (CM-polyI:C) or TNF-α (CM-TNF-α) when compared with media from untreated cultures.

As shown in Figure 8A-E, the mRNA levels of IL-17A, IL-17F, IL-22, CCL-20 and STAT3 were significantly higher in $CD4^+$ T cells treated with CM-polyI:C or CM-TNF-α for 4 days compared with the control medium or conditioned media of HCEC culture without any stressors (CM-Control, all $P<0.05$, n=3). IL-17 protein levels in the supernatants of $CD4^+$ T cells exposed to CM-polyI:C or CM-TNF-α for 4 days (Figure 8F) were also significantly higher than the media (both $P<0.01$, n=3) and CM ($P<0.05$, n=3) controls. Furthermore, the number of IL-17-producing cells differentiated from $CD4^+$ T cells, determined by ELISPOT bioassay (Figure 8G, 8H), displayed the same pattern to the induction of IL-17 mRNA and protein. The numbers of IL-17-producing cells were stimulated by CM-polyI:C or CM-TNF-α, to the levels similar to that seen in the 3-cytokine system (TGF-β1+IL-6+IL-23), suggesting that cytokines in the conditioned media of HCECs exposed to polyI:C or TNF-α were capable to promote Th17 cell expansion to levels induced by IL-6+TGF-β1+IL-23. It suggests that the Th17 cells can be indeed promoted by factors produced by corneal epithelium in response to a variety of inflammatory stimuli [97].

8. Th17-mediated inflammation in dry eye

Dry eye is the second most common problem of patients seeking eye care, and is characterized by eye irritation symptoms and blurred vision. The prevalence of dry eye

