Expression of the interleukin-4 receptor α in human conjunctival epithelial cells

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ABSTRACT

Aim To investigate the expression and function of interleukin-4 receptor α (*IL-4Rα*) in human conjunctival epithelial cells (HCjECs).

Methods The presence of *IL-4Rα* mRNA and protein was examined by reverse transcription (RT) PCR and immunohistology, respectively. Cell surface expression was examined by flow cytometry. The effects of interleukin (IL)-4 or IL-13 on the tyrosine phosphorylation of signal transducer and the activator of transcription 6 (STAT6) were evaluated by immunoblot analyses. The transcripts upregulated upon IL-4 stimulation were examined using GeneChip, and confirmed by quantitative RT-PCR.

Results *IL-4Rα* mRNA and protein were detected in human conjunctival epithelium. *IL-4Rα* protein was expressed on the cell surface of HCjECs. IL-4 and IL-13 induced tyrosine phosphorylation of STAT6. GeneChip analysis showed that nine transcripts were upregulated more than fourfold by IL-4 stimulation in the primary HCjECs from two individuals. Quantitative RT-PCR assay confirmed the upregulation of these transcripts: *lecithin retinol acyltransferase (LRAT), calpain (CAPN14), tumour necrosis factor alpha-induced protein 6 (TNFAIP6), RAS guanyl-releasing protein 1 (RASGRP1), endothelin receptor type A (EDNRA), hyaluronan synthase 3 (HAS3), cathepsin C (CTSC), carbonic anhydrase II (CA2)* and *cytokine-inducible SH2-containing protein (CISH)*.

Conclusions HCjECs expressed functioning *IL-4Rα*, and IL-4 stimulation induced the expression of several genes.

INTRODUCTION

Interleukin-4 receptor α (*IL-4Rα*), a component of the interleukin (IL)-4 and the IL-13 receptor, is essential for both IL-4 and IL-13 signalling. The type I IL-4 receptor is composed of two subunits, an α subunit (*IL-4Rα*), which binds IL-4 and transduces its growth-promoting and transcription-activating functions, and a γc subunit, common to several cytokine receptors, that amplifies signalling of *IL-4Rα*. The IL-13 receptor is composed of the *IL-4Rα* chain and the IL-13Rα1 chain.

IL-4R is representative of the candidate genes for allergic diseases such as atopy and asthma, which are biologically linked to T-helper type 2 (Th2) cytokine-driven inflammatory mechanisms.[1 2] Furthermore, we previously reported that IL-4R is also representative of the candidate genes for Stevens–Johnson syndrome (SJS)/toxic epidermal necrolysis (TEN) with ocular complications.[3 4] Although there are many reports regarding *IL-4Rα* expression and function of human corneal fibroblasts[5–7] and human conjunctival fibroblasts,[8 9] there have been no reports of *IL-4Rα* expression in relation to the function of human ocular surface epithelial cells.

In this study, we examined the expression and function of IL-4R in the conjunctival epithelium of the human ocular surface.

MATERIALS AND METHODS

Human conjunctival epithelial cells

For reverse transcription (RT)-PCR, assay we obtained human conjunctival epithelial cells (HCjECs) from healthy volunteers by brush cytology using the previously described method.[10] Briefly, a tiny brush (Cytobrush S; Medscand AM, Malmo, Sweden) was used to scrape epithelial cells from the bulbar conjunctiva. For flow cytometric analysis, immunoblot analyses, GeneChip analysis and real-time quantitative PCR, primary HCjECs were harvested from conjunctival tissue obtained at conjunctivochalasis surgery and cultured using the previously described method.[10] Briefly, conjunctival tissues were washed and immersed for 1 h at 37°C in 1.2 U/ml purified dispase (Roche Diagnostic Ltd, Basel, Switzerland), and epithelial cells were detached, collected and cultured in low-calcium defined keratinocytes–serum free medium (SFM) with defined growth-promoting additives including insulin, epidermal growth factor, fibroblast growth factor and 1% antibiotic–antimycotic solution. By use of this method, the cell colonies usually became obvious within 3 to 4 days. After reaching 80% confluence in 7 to 10 days, the cultured HCjECs were used in subsequent procedures. When we confirmed the purity of the cells by immunohistochemical staining of vimentin, which is a marker of fibroblasts, vimentin-positive cells were scarcely found in the cultured HCjECs (Supplemental figure). For immunohistological analysis, we also used conjunctival tissue obtained at the time of conjunctivochalasis surgery. All donor specimens used in this study had no atopic history.

RT-PCR

We analysed HCjECs for *IL-4Rα* mRNA expression as described in our previous study.[11] Briefly, total RNA was isolated from HCjECs and human peripheral mononuclear cells using Trizol Reagent (Life Technologies, New York, USA) according to the manufacturer's instructions. For the RT reaction we used the SuperScript Preamplification kit (Invitrogen Corporation, Carlsbad, California, USA). PCR amplification was with DNA polymerase (cTaq; Toyobo Co., Ltd, Osaka, Japan); the conditions were 38 cycles at 95°C for 1 min, followed by 95°C for 30 s, 55°C for 30 s and 72°C for 1 min, with final extension at 72°C for 2 min on a commercial PCR machine (GeneAmp; PE Applied Biosystems, Foster City,

California, USA). The specific primers for IL-4R were 5′-GACC-TGGAGCAACCCGTA TC-3′ and 5′-CATAGCACAACAGGC-AGACG-3, and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were 5′-CCATCACCATCTTCCAGGAG-3′ and 5′-CCTGCTTCACCACCTTCTTG-3. RNA integrity was assessed electrophoretically in ethidium bromide-stained 1.5% agarose gels.

Immunohistological study of *IL-4Rα* in human conjunctival sections

Serial sections of human conjunctiva were prepared from samples obtained at the time of conjunctivochalasis surgery. The sections were fixed for 30 min with methanol, and then incubated overnight in a moist chamber at 4°C with mouse anti-human *IL-4Rα* monoclonal antibody (mAb; R&D Systems, Inc., Minneapolis, Minnesota, USA) or isotype control mouse IgG2a (DakoCytomation, Kyoto, Japan), and then washed in PBS(−). Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Inc., Eugene, Oregon, USA) was applied for 1 h at room temperature; the slides were then washed, followed by the application of anti-fade mounting medium with Propidium iodide (PI) (Vectashield; Vector Laboratories, Inc., Burlingame, California, USA).

Flow cytometric analysis

Primary HCjECs were analysed for cell surface expression of *IL-4Rα* by flow cytometry as previously described.[11] Briefly, primary HCjECs were treated with 0.02% EDTA, and cells were incubated with mouse anti-human *IL-4Rα* monoclonal antibody (mAb; R&D Systems) or isotype control mouse IgG2a (Dako-Cytomation) for 30 min at 4°C. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes) was used as the secondary antibody. Stained cells were analysed with a FACS Calibur (Becton, Dickinson and Co., San Jose, California, USA); data were analysed using Cellquest software (Becton, Dickinson and Co.).

Immunoblot analysis of STAT6 phosphorylation

The tyrosine phosphorylation of the signal transducer and the activator of transcription 6 (STAT6) in the primary HCjECs was examined by immunoblot analysis. Primary HCjECs were incubated with 10 ng/ml IL-4 or IL-13 for various lengths of time, then washed twice and lysed by scraping in 0.2 ml of ice-cold CelLytic M Cell Lysis Reagent (Sigma-Aldrich Corp., St Louis, Missouri, USA).

Sodium dodecyl sulphate (SDS)—polyacrylamide gel electrophoresis was performed using the NuPAGE electrophoresis system (Invitrogen) according to the manufacturer's instructions. The separated proteins were transferred to a polyvinylidene difluoride membrane using the iBlot Gel Transfer Device (Invitrogen) according to the manufacturer's instructions. The membrane was incubated in 5% skimmed milk with 0.1% Tween 20/TBS buffer for 1 h at room temperature for blocking, then subjected to immnoblot analysis with rabbit anti-phospho STAT6 or anti-STAT6 polyclonal antibodies (Cell Signalling Technology, Inc., Danvers, Massachusetts, USA) for first antibodies and HRP-conjugated donkey anti-rabbit IgG antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for second antibodies. For the detection of the proteins, ECL Plus Western Blotting Detection System (GE Healthcare) was used.

Gene expression analysis

Gene expression profiles were investigated using a high-density oligonucleotide probe array, GeneChip, of Human Genome U133 Plus 2.0 (Affymetrix, Inc., Santa Clara, California, USA). Total RNA was extracted by use of the Qiagen RNeasy kit (Qiagen, Valencia, California, USA). cRNA preparation and target hybridisation were done according to the Affymetrix GeneChip technical protocol. The DNA chips were scanned with a specially designed confocal scanner (GeneChip Scanner 3000; Affymetrix). Array data analysis done was with Affymetrix GeneChip operating software (GCOS) version 1.0 (Affymetrix); this software analyses image data and computes an intensity value for each probe cell. To quantitate RNA abundance, the average-difference values (ie, gene expression levels) representing the perfect match—mismatch for each gene-specific probe family were calculated, and the fold-changes in the average-difference values were determined according to Affymetrix algorithms and procedures.

Real-time quantitative PCR

Real-time quantitative PCR was performed on an ABI-prism 7700 (Applied Biosystems) according to previously described procedure.[11] The primers and probes were purchased from Applied Biosystems. The quantification data were normalised to the expression of the housekeeping gene *GAPDH*.

Data analysis

Data were expressed as mean±SE and evaluated by Student t test using the Excel program.

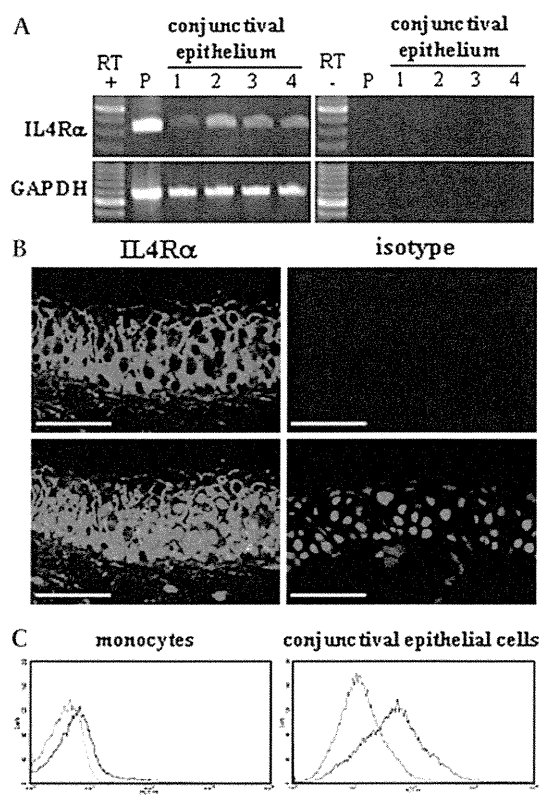

Figure 1 (A) Reverse transcription (RT) PCR analyses of the expression of *IL-4Rα*-specific mRNA in human conjunctival epithelium. As a positive control, mRNA isolated from human peripheral-blood mononuclear cells was subjected to RT-PCR (left column). RT− indicates data were obtained without RT (controls). (B) Immunohistological analysis for *IL-4Rα* in human conjunctival epithelium. Bound antibodies were visualised by Alexa Fluor 488 goat anti-mouse IgG, and the nuclei were visualised by PI staining. Each bar represents a length of 50 μm. (C) Flow cytometry analysis of cell surface expression of *IL-4Rα* in human conjunctival epithelium. The histogram data are representative of three separate experiments (solid line, *IL-4Rα* antibody; dotted line, isotype control).

