TABLE 1. SIS of a Comprehensive Data Set of 13 Immune-Related Genes

Symbol	*PTGER3*	*IL13*	*TLR3*	*FasL*	*IL4R*	*MAIL*
Name	Prostaglandin E receptor EP3	Interleukin 13	Toll-like receptor 3	Fas ligand	Interleukin 4 receptor	Nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitor, zeta
rs. number	rs17131450	rs1800925	rs3775296	rs3830150	rs1805010	rs3821727
	rs5702	rs20541	rs3775295	rs2859247	rs1805015	rs677011
	rs1325949	rs1295685	rs3775294	rs2639614	rs1801275	rs595788
	rs7543182		rs3775293	rs929087		rs3217713
	rs7555874		rs3775292			rs14134
	rs4147114		rs3775291			rs622122
	rs1327464		rs3775290			rs2305991

Symbol	*IL4*	*IL1A*	*TLR2*	*TLR5*	*PTGER4*	*Chr5p13*	*GNLY*
Name	Interleukin 4	Interleukin 1 alpha	Toll-like receptor 2	Toll-like receptor 5	Prostaglandin E receptor 4	Genes in cytogenetic band chr 5p13	Granulysin
rs. number	rs2243250	rs2071376	rs3804100	rs2072493	rs1494558	rs6871834	rs3755007
		rs2071375	rs3804099	rs5744168			
		rs2071373					
		rs1894399					
		rs1609682					

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ANALYSIS OF FUNCTIONAL INTERACTIONS BETWEEN TLR3 AND EP3 (*PTGER3* PROTEIN) USING THE MURINE EXPERIMENTAL ALLERGIC CONJUNCTIVITIS MODEL

We reported previously that conjunctival eosinophilic infiltration in EAC was significantly more marked in EP3 knockout (k/o) mice[29] and significantly less marked in TLR3 k/o mice than in wild-type mice.[30] Together with the significant interaction between the *PTGER3* and *TLR3* loci and the opposite roles of EP3 and TLR3 in allergic conjunctivitis, we hypothesized there was an unknown functional interaction between EP3 and TLR3. Therefore, we examined the functional interactions between EP3 and TLR3 using *EP3* k/o, *TLR3* k/o and *EP3/TLR3* double k/o mice in addition to our model of murine experimental allergic conjunctivitis (EAC). We compared conjunctival eosinophil infiltration in wild-type, *EP3* k/o, *TLR3* k/o, and *EP3/TLR3* double k/o mice. Sensitization of mice by intracutaneous and intraperitoneal injection of ragweed without challenge (ragweed-containing eye drops) did not affect the number of eosinophils. However, after sensitization and challenge, the number of eosinophils in the lamina propria mucosae of the conjunctiva significantly increased in all mice. Eosinophil numbers in *EP3* k/o mice were significantly higher after sensitization and challenge and significantly lower in *TLR3* k/o than in wild-type mice, as reported previously.[29,30] Furthermore, in *EP3/TLR3* double k/o mice, the number of eosinophils in the lamina propria mucosae of the conjunctiva decreased to a level similar to *TLR3* k/o mice and was significantly lower than in *EP3* k/o mice and wild-type mice (Fig. 3).[28] These results suggest that EP3 negatively regulates the eosinophilic infiltration of EAC induced by TLR3, which causes reduced eosinophilic conjunctival inflammation in *EP3/TLR3* double k/o mice, although pronounced eosinophilic conjunctival inflammation has also been observed in *EP3* k/o mice.[28]

TABLE 2. Susceptible Interactions Between Loci Detected by Iterative SIS

Locus 1	Locus 2	Odds Ratio	95% confidence interval	*P*
PTGER3 rs4147114 (GC)	*TLR3* rs3775296 (TT)	25.3	3.2–203	0.0000527
PTGER3 rs4147114 (GC)	—	2.66	1.4–5.0	0.0023
—	*TLR3* rs3775296 (TT)	5.35	2.0–14.1	0.00025
HLA-A*02:06	*IL1α* rs1609682 (CA)	9.66	2.0–47.0	0.00193
HLA-A*02:06	—	3.46	1.8–6.8	0.0002
—	*IL1α* rs1609682 (CA)	—	—	0.31