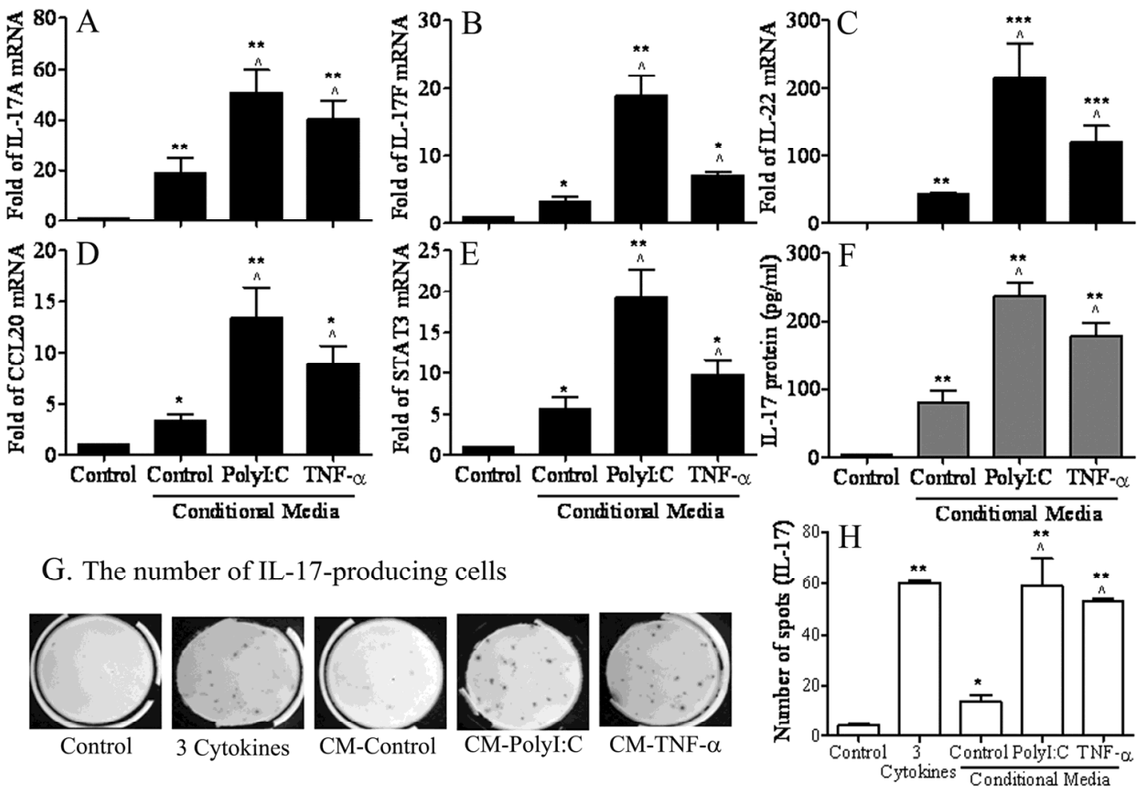

Fig. 8. Induction of Th17 differentiation of murine CD4+ T cells cultured in RPMI media containing 50% conditioned media (CM) of HCECs. **A-E.** Real-time PCR data showing the relative fold of mRNA of Th17 associated cytokines (IL-17A, IL-17F, IL-22, CCL-20) and regulator STAT3 in CD4+ T cells incubated for 4 days with 50% of conditioned media of HCECs irritated by polyI:C (CM-PolyI:C) or TNF-α (CM-TNF-α) for 48 hrs. **F.** Luminex immunobead assay showing IL-17 concentration in the supernatant of CD4+ T cells receiving the same treatment for 4 days. **G & H.** ELISPOT bioassay showing the spots/3x10^5 cells/well, representing the numbers of IL-17-producing T cells, in CD4+ T cells treated with CM-PolyI:C, CM-TNF-α, or 3 cytokines (TGF-β1+IL-6+IL-23) for 7 days. Results shown are mean ± SD of 3-5 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ each treated groups vs. media control. ^, $P < 0.05$; ^^, $P < 0.01$; ^^^, $P < 0.001$ CM-PolyI:C or CM-TNF-α groups vs. CM-control group.

increases with age, 6% at the age of 40, and 15-25% in the population over the age of 65. Among dry eye patients, 11% have been estimated to have the systemic autoimmune condition Sjögren's syndrome, a severe and potentially blinding condition. Dry eye is a potent stimulus of both innate and adaptive immune systems. At the nexus of the dry eye inflammatory/immune response is the dynamic interplay between the ocular surface epithelia and bone marrow derived immune cells. On the one hand, the ocular surface epithelial cells play a key initiating role in this inflammatory reaction, while on the other hand they are targets of cytokines that are produced by activated T cells that are recruited to the ocular surface in response to dry eye.

Dry eye has been demonstrated to cause inflammation of the ocular surface, evidenced by increased levels of inflammatory cytokines (IL-1, IL-6, and TNF-α) in the tear fluid, corneal and conjunctival epithelium, and an increased infiltration of dendritic cells and T lymphocytes in the conjunctiva [102-105]. Recently, increased levels of IL-17, IL-23 and IL-6 were found in saliva and salivary glands biopsies obtained from patients with the severe autoimmune dry eye condition, Sjögren's syndrome [106]. The increased matrix metalloproteinase (MMP) 9 and disrupted barrier function were observed in human [107] and murine dry eye [108]. Recently, our group has found increased expression of Th17 associated cytokines and IL-17-producing cells in human and experimental murine dry eye [108, 109]. Increased expression of Th17 cytokine IL-17A was observed in corneal and conjunctival epithelia of the dry eye mice. Since IL-17A is produced by T cells, not by epithelial cells, the Th17 reaction of the ocular surface is likely due to $CD4^+$ T cells, which have previously been found to infiltrate ocular surface tissues following experimental desiccating stress [110, 111]. Antibody neutralization of IL-17 ameliorated experimental dry eye-induced corneal epithelial barrier dysfunction and decreased the expression of MMP-3 and -9 [108]. These findings provide clear evidence that changes in the ocular surface environment, such as Th17-inducing cytokines, following desiccating stress are capable of inducing Th17 differentiation [112], which plays an important role in dry eye disease.

