ABSTRACT

Ocular allergy is one of the most common allergic diseases with an increasing prevalence worldwide. Pollen is a ubiquitous allergen that affects a large population with allergic diseases including allergic conjunctivitis. However, the underlying molecular mechanism by which pollen induces Th2-dominant allergic inflammation remains poorly understood. This chapter summarized recent advance in ocular allergic diseases based on literature review and our investigations.

The concept of innate immunity has been largely changed. The innate immunity has been expanded to recognize and respond to environmental pathogens other than conserved microbe, such as pollen. Epithelium is known to play a vital role in innate immunity and serves as a bridge
linking innate to adaptive immune responses. The epithelium-derived pro-allergic cytokines, thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33 have been recognized as key initiators in allergic inflammation. Allergic diseases have been seen as epithelial disorders both structurally and functionally.

Our recent studies have developed a novel pollen/TLR4 concept, the innate immunity pathways, where short ragweed (SRW) pollen triggers Th2-dominent allergic inflammation via TLR4-dependent innate immunity by mucosal epithelium that produces pro-allergic cytokines, TSLP and IL-33. First, we have revealed that the induction of TSLP and IL-33 by ocular epithelium is mediated by TLR-dependent innate pathways in response to microbial components and SRW pollen. Second, we have identified that dendritic cells not only respond to but also produce TSLP and IL-33 via TLRs/NFκB-mediated pathways, which suggests a novel mechanism by which local inflammatory response may be amplified by dendritic cells via a autocrine regulation of TSLP/TSLPR and IL-33/ST2 loops. Finally, we uncovered two novel allergic signaling pathways, TSLP/OX40L/OX40/Th2 and IL-33/ST2/Th2, initiated by mucosal epithelium via TLR4-dependent innate response in SRW pollen-induced allergic conjunctivitis. Discovery of a novel pollen/TLR4 concept in allergic inflammation may create new molecule targets and therapeutic potential to prevent or cure allergic diseases.

Keywords: Pollen; Allergy; Conjunctivitis; Toll-like receptor; Thymic stromal lymphopoietin; IL-33; Innate immunity

INTRODUCTION

Ocular allergy is one of the most common allergic diseases with an increasing prevalence worldwide, estimated to affect 15–25% of the US population [1,2] and as much as 50% of the Europe population, possibly due to the introduction of ragweed in 2009 [3]. Ocular allergy manifests vary from benign seasonal allergic conjunctivitis, perennial allergic conjunctivitis and giant papillary conjunctivitis to the chronic, morbid, and potentially sight-threatening vernal keratoconjunctivitis, atopic keratoconjunctivitis and contact blepharoconjunctivitis [4].

Ocular allergy is a complex inflammatory disease involving multiple target tissues. Allergens, such as pollen, interact first with the corneal and conjunctival epithelial cells by innate immunity, then crosses the epithelium into the stroma where activation of immune cells including mast cells, eosinophils, dendritic cells, T cells and macrophages occurs. Much of research efforts have focused on the cells and molecules that mediate adaptive immunity, and identified T help type 2 (Th2) cell dominant disorders in most allergic inflammatory diseases [5,6]. However, the underlying mechanism for initiation of this adaptive immune disorder by mucosal epithelium remains a relative mystery.

Increasing evidence suggests that the concept of innate immunity has been largely changed. Traditionally, the essential role of innate immunity is recognition of invading microorganisms,
a process mediated by germ line-encoded pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), NOD-like receptors (NLRs) [7,8] etc. Recent breakthroughs reveal that the innate immunity has been expanded to recognize and respond to environmental pathogen associated molecular patterns (PAMPs) other than conserved microbial components, such as pollen.