RESULTS

Expression of *IL-4Rα* in human conjunctival epithelium

IL-4Rα-specific mRNA expression was present in HCjECs harvested by brush cytology from healthy volunteers who had no atopic history (figure 1A). This finding demonstrates that the *IL-4Rα* gene is constitutively expressed in normal HCjECs. We subjected conjunctival tissues to immunohistochemical study to determine the presence and localisation of *IL-4Rα* expression in stratified conjunctival epithelium. *IL-4Rα* protein was consistently and abundantly expressed at the cell membrane in human conjunctival epithelium and was located in cells from the basal to the superficial layer of the conjunctival epithelium (figure 1B). The *IL-4Rα* protein was also expressed at the cell surface by primary HCjECs harvested from conjunctival tissue at levels higher than comparable monocytes to lymphocytes (figure 1C2). Our results showed that *IL-4Rα* mRNA and protein were present in human conjunctival epithelium.

IL-4- and IL-13-induced phosphorylation of STAT6 in HCjECs

STAT proteins are activated by tyrosine phosphorylation in cells treated with cytokines. It is well known that STAT6 is activated with IL-4 or IL-13, which are ligands of *IL-4Rα*. To determine whether IL-4 and IL-13 activates STAT6 in HCjECs, we examined the effects of these cytokines on the tyrosine phosphorylation of STAT6. Primary HCjECs were incubated for various lengths of time with IL-4 (10 ng/ml) or IL-13 (10 ng/ml), then lysed and subjected to immunoblot analysis. Both IL-4 and IL-13 induced the tyrosine phosphorylation of STAT6 in a time-dependent manner (figure 2). These effects were apparent as early as 15 min after exposure to IL-4 or IL-13, and were most apparent after 60 min. Thus, these findings show that STAT6 protein is activated by IL-4 or IL-13 in HCjECs, suggesting that HCjECs express functioning *IL-4Rα*.

Upregulation of transcripts upon IL-4 stimulation

Moreover, to examine the function of *IL-4Rα*, we performed gene expression analysis of primary HCjECs from two individuals that were, or were not, cultured with 10 ng/ml IL-4 for 3 h using GeneChip. We found nine transcripts that were upregulated more than fourfold upon IL-4 stimulation in the primary HCjECs from two individuals (table 1). These transcripts were *lecithin retinol acyltransferase (LRAT), calpain (CAPN14), tumour necrosis factor alpha-induced protein 6 (TNFAIP6), RAS guanyl-releasing protein 1 (RASGRP1), endothelin receptor type A (EDNRA), hyaluronan synthase 3 (HAS3), cathepsin C (CTSC), carbonic anhydrase II (CA2)* and *cytokine-inducible SH2-containing protein (CISH)* (table 1). Quantitative RT-PCR assay confirmed the upregulation of these nine transcripts (figure 3).

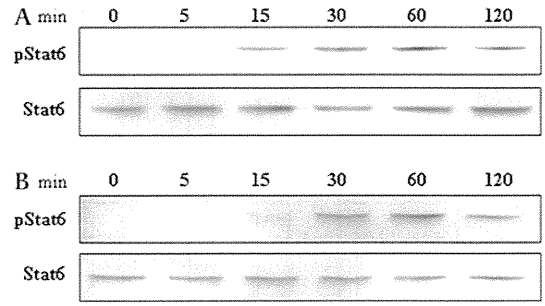

Figure 2 Effect of IL-4 (A) or IL-13 (B) on the tyrosine phosphorylation of STAT6 in human conjunctival epithelial cells. Data are representative of three independent experiments.

DISCUSSION

To the best of our knowledge, this is the first study to document the expression and function of *IL-4Rα* on human conjunctival epithelium. Our results showed that *IL-4Rα* mRNA and protein were detected in human conjunctival epithelium and that *IL-4Rα* protein was expressed on the cell surface. IL-4 and IL-13, which are ligands of *IL-4Rα*, induced tyrosine phosphorylation of STAT6. Moreover, nine transcripts were confirmed to be upregulated upon IL-4 stimulation in primary HCjECs. Thus, HCjECs might express functioning *IL-4Rα*.

Others have reported that *IL-4Rα* was expressed on human corneal fibroblasts[5–7] and human conjunctival fibroblasts.[8 9] In human corneal fibroblasts, IL-4 stimulation with tumour necrosis factor (TNF)-α induced eotaxin and thymus- and activation-regulated chemokine (TARC) production.[9] In human conjunctival fibroblasts, IL-4 stimulation induced the production of extracellular matrix proteins such as collagen I, collagen III and fibronectin,[8] These reports suggest that fibroblasts play a central role in the induction and amplification of ocular allergic inflammation. On the other hand, it has been reported that IL-4 (with or without TNF-α) stimulation of human corneal epithelial cells did not induce the production of eotaxin or TARC.[9] We confirmed that IL-4 stimulation (with or without TNF-α) of HCjECs did not result in their induction (data not shown).

Our findings indicate that HCjECs express functioning *IL-4Rα* and that IL-4 stimulation could induce the expression of various

Table 1 Gene expression upregulated by IL-4 stimulation

Probe-set ID	Gene name (gene symbol)	Ratio IL-4$^+$/IL-4$^-$ Case 1	Case 2
220317_at	Lecithin retinol acyltransferase (LRAT)	52.0	5.7
1557321_a_at	Calpain 14 (CAPN14)	22.6	6.5
206026_s_at	Tumour necrosis factor, alpha-induced protein 6 (TNFAIP6)	13.0	7.0
205590_at	RAS guanyl-releasing protein 1 (RASGRP1)	9.2	5.3
204463_s_at	Endothelin receptor type A (EDRA)	7.5	4.0
204464_s_at	Endothelin receptor type A (EDRA)	7.0	4.6
223541_at	Hyaluronan synthase 3 (HAS3)	7.0	6.0
231234_at	Cathepsin C (CTSC)	6.1	6.5
209301_at	Carbonic anhydrase II (CAII)	5.7	10.6
225646_at	Cathepsin C (CTSC)	4.6	5.7
223377_x_at	Cytokine-inducible SH2-containing protein (CISH)	4.3	7.0

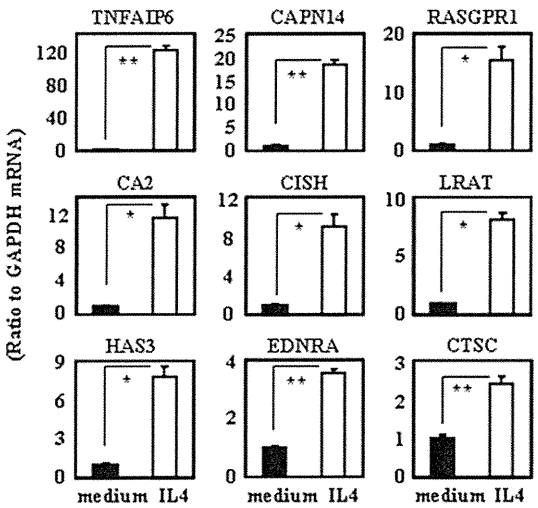

Figure 3 mRNA expression of the nine transcripts in human conjunctival epithelial cells exposed to 10 ng/ml IL-4 for 6 h. The quantification data were normalised to the expression of the housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. The y-axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show the mean±SEM from one experiment carried out in four wells per group (*p<0.01, **p<0.001).

genes such as *LRAT, CAPN14, TNFAIP6, RASGRP1, EDNRA, HAS3, CTSC, CA2* and *CISH*.

LRAT is one of the enzymes involved in vitamin A metabolism, and is reportedly upregulated by cellular differentiation in human keratinocytes.[12] RASGRP1 is one of the diacylglycerol/phorbol ester receptors, and its overexpression reportedly inhibits the expression of differentiation markers in keratinocytes.[13] EDNRA is one of the receptors of endothelin-1, and it reportedly induces epithelial–mesenchymal transition in alveolar epithelial cells.[14] HAS3 is one of the hyaluronan synthases, and hyaluronan reportedly controls epithelial proliferation and regeneration.[15] Thus, these four genes may be associated with processes related to epithelial differentiation or proliferation.

On the other hand, TNFAIP6 and CISH are anti-inflammatory molecules. TNFAIP6 is an anti-inflammatory protein present in bronchoalveolar lavage fluid from patients with asthma; its level is reportedly increased after allergen challenge.[16] CISH is one member of the suppressors of the cytokine signalling family of proteins, and is reportedly an important negative regulator for inflammatory cytokine signalling.[17]

CA2 is a one of the carbonic anhydrases and is thought to be related to the transport of fluids and ions.[18] CTSC, a member of the cathepsin family, is upregulated in bronchial biopsies tissues of asthma patients; however, its function remains unknown.[19] CAPN14 is a newly discovered member of the calpain family that functions as calcium-dependent cysteine proteases; its function is also not known at present.[20] Intriguingly, two of these nine transcripts, *TNFAIP6*[16] and *CTSC*,[19] have been reported to be upregulated in asthma patients. We speculate that their upregulation in bronchoalveolar lavage or biopsy tissues obtained from these asthma patients may be attributable to being derived from epithelial cells.

Although fibroblasts could produce chemokines such as eotaxin and TARC, and were able to augment allergic inflammation, it is possible that epithelial cells might suppress allergic inflammation via TNFAIP6 and CISH. Furthermore, conjunctival epithelial cells might contribute to the cellular differentiation or proliferation. Although the functions of *IL-4Rα* in conjunctival epithelial cells remain unclear, it is possible that epithelial cells might have different roles from fibroblasts in ocular surface inflammation. We previously showed that prostaglandin (PG) E receptor subtype EP3 is expressed on the ocular surface, and that the PGE_2–EP3 pathway in conjunctival epithelium acts as a negative regulator in allergic conjunctivitis.[21] Obviously, ocular surface epithelial cells play important roles in allergic inflammation, yet the role of conjunctival epithelial cells in allergic inflammation remains to be elucidated.

In summary, we demonstrated that HCjECs expressed functioning *IL-4Rα* and that IL-4 stimulation could induce the expression of various genes, for example, cellular differentiation-related molecule genes such as *LRAT, RASGRP1* and *EDNRA*, and anti-inflammatory molecule genes such as *TNFAIP6* and *CISH*.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Institutional Review Board at Kyoto Prefectural University of Medicine, Kyoto, Japan. All experimental procedures were conducted in accordance with the tenets of the Declaration of Helsinki.

Provenance and peer review Not commissioned; externally peer reviewed.

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Comment. Topical interferon was first described by Maskin[1] in 1994 as being effective in the treatment of ocular neoplasia. A limited number of cases in the literature also show the cytostatic effect of ATRA on ocular surface dysplasia.[4]

Our early experience with topical ATRA alone was consistent with early reports of effectiveness, with no response occurring in certain patients. Our early experience with topical interferon alfa-2b demonstrated a more consistent clinical response, and recent studies have documented an 80% treatment efficacy using topical interferon alfa-2b.[2] Mitomycin C and fluorouracil are alternative topical therapies for ocular surface dysplasia. However, interferon has fewer ocular adverse effects compared with these topical chemotherapeutic agents.[2] Retinoic acid is known to irritate the conjunctiva in higher doses.[4]

In our patient, neither ATRA nor interferon alfa-2b alone was effective in slowing growth of the ocular lesion. Longer treatment with interferon alfa-2b may have led to a better response. The rapid clinical response to the combined treatment with topical interferon alfa-2b and ATRA seems remarkable. However, previous studies have described the synergistic effects of interferon alfa-2b and ATRA in combination, both in vitro and in vivo. These same studies, although not of an eye or eye model, reported that ATRA can permit growth inhibition by interferons in interferon-unresponsive cells.[5]

Prospective studies with more patients and longer follow-up are needed to confirm the treatment efficacy and safety profile of this combination therapy as a well-tolerated alternative to topical mitomycin C and fluorouracil. Appropriate further studies may reveal a benefit for both dysplastic and neoplastic lesions.