Th17 differentiation was also found to be mediated through a dendritic cell-mediated pathway. DCs have an important function in Th17 cell differentiation. They are antigen-presenting cells specialized to activate $CD4^+$ T cells and through their interaction with $CD4^+$ T cells to initiate primary immune responses. Furthermore, when primed, certain DCs express a high-level of Th17 inducing cytokines, including IL-6, TGF-β, IL-23 and IL-1 [113].

We found that efficient differentiation of $CD4^+$ T cells to IL-17 producers required the combination of ocular surface epithelium from dry eye mice and dendritic cells. $CD4^+$ T cells that were co-cultured with ocular epithelial explants from desiccating stress-induced dry eye mice and dendritic cells were found to express increased mRNA levels of Th17 cytokines (IL-17A, IL-17F, IL-22) and chemokine (C-C motif) ligand 20 (CCL20) (Figure 9A), as well as to produce and release IL-17A (Figure 9B & 9D), but not Th1 cytokine IFN-γ (Figure 9C). Exposure of dendritic cells to conditioned media from ocular surface explants of dry eye mice did not sufficiently activate these cells to promote T cell differentiation. The possible explanations for these findings include the need for direct contact between these cells, more efficient activation of cytokines such as TGF-β1 produced by the ocular surface epithelial cells or insufficient concentrations of Th17 inducing factors in the explants conditioned media.

The transcription factor RORγt was identified as a candidate master regulator that drives Th17 cell lineage differentiation [96]. Expression of RORγt is induced by TGF-β or IL-6, and overexpression of γt was found to promote Th17-cell differentiation when both Th1- and Th2-cell differentiations were blocked. In a model of experimental autoimmune encephalomyelitis, mice with RORγt-deficient T cells were found to have attenuated autoimmune disease and lacked tissue-infiltrating Th17 cells [96]. We found robust up-regulation (up to 100 fold) of the level of Th17 cell transcription factor-RORγt in T cells co-cultured with desiccated ocular surface tissues and dendritic cells (Figure 9E). This provides further evidence of the potent Th17 prone environment induced by desiccation.

