

Toll-like receptor 4 signalling attenuates experimental allergic conjunctivitis

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Summary

Allergic conjunctivitis from an allergen-driven T helper type 2 (Th2) response is characterized by conjunctival eosinophilic infiltration. Association between signalling through Toll-like receptor 4 (TLR-4) and adaptive immune responses has been observed in allergic airway disease. We examined whether administration of bacterial lipopolysaccharide (LPS), a prototypic bacterial product that activates immune cells via TLR-4, could affect the development of allergic conjunctivitis and modify the immune response to ovalbumin (OVA) allergen in an experimental allergic conjunctivitis (EAC) model. Mice were challenged with two doses of OVA via conjunctival sac after systemic challenge with OVA in alum. Several indicators for allergy were evaluated in wild-type and TLR-4^{-/-} mice with or without adding of different doses of LPS into OVA in alum. Mice challenged with OVA via conjunctival sac following systemic challenge with OVA in alum had severe allergic conjunctivitis. Of interest, LPS administration markedly suppressed immunoglobulin (Ig)E-mediated and eosinophil-dependent conjunctival inflammation. In addition, mice sensitized with OVA plus LPS had less interleukin (IL)-4, IL-5 and eotaxin secretion than mice sensitized with OVA only. The suppression of allergic response by LPS administration was due to Th1 shift. In contrast, the presence of LPS during sensitization with OVA had no effect on severity of allergic conjunctivitis and Th2 responses in TLR4-4^{-/-} mice. Our findings demonstrate, for the first time, that LPS suppresses Th2 responses via the TLR-4-dependent pathway in the EAC model.

Keywords: allergic conjunctivitis, eosinophil, LPS, Th2-type immune response, TLR-4

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Introduction

Allergic conjunctivitis (AC) describes a group of conditions, ranging in severity from mild to severe [1]. The immunopathogenic mechanisms in these allergic disorders involve a combination of immunoglobulin (Ig)E-mediated and T helper type 2 (Th2) cell-mediated responses [2–4]. The IgE-mediated conjunctival allergic reaction can be reproduced easily by specific conjunctival provocation [5], which induces an early reaction followed by a predominant infiltration of eosinophilic inflammatory cells [6,7]. Eosinophils are the hallmark of allergic disease, particularly in severe chronic ocular allergy, where they are found easily in quantity in tears and tissues [8,9]. The release of eosinophil granule proteins is implicated in the pathogenesis of conjunctival inflammation [10].

The Th2 hypothesis of allergic disease such as asthma was first suggested by Mosmann in 1989 [11,12]. The Th2 hypothesis states that asthma is caused by an increase in Th2 response in combination with a decrease in Th1 response. Although a wealth of animal data support the Th2 hypothesis, studies in humans, however, have found that both Th1 and Th2 cytokines are elevated in the blood and airways of asthma patients [13,14].

Epidemiological evidence indicates that endotoxin lipopolysaccharide (LPS), a cell-wall component of Gram-negative bacteria that activates immune cells via the transmembrane Toll-like receptor 4 (TLR-4) [15,16], can influence the development of asthma [17–19]. LPS is ubiquitous in the environment and is often present in polluted air as well as in organic or household dusts [20]. The role of endotoxin exposure in asthma development in children has

been controversial, with studies indicating either a protective role through Th1 induction or an exacerbating effect on asthma severity [21–23].

In this study, we evaluated the hypothesis that allergen sensitization with LPS influences adaptive immune response to conjunctival allergen challenge. To address directly the role of LPS for Th2 sensitization in the induction of allergic conjunctival responses, murine models of Th2-type conjunctival inflammation were prepared by intraperitoneal (i.p.) allergen sensitization using different LPS doses. We then evaluated the roles of TLR-4 signalling on the development of allergic conjunctivitis using these mouse models.

Materials and methods

Animals

Throughout the study, we followed the protocol of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Wild-type BALB/c mice (4–5-week-old females) were purchased from Charles River Laboratories (Orient Co., Sungnam, Korea). TLR-4 gene-deficient (TLR-4^{-/-}) mice on a BALB/c background were kindly provided by Professor Shizuo Akira (Osaka University, Japan). All mice were main-

tained under pathogen-free conditions at the animal facilities of the Catholic University of Korea (Seoul, Korea), where they received sterilized food and water *ad libitum*.