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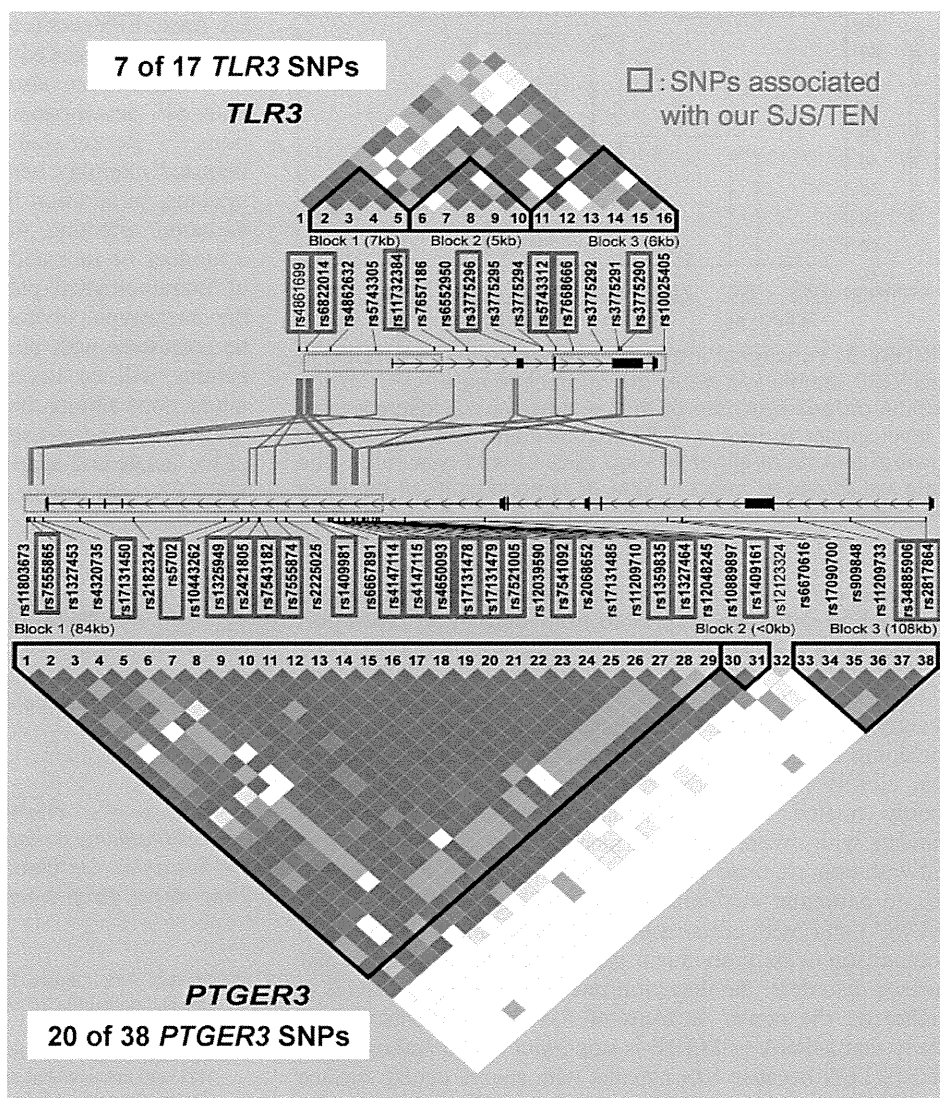
FIGURE 2. Additional analysis of *PTGER3* and *TLR3* SNPs and linkage disequilibria around *TLR3* and *PTGER3* loci. Seven of 17 *TLR3* SNPs and 20 of 38 *PTGER3* SNPs were associated with SJS with ocular surface complications. Linkage disequilibria around *TLR3* and *PTGER3* loci show 3 solid-spine linkage disequilibria blocks in each locus. Iterative SIS identified 14 variables with nonzero regression coefficients that were localized between *TLR3* and *PTGER3* as if connecting the 5' region of *TLR3* in block 1 and the 3' region of *PTGER3* in block 1. Reprinted from Ueta et al[28] with permission from Elsevier.

We found significant interactions between SNPs in the *EP3* and *TLR3* genes using high-dimensional variable selection methods such as iterative SIS.[28] Furthermore, we experimentally demonstrated the functional interaction of EP3 and TLR3 using our EAC model.[28]

TLRs are well-known key receptors of the innate immune system.[31] TLR3 recognizes double-stranded RNA, a component of the life cycle of most viruses, that has similar effects to polyinosinic:polycytidylic acid (poly I:C). We previously reported that TLR3 positively regulates the late-phase reaction of EAC.[30] Eosinophilic conjunctival inflammation was reduced in *TLR3* k/o mice and aggravated in *TLR3* transgenic mice, suggesting that TLR3 may play an important role in allergic inflammation.[30]