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Prostaglandin E Receptor 4 Expression in Human Conjunctival Epithelium and Its Downregulation in Devastating Ocular Surface Inflammatory Disorders

Prostanoids are a group of lipid mediators that form in response to various stimuli, including prostaglandin (PG) D_2 (PGD_2), PGE_2, $PGF_{2\alpha}$, PGI_2, and thromboxane A_2. There are 8 types of prostanoid receptors that are conserved in mammals ranging from mice to humans: the PGD receptor, 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor, the PGI receptor, and the thromboxane A receptor.[1] In regard to PGE receptor subtype EP4, it was reported that EP4 messenger RNA was present in the intestinal epithelium[2] and that EP4 maintained intestinal homeostasis and downregulated immune response.[3] Like the intestine, the ocular surface is also one of the mucosa that are in contact with commensal bacteria. In this study, we examined the expression of EP4 in human conjunctival epithelium and compared its expression between various ocular surface diseases.

Methods. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine, Kyoto, Japan. For reverse transcription–polymerase chain reaction assay, we obtained human conjunctival epithelial cells from healthy volunteers by brush cytology using previously described methods.[4] The primers were (forward) 5′-TCA ACC ATG CCT ATT TCT ACA GCC ACT ACG-3′ and (reverse) 5′-AGG TCT CTG ATA TTC GCA AAG TCC TCA GTG-3′ for human *PTGER4* and (forward) 5′-CCA TCA CCA TCT TCC AGG AG-3′ and (reverse) 5′-CCT GCT TCA CCA CCT TCT TG-3′ for human *GAPDH*. For immunohistochemistry, we used nearly normal bulbar conjunctival tissues obtained during surgery for conjunctivochalasis as a control, and human conjunctival tissues were also prepared from samples obtained during surgery to reconstruct the ocular surface such as treatment for various ocular surface diseases including Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), ocular cicatricial pemphigoid (OCP), and pterygium. For EP4 staining, we used the rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan).

Results. The presence of *PTGER4* messenger RNA and EP4 protein in human conjunctival epithelium was examined by reverse transcription–polymerase chain reaction and immunohistological analysis, respectively. The *PTGER4* messenger RNA was detected in normal human conjunctival epithelium (**Figure**, A). The sequences obtained from these polymerase chain reaction products were identical to the human *PTGER4* complementary DNA sequence. The EP4 protein was also detected in the nearly normal conjunctival epithelium obtained from the patients with conjunctivochalasis (Figure, B). Next, we examined the conjunctival tissues with various ocular surface diseases. The EP4 protein was detected in conjunctival epithelium from patients with pterygium as well as in the conjunctival epithelium from

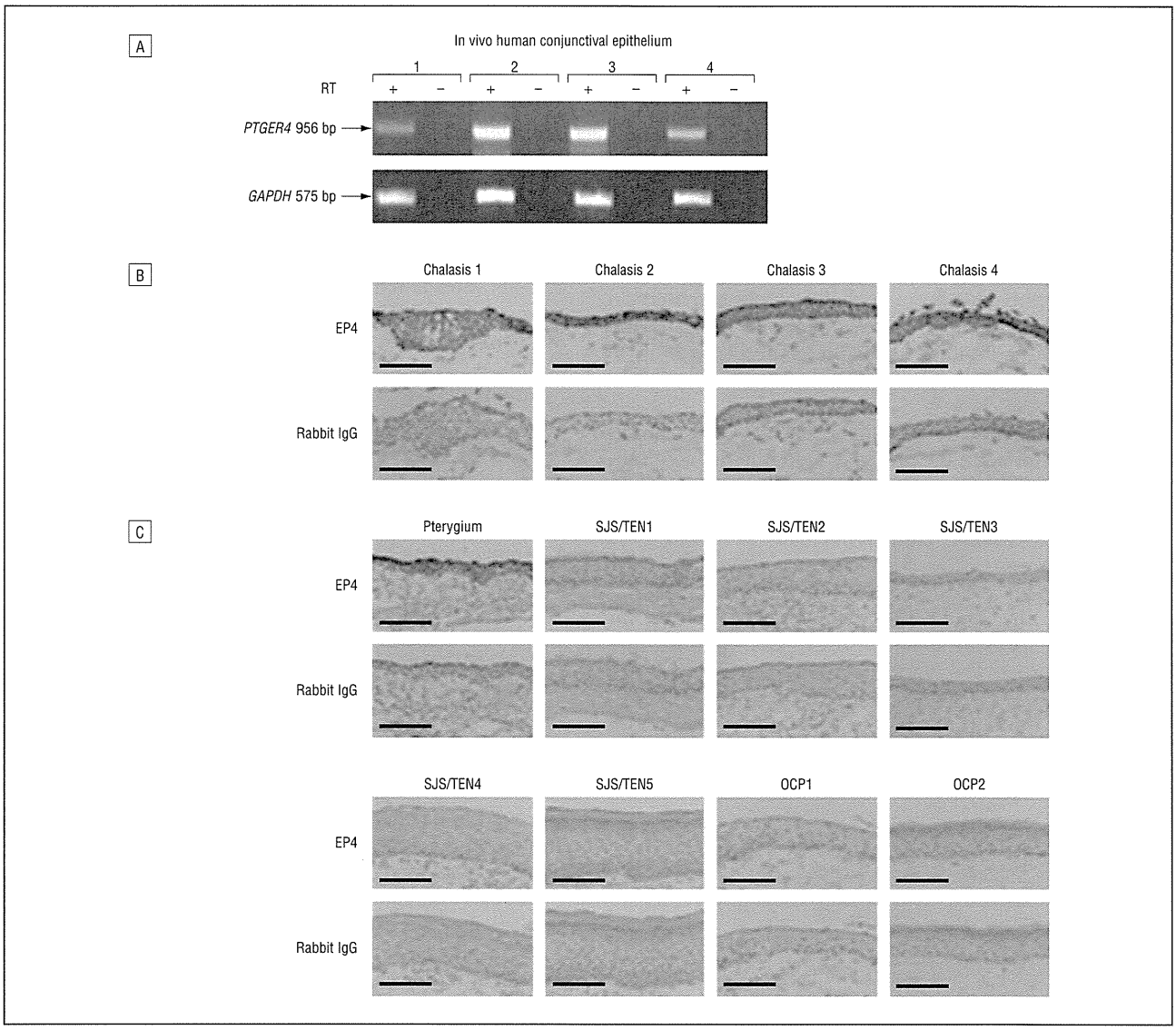

Figure. Polymerase chain reaction and immunohistological analysis results. A, Reverse transcription (RT)–polymerase chain reaction analyses of the expression of *PTGER4*-specific messenger RNA in human conjunctival epithelium derived from 4 volunteers. bp indicates base pairs. Immunohistological analysis for prostaglandin E receptor 4 (EP4) in human conjunctival epithelium in nearly normal conjunctival tissues with conjunctivochalasis (B) and in conjunctival tissues with various ocular surface diseases such as pterygium, Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN), and ocular cicatricial pemphigoid (OCP) (C). Scale bars indicate 100 μm. For EP4 staining, we used the rabbit polyclonal antibody to EP4 (Cayman Chemical Co, Ann Arbor, Michigan) and Biotin-SP–conjugated AffiniPure F(ab')2 fragment donkey antirabbit IgG (H + L) (Jackson Immuno Research, Baltimore, Maryland) as the secondary antibody; then, Vectastain ABC reagents (Vector Laboratories, Inc, Burlingame, California) were used for increased sensitivity with peroxidase substrate solution (DAB substrate kit; Vector Laboratories, Inc) as a chromogenic substrate.

control patients with conjunctivochalasis. However, we did not detect EP4 immunoreactivity in the conjunctival epithelium from patients with SJS/TEN or OCP (Figure, C). Our results showed that EP4 is strongly downregulated in the conjunctival epithelium of tissues with devastating ocular surface disorders such as SJS/TEN and OCP, although it is usually expressed in human conjunctival epithelium.

Comment. To our knowledge, this is the first documentation regarding downregulation of EP4 expression in human conjunctival epithelium in tissues with devastating ocular surface inflammatory disorders, although there were reports of expression of EP receptors in ocular tissues.[5] Kabashima et al[3] reported that EP4 deficiency impaired mucosal barrier function and aggregation of neutrophils and lymphocytes in the colon and that administration of an EP4-selective agonist to wild-type mice ameliorated severe colitis; they concluded that EP4 maintains intestinal homeostasis. On the other hand, Yao et al[6] recently reported that PGE_2 acts on its receptor EP4 on T cells and dendritic cells and promotes immune inflammation.

In human conjunctival tissues, the EP4 protein was detected in only epithelial cells but not infiltrating cells into subconjunctival tissues. Because there is mucosal inflammation on the ocular surface even in patients with chronic-phase SJS/TEN or OCP, we suspect that the downregulation of EP4 expression in conjunctival epithelium might be associated with the ocular surface inflam-

mation in patients with SJS/TEN or OCP and that there is a possibility that EP4 in human normal conjunctival epithelium suppresses the ocular surface inflammation.

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Subconjunctival Mycetoma as an Unusual Cause of Tears With Black Deposits

Ocular mycosis is a rare condition that is usually related to ocular trauma, preexisting ocular disease, or immunocompromised states. We report a case of subconjunctival mycetoma secondary to *Exophiala dermatitidis* in a healthy middle-aged woman with recalcitrant ocular inflammation and black deposits in her tears.

Report of a Case. A 44-year-old woman had recurrent discharge from her right eye and black deposits in her tears for 2 years. Her symptoms persisted despite the use of topical antibiotics, steroids, and antihistamine. She was otherwise healthy and was not receiving any systemic or other topical medication. She denied any history of ocular trauma or surgery. She did not use contact lenses or eye makeup.

On examination, her general condition was excellent. Her visual acuity, intraocular pressure, and fundi were all normal. There was no eyelid swelling or erythema. On everting the right upper eyelid, some subconjunctival black deposits were noted (**Figure**, A). During biopsy, the conjunctiva was incised and multiple black, mulberry-like concretions extruded with mucoid discharge (Figure, B). Topical chloramphenicol, 0.5%, with dexamethasone sodium phosphate, 0.1%, eyedrops were prescribed postoperatively. Histopathological evaluation of these concretions showed large amounts of fungal hyphae (Figure, C and D) with chronic inflammation over the conjunctiva. The diagnosis was subconjunctival mycetoma. Initial culture results for fungal growth were negative, but further evaluation with 28S ribosomal RNA gene sequencing identified the causative organism as *E dermatitidis*. At subsequent follow-up visits, the patient had complete resolution of symptoms. Topical antifungal treatment was not given as she was asymptomatic and there was no recurrence of mycetoma at month 3 after débridement.