Removal of lipopolysaccharides from ovalbumin

Chicken OVA (Sigma Aldrich, St Louis, MO, USA) was diluted in phosphate-buffered saline (PBS) (2 mg/ml) and endotoxin in the OVA was reduced with Detoxi-Gel (Pierce, Rockford, IL, USA). The endotoxin level of purified OVA was below the limit of detection in the limulus amoebocyte lysate assay kit (Sigma Aldrich) (<0.1 EU).

Protocol for mouse models of experimental allergic conjunctivitis (EAC)

To generate experimental allergic conjunctivitis (EAC), mice were sensitized i.p. with 1 µg of OVA (grade V; Sigma Aldrich) and 200 µl of 1.5% aluminium hydroxide (ALUM; Pierce) with or without different doses of LPS from *Escherichia coli* (Sigma Aldrich) on days 0 and 7, and then challenged twice in the conjunctival sac with 250 µg of OVA on days 14 and 17 (Fig. 1a) [24,25]. Control mice were given ALUM in sensitization stages and PBS in place of OVA in challenge stages. Twenty-four h after the final challenge with

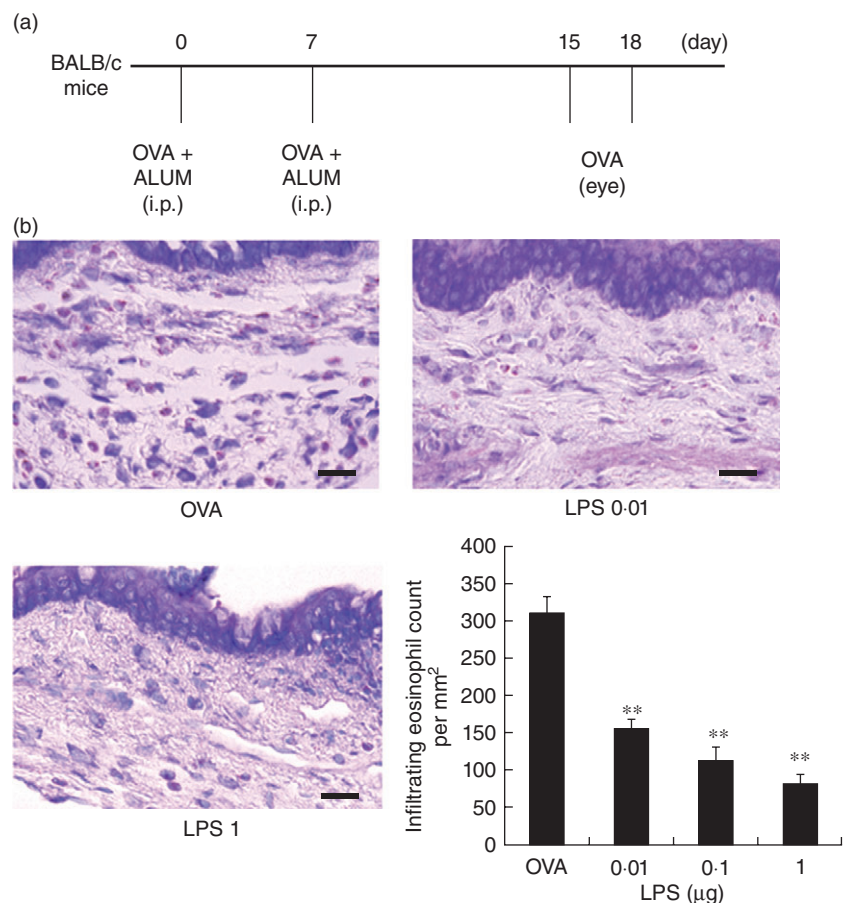


Fig. 1. Lipopolysaccharide (LPS) administration prevents the development of allergic conjunctival inflammation. (a) Experimental protocol. BALB/c mice were injected intraperitoneally (i.p.) with 1 µg ovalbumin (OVA) and 200 µl of 1.5% aluminium hydroxide (ALUM) with or without different doses of LPS (0.01, 0.1 or 1 µg) on days 0 and 7. The mice were challenged with OVA via conjunctival sac on days 15 and 18. (b) Infiltration of eosinophils into the conjunctiva decreased in OVA plus LPS-sensitized mice. Bars = 20 µm. ***P* < 0.01 (*n* = 6 in each group, three independent experiments).