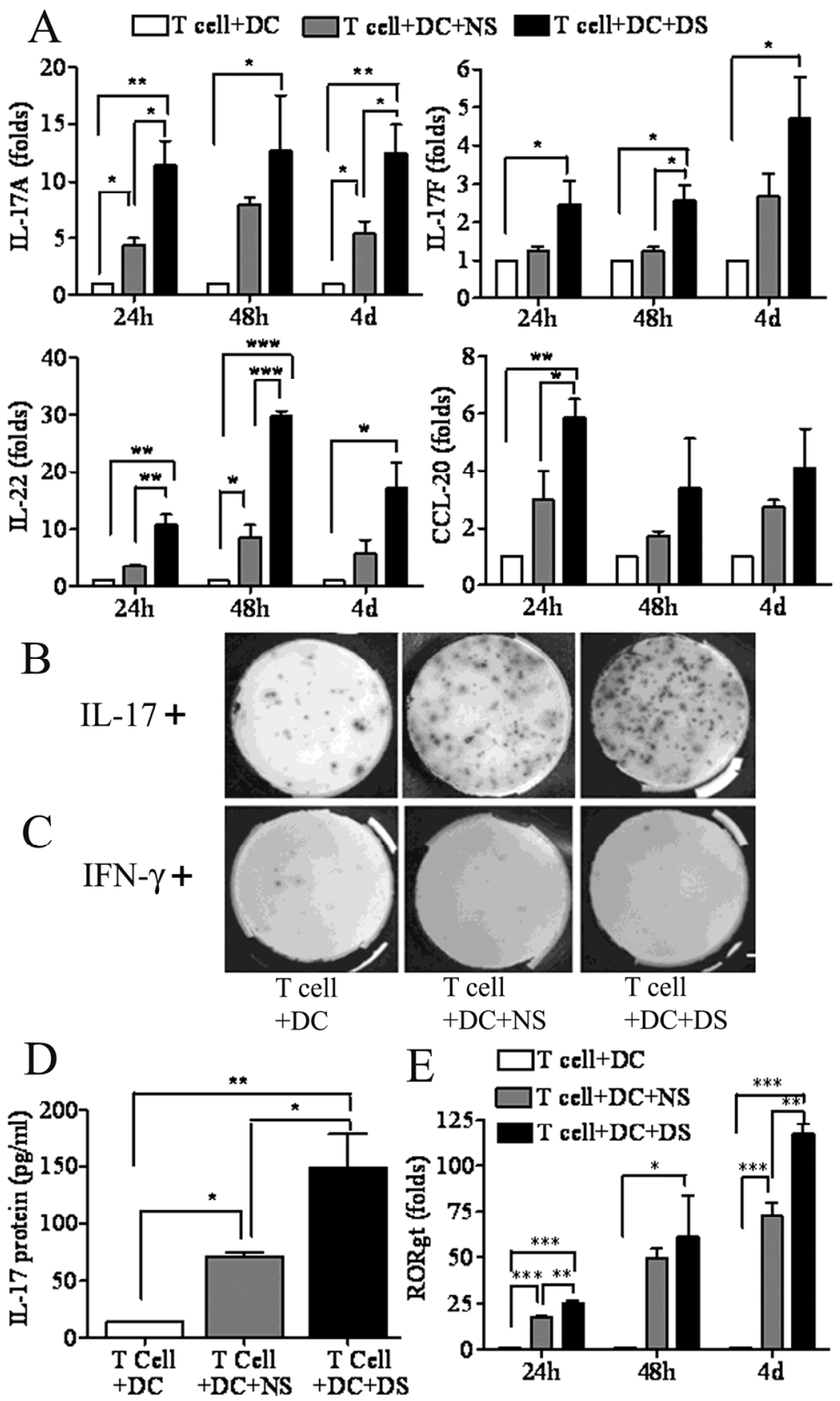

Fig. 9. Th17 differentiation of CD4+ T cells induced by the co-culture with dendritic cells (DCs) in the presence of cornea and conjunctival tissues of C57BL/6 mice subjected to desiccating stress. A. Real-time PCR data showing the relative mRNA expression (*x*-fold) of Th17 cytokines (IL-17A, IL-17F, IL-22, CCL-20) in CD4+ T cells (3x10^5 cells/ well) co-cultured for 1, 2, 4 days with DCs (T cell+DC), or with DCs and cornea and conjunctival explants from non-stressed control mice (T cell+DC+NS) or from mice desiccating stressed for 10-day (T

cell+DC+DS), (n=4). B & C. ELISPOT bioassay showing the numbers of IL-17 or IFN-γ-producing cells in these 3 groups. D. IL-17 concentration in the supernatant of $CD4^+$ T cells co-cultured with DCs for 4 days in absence or presence of corneal and conjunctival explants from non-stressed and 10 day desiccating stressed mice. E. Real-time PCR showing the relative mRNA expression of RORγt in $CD4^+$ T cells co-cultured for 1, 2, 4 days in 3 conditions. Data are presented as mean ± SD of 3 or 4 independent experiments. *P<0.05, **P<0.01, ***P<0.001.