Comment. Tears with black deposits are extremely rare. In our case, we initially thought the black deposits were either foreign bodies or adrenochrome deposits, but they proved to be shedding from the subconjunctival mycetoma. Patients with tears with black deposits should therefore be evaluated for the presence of subconjunctival mycetoma. A similar clinical entity termed *melanodacryorrhea* (black tears) is caused by extraocular extension of uveal melanoma.[1]

In immunocompetent subjects, fungal infection can remain superficial and localized as illustrated in our case. Subconjunctival mycetoma has been reported after subtenon corticosteroid injection in an immunocompromised host[2] and in an immunocompetent woman with no risk factors, similar to our patient.[3] The *Exophiala* species are dematiaceous mold commonly recovered from soil, plants, water, and decaying wood materials. This strain of black yeasts has been reported to cause deep infection (especially in the lung), cutaneous infection involving skin and mucous membranes, and subcutaneous infection manifested as mycetoma.[4] *E dermatitidis* has been described as the causative agent in fungal keratitis that occurred after keratoplasty[5] and laser in situ keratomileusis,[6] but to our knowledge it has not been reported to cause subconjunctival mycetoma.

Treatments described for subconjunctival mycetoma are diverse, ranging from aggressive topical and systemic antifungal treatments following surgical intervention[2] to surgical débridement alone.[3] A study by Zeng et al[4] evaluated the activity of amphotericin B, itraconazole, voriconazole, and posaconazole against *E dermatitidis* and reported that all 4 antifungal agents have low minimum inhibitory concentrations (range, 0.03-0.5). However, data on correlation between in vitro and in vivo susceptibility are unavailable.

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REVIEW

Innate Immunity of the Ocular Surface

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Keywords: Inflammation, innate immunity, ocular surface epithelium, single-nucleotide polymorphism (SNP), Toll-like receptor (TLR)

Introduction

The ocular surface epithelium serves as the defensive front line of the innate immune system. While the detection of microbes is arguably the most important task of the immune system, an exaggerated host defense reaction to endogenous bacterial flora may initiate and perpetuate inflammatory mucosal responses.

The ocular surface epithelium can produce inflammatory cytokines such as interleukin (IL)-1α, tumor necrosis factor α, IL-6, and IL-8.[1] Therefore, ocular surface epithelium can theoretically respond to various pathogens, resulting in inflammation. At the same time, there are commensal bacteria on the ocular surface as well.[2] Although ocular surface epithelia are in constant contact with bacteria and bacterial products, a healthy ocular surface is not inflammatory. Our group has been engaged in studying pathogen recognition of the ocular surface epithelium under the assumption that it possesses a unique innate immune mechanism to regulate microbe-induced inflammation.

Recognition of Pathogen-Associated Molecular Patterns

The ability of cells to recognize pathogen-associated molecular patterns depends on the expression of a family of Toll-like receptors (TLRs).[3] The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon (IFN) α/β. For example, TLR2 recognizes lipoproteins and peptidoglycan (PGN), both components of the Gram-positive bacterial cell wall. In addition, TLR2 forms a heterodimer with either TLR1 or TLR6. TLR5 recognizes flagellin, a component of bacterial flagella. Both TLR7 and TLR8 recognize viral single-stranded RNA. TLR9 recognizes CpG DNA contained in both bacteria and viruses. TLR4 recognizes lipopolysaccharide (LPS), a component of the Gram-negative bacterial cell wall. TLR3 recognizes viral double-stranded RNA.

Our group first examined whether human ocular surface epithelium expresses specific mRNA for TLRs 1–10. TLR-specific reverse transcription-polymerase chain reaction (RT-PCR) showed that mRNA from all but TLR8 was present in normal human corneal epithelium and that all TLR1–10-specific mRNA expression was present in the human conjunctival epithelium.[4,5]

One of the TLR2 ligands, PGN, is a major component of the Gram-positive bacteria cell wall. On the other hand, LPS, a TLR4 ligand, is a component of the Gram-negative bacteria cell wall. In providing an immune response, both PGN and LPS can induce the production of inflammatory cytokines such as IL-6 and IL-8. We stimulated cultured primary human corneal epithelial cells with the TLR4 ligand LPS and the TLR2 ligand PGN. However, the cells did not respond to either LPS or PGN.[6] Monocytes can phagocytose LPS intracellularly, but owing to their lack of phagocytotic activity corneal epithelial cells cannot. We therefore used the transfection agent DOTAP to force the introduction of LPS intracellularly into human corneal epithelial cells. However, even when LPS was present in the cytoplasm of the corneal epithelial cells, the cells did not respond to its introduction.[6] Yet, unlike our results, other groups report

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both TLR2 and TLR4 of the corneal epithelium of mammalian cornea responding to their ligands.[7,8]

TLR3 recognizes viral double-stranded RNA, which almost all viruses synthesize at the time of duplication. Since viral double-stranded RNA is mimicked by polyinosinic:polycytidylic acid (polyI:C), the ligand for TLR3, and we used polyI:C in our experiments to stimulate both human peripheral mononuclear cells and primary human corneal epithelial cells. In the human peripheral mononuclear cells, LPS but not polyI:C stimulation significantly increased the production of IL-6 and IL-8 (Fig. 1A). On the other hand, in human corneal epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8, whereas LPS treatment did not (Fig. 1A).[4] IFN-β is controlled by TLR3 signaling. Thus, IFN-β-specific mRNA was significantly elevated in the polyI:C-stimulated cells. However, and quite surprisingly, IFN-β-specific mRNA expression was markedly higher in human corneal epithelial cells than in human peripheral mononuclear cells (Fig. 1A).[4] PolyI:C stimulation also upregulated mRNA expression of the antiviral chemokine IFN-γ inducible protein 10 (IP-10), myxovirus resistance gene A, and 2′,5′-oligoadenylate synthetase.[9]

TLR5 recognizes flagellin, which is the bacteria flagella protein. Flagella are present mainly on Gram-negative bacteria. *Pseudomonas aeruginosa* contributes to the inflammatory response of the human corneal epithelium.[10] Ocular surface-related bacteria with flagella include *P. Aeruginosa* as a pathogen, and *Bacillus subtilis* as a nonpathogen. Third, we stimulated both human peripheral mononuclear cells and primary human corneal epithelial cells by various kinds of flagellin as the ligand of TLR5. In this experiment, flagellin derived from the ocular pathogenic *P. aeruginosa* and from the ocular nonpathogenic *B. subtilis* were used. Moreover, the intestinal, but not ocular, pathogen *Salmonella typhimurium* derived from flagellin was also used. All flagellin stimulation of the human peripheral mononuclear cells significantly increased the production of IL-6 and IL-8. On the other hand, only the ocular pathogenic *P. aeruginosa*-derived flagellin significantly induced the secretion of IL-6 and IL-8 in human corneal epithelial cells, while ocular nonpathogenic *B. subtilis*-derived flagellin and intestinal pathogenic *S. typhimurium*-derived flagellin did not induce any secretion at all.[11]

Another type of ocular surface epithelial cells, human conjunctival epithelial cells, did not secrete any IL-6 or IL-8 in response to LPS stimulation. They can secrete both IL-6 and IL-8 in response to the ocular pathogenic *P. aeruginosa*-derived flagellins only, but not to non-ocular pathogenic *S. typhimurium*-derived flagellins and ocular nonpathogenic *B. subtilis*-derived flagellins.[4]

Our immunohistochemical studies showed that TLR2, -3, and -4 proteins were located in cells ranging from the basal to the superficial layer of both the corneal and conjunctival epithelium.[1] TLR5 protein was consistently and abundantly expressed only at basal and wing sites in stratified corneal and conjunctival epithelium, indicating a spatially selective presence on the basolateral but not the apical side (Fig. 1B).[1,4,11]

Ocular surface epithelial cells respond to the flagellin derived from ocular-pathogenic bacteria through TLR5 and produce inflammatory cytokines. However, the superficial ocular surface epithelial cells do not express TLR5. Therefore, it is reasonable to speculate that TLR5 of ocular surface epithelium cannot function in a healthy ocular surface without any epithelial defect.[1,4,11]

In summary, immune competent cells such as macrophages may recognize various microbial components through TLRs, induce the inflammation, and then exclude the microbes. On the other hand, ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. The difference between macrophages and ocular surface epithelial cells might be due to the dissimilarity in the coexistence with commensal bacteria (Fig. 1C). The unique innate immune response of the ocular surface epithelium might contribute to its coexistence with commensal bacteria.[1]

Ocular Surface Inflammation

We also hypothesized that an abnormality in the proper innate immunity of the ocular surface may result in ocular surface inflammation.

IκBζ is induced by diverse pathogen-associated molecular patterns and regulates nuclear factor (NF)-κB activity.[12] Thus, IκBζ is important for TLR/IL-1 receptor signaling, which is essential for an innate immune response. IκBζ knockout (KO) mice expressly exhibit severe, spontaneous ocular surface inflammation accompanied by the eventual loss of almost all goblet cells.[13] Moreover, Balb/c-background IκBζ KO mice exhibit not only spontaneous ocular surface inflammation but also spontaneous perioral inflammation (Fig. 2).[14] IκBζ induced by diverse pathogen-associated molecular patterns regulates NF-κB activity, possibly to prevent excessive inflammation in the presence of bacterial components. The spontaneous ocular surface inflammation observed in IκBζ KO mice suggests that dysfunction/abnormality of innate immunity can play a role in ocular surface inflammation.[1]

We considered the possibility of an association between a disordered innate immune response and Stevens-Johnson syndrome (SJS), a severe ocular-surface inflammatory disease. Under the hypothesis of a disordered innate immune response in SJS, we performed gene expression analysis of monocytes, cells that are essential for innate immunity. We found differences in IL-4R gene expression; upon LPS stimulation, it was downregulated in SJS/toxic epidermal necrolysis (TEN) patients and slightly upregulated in the controls.[1] After culture for 1 h without LPS, the expression of IκBζ- and IL-1α-specific mRNA was lower in monocytes from SJS/TEN patients than in those from normal controls.[1] Our findings suggest that the reduced