OVA, mice were given a fatal dose of ketamine. Blood was collected and serum prepared.

Evaluation of conjunctival inflammation

The eyes, including eyelids and the conjunctivas, were exenterated. They were harvested and fixed in 10% buffered formalin, cut into horizontal 4- μ m-thick sections and stained with acid-Giemsa stain for detection of eosinophils [26]. OVA-specific allergic conjunctivitis in this mouse model is developed in an eosinophil-dependent manner, not in mast cells [24,25]. In each section, infiltrating cells in the lamina propria mucosae of the tarsal and bulbar conjunctivas were counted by two blinded observers, as described in detail elsewhere [27,28]. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. The data are presented as a mean $\pm$ standard deviation (s.d.) per slide.

Enzyme-linked immunosorbent assay (ELISA) for OVA-specific IgE antibodies in serum

Twenty-four h after OVA challenge of immunized mice, blood was collected and serum prepared. Briefly, the immunoplates (Nalge Nunc International, Naperville, IL, USA) were coated with OVA (1 mg/ml) for OVA-specific IgE antibody detection overnight at 4°C. After blocking with 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, serial dilutions of serum samples were added and incubated for 4 h at room temperature. The plates were then washed with PBS plus 0.05% Tween (PBS/T) and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated rat anti-mouse IgE antibodies (Southern Biotech, Birmingham, AL, USA). After washing with PBS/T, colour reaction was developed with 3, 3', 5, 5'-tetramethylbenzidine (TMB; Moss Inc., Pasadena, CA, USA) and stopped with 0.1 N HCl.

Lymphoid cell culture

Spleens and cervical lymph nodes (CLN) were harvested, and a single-cell suspension was cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 mM 2-mercaptapurine and 10% heat-inactivated fetal calf serum (FBS) (all from Invitrogen Life Technologies, Carlsbad, CA, USA). Cells were cultured at 5×10^6 cells/ml (spleen) or 1×10^6 cells/ml (CLN) with 1 mg/ml OVA for 96 h in 96-well plates (Nunc, Rochester, NY, USA). Supernatants were analysed for cytokine concentrations.

Cytokine analysis

Cytokine level (eotaxin) of serum was assayed using ELISA kits (R&D Systems, Minneapolis, MN, USA). Cytokine levels [including interleukin (IL)-4, IL-5 and interferon (IFN)- γ] of culture supernatants of antigen-stimulated cells isolated

from spleen and CLN were assayed using BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2 Cytokine kit (BD Biosciences, San Diego, CA, USA).

Statistical analysis

Statistical significance between groups was examined by one-way analysis of variance (ANOVA) followed by Tukey's test using SPSS version 12.0. We regarded $P < 0.05$ as significant, $P < 0.01$ highly significant and $P < 0.001$ extremely highly significant. Data are representative of three independent experiments with six mice in each group.

Results

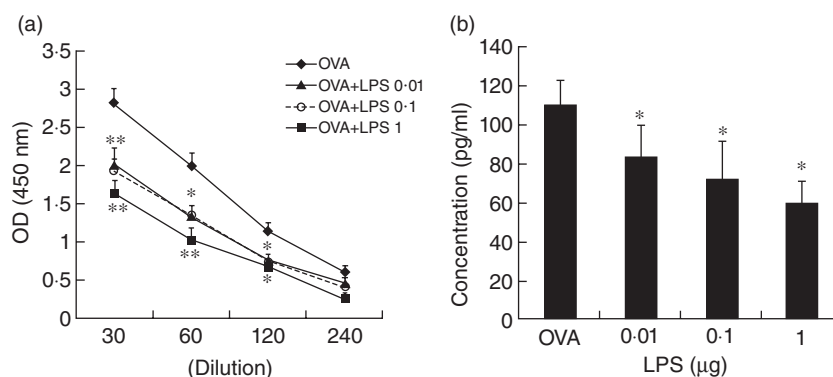
Allergen sensitization with LPS attenuates OVA-induced allergic conjunctival inflammation in EAC