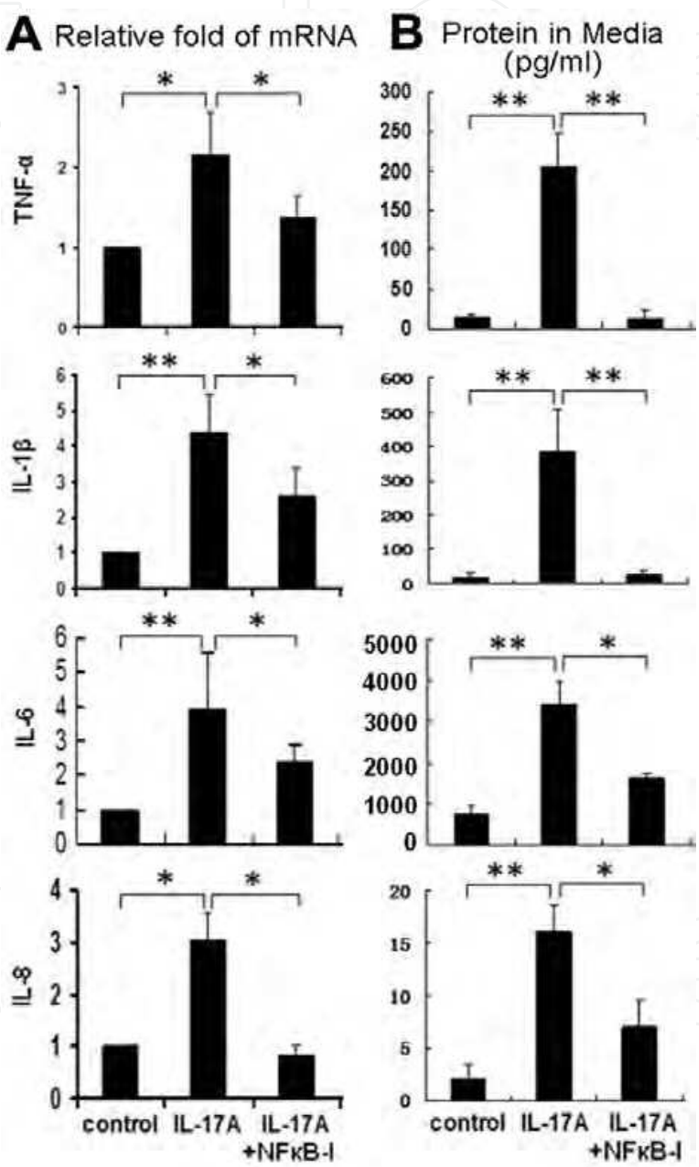

Fig. 10. The inflammatory effects of IL-17 on HCECs. Primary HCECs (5×10^5 cells/well) were treated with recombinant human IL-17A at 10 ng/ml for 4-48 hours with or without NF-κB activation inhibitor quinazoline (NFκB-I, 5μM). The pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokine IL-8 were measured by RT-qPCR for mRNA (**A**) and by ELISA or Luminex immunobead assays in culture supernatants (**B**). Results shown are mean ± SD of 4 independent experiments. * $P < 0.05$, ** $P< 0.01$.

IL-17 producing T cells are distinct from Th1 cells. Analysis of the expression of transcription factors showed clearly that IL-17-producing T cells expressed neither GATA-3 nor T-bet and its target Hlx, which were typically expressed by IFN-γ-producing Th1 cells [88]. In the T cells co-cultured with desiccated ocular surface tissues and dendritic cells, we observed the lower expression of Th1 associated factors (IL-2, T-bet) and Th2 associated factors (IL-4, IL-13, GATA-3). There was no change in production of IFN-γ and IL-12 transcripts as well as in the number of IFN-γ-producing $CD4^+$T cells in this co-culture system. Taken together, these findings indicate that desiccating stress may selectively promote the Th17 pathway, a finding that is consistent with the increased level of IL-17 in dry eye disease [108].

IL-17 initiates pro-inflammatory effects by binding to the IL-17 receptor (IL-17R), which is expressed by a variety of cell types including epithelial, endothelial, and fibroblastic stromal cells [81, 114]. As shown in Figure 10, recombinant human IL-17A (10 ng/ml) significantly increased mRNA levels (2-4 fold) of proinflammatory cytokines (TNF-α, IL-1β and IL-6) and chemokine IL-8 expressed by HCECs. These stimulatory responses to IL-17A were confirmed by 4-7 fold increases at protein levels. The stimulated production of these inflammatory cytokines and chemokine was significantly suppressed at both mRNA (all $P<0.05$, n=4) and protein levels ($P<0.05$ or 0.01) by NF-κB activation inhibitor quinazoline [115], indicating NF-κB pathway is involved in the inflammatory effect of IL-17 on mucosal epithelium.