The advances in innate immunity have also changed the view on epithelial cells. In addition to a long-recognized property of their physical barrier function, epithelial cells are known to play a vital role in innate immunity and serve as a bridge linking innate to adaptive immune responses [9]. A recently major breakthrough has further uncovered that epithelium-derived pro-allergic cytokines, thymic stromal lymphopoietin (TSLP) [10-12] and interleukin (IL) 33 [13,14] are key initiators in allergic inflammatory diseases. Thus, allergic disease is increasingly being seen as an epithelial disorder both structurally and functionally.

Pollen is a ubiquitous allergen that affects a large population with allergic diseases. 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 remains poorly understood. This represents an important challenge for us to resolve allergen-driven inflammation, which potentially leads to recurrent or chronic allergic diseases. Ambrosia artemisiifolia short ragweed (SRW) is the most widespread plant in North America and Europe [3]. SRW pollen induced allergic conjunctivitis is a good model to study allergic diseases [15]. Our recent studies have developed a novel pollen/TLR4 concept, the innate immunity pathways, where SRW pollen triggers Th2-dominant allergic inflammation via TLR4-dependent innate immunity by mucosal epithelium that produces pro-allergic cytokines, TSLP [16] and IL-33[17].

**POLLEN/TLR4 INNATE IMMUNITY SIGNALING INITIATES TSLP/OX40L/OX40 MOLECULAR PATHWAYS IN ALLERGIC CONJUNCTIVITIS**

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 [10,11,18]. Compelling evidence demonstrates that TSLP represents one of the master switches initiating allergic inflammation at the interface between epithelial cells and dendritic cells, and has a determinant role in atopic dermatitis, asthma and ocular allergy [16,19,20].

**Large Induction of TSLP by Ocular Epithelium via TLR-dependent Innate Pathways in Response to Microbe and SRW Pollen**

TSLP is produced primarily by epithelial cells in the lungs, gut, and skin, although fibroblasts, smooth muscle cells, and mast cells all have the potential to produce TSLP [21-23]. Recent work
has shown that TSLP levels increased at sites of inflammation. For example, airway epithelium from asthmatics showed increased TSLP expression that supports a role of TSLP in promoting Th2 allergic inflammation. CD4+ T cells, primed by TSLP-treated dendritic cells, produce the proallergic cytokines IL-4, IL-13, IL-5, and TNF-α, but not IL-10 and IFNγ [24].

Using fresh human corneoscleral tissue and primary cultured human corneal epithelial cells (HCECs), our study has revealed that TSLP is expressed by normal corneal epithelium and is largely induced by microbial components, inflammatory cytokines and Th2 cytokines through TLR and NF-kB signaling pathways [23]. As shown in Figure 1A, TSLP mRNA expression and protein production were found to be largely induced by polyI: C, flagellin and FSL-1, the ligands for TLRs 3, 5 and 6, representing viral dsRNA and the bacterial components flagellin and lipopeptides, 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 inhibited TSLP expression. The pattern of TLR-dependent TSLP induction was further evidenced by ex vivo human corneal tissues exposed to polyI: C and flagellin, suggesting that HCECs are able to rapidly initiate an innate immune response to virus or bacteria by producing TSLP.

Interestingly, our studies provided first evidence that SRW pollen stimulates TSLP expression and production by ocular surface epithelia via TLR4 innate immune pathways in an in vitro culture model of primary HCECs and an in vivo mouse model of topical challenge treated with aqueous extract of defatted SRW pollen (SRWe) [16]. As shown in Figure 1B, we observed that SRWe dose-dependently stimulated TSLP expression and production in the human culture model, and this stimulation was blocked by the TLR4 antibody and NF-kB activation inhibitor quinazoline. In an in vivo topical challenge mice, SRWe stimulated TSLP production in a dose-dependent fashion, and the stimulation was largely blocked by exogenous TLR4 antibody or by deletion of Tlr4 or MyD88 genes (Figure 1C). This finding supports our hypothesis that SRW pollen serving as a functional TLR4 agonist stimulates TSLP production via NF-kB signaling pathway. These findings provide new evidence that corneal epithelial cell-derived TSLP may serve as a potential link between the innate and adaptive immune responses in allergic inflammatory disease.
Figure 1: TSLP induction by ocular epithelium via TLR-dependent innate pathways in response to microbial components and SRW pollen. A. Concentration-dependent induction of TSLP by HCECs exposed to 5-50 µg/ml polyI:C, 0.1-10 µg/ml of flagellin or FSL-1 for 4 hours for TSLP mRNA or 48 hours for TSLP protein in the supernatants. B. SRW induces TSLP mRNA and protein by HCECs through TLR4 and NF-κB signaling pathways. C. SRW induces TSLP mRNA and protein by murine corneal and conjunctival epithelia through TLR4-and MyD88-dependent pathway.
Potential Link between Bacteria and Allergy by TSLP-Producing Dendritic Cells