To investigate the notion that innate immune response induced by LPS modulated adaptive immune responses to conjunctival allergens, BALB/c mice were sensitized i.p. with LPS-depleted OVA in combination with 0.01, 0.1 or 1 μ g of LPS at days 0 and 7, and then challenged via conjunctival sac with OVA alone twice, as shown in Fig. 1. In terms of conjunctival inflammation, histological findings demonstrated significant infiltration of eosinophils in the conjunctiva of OVA-challenged mice after systemic priming (Fig. 1). Interestingly, eosinophils in conjunctiva after allergen challenge were decreased in mice sensitized with OVA plus LPS in a dose-dependent manner. Eosinophil counts were lower in mice sensitized with OVA plus high-dose (0.1 or 1 μ g) LPS than in mice sensitized with OVA plus low-dose (0.01) LPS (Fig. 1). Animals injected with these doses of LPS did not show any discrete signs of toxicity such as diarrhoea, curly hair and lethargy. These results indicate that the presence of LPS during OVA sensitization suppressed dose-dependently the development of OVA-induced allergic conjunctivitis, which was mediated by eosinophils.

Selective reduction of IgE antibody and eotaxin in serum of OVA plus LPS sensitized mice

In order to clarify further the severity of EAC in mice sensitized with OVA plus LPS, we assessed levels of OVA-specific IgE antibodies in sera. A combination of systemic priming and local boosting with OVA resulted in significantly higher levels of antigen-specific IgE antibody secretion (Fig. 2a). However, the presence of LPS during OVA sensitization led to a profound decrease of antigen-specific IgE antibody secretion (Fig. 2a). Once we determined that conjunctiva of OVA-challenged mice had brisk numbers of eosinophils, we sought to determine whether eotaxin levels were similarly enhanced. We found significantly higher levels of eotaxin in sera of allergic conjunctivitis-induced mice than in PBS-

Fig. 2. Selective reduction of immunoglobulin (Ig)E antibody and eotaxin secretion in sera of ovalbumin (OVA) plus lipopolysaccharide (LPS)-sensitized mice. (a) The presence of LPS during OVA sensitization led to a profound decrease of antigen-specific IgE antibody secretion in serum in a dose-dependent manner. (b) Eotaxin levels were decreased in serum of OVA plus LPS-sensitized mice in a dose-dependent manner. * $P < 0.05$; ** $P < 0.01$ ($n = 6$ in each group, three independent experiments).



challenged control mice (Fig. 2b). The presence of LPS during OVA sensitization led to a marked reduction of eotaxin levels in sera when compared to the OVA-only sensitization group (Fig. 2b). Taken together, analyses of IgE and eotaxin levels in mice sensitized with OVA plus LPS suggest possible prevention of the development of Th2-mediated allergic inflammation by LPS.

Th2 type cytokine responses are inhibited selectively in the presence of LPS during OVA sensitization in EAC

To determine the role of the presence of LPS during OVA sensitization in Th cell polarization, Th1- and Th2-type cytokine secretions by mononuclear cells were analysed. Splenocytes isolated from mice with allergic conjunctivitis elicited significantly higher levels of Th2-type cytokine (i.e.

IL-4 and IL-5) and Th1-type cytokine (i.e. IFN- γ) after restimulation with OVA *in vitro* than those from the PBS control group (data not shown). As expected, splenocytes isolated from OVA plus LPS-sensitized mice produced significantly less IL-4 and IL-5 compared with those from OVA-only sensitized mice (Fig. 3a). In contrast, the presence of LPS during OVA sensitization led to a marked enhancement in IFN- γ synthesis by splenocytes from mice with allergic conjunctivitis (Fig. 3a). Additionally, mononuclear cells isolated from CLN from OVA plus-LPS sensitized mice also produced significantly lesser levels of Th2-type cytokine compared with those from OVA-only sensitized mice (Fig. 3b). Taken together, these data demonstrate that the presence of LPS during OVA sensitization is committed to the generation of preferential Th1 type responses.