Prostanoids, that is, the prostaglandins (PGs) and the thromboxanes (TXs), including PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TXA_2, are a group of lipid mediators that are formed in response to various stimuli. They are released extracellularly immediately after their synthesis and act by binding to a G-protein–coupled rhodopsin-type receptor on the surface

of target cells. Eight types of prostanoid receptors are conserved in mammals from mice to humans: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP).[32] PGE_2–EP3 signaling is reported to inhibit keratinocyte activation and exert anti-inflammatory actions in mouse contact hypersensitivity.[33] We also previously reported that PGE_2 acts as a ligand for EP3 in conjunctival epithelial cells and downregulates the progression of murine EAC.[29] In addition, an EP3 agonist suppressed the production of thymic stromal lymphopoietin (TSLP) induced by poly I:C stimulation in human conjunctival epithelial cells, suggesting that the PGE_2–EP3 pathway might function to suppress the development of human allergic conjunctivitis through inhibition of TSLP production.[34]

We considered the possibility of an association between SJS/TEN with severe ocular surface complications and a disordered innate immune response.[11,12,35] Our hypothesis was based on an association between the onset of SJS/TEN and viral infection because many SJS/TEN patients with
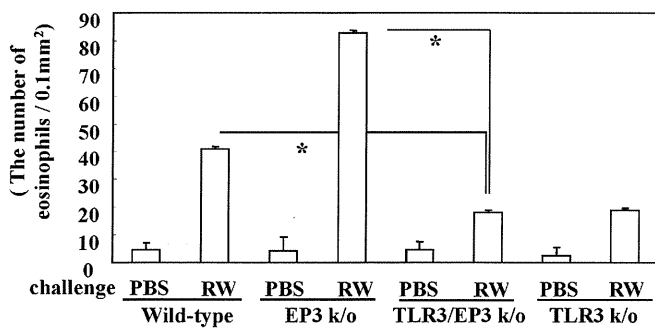
FIGURE 3. Functional interaction between EP3 and TLR3. In *EP3/TLR3* double k/o mice, the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to that in *TLR3* k/o mice and was significantly lower than that of either EP3 k/o mice or wild-type mice. The data are shown as mean ± SEM of samples from all the mice examined. [Wild type: PBS treated n = 24, ragweed (RW) treated n = 28; *EP3* k/o mice: PBS treated n = 23, RW treated n = 25; *EP3/TLR3* double k/o mice: PBS treated n = 4, RW treated n = 11; *TLR3* k/o mice: PBS treated n = 12, RW treated n = 12.] *P < 0.0005. Reprinted from Ueta et al[28] with permission from Elsevier.

severe ocular surface complications exhibit prodromata, including nonspecific fever, coryza, and sore throat ailments that closely mimic upper respiratory tract infections commonly treated with antibiotics.[12] In addition, SJS/TEN patients with severe ocular surface complications often present with opportunistic bacterial infections of the ocular surfaces, in particular methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE). In comparison with individuals with other devastating ocular surface disorders, the detection rate of MRSA and MRSE is higher on the ocular surfaces of SJS/TEN patients.[36] It is likely that MRSA or MSRE is important in the pathogenesis of SJS/TEN because SJS patients with severe ocular surface complications often have severe inflammation of the ocular surface when MRSA or MRSE is present.[11,35,37] Notably, elderly individuals who are hospitalized have no ocular surface inflammation, even when MRSA or MRSE is present on the ocular surface. The ocular surface inflammation of SJS patients is also greatly reduced after treatment with antibiotics against MRSA or MRSE. Finally, SJS/TEN patients present with persistent inflammation of the ocular surfaces harboring commensal bacteria.[11,35]

Under the hypothesis of a disordered innate immune response in SJS/TEN, we have performed SNP association analysis of candidate genes in Japanese SJS/TEN patients with severe ocular complications and documented associated polymorphisms in the genes encoding TLR3[12] and other genes.[11,21–23,35] Furthermore, we performed a genome-wide association study and found associations between the *PTGER3* gene SNPs and SJS/TEN accompanied by severe ocular complications.[24]

Although the possibility that EP3 may be related to innate immunity has not been considered, interactions between TLR3 and EP3 were demonstrated by statistical analysis, and functionally, using *EP3* k/o, *TLR3* k/o, and *EP3/TLR3* double

k/o mice. EP3 seems to negatively regulate the ocular surface inflammation induced by TLR3.[28]

HLA-A has also been shown to be strongly associated with SJS accompanied by severe ocular surface complications.[20,38] Recent studies have highlighted a previously unanticipated interplay between the innate and adaptive immune systems. In addition to the well-established roles of genetic variation affecting the major histocompatibility complex, a number of rare and common variants that affect a range of immunological pathways exert important influences on the phenotypic diversity of various diseases,[39] which may be consistent with our hypothesis. Although further investigations will be necessary to elucidate the mechanism of interactions among these molecules, that is, direct or indirect, our present findings shed light on the substantial involvement of the innate and adaptive immune systems in the etiology of SJS/TEN with severe ocular surface complications.