It has been well documented that epithelial cells are major producer of TSLP, and DCs express abundant TSLP receptor (TSLPR) to respond to TSLP activation [25,26]. Until very recently, TSLP was found to be expressed by DCs [27,28]. However, the association and mechanism of bacterial pathogens linking to the allergic inflammation have not been well elucidated. Our study has demonstrated that DCs not only respond to TSLP but also produce TSLP through autocrine regulation [29].

As shown in Figure 2 A & B, LPS and flagellin significantly induced TSLP mRNA expression to 10-14 fold (P<0.01) in dose-dependent fashion; pam3 CSK4, polyI:C, FSL-1 and R837 also up regulated TSLP mRNA by 3-5 fold (P<0.05); while PGN, ssRNA and C-CpG-ODN did not significantly induce TSLP expression. 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 (Figure 2C). 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 (Figure 2D). The immune fluorescent 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 (Figure 2E), when compared with untreated DCs.

Myeloid differentiation primary-response protein 88 (MyD88) is a universal adapter protein necessary for response to most TLRs except TLR3 [30,31]. We incubated bone marrow-derived DCs from BALB/c mice with flagellin and observed the significantly increased mRNA expression of MyD88. We then cultured 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 Figure 2F, TSLP induction, as evaluated by immunofluorescent staining, was significantly increased by flagellin in DCs from wild type mice, but not in DCs of MyD88−/− knockout mice. These findings demonstrate that flagellin promotes TSLP production via TLR-mediated signaling pathways.
**Figure 2:** Murine dendritic cells (DCs) produce TSLP via TLR/MyD88-dependent pathway in response to microbial pathogens. A. TSLP mRNA expression 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. B. The time course and dose response of TSLP mRNA by DCs exposed to LPS or flagellin. C and D. TSLP protein production in culture medium or cell lysates of DCs treated with LPS (1μg/ml) or flagellin (10μg/ml) for 24 h respectively. E. Representative images showing TSLP localization (green) in DCs exposed to LPS (1μg/ml) or flagellin (10μg/ml) for 24 h by immune fluorescent staining with PI (Red) as nuclear counterstaining. F. TSLP production was evaluated by immune fluorescent staining (Green) with PI counterstaining (Red) in DCs from MyD88+/+ and MyD88−/− mice treated with flagellin (10μg/ml) for 24 h. Each bar in the diagrams represents mean ± SD of three to five independent experiments. *P<0.05; **P<0.01.
We further investigated whether flagellin promoted TSLP production in DCs via NFκB-mediated signaling pathways. The mRNA expression of NFκB1, NFκB2, and RelA 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. Immuno fluorescent staining revealed that NFκB-p65 protein was mainly located in cytoplasm in untreated control DCs, but markedly translocated from cytoplasm to nucleus in response to flagellin, indicating NFκB signaling activation. Interestingly, the stimulated mRNA expression of MyD88, NFκB1 and NFκB2, the nuclear translocation of NFκB p65, and the increased TSLP production by flagellin were significantly blocked by TLR5 antibody or NFκB activation inhibitor quinazoline [29].