These findings demonstrate that desiccating stress stimulates the expression and production of Th17 inducing cytokines by corneal and conjunctival epithelia, and that desiccation creates an environment promoting Th17 differentiation through a dendritic cell-mediated pathway. We hypothesize that Th17 inducing cytokines produced by the ocular epithelium may participate in Th17 differentiation in three ways: (1) activation of immature dendritic cells on the ocular surface; (2) direct transfer to the lymph node in lymphatic liquid; or (3) direct promotion of differentiated Th17 cells that infiltrate the ocular surface.

9. Conclusion

This chapter focused on recent breakthroughs in ocular mucosal immunology, including the discoveries of TLR signaling in innate immunity, novel epithelium-derived pro-allergic cytokines TSLP and IL-33, and a new Th17 cell population in adaptive immunity. One of important breakthroughs is a discovery of a novel mechanism by which short ragweed pollen, serving as a functional TLR4 agonist, induces TSLP/OX40L/OX40 signaling to trigger Th2-dominant allergic inflammation via TLR4-dependent innate immunity pathways. All these advances provide compelling evidence that mucosal epithelium actively participate, as initiators, mediators and regulators, in innate and adaptive immune responses for host defense, in addition to physical barrier function. These novel signaling molecules may be critical for allergic, inflammatory and autoimmune diseases on mucosal ocular surface, and may become potential molecular targets for new therapies to treat these ocular diseases.

10. Abbreviations used in this chapter

CLN: cervical lymph nodes; **DAMP**: damage-associated molecular patterns; **DC**: dendritic cell; **dsRNA**: double stranded RNA; **EAC**: experimental allergic conjunctivitis; **HCECs**:

human corneal epithelial cells; **IL**: interleukin; **MyD88**: myeloid differentiation primary response gene 88; *MyD88-/-*: *MyD88* knockout mice; **LPS**: lipopolysaccharide; **NF-κB**: nuclear factor kappa B; **PBS**: phosphate buffered saline; **PGN**: peptidoglycan; **PAMP**: pathogen-associated molecular patterns; **PCR**: polymerase chain reaction; **polyI:C**: polyinosinic-polycytidylic acid; **PRR**: pattern recognition receptor; **R837**: imiquimod; **RT-qPCR**: reverse transcription and quantitative real-time PCR; ROR: retinoic-acid-receptor-related orphan receptor; **SRW**: short ragweed; **SRWe**: aqueous extract of defatted short ragweed pollen; **ssRNA**: single stranded RNA; **TGF-β**: transforming growth factor; **Th1**: T helper cell type 1; **Th17**: T helper cell producing IL-17 family; **Th2**: T helper cell type 2; **TLR**: Toll-like receptor; *Tlr4-d*: *Tlr4* gene deficient; **TRIF**: TIR-domain-containing adaptor inducing interferon; **TSLP**: thymic stromal lymphopoietin; **TSLPR**: TSLP receptor.

11. References

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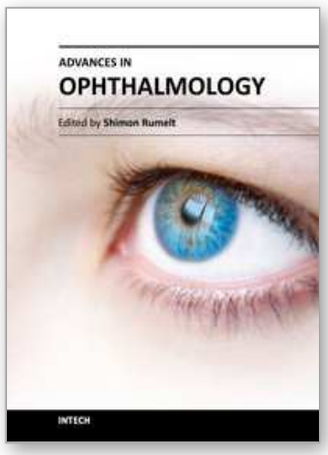

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This book focuses on the different aspects of ophthalmology - the medical science of diagnosis and treatment of eye disorders. Ophthalmology is divided into various clinical subspecialties, such as cornea, cataract, glaucoma, uveitis, retina, neuro-ophthalmology, pediatric ophthalmology, oncology, pathology, and oculoplastics. This book incorporates new developments as well as future perspectives in ophthalmology and is a balanced product between covering a wide range of diseases and expedited publication. It is intended to be the appetizer for other books to follow. Ophthalmologists, researchers, specialists, trainees, and general practitioners with an interest in ophthalmology will find this book interesting and useful.

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