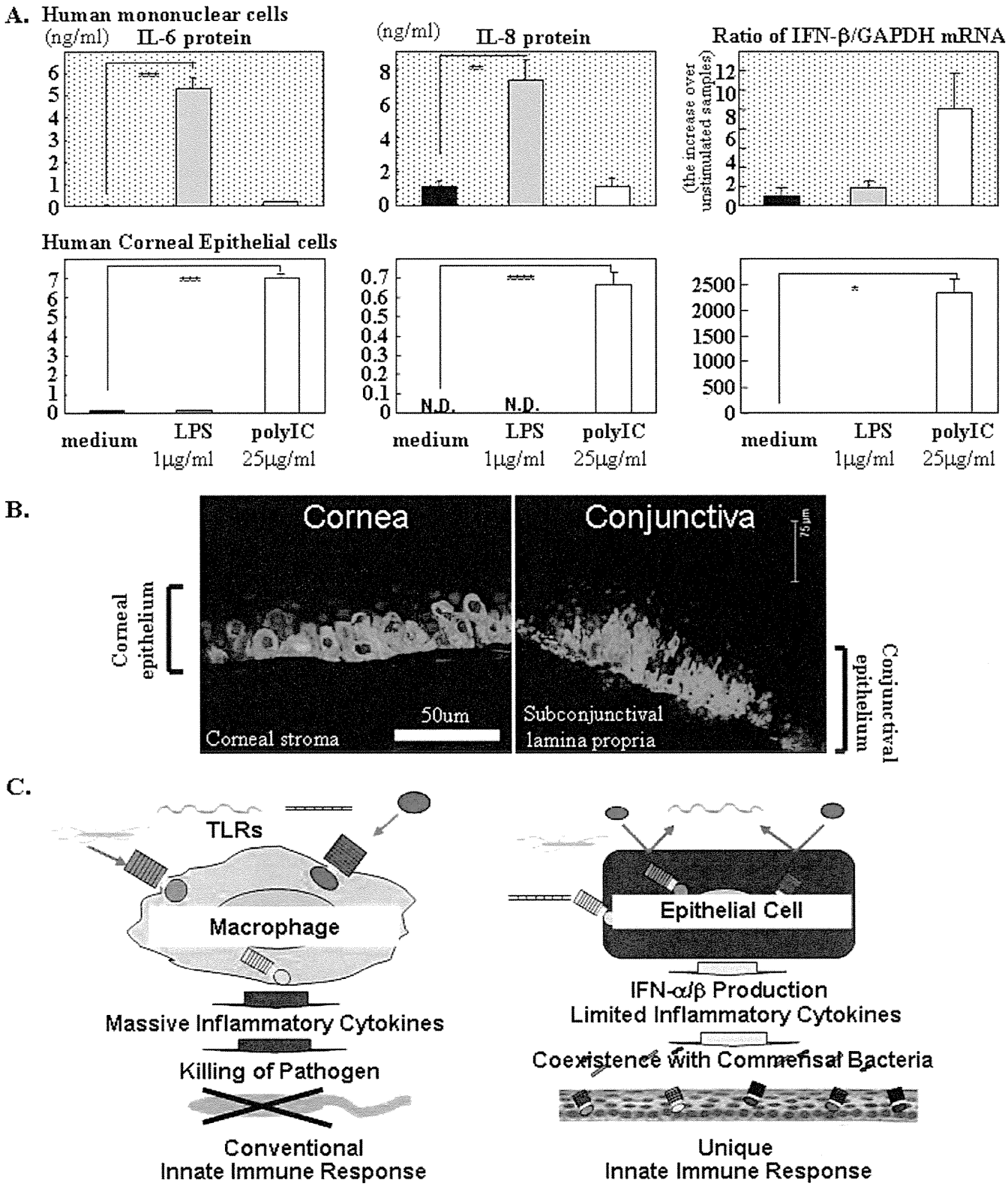

Figure 1. **A** In human peripheral mononuclear cells, lipopolysaccharide (*LPS*) but not polyinosinic:polycytidylic acid (*polyI:C*) stimulation significantly increased the production of interleukin (*IL*)-6 and IL-8. On the other hand, in human corneal epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8, while LPS treatment did not. Interferon (*IFN*)-β-specific mRNA expression was markedly higher in human corneal epithelial cells than in human peripheral mononuclear cells, although IFN-β-specific mRNA was significantly elevated in polyI:C-stimulated cells (modified with permission from M. Ueta[1,5]). **B** Human ocular surface epithelium expresses toll-like receptor (*TLR*) 5 protein. Bound antibodies were visualized by Alexa Fluor 488 donkey anti-mouse IgG and nuclei by PI staining. TLR5 was detected only at basal and wing sites of corneal and conjunctival epithelium, indicating its spatially selective presence on the basolateral but not the apical side (modified with permission from M. Ueta,[1] Kojima et al.,[4] and Hozono et al.[11]). **C** Immune competent cells such as macrophages could recognize various microbial components through TLRs and induce the inflammation and then exclude the microbes. On the other hand, ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. The difference between macrophages and ocular surface epithelial cells might be due to the dissimilarity in the coexistence with commensal bacteria. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

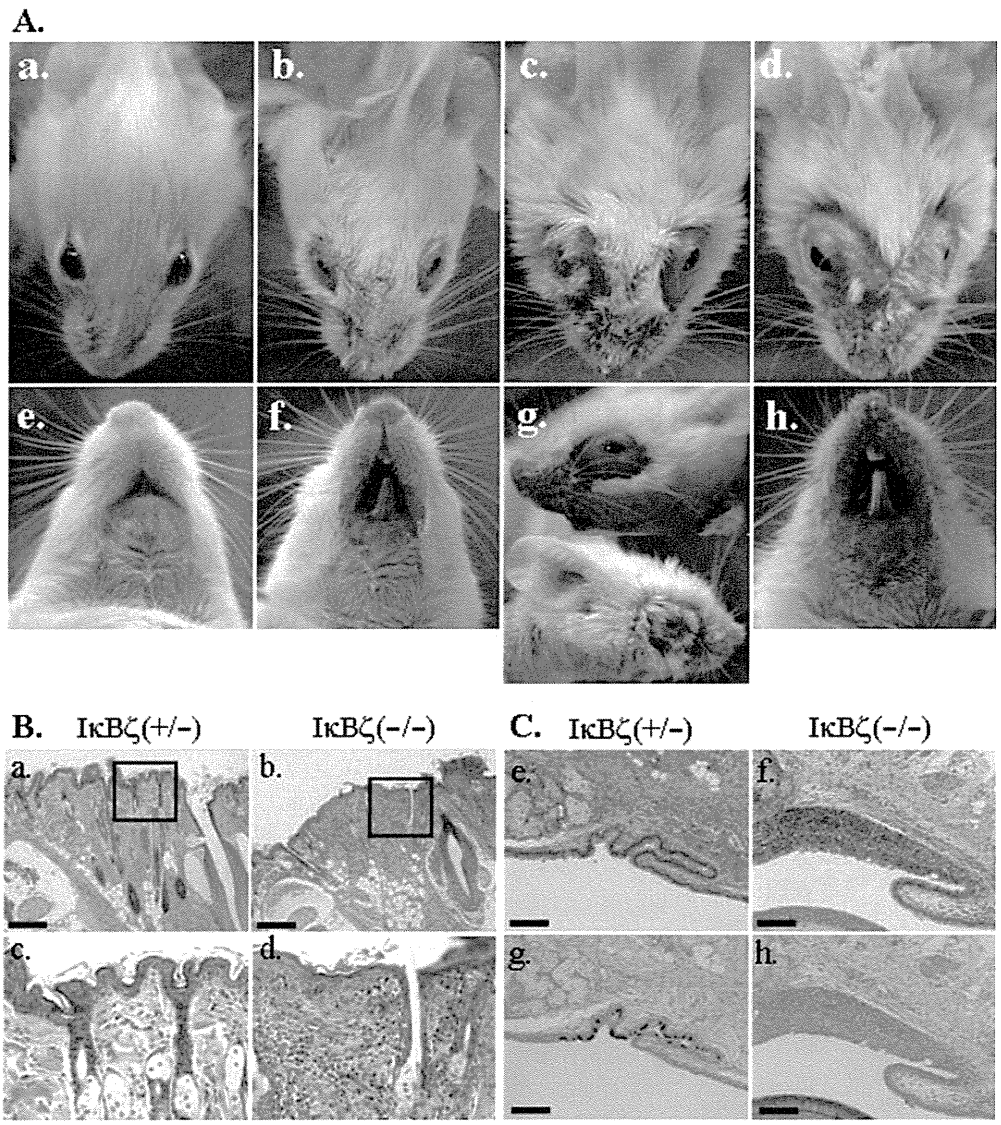

Figure 2. A Kinetic monitoring of the inflammatory phenotype in the eyes and perioral skin of IκBζ–/– mice. Photographs show the face and perioral skin of IκBζ–/– mice. *a*, *e*, at 3 weeks of age (before onset); i, *f*, at 9 weeks of age (4 weeks postonset); *c*, *g*, at 13 weeks of age (8 weeks postonset); *d*, *h*, at 15 weeks of age (10 weeks postonset). **B** Histological analysis of the perioral skin of IκBζ–/– mice. The perioral skin of 6-week-old IκBζ+/– (a, c) and IκBζ–/– (b, d) mice 2 weeks after symptom onset. Enlargements of the *boxed* lesions in *a* and *b* are shown in *c* and *d*. H&E stains. Each bar represents a length of 200 μm. **C** Histological analysis of eyelids of IκBζ–/– mice. The eyelids of 6-week-old IκBζ+/– (*e*, *g*) and IκBζ–/– (f, h) mice 2 weeks after symptom onset. H & E- (e, f) and PAS periodic acid-Schiff (g, *h*) stains. Each bar represents a length of 200 μm (modified with permission from Ueta et al.[14]).

expression of IκBζ and IL-1α genes may play an important role in the pathophysiology of SJS.[1]

While SJS can be induced by specific drugs, not all individuals treated with those drugs develop SJS. As the incidence of SJS is very low, we suspected a genetic predisposition and performed a single-nucleotide polymorphism (SNP) association analysis using candidate genes associated with innate immunity, apoptosis, or allergy.

We found that the TLR3 SNP rs.3775296[15] and the IL-4R SNP rs.1801275 (Gln551Arg)[16] were both strongly associated ($P < 0.0005$), that the FasL SNP rs.3830150 was mildly associated ($P < 0.005$),[17] and that the IL-13 SNP rs.20541 (Arg110Gln)[18] and the IκBζ SNP rs.595788[1] were weakly associated ($P < 0.05$) with SJS/TEN with ocular surface complications. On the basis of the considerations presented here, we suggest that viral infection or drugs may trigger a disorder in the host innate immune response and that the triggering event is followed by aggravated inflammation of the mucosa, ocular surface, and skin.

In summary, we posit the possibility of an association between disordered innate immunity and ocular surface inflammation.

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Ocular Surface Inflammation Mediated by Innate Immunity

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Abstract: This review addresses three subjects: the innate immunity of the ocular surface epithelium, innate immunity and ocular surface inflammation, and Stevens-Johnson syndrome (SJS) and abnormality of innate immunity. In innate immunity of the ocular surface epithelium, ocular surface epithelial cells respond selectively to microbial components and induce limited inflammation, whereas immune-competent cells such as macrophages can recognize various microbial components through Toll-like receptors (TLRs) and induce inflammation to exclude the microbes. The difference between macrophages and ocular surface epithelial cells may be caused by the dissimilarity in the degree of coexistence with commensal bacteria. The unique innate immune response of ocular surface epithelium might contribute to coexistence with commensal bacteria. In innate immunity and ocular surface inflammation, we speculate that an abnormality in the proper innate immunity of the ocular surface may result in ocular surface inflammation. Our investigation shows that TLR3 positively regulates the late-phase reaction of experimental allergic conjunctivitis, which causes reduced eosinophilic conjunctival inflammation in TLR3KO (knockout) mice and pronounced eosinophilic conjunctival inflammation in TLR3Tg mice. We also demonstrate that human ocular surface epithelial cells can be induced to express many transcripts, including antiviral innate immune response-related genes and allergy-related genes, through polyI:C stimulation. Furthermore, we show that IκBζ KO mice exhibit severe, spontaneous ocular surface inflammation accompanied by the eventual loss of almost all goblet cells and spontaneous perioral inflammation. IκBζ is induced by diverse pathogen-associated molecular patterns and regulates nuclear factor-κB activity, possibly to prevent excessive inflammation in the presence of bacterial components. The spontaneous ocular surface inflammation observed in IκBζ KO mice suggested that dysfunction/abnormality of innate immunity can play a role in ocular surface inflammation. In SJS and abnormality of innate immunity, we considered the possibility that there may be an association between SJS and a disordered innate immune response. In gene expression analysis of $CD14^+$ cells, we found that *IL4R* gene expression was different in patients with SJS/toxic epidermal necrolysis (TEN) and controls on lipopolysaccharide stimulation, being downregulated in patients with SJS/TEN and slightly upregulated in the controls. The expression of *IκBζ*- and *interleukin (IL)-1α*-specific mRNA in patients with SJS/TEN was lower than in normal controls after 1-hour culture. Although SJS/TEN can be induced by drugs, not all individuals treated with these drugs developed SJS/TEN. Because the incidence of SJS/TEN is very low, we suspected a genetic predisposition and performed single-nucleotide polymorphism (SNP) association analysis using candidate genes associated with innate immunity, apoptosis, or allergy. We found that *TLR3* SNP rs.3775296 and *IL4R* SNP rs.1801275 (Gln551Arg) were strongly associated ($P<0.0005$) with SJS/TEN with ocular surface complications, *FasL* rs.3830150 SNP was mildly associated ($P<0.005$), and *IL13* rs.20541 (Arg110Gln) and *IκBζ* SNP rs.595788G/A exhibited a weak association ($P<0.05$). Genetic and environmental factors may play a role in an integrated cause of SJS, and there is the possibility of an association between SJS and a disordered innate immunity.