TSLP has been known to prime or activate myeloid DCs to produce OX40L that triggers a Th2 response. We have observed that TSLP promotes DC mature with increased expression of CD40, CD80, and OX40L. TSLP-producing DCs were identified in ocular surface conjunctiva and draining cervical lymph nodes (CLN) from two murine models of topical challenge with LPS or flagellin, and experimental allergic conjunctivitis (EAC) in BALB/c mice. The findings reveal that DCs not only respond to TSLP, but also produce TSLP in response to microbial pathogens. The production of TSLP by DCs might amplify local allergic inflammatory response via a novel autocrine mechanism, suggesting a potential link between bacterial pathogens to allergic inflammation.

**TLR4-Dependent TSLP/OX40L/OX40 Signaling Pathway in Short Ragweed Pollen-induced Allergic Conjunctivitis**

Many studies have focused on the allergen-induced inflammation, and TSLP has been identified to trigger Th2-dominant allergic inflammation in clinical patients with atopic dermatitis, asthma, allergic rhinitis and conjunctivitis [32-37]. However, the mechanisms leading to resolution of pollen-driven allergic inflammation remain poorly understood. This represents an important challenge because failure to resolve pollen-driven inflammation potentially leads to recurrent or chronic allergic diseases.

Pollen is a ubiquitous allergen that affects a large population, and Ambrosia artemisiifolia short ragweed is the most widespread plant in North America and Europe. SRW pollen and extracts have been widely used for allergic models including asthma, rhinitis, skin and ocular allergy in mouse, guinea-pig, dog and other animal species [15,38-40]. However, there was no report showing that SRW pollen triggers TSLP signaling in these allergic models. Using multiple in vivo mouse and in vitro culture models, we have recently uncovered a previously unknown mechanism by which pollen induces TSLP-triggered Th2-dominant inflammation through a TLR4-dependent innate pathway [16,41].

The animal model of EAC was first established by Magone et al in 1998 using a single topical challenge with SRW pollen in SRW-sensitized mice [15]. This murine model incorporates the
clinical, cellular, and humoral parameters of allergic conjunctivitis, including a ragweed-induced Th2 cytokine production by lymphocytes. This model was modified by Stern and colleagues using repeated ocular exposure to SRW [38], which resulted in the development of late phase conjunctival inflammation and displayed parameters indicative of a Th2 allergic conjunctivitis through chronic ocular exposure to SRW allergens. Using the same repeated ocular exposure to SRW for 3 consecutive days, we were able to induce allergic conjunctivitis in SRW-sensitized mice with similar symptoms and signs as described in the previous reports [15,38].

In this EAC model, TSLP expression at mRNA and protein levels were significantly up regulated in the corneal and conjunctival epithelia from SRW mice compared with the PBS alone and untreated control groups. The increased mRNA levels and immune reactivity of CD11c, TSLPR and OX40L were observed in the ocular surface, primarily in the stroma subjacent to conjunctival epithelia, where large amount of infiltrated CD4+ Th2 cells were observed as evidenced by increased expression and immune reactivity of CD4, IL-4, IL-5 and IL-13 in the conjunctival tissues. The stimulated expression of OX40, STAT6 and GATA3 by the cells in conjunctiva and draining CLNs provides evidence that Th2-dominant pathway was activated in EAC mice [16,41]. These findings demonstrate that TSLP-activated DCs, through OX40L/OX40 interaction, prime the CD4+ T cells to differentiate to Th2 cytokine-producing cells by activation of IL-4-dependent transcription factors GATA-3 and STAT6 in this EAC model induced by SRW pollen.