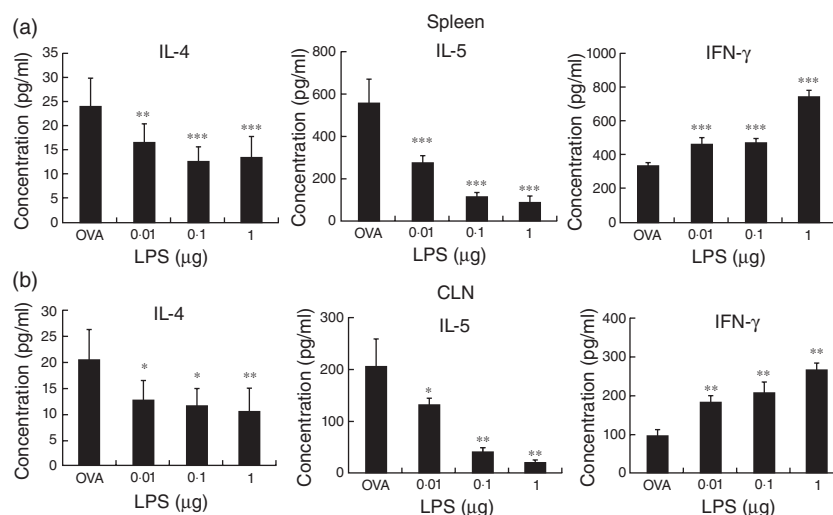


Fig. 3. The presence of lipopolysaccharide (LPS) during ovalbumin (OVA) sensitization reduced T helper type 2 (Th2) and enhanced Th1-immune responses in an OVA-induced allergic conjunctivitis model. Splenocytes and monocytes from cervical lymph nodes (CLN) were harvested on day 19 and cultured with 1 mg/ml OVA *in vitro*, as described in Materials and methods. Culture supernatants were collected at 96 h and cytokine concentration was assayed using fluorescence activated cell sorter. (a) Splenocytes from OVA plus LPS-sensitized mice produced significantly less interleukin (IL-4) and IL-5 (Th2-type cytokine) and more interferon (IFN)- γ (Th1-type cytokine) compared with those from OVA-sensitized mice. (b) Monocytes from CLN from OVA plus LPS-sensitized mice also produced significantly less IL-4 and IL-5 (Th2-type cytokine) and more IFN- γ (Th1-type cytokine) compared with those from OVA-sensitized mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 6$ in each group, three independent experiments).

Effect of the presence of LPS during OVA sensitization on development of OVA-induced allergic conjunctivitis is dependent on TLR-4 activation

In order to address a role of TLR-4 on suppression of allergic conjunctivitis in the presence of LPS during OVA sensitization, TLR-4^{-/-} mice were sensitized and challenged with OVA, as described above. As shown in Fig. 4, the severity of allergic conjunctival inflammation and degree of Th2-type immune response was decreased in TLR-4^{-/-} mice following systemic priming and local boosting with OVA compared to wild-type mice. These findings indicate that TLR-4 is required for the optimal development of Th2 immune responses in the EAC model. Most interestingly, attenuation of allergic conjunctival inflammation by LPS during OVA sensitization was abrogated in TLR-4^{-/-} mice. TLR-4^{-/-} mice sensitized with OVA plus LPS showed conjunctival eosinophil infiltration indistinguishable from those of OVA-only sensitized TLR-4^{-/-} mice (Fig. 4a). These results indicate clearly that LPS during OVA sensitization does not affect the severity of OVA-induced allergic conjunctivitis in TLR-4^{-/-}

mice and imply that attenuation of allergic conjunctival inflammation by the presence of LPS during OVA sensitization is critically dependent on TLR-4 activation.

We investigated further the role of TLR-4 on the immunological effect of LPS during OVA sensitization in EAC. LPS-mediated reduction of OVA-specific IgE antibody secretion was abrogated in TLR-4^{-/-} mice with allergic conjunctivitis (Fig. 4b). Further, LPS-mediated reduction of IL-4, and IL-5 secretion from splenocytes and mononuclear cells from CLN were not observed in TLR-4^{-/-} mice with allergic conjunctivitis (Fig. 4c). Overall, the presence of LPS during OVA sensitization did not affect the Th2-type immune responses of TLR-4^{-/-} mice with allergic conjunctivitis. These results indicate that the effect of LPS during OVA sensitization in allergic conjunctivitis is critically dependent on TLR-4 activation.