Key Words: Innate immunity—Ocular surface—Epithelium—Toll-like receptors—Stevens-Johnson Syndrome

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INNATE IMMUNITY OF OCULAR SURFACE EPITHELIUM

The ocular surface epithelium not only forms a physical barrier against the external environment but also serves a critical function as the defensive front line of the innate immune system. The ocular surface manifests many nonspecific defense mechanisms against microbes, e.g., lysozyme, lactoferrin, IgA in tear fluids, and all isoforms of human beta-defensins found in the ocular surface epithelium. Furthermore, ocular surface epithelium can produce inflammatory cytokines such as interleukin (IL)-6, IL-8, IL-1α, and tumor necrosis factor-α. Therefore, ocular surface epithelium can theoretically respond to various pathogens, resulting in inflammation. On the other hand, an exaggerated host defense reaction to endogenous bacterial flora may initiate and perpetuate inflammatory mucosal responses, although the detection of microbes is arguably the most important task of the immune system. There are commensal bacteria on the ocular surface and other mucosa. When we harvested commensal bacteria from the conjunctival sacs of 42 healthy volunteers, *Staphylococcus epidermidis* bacteria were isolated from 45% of the volunteers and *Propionibacterium acnes* bacteria from 31%.[1] Although the ocular surface epithelium is in constant contact with bacteria and bacterial products, the healthy ocular surface is not inflammatory.

Innate immunity, the early host defense against microbes, is primarily studied in host immune-competent cells such as macrophages. The ability of cells to recognize pathogen-associated molecular patterns (PAMPs) depends on the expression of a family of Toll-like receptors (TLRs).[2] Macrophages recognize and phagocytose microbes such as bacteria and produce inflammatory cytokines and chemokines, thus resulting in inflammation. These cells also activate

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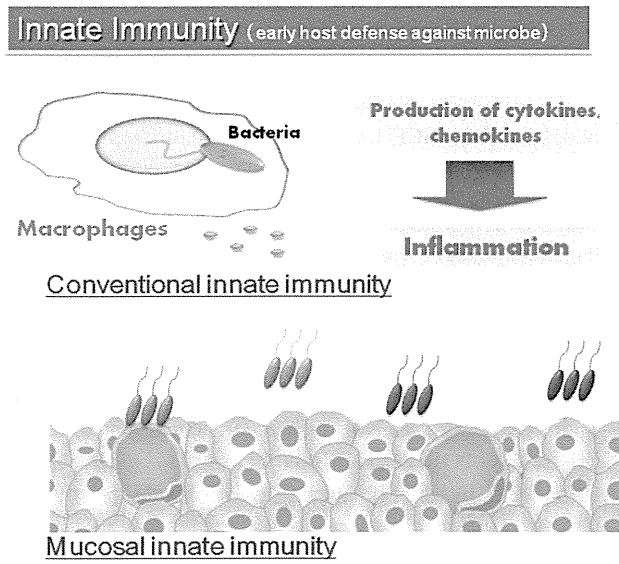

FIG. 1. Mucosal innate immunity with the presence of commensal bacteria seems to be different from conventional innate immunity. Conventional innate immunity: macrophages recognize and phagocytose microbes such as bacteria and produce inflammatory cytokines and chemokines, thus resulting in inflammation. Mucosal innate immunity: mucosal epithelium neither usually respond to resident commensal bacteria nor induce inflammation under normal conditions.

adaptive immunity. However, it is now clear that the innate immunity of the mucosa in contact with commensal bacteria differs from conventional innate immunity (Fig. 1).[3] The ocular surface is one of the mucosa that is in contact with commensal bacteria. The ocular surface epithelium neither usually respond to resident commensal bacteria nor induce inflammation under normal conditions. Therefore, we speculate that the ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes.[4–6]

The TLRs are important molecules associated with innate immunity, and the first line of defense against infection comprises evolutionarily conserved sets of TLR molecules. The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon (IFN)-α/β. For example, TLR2 recognizes lipoprotein or peptidoglycan, identifies components of the gram-positive bacterial cell wall, and forms a heterodimer with TLR1 or TLR6; TLR3 also recognizes viral double-strand (ds) RNA. The TLR4 recognizes lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall; TLR5 recognizes flagellin, a component of bacterial flagella; TLR7 and TLR8 recognize viral single-stranded RNA, and TLR9 recognizes bacterial or viral CpG DNA (Fig. 2).

Our study aimed to determine whether the human ocular surface epithelium expresses specific mRNA for *TLRs 1* to *10*. The results revealed that *TLR1*- to *10*-specific mRNA expression was present in human conjunctival epithelium and *TLR1*- to *7*- and *TLR9*- and *TLR10*-specific mRNA was found in human corneal epithelium (Fig. 3).[4,5,7,8]

The TLR3 recognizes the viral dsRNA synthesized by almost all viruses at the time of duplication. Because polyI:C mimics viral dsRNA, we used it in our experiments. We stimulated human peripheral mononuclear cells (HPMC), primary human corneal epithelial cells (PHCEC), and primary human conjunctival epithe-

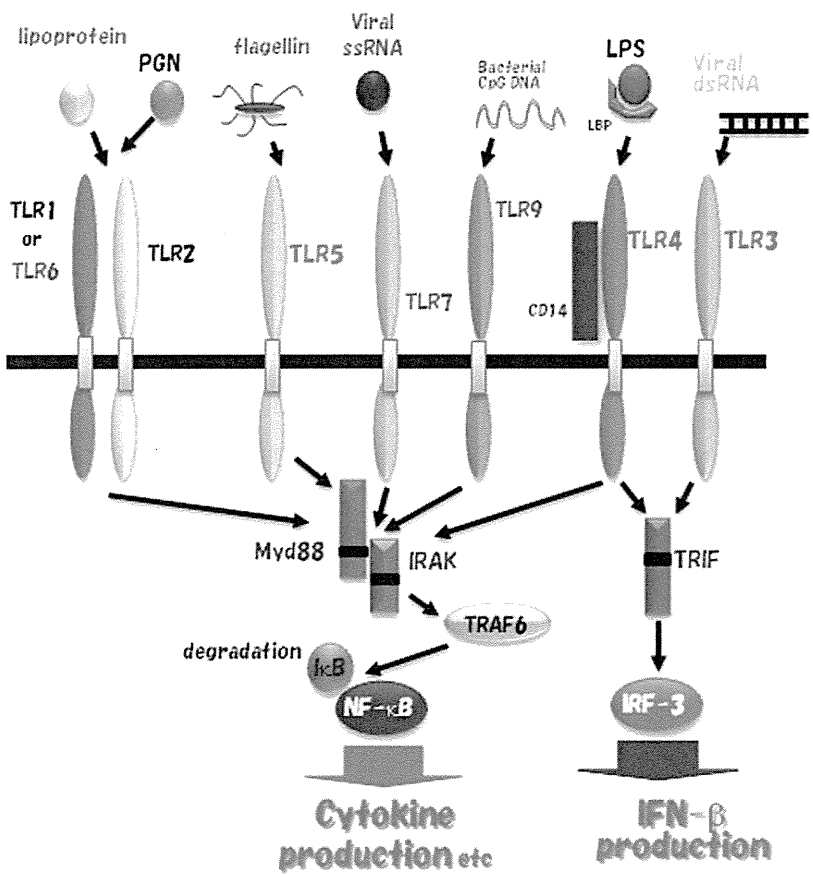

FIG. 2. Function of TLRs. The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon α/β.

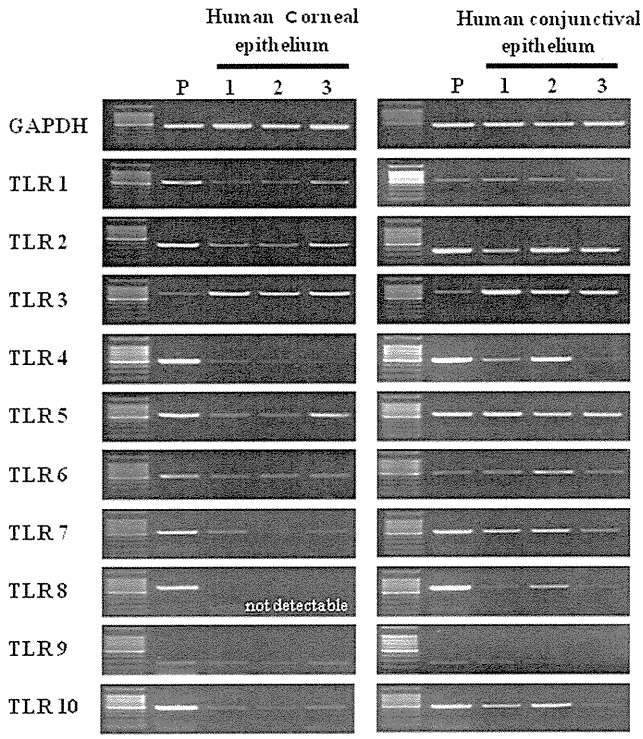

FIG. 3. Human ocular surface epithelium expresses *TLR*-specific mRNA. *TLR1*- to *10*-specific mRNA expression was present in human conjunctival epithelium, and *TLR1*- to *7*- and *TLR9*- and *10*-specific mRNA was found in human corneal epithelium. The positive control (P) was human mononuclear cells. Reprinted with permission from Ueta M. Innate immunity of the ocular surface and ocular surface inflammatory disorders. *Cornea* 2008;27(suppl 1):S31–S40. © 2008 by Lippincott Williams & Wilkins.

lial cells (PHCjEC) with polyI:C, the ligand of TLR3. In the primary human ocular surface epithelial cells, PHCEC and PHCjEC, but not in HPMC, stimulation with polyI:C significantly induced the secretion of IL-6 and IL-8 (Fig. 4A).[4,5,8] These findings were confirmed at the mRNA expression level; in PHCEC and PHCjEC, but not in HPMC, polyI:C stimulation resulted in the increased expression of *IL-6*- and *IL-8*-specific mRNA (Fig. 4B).[4,5,8] Because IFN-β is controlled by TLR3 signaling, *IFN-β*-specific mRNA was significantly increased in polyI:C-stimulated cells; its expression was markedly higher in PHCEC than in PHCjEC or HPMC (Fig. 4C).[4,5,8] Interestingly, TLR3 is expressed on the cell surface of PHCEC,[8] PHCjEC,[5] endothelial cells,[9] and fibroblasts.[10] In dendritic cells, TLR3 is reportedly localized to endosomes.[11]