To explore whether SRW pollen stimulates TSLP through TLR4-dependent innate response, we sensitized and topically challenged the TLR4-deficient (Tlr4-d) and MyD88 knockout (MyD88−/−) mice with SRW pollen (Figure 3). 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. The SRW topical challenges triggered the typical allergic signs and scratching behavior in wild type MyD88+/+ 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 and protein levels. However, the clinical allergic signs and stimulated production of TSLP signaling molecules and Th2 cytokines were dramatically reduced or eliminated in SRW challenged MyD88−/− mice. These findings suggest that TLR4-dependent TSLP signaling was involved in the SRW pollen-induced allergic inflammation.
Figure 3: The stimulated production of TSLP signaling proteins and Th2-dominant inflammation in SRW-induced EAC model requires TLR4 and MyD88. A, C. immuno histochemical staining of TSLP signaling molecules and Th2 cytokines on cornea and conjunctiva (Conj) of wild type and Tlr4 deficient BALB/c mice (A), and C57BL/6 based wild type MyD88+/+ and MyD88−/− mice (C), challenged by SRW pollen, with PBS-treated mice as controls. B, D. immune fluorescent staining of TSLP activated signals, TSLPR, OX40L and OX40 in CLN of different strains as described above. Bar: 20µm; Arrows: red or red brown positive staining signals.
In conclusion, these findings for the first time uncovered a novel phenomenon 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. These novel findings shed light on the understanding of innate mucosal epithelial immunity involved in allergic inflammation, and may create new therapeutic targets to treat allergic disease.

POLLEN/TLR4 INNATE IMMUNITY SIGNALING TRIGGERS IL-33/ST2/TH2 PATHWAYS IN ALLERGIC CONJUNCTIVITIS

A recently major breakthrough has been explored that epithelium-derived pro-allergic cytokines are key initiators in allergic inflammatory diseases. In addition to TSLP, IL-33 is another novel pro-allergic epithelial cytokine. IL-33, a newly discovered IL-1 family cytokine, was recently identified as a functional ligand to IL-1 receptor like 1 (IL1RL1), also referred as to ST2, which has been well known as a receptor on Th2 cells to mediate allergic inflammatory diseases [42-45]. IL-33 has been now recognized to trigger asthma, rhinitis, atopic dermatitis and allergic conjunctivitis [46-51]. Recently, the role of IL-33 in initiating allergic inflammation has been investigated in pollen-induced mouse models of allergic conjunctivitis or allergic rhinitis [17,52,53].

IL-33 Induction by Ocular Epithelium via TLR-dependent Innate Pathways in Response to Microbe and SRW Pollen

Mucosal epithelium functions not only as a physical barrier, but also as a regulator of innate and adaptive immune responses against foreign substances and microorganisms. In particular, epithelial cells have been directly implicated in Th2 responses, serving as a critical interface between innate immunity and adaptive immune response. TLR activation by pathogens on the ocular surface would result in innate immune responses that stimulate production of inflammatory cytokines and chemokines, as well as promote immune cell infiltration into the area to alleviate the microbial load and resolve the infection.

IL-33 is produced mainly by epithelial and endothelial cells, fibroblast, and others [14,54,55]. By binding to ST2 receptor, IL-33 can activate Th2 cells and mast cells to secrete Th2 cytokines and chemokines that lead to severe pathological changes in mucosal organs [54]. IL-33 expression has been found to be up-regulated by stimulation with inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and IL-1β [56]. However, it is still unclear how IL-33 is induced by environmental allergens, and how microbial components play a role in initiating and developing allergic diseases.

We have investigated the TLR-mediated induction of IL-33 and the signaling pathway in human ocular surface mucosa that responds to microbial components, and explored the potential role of an innate immune response of IL-33 in Th2-dominant allergic inflammation [57].

Primary HCECs were challenged by a variety of extracted or synthetic microbial components, representing the ligands respective to TLRs 1-9 for 4-48 hours. IL-33 expression at the mRNA and protein levels was evaluated by RT and real-time PCR and ELISA, respectively. As shown...
in Figure 4 A and B, the expression of pro allergic cytokine IL-33 in untreated primary HCECs was at a relatively low level, but was largely induced after exposure to certain viral or bacterial components. The mRNA expression and protein production of IL-33 was largely induced by polyI:C, 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 the most robust IL-33 inducers, stimulating IL-33 production by 7- and 4-fold, respectively, in HCECs. The expression of IL-33 mRNA was induced to peak levels at 8 hours in a concentration-dependent fashion by several microbial ligands, including polyI: C.