Discussion

Our findings provide strong evidence for an immunopathological role of LPS in the development of allergic conjunc-

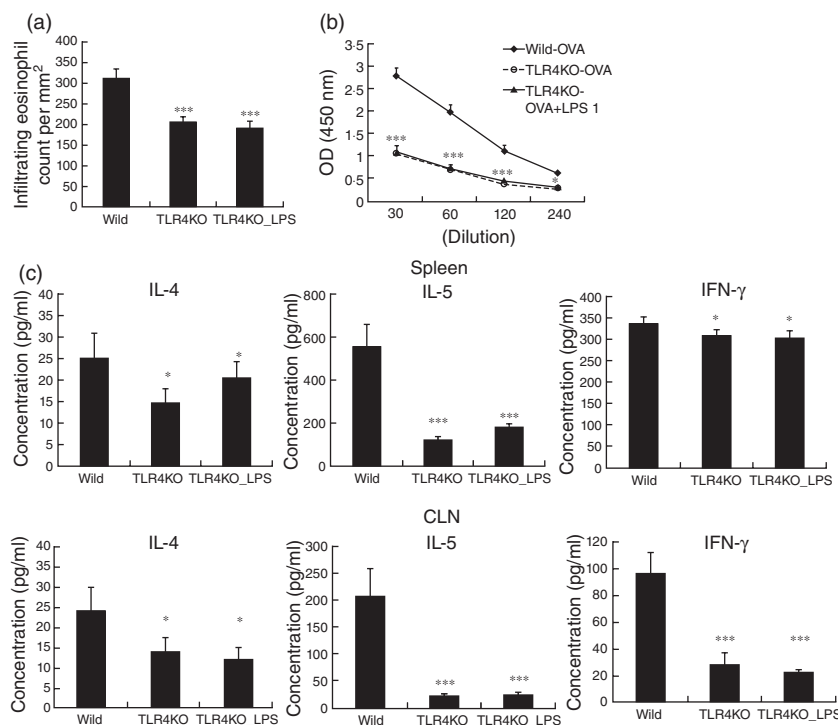


Fig. 4. Ovalbumin (OVA)-specific T helper type 2 (Th2) responses and allergic conjunctival inflammation were decreased in Toll-like receptor (TLR)-4^{-/-} mice compared with wild-type animals. The presence of lipopolysaccharide (LPS) during OVA sensitization did not affect the severity of allergic conjunctivitis and Th2-type immune responses in the TLR-4^{-/-} OVA-induced allergic conjunctivitis model. (a) TLR-4^{-/-} mice were sensitized and challenged as described in Fig. 1. TLR-4^{-/-} mice had a dramatic reduction in the numbers of eosinophils in conjunctiva compared with wild-type animals. Counts of eosinophils were not statistically different between OVA plus LPS-sensitized and OVA-sensitized groups in TLR-4^{-/-} mice ($P > 0.05$). (b) TLR-4^{-/-} mice had a lower level of OVA-specific immunoglobulin (Ig)E antibody. The presence of LPS during OVA sensitization did not reduce OVA-specific IgE antibody secretion in serum ($P > 0.05$). (c) TLR-4^{-/-} mice had a reduction of Th2-type cytokine production. There were no significant differences in the level of Th2-type cytokine production from splenocytes and monocytes from cervical lymph nodes (CLN) between OVA plus LPS-sensitized and OVA-sensitized groups in TLR-4^{-/-} mice ($P > 0.05$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ ($n = 6$ in each group, three independent experiments).

tivitis in the OVA-induced allergic conjunctivitis model. The present work provides data indicating that co-adsorption of TLR-4 agonist LPS with allergen to alum impairs the development of Th2-driven allergic conjunctival inflammation. LPS induced a Th1 polarization as reflected in the cytokine profile and production of antigen-specific IgE antibody, characterized by the activation of TLR-4 signalling. Blockade of TLR-4 signalling resulted in abrogation of a significant decrease of allergic conjunctival inflammation and Th2-type immune responses.