The LPS, a TLR4 ligand, is present in the cell walls of gram-negative bacteria. Although LPS stimulation significantly increased the production of IL-6 and IL-8 in HPMC, it failed to induce the production of inflammatory cytokines such as IL-6 and IL-8 in the human ocular surface epithelial cells, PHCEC, and PHCjEC (Fig. 3).[4–6] Monocytes, but not PHCEC, can phagocytose LPS. We used the transfection agent DOTAP Liposomal Transfection Reagent (Roche, Mannheim, Germany) to force the intracellular introduction of LPS into PHCEC. However, even in the presence of LPS in the cytoplasm of PHCEC, they did not respond to LPS stimulation.[6]

The TLR5 recognizes flagellin, the bacterial flagella protein. Flagella are primarily present on gram-negative bacteria. Ocular surface-related bacteria with flagella include pathogenic *Pseudomonas aeruginosa* and nonpathogenic *Bacillus subtilis*. We stimulated HPMC, PHCEC, and PHCjEC with different flagellins and TLR5 ligands; we used flagellin from *P. aeruginosa* and from *B. subtilis*. We also used flagellin from *Salmonella typhimurium*, which is an intestinal but not an ocular pathogen. In HPMC, all flagellin stimulation significantly increased the production of IL-6 and IL-8.[4,5,7,12] On the other hand, in PHCEC and PHCjEC, only flagellin derived from the ocular pathogen *P. aeruginosa* significantly induced the secretion of IL-6 and IL-8 and not flagellin derived from ocular nonpathogenic *B. subtilis* and intestinal pathogenic *S. typhimurium*.[4,5,7,12] We confirmed these findings at the mRNA expression level. In PHCEC and PHCjEC, only ocular pathogenic *P. aeruginosa*-derived flagellin resulted in a significant increase in the expression of *IL-6*- and *IL-8*-specific mRNA.[4,5,7,12] Interestingly, *P. aeruginosa*- and *S. typhimurium*-derived flagellin exhibit identical potency in inducing IL-8 protein production by cells from the human intestinal epithelial cell line HT29 (Fig. 5A).[12]

Our immunohistochemical studies showed that TLR3 and TLR4 proteins were located in cells from the basal to the superficial layer of the corneal and conjunctival epithelia.[4,5] The TLR5 proteins were present only at basal and wing sites, indicating a spatially selective presence on the basolateral but not the apical side (Fig. 5B).[4,5,7,12] Ocular surface epithelial cells respond to the flagellin derived from ocular pathogenic bacteria through TLR5 to produce inflammatory cytokines. However, superficial ocular surface epithelial cells do not express TLR5. Therefore, it is reasonable to speculate that TLR5 of ocular surface epithelium is not functional on a healthy ocular surface free of epithelial defects (Fig. 5C).[4,5,7,12]

Immune-competent cells such as macrophages do recognize various microbial components via TLRs, induce inflammation and then exclude microbes, whereas ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. This difference in the action of macrophages and ocular surface epithelial cells may be caused by dissimilarities in their coexistence with commensal bacteria. Thus, the unique innate immune response of the ocular surface epithelium may contribute to its coexistence with commensal bacteria (Fig. 6).[4,5]

INNATE IMMUNITY AND OCULAR SURFACE INFLAMMATION

Furthermore, we also speculate that an abnormality in the proper innate immunity of the ocular surface may result in ocular surface inflammation because inflammatory bowel disease is thought to result from an abnormal response to the gut microbiota.

TLR3 and Allergy

The TLRs are well-known key receptors of the innate immune system. The TLR3 recognizes dsRNA, a component of the life cycle of most viruses, mimicking polyI:C.[13] The TLR3 is expressed most intensely in ocular surface epithelium and more intensely than mononuclear cells.[4,5,8]

Although a relationship between viral infection and allergic inflammation has been reported, the function of TLR3 in allergic

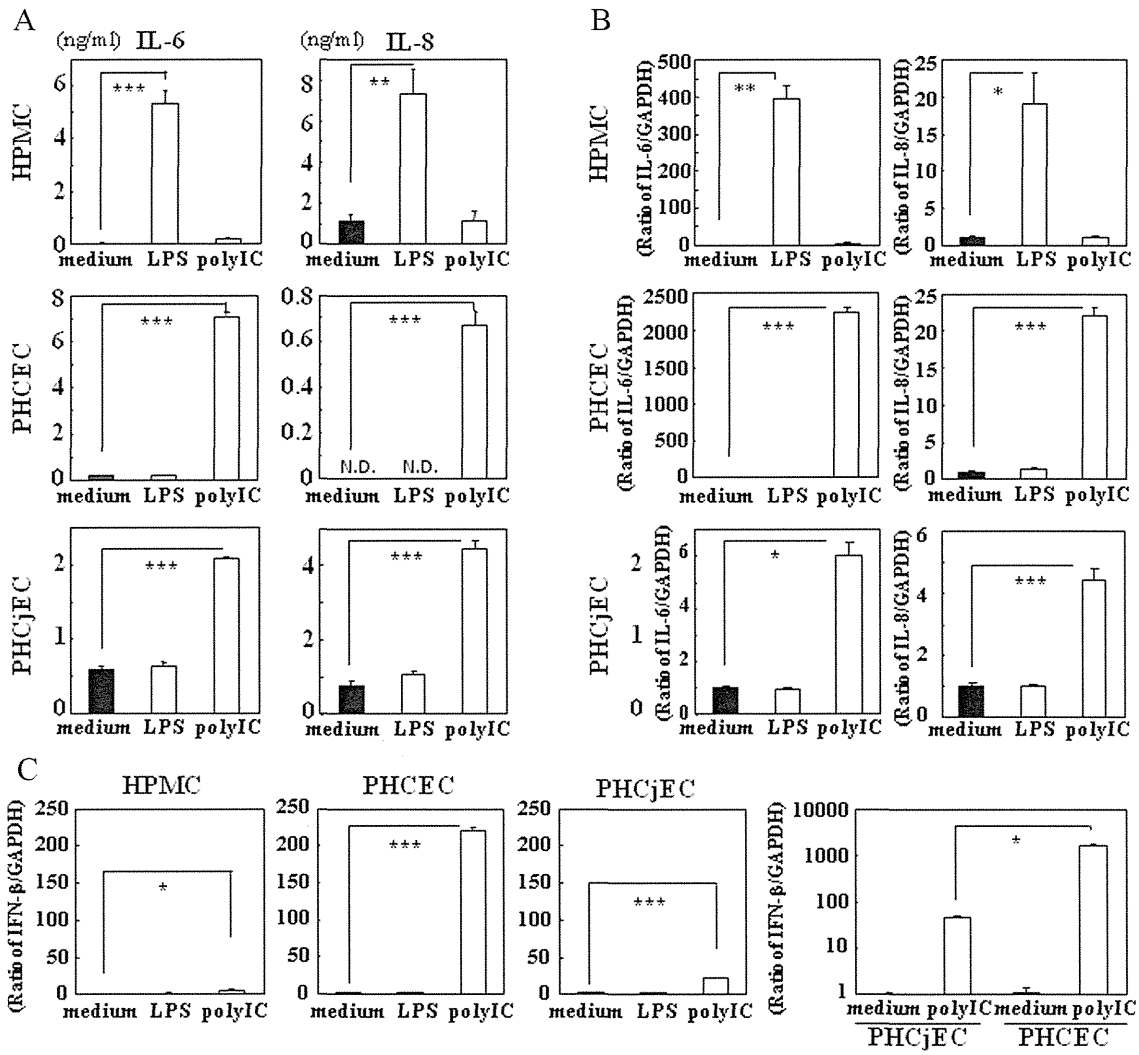

FIG. 4. Responsiveness against polyI:C, the TLR3 ligand, of PHCEC and PHCjEC. (A), Production of IL-6 and IL-8. The HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 24 hours to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). (B), Expression of *IL-6* and *IL-8* specific mRNA. HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 6 hours (HPMC, PHCEC) or 3 hours (PHCjEC) to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). The y-axis for the ratio of *IL-6* or *IL-8/GAPDH* mRNA shows an increase in specific mRNA over unstimulated cell samples. (C), Expression of *IFN-β*-specific mRNA. HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 3 hours to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). The y-axis for the ratio of *IFN-β/GAPDH* mRNA shows an increase in specific mRNA over unstimulated samples of each cells or PHCjEC. Reprinted with permission from Ueta M, Kinoshita S. Innate immunity of the ocular suface. *Brain Res Bull* 2010;81:219–228. © 2010 by Elsevier Inc.

inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions; the degree of eosinophil infiltration in the conjunctiva reflects the degree of its late-phase reaction. Using our murine model of experimental allergic conjunctivitis (EAC) (Fig. 7A)[14] and *TLR3* knockout (KO) and *TLR3* transgenic (Tg) mice (*TLR3*KO and *TLR3*Tg, respectively), we have directly assessed the role of TLR3 in conjunctival eosinophil infiltration.

In our model of murine EAC, the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in mice after sensitization and challenge, although sensitization without challenge had no effect. Sensitization with short-ragweed pollen induced short-ragweed pollen-specific immune responses equally in wild-type, *TLR3*KO, and *TLR3*Tg mice; sensitization also produced an increase in IgE and IgG_1 antigen-specific antibody responses. This effect was similar in magnitude in all three groups of mice. Comparing the number of eosinophils in the lamina propria mucosae of the conjunctiva in *TLR3*KO and wild-type mice revealed significantly lower levels in *TLR3*KO than in wild-type mice (Fig. 7B).[14] Moreover, comparing eosinophil infiltration in *TLR3*Tg and wild-type mice revealed that the numbers of eosinophils in *TLR3*Tg mice after sensitization and challenge were significantly larger than in wild-type mice (Fig. 7B).[14] These results suggest that *TLR3* positively regulates the late-phase reaction of EAC, which causes reduced eosinophilic conjunctival inflammation in *TLR3*KO mice and pronounced eosinophilic conjunctival inflammation in *TLR3*Tg mice.[14]