To further investigate the cellular location and stimulation of IL-33 protein ex vivo, fresh human corneal tissues were incubated ex vivo with polyI: C or flagellin for 48 hours. Immunohistochemical staining showed that IL-33 protein was normally produced by certain basal epithelial cells, and mainly located in the nucleus and cytoplasm in the untreated corneal epithelial tissues. Stronger staining throughout multiple layers of corneal epithelium was observed in the donor tissues exposed to polyI: C (50µg/mL) or flagellin (10µg/mL) for 48 hours (Figure 4C). The specificity of the TLR-dependent response by HCECs was also confirmed by significant inhibition of polyI:C- or flagellin-stimulated IL-33 expression following pretreatment with TLR3 or TLR5 antibodies, respectively. IL-33 induction was further identified to be mediated by the TLR and NF-κB signaling pathways. 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.

Furthermore, we demonstrated that the pollen/TLR4 signaling is essential or indispensible for IL-33 induction by SRWe using an in vitro culture model of primary HCECs. As shown in Figure 4D, IL-33 expression at mRNA and protein levels was concentration-dependently upregulated by SRWe (1-50µg/ml) in primary HCECs and this stimulation was significantly blocked by a neutralizing antibody against human TLR4, but not by its isotype mouse IgG2a k (Figure 6). Furthermore, SRWe-stimulated IL-33 expression was also significantly inhibited by quinazoline, a NF-κB Activation Inhibitor. The findings provide novel evidence that pollen/TLR4 signaling induces IL-33 expression via NF-κB pathway.
Figure 4: TLR-dependent induction of IL-33 by microbial ligands and SRWe in primary HCECs. A. IL-33 mRNA levels after stimulation by 50μg/ml polyI:C or 10μg/ml of Pam3CSK4, PGN, polyI:C, LPS, flagellin, FSL-1, R-837, ssRNA40 or C-CpG-ODN. B. IL-33 protein concentrations by ELISA in cell lysates treated with various TLR ligands for 48 hours. C. IL-33 induction in an ex vivo human corneal tissues evaluated by immunohistochemical staining. D. IL-33 mRNA and protein levels were induced by SRWe in HCEC culture with or without pre-incubation of mouse TLR4 antibody (5μg/ml), isotype mouse IgG2a k, or NF-kB Activation Inhibitor quinazoline (NI,10μM).

Results shown are the mean ± SD of 3-5 separate experiments. *P< 0.05; **P < 0.01.
Autocrine Regulation of IL-33/ST2 Signaling of Dendritic Cells in Allergic Inflammation

DCs are the most potent professional antigen presenting cells linking innate and adaptive immune response. DCs express a variety of TLRs, which recognize conserved microbial components and play an important role in the mucosal innate immune system [58]. Mucosal surfaces contain resident DCs capable of sensing the external stimuli and mounting local responses upon recognition of invading microorganisms [59,60]. However, it is not clear that DCs produce IL-33 via TLR-mediated innate immunity signaling. Our recent study explored a novel phenomenon that DCs produce IL-33 in response to microbial pathogens, which may play an important role in amplifying the local allergic inflammatory response through a potential autocrine regulatory mechanism.

When incubated the murine bone marrow-derived DCs with TLR ligands 1-9, several TLR ligands, especially LPS and flagellin, the ligands to TLR4 and TLR5 respectively, were observed to significantly stimulate IL-33 expression by mouse DCs at both mRNA and protein levels, as determined by RT-qPCR, ELISA, Western blotting and immune fluorescent staining respectively (Figure 5A-E).