The main pathophysiological changes in conjunctival allergic reactions include increased levels of IgE antibody in serum and infiltration of eosinophils into the conjunctiva [1,8]. These pathological processes of allergic reaction are thought to be mediated by Th2-type cells, which produce preferentially IgE-enhancing cytokines such as IL-4 and IL-5 [2,3,29,30]. Indeed, our results provide direct evidence that cytokine synthesis by splenocytes and mononuclear cells from CLN are predominantly of the Th2 type (IL-4 and IL-5). Such Th2-type cytokine synthesis contributes to the high levels of IgE antibody and eotaxin production and infiltration of eosinophils into the conjunctiva of OVA-challenged mice.

Increasing evidence suggests that exposure to microbial stimuli, acting via the innate immune system, can influence adaptive immune responses to allergens and development of allergic disease. Endotoxin LPS is ubiquitous in the environment and is often present in polluted air as well as in organic or household dust [20]. Experimental data obtained with the murine OVA model of asthma indicated that exposure to LPS could either protect against asthma or exacerbate it [21–23]. The role of LPS exposure in the induction of asthma appears to be complex, reflecting the timing of exposure as well as the particular parameters of Th2-mediated pathology. Previous reports that examined the effects of TLR-4 agonists administered systemically on experimental allergic airway disease down-modulate allergic lung disease and prevent the development of polarized Th2-cell-mediated airway inflammation [20,31,32]. Our study demonstrated for the first time that LPS suppresses Th2 responses via the TLR-4-dependent pathway in EAC model. Sensitization with OVA plus LPS co-adsorbed on to alum in a dose-dependent manner impaired OVA-induced Th2-mediated allergic responses such as eosinophilic infiltration into conjunctiva, Th2-type cytokine and eotaxin secretion, and serum levels of OVA-specific IgE antibodies. Although Th1-affiliated cytokine increased, investigation into the conjunctiva revealed that LPS did not induce a Th1 pattern of inflammation in conjunctiva. In contrast, LPS administration during the allergen sensitization period did not affect the severity of allergic conjunctivitis and Th2 immune responses in TLR-4^{-/-} mice.

Our study demonstrated decreased OVA-specific Th2 responses and allergic conjunctival inflammation in TLR-4^{-/-} mice compared with wild-type animals. Previous reports in

asthma models have indicated that TLR-4 is required for optimal Th2 responses to allergens [33]. TLR-4^{-/-} mice had a dramatic reduction in the numbers of eosinophils in conjunctiva. Reduced inflammation with eosinophils, which in this model is dependent on the production of Th2 cytokine, suggested a reduction in Th2 responses. A reduction of Th2 responses in TLR-4^{-/-} mice was supported by the substantially lower circulating level of OVA-specific IgE. Importantly, when splenocytes and mononuclear cells from CLN were cultured in the presence of OVA *in vitro*, cells from TLR-4^{-/-} mice produced significantly lower amounts of IL-4 and IL-5 compared with wild-type mice. There was also a significant reduction in IFN- γ production by cells from TLR-4^{-/-} mice. These results suggest that allergen-induced Th2 responses were compromised in the absence of signalling via TLR-4. Th2 cell priming using the adjuvant alum and cell recruitment to the eye is impaired in TLR-4^{-/-} mice. Taken altogether, OVA-induced allergic conjunctivitis and immune responses to OVA are dependent on TLR-4 signalling, and the effect of LPS administration systemically and its mechanism of action in EAC appear to be TLR-4-dependent.

In summary, we found that induction of innate immunity through TLR-4 stimulation can play an important role in Th2-associated allergic conjunctivitis. Our results demonstrate that through TLR-4 signalling, LPS attenuates eosinophil infiltration into the conjunctiva by a decreasing Th2 immune response in mice. Therefore, it seems reasonable to consider that LPS might prevent the development of polarized Th2 cell-mediated conjunctival inflammation via TLR-4-dependent signalling pathways in humans.

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Disclosure

The authors have no financial support and no financial or proprietary interests in this study.

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