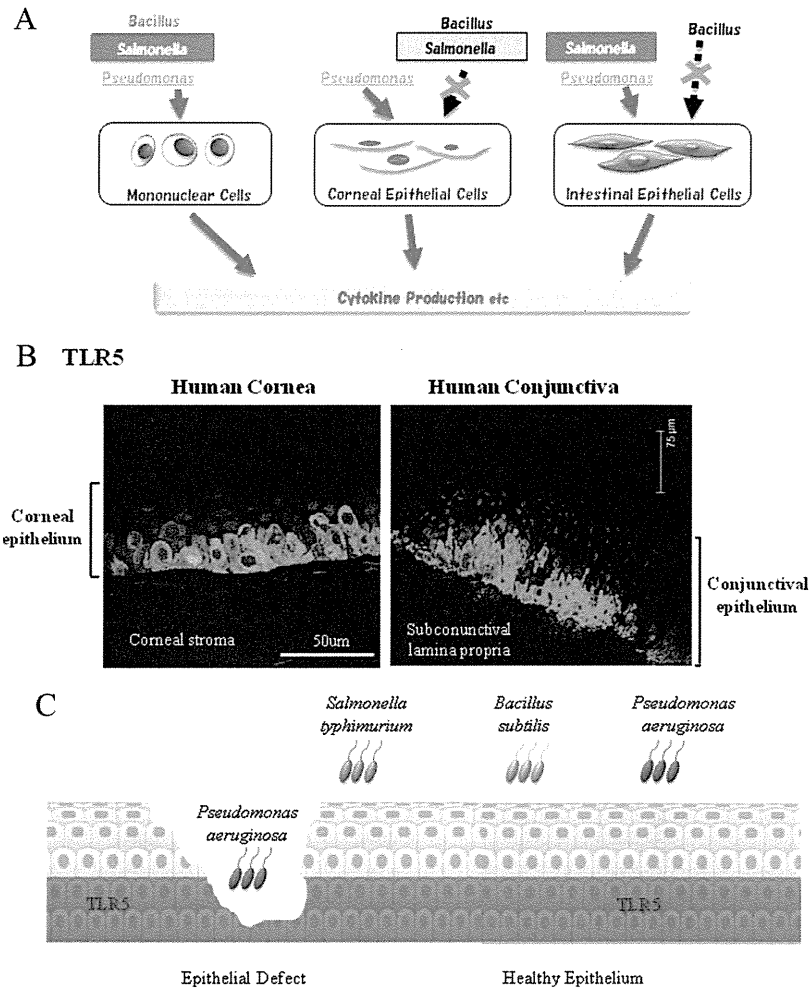

FIG. 5. Responsiveness against various flagellins, the TLR5 ligands, of PHCECs and PHCjEC. (A), In HPMC, all flagellin stimulation significantly increased the production of proinflammatory cytokine such as IL-6 and IL-8. On the other hand, in PHCEC and PHCjEC, only flagellin derived from the ocular pathogen *P. aeruginosa* significantly induced the secretion of proinflammatory cytokine and not flagellin derived from ocular nonpathogenic *B. subtilis* and intestinal pathogenic *S. typhimurium*. In human intestinal epithelial cell line HT29, *P. aeruginosa*- and *S. typhimurium*-derived flagellin induced the secretion of proinflammatory cytokine. (B), Expression of TLR5 protein in ocular surface epithelium. TLR5 proteins were present only at basal and wing sites. Human corneal tissues were obtained from corneal buttons of a patient undergoing corneal transplantation for early-stage bullous keratopathy; human conjunctival tissues were obtained at the time of conjunctivochalasis surgery. Isotype control incubation was the negative control. Bound antibodies were visualized by Alexa Fluor 488 donkey anti-mouse IgG−, nuclei by propidium iodide staining. Each bar represents a length of 50 μm in corneal epithelium or 75 μm in conjunctival epithelium. (Modified with permission from Ueta M. Innate immunity of the ocular surface and ocular surface infammatory disorders. *Cornea* 2008;27(suppl 1):S31–S40.) (C) Function of TLR5 on an ocular surface. Ocular surface epithelial cells respond to the flagellin derived from ocular pathogenic bacteria through TLR5 to produce inflammatory cytokines. However, superficial ocular surface epithelial cells do not express TLR5. Therefore, TLR5 of ocular surface epithelium might be not functional on a healthy ocular surface free of epithelial defects.

We previously reported that mast cells do not play an essential role in the development of eosinophilic conjunctival inflammation in the late-phase reaction because mast cell-deficient mice exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation similar to that seen in their congenic littermates (Fig. 8).[15] We also suggested that conjunctival epithelial cells may be implicated in the eosinophilic conjunctival inflammation seen in allergic conjunctivitis. The previous report raises a possibility that the ocular surface epithelial cells regulate the inflammation of allergic conjunctivitis.[15]

Elsewhere, we showed that EP3 is expressed in the ocular surface epithelium (Fig. 9A),[14] and that the PGE_2-EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis; *Ptger3*$^{-/-}$ mice demonstrated significantly increased eosinophil infiltration in conjunctiva after short-ragweed pollen-challenge compared to wild-type mice (Fig. 9B).[14] It is evident that ocular surface epithelial cells regulate the inflammation of allergic conjunctivitis.[14]

We previously found that stimulation with polyI:C elicited increased mRNA expression of *IL-6*, *IL-8*, and *IFN-β* in PHCjECs as well as in PHCEC.[4,5,8] Moreover, to examine the comprehensive effects of polyI:C stimulation of PHCjECs, we performed gene expression analysis of PHCjECs from two individuals that were or were not cultured with 25 μg/mL polyI:C.

Our results showed that polyI:C stimulation may induce upregulation of many transcripts (150 transcripts were upregulated more than threefold); 47 transcripts were upregulated more than 10-fold on polyI:C stimulation of the PHCjECs from two individuals. These included 11 transcripts: *CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, retinoic acid-inducible gene (RIG)-I,* and *MDA5*, the upregulation of which was confirmed by quantitative real-time polymerase chain reaction (RT-PCR).[16]

Thus, although *CXCL11*,[17–19] *CXCL10*,[17–19] *IL28A*,[20,21] *CCL5*,[22,23] *CCL4*,[22,23] and *CCL20*[22,23] are innate immune response-related genes, they have also been reported to be upregulated in allergic diseases. *IL7R*,[24] *TSLP*,[24–26] and *ICAM-1*[27] are allergy-related genes. At least 9 of the 47 transcripts that were found to be upregulated more than 10-fold on polyI:C stimulation of the PHCjECs from two individuals might be associated with allergy.

The significant upregulation of these genes, which is increased in allergic diseases via polyI:C, might be consistent with our previous finding that TLR3 positively regulates the late-phase reaction of EAC in a mouse model. Our results show that TLR3 of conjunctival epithelium may not only induce antiviral innate immune responses but also regulate the allergic reactions.

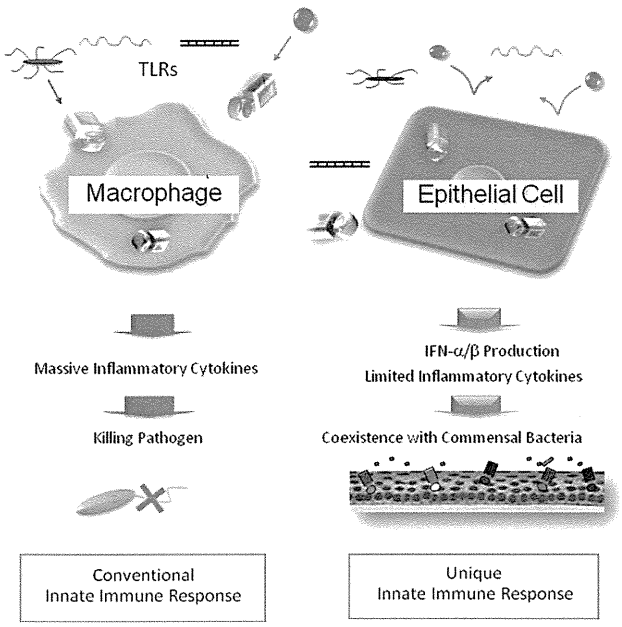

FIG. 6. Unique innate immune response of the ocular surface epithelium. Immune-competent cells such as macrophages do recognize various microbial components via TLRs, induce inflammation, and then exclude microbes, whereas ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. This difference in the action of macrophages and ocular surface epithelial cells may be caused by dissimilarities in their coexistence with commensal bacteria.

On the other hand, our results showed that RIG-I and MDA5, which are reportedly implicated in viral dsRNA recognition, are also remarkably upregulated by polyI:C stimulation of PHCjECs. We previously reported that TLR3 was the most intensely expressed among TLR1 to 10 in ocular surface epithelial cells and speculated that TLR3 mainly contributes to polyI:C inducible responses in ocular surface epithelial cells. However, in this study, we found that new receptors that recognize dsRNA and polyI:C, *RIG-I* and *MDA5* are also expressed in PHCjECs and are upregulated by polyI:C stimulation. Although the TLR family detects PAMPs either on the cell surface or in the lumina of intracellular vesicles such as endosomes or lysosomes, recent studies have confirmed the existence of a cytosolic system for detecting intracellular PAMPs. These cytosolic pattern recognition receptors include RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain–like receptors. RLRs belong to the RNA helicase family that specifically detects RNA species derived from viruses in the cytoplasm and coordinates antiviral programs via type I IFN induction. RIG-I and MDA5 are RLRs. Further investigation is required to resolve how these receptors contribute to polyI:C-inducible responses.

We also examined whether these 11 transcripts could be upregulated on polyI:C stimulation in PHCEC to perform a quantitative RT-PCR assay. Our results showed that polyI:C stimulation upregulated these 11 transcripts (*CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I,* and *MDA5*) in PHCEC (Fig. 10). The actual role of TLR3 in ocular surface inflammation must be further investigated.

In summary, we demonstrated that human ocular surface epithelial cells can be induced by polyI:C stimulation to express many

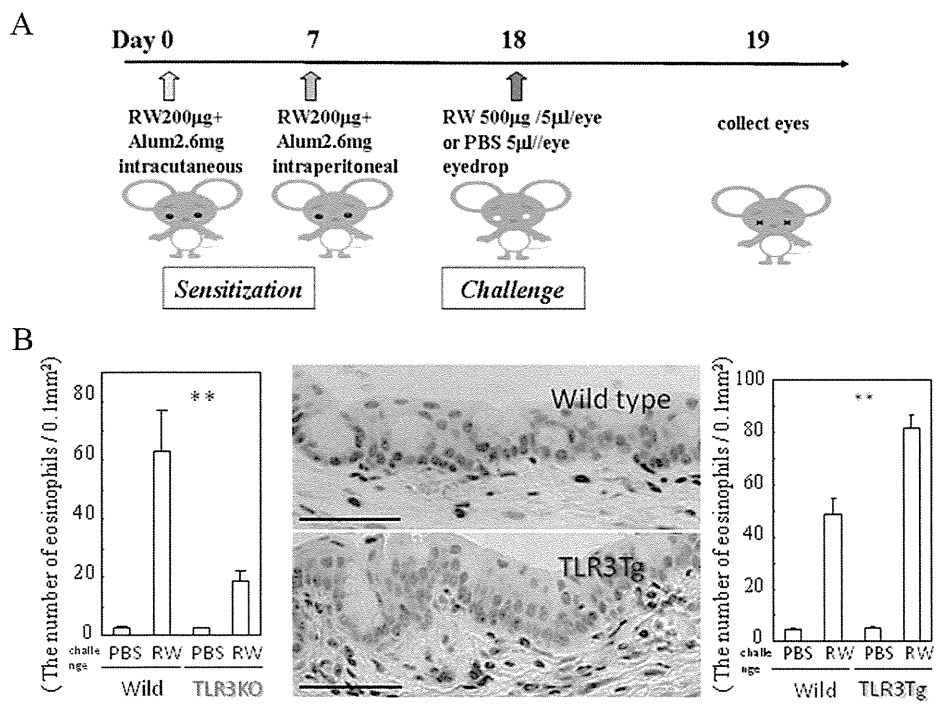

FIG. 7. (A) Our murine model of EAC. (B) Eosinophilic conjunctival inflammation in TLR3KO and TLR3Tg mice. The infiltration of eosinophils into the conjunctiva of wild-type and TLR3Tg mice was detected with Luna's method. Scale bars, 50 μm. Reprinted with permission from Ueta M, Uematsu S, Akira S, et al. Toll-like receptor 3 enhances late-phase reaction of experimental allergic conjunctivitis. *J Allergy Clin Immunol* 2009;123:1187–1189. © 2009 by Elsevier Inc.