Using a topical challenge mouse model with LPS and flagellin, we identified that DCs produce IL-33 in vivo (Figure 5F). The infiltrated CD11c+ DCs that produce IL-33 were observed in the conjunctiva challenged with LPS or flagellin. We further found that CD11c+ DCs migrated to CLNs and expressed high level of IL-33. The double reactive cells (IL-33+CD11c+), the most should be DCs, in CLN significantly increased 3.7-4.5 fold in LPS or flagellin challenged mice, respectively.

Furthermore, IL-33-producing DCs were observed to accumulate in the ocular surface and the draining CLNs in a murine EAC model, as evaluated by double staining with CD11c and IL-33 antibodies (Figure 5G). The double reactive cells (IL-33+CD11c+) in CLN also increased significantly by 5.2 fold in EAC mice.
Figure 5: Autocrine regulation of IL-33/ST2 signaling by dendritic cells (DCs) in response to microbial pathogens. A. IL-33 mRNA expression by 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 8 h. B. The time course and dose response of IL-33 mRNA by DCs exposed to LPS or flagellin. C and D. IL-33 protein production in cell lysates of DCs treated with LPS (1μg/ml) or flagellin (10μg/ml) for 24 h by ELISA and western blotting, respectively. E. Representative images showing IL-33 immuno reactivity (green) in DCs exposed to LPS or flagellin for 24 h with PI (Red) as nuclear counterstaining. F. DCs produce IL-33 in murine conjunctiva (Conj) and cervical lymph nodes (CLN) in vivo by double immuno fluorescent staining with CD11c (Red) and IL-33 (Green) using DAPI counterstaining (Blue). G. DCs produce IL-33 in EAC mice. Each bar in the diagrams represents mean ± SD of 3-5 independent experiments. *P<0.05; **P<0.01.
IL-33 induction by DCs in response to microbial pathogens was identified via TLR/NF-κB signaling pathways. Using flagellin as a model, we observed that flagellin significantly stimulated the expression of MyD88, NF-κB, NF-κB2, RelA and IL-33, as well as NF-κB activation with p65 nuclear translocation. The stimulated IL-33 production by flagellin was markedly blocked by TLR5 antibody and NF-κB inhibitor. Furthermore, the IL-33 induction by flagellin was significantly reduced in DCs derived from MyD88−/− mice when compared with their wild-type littermate.

As DCs could express ST2 [61], our finding that DCs also produce IL-33 suggests a potential autocrine mechanism. We further observed that exogenous IL-33 stimulated expression of costimulatory molecules, CD40, CD80, and OX40L, as well as Th2 inflammatory cytokines in DCs, while these stimulatory effects were significantly blocked by ST2 antibody or soluble ST2 protein. DCs not only respond to IL-33 but also produce IL-33 in allergic condition. These findings suggest a novel mechanism by which local inflammatory response may be amplified by IL-33-producing DCs via a potential autocrine regulation, which may provide a therapeutic potential to treat ocular allergic disease through a local blockade of IL-33.

**TLR4-dependent IL-33/ST2/Th2 Signaling Pathway in Short Ragweed Pollen-induced Allergic Conjunctivitis**

Based on epithelial origin and induction by innate immune response, IL-33 is well qualified to serve as another pro-allergic cytokine that initiates Th2-dominant allergic inflammation via pollen/TLR4-depedent signaling pathway. Our recent investigation confirmed our hypothesis [17].

Firstly we demonstrated the IL-33/ST2 signaling pathway that triggers Th2-dominant allergic inflammation in a well-characterized murine EAC model by SRW pollen [15,38,39]. SRW has been widely used for allergic models including asthma, rhinitis and skin allergy, in mouse, guinea-pig, dog and other animal species [6,62,63]. However, there is no report showing how SRW pollen triggers IL-33/ST2 signaling although ST2 has been well known as a receptor on Th2 cells to mediate allergic inflammation [13,43,54,64]. Our study have shown that IL-33 expression was largely induced in corneal and conjunctival epithelia at both mRNA and protein levels, the infiltrated CD4+ T cells in conjunctiva were largely in Th2 lineage expressing IL-4, IL-5 and IL-13 with activated Th2 cell receptors ST2 and IL1RAP (Figure 6 A-B), and CD4+ T cells in the ocular draining CLNs were also differentiated to Th2 cells in response to ocular surface epithelium-derived IL-33 in an EAC model induced by SRW pollen (Figure 6C). To explore the direct effects of IL-33 on T cells, we further showed that mrIL-33 dose-dependently stimulated expression at both mRNA and protein levels of Th2 cytokines IL-4, IL-5, and IL-13.

Secondly, we demonstrated that IL-33/ST2 allergic pathway is initiated by pollen/TLR4 innate immunity signaling in Th2-dominant inflammation. This novel mechanism was revealed by strong evidence that all clinical signs of allergic conjunctivitis, stimulated expression of IL-33 by ocular epithelium, up regulated Th2 cell receptors ST2 and IL1RAP, as well as Th2 cytokine-
dominant inflammation were only observed in EAC model of wild type BALB/c and $\text{MyD88}^{+/+}$ mice, but not in $\text{Tlr4-d}$ or $\text{MyD88}^{-/-}$ mice with the same treatment by SRW sensitization and ocular surface challenge (Figure 6).

**Figure 6:** The stimulated production of IL-33 in SRW-induced EAC model requires TLR4 and MyD88. A & B. Immunohistochemical staining of IL-33 and its signaling molecules and Th2 cytokines on cornea and conjunctiva (**Conj**) in wild type BALB/c and $\text{Tlr4-d}$ mice (A), as well as in $\text{MyD88}^{+/+}$ and $\text{MyD88}^{-/-}$ mice (B), challenged by SRW pollen, with PBS-treated mice as controls. C. Immunofluorescent staining of Th2 cell markers and cytokines in CLNs in EAC mice. Bar: 20µm; Arrows: positive staining signals.
Thirdly, we demonstrated the direct evidence that SRW stimulates IL-33 expression and production by ocular surface epithelium via TLR4-dependent innate immunity signaling using an in vivo mouse model with SRWe topical challenge. Our results showed that the aqueous protein extract of defatted SRW pollen significantly increased IL-33 expression at mRNA and protein levels in a concentration-dependent manner, and this stimulation was largely suppressed by exogenous TLR4 antibody in wild BALB/c mice. The direct stimulation of IL-33 expression by SRWe in corneal and conjunctival epithelia was only observed in the topical challenge model of wild type BALB/c and MyD88+/+ mice, but not in Tlr4-d or MyD88−/− mice.

In conclusion, we have uncovered another novel mechanism by which pollen/TLR4 innate immunity signaling initiates IL-33/ST2 allergic pathway that triggers Th2-dominant inflammation in pollen induced allergic diseases. Together with TSLP, these findings provide new evidence that allergic conjunctivitis appears to be a mucosal epithelial disorder; and the innate immunity of epithelial cells not only recognize microbial invasion, but is also capable to respond to pollen components from plants. This discovery will shed light on the understanding of mucosal innate immunity involved in allergic inflammation, and may create new therapeutic targets to prevent and cure allergic disease.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health, National Eye Institute grants R01 EY023598 (DQL), an unrestricted grant from Research to Prevent Blindness, the Oshman Foundation and the William Stamps Farish Fund.

References


45. Dinarello CA. An IL-1 family member requires caspase-1 processing and signals through the ST2 receptor. Immunity. 2005; 23: 461-462.


53. Gluck J, Rymarczyk B, Rogala B. Serum IL-33 but not ST2 level is elevated in intermittent allergic rhinitis and is a marker of the disease severity. Inflammation research : official journal of the European Histamine Research Society. 2012; 61: 547-550.

54. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. 2005; 23: 479-490.