CONCLUSIONS

In conclusion, functional interactions between TLR3 and EP3, supported by their epistatic interactions, were demonstrated, and confer an increased risk for SJS with severe ocular surface complications.[28]

ACKNOWLEDGMENTS

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Ocular surface inflammation is regulated by innate immunity

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ABSTRACT

On the ocular surface, as in the intestines and airway, the surface epithelium serves a critical function as the front-line defense of the mucosal innate immune system. Although the detection of microbes is arguably the most important task of the immune system, an exaggerated epithelial host defense reaction to endogenous bacteria may initiate and perpetuate inflammatory mucosal responses.

In this review we first describe commensal bacteria found on the ocular surface, which is in contact with the ocular surface epithelium. We also discuss the innate immunity of the ocular surface epithelium and we present the allergic reaction regulated by ocular surface epithelial cells. We address ocular surface inflammation due to disordered innate immunity and we present our hypothesis that the onset of Stevens-Johnson syndrome (SJS) with severe ocular surface complications, a devastating ocular surface inflammatory disease, is strongly associated with abnormality of the innate immune system.

In this review we raise the possibility that some ocular surface inflammatory diseases are pathogenetically related with a disordered innate immune response.

Focusing on the innate immunity of the ocular surface might help to elucidate the pathogenesis of various ocular surface diseases.

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Contents

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 [1] Percentage of work contributed by each author in the production of the manuscript is as follows: Mayumi Ueta 95% Shigeru kinoshita 5%.

1. Introduction

On the ocular surface, as in the intestines and airway, the surface epithelium serves a critical function as the front-line defense of the mucosal innate immune system (Haynes et al., 1999; Streilein, 2003). Epithelial cells lining mucosal surfaces play a pivotal role in innate immunity; upon challenge they secrete chemokines and other immune mediators. The ocular surface epithelium also features all isoforms of human beta defensins and can produce inflammatory cytokines such as interleukin (IL)-1α, IL-1β, tumor necrosis factor (TNF)α, IL-6, and IL-8. Anti-microbial molecules such as IgA, lysozyme, and lactoferrin are found in tear fluids. Goblet cells in conjunctival epithelium produce mucin. Thus, the ocular surface possesses many non-specific defense mechanisms against microbes (Fig. 1).

Although the detection of microbes is arguably the most important task of the immune system, an exaggerated epithelial host defense reaction to endogenous bacteria may initiate and perpetuate inflammatory mucosal responses (Bouma and Strober, 2003; Strober, 2004; Strober et al., 2002).

In this review we first describe commensal bacteria found on the ocular surface, which is in contact with the ocular surface epithelium. We also discuss the innate immunity of the ocular surface epithelium and we present the allergic reaction regulated by ocular surface epithelial cells. We address ocular surface inflammation due to disordered innate immunity and we present our hypothesis that the onset of Stevens-Johnson syndrome (SJS) with severe ocular surface complications, a devastating ocular surface inflammatory disease, is strongly associated with abnormality of the innate immune system.

We propose that the pathogenesis of some human ocular surface inflammatory diseases is related to a disordered innate immune response.

2. Commensal bacteria on the ocular surface

2.1. Commensal bacteria

Bacterial flora comprised of gram-positive and gram-negative organisms can be found on the skin and in mucosal tissues. The ocular surface and other mucosal tissue are host to commensal bacteria (Doyle et al., 1995; Hara et al., 1997; Ueta et al., 2007a). To examine these organisms present on the ocular surface we harvested commensal bacteria from the lower conjunctival sacs of 42 healthy volunteers using CultureSwab (Becton Dickinson, Brescia, Italy) without touching the lids. Staphylococcus epidermidis (S. epidermidis) was isolated from 45% of the volunteers and Propionibacterium acnes (P. acnes) from 31% (Ueta et al., 2007a). Interestingly, although the ocular surface epithelium is in constant contact with bacteria and bacterial products, the healthy ocular surface is not in an inflammatory state. The ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes (Fig. 2).

2.2. Polyclonality of S. epidermidis

In humans, the predominant staphylococcus species S. epidermidis is widely distributed over the body surface (Kloos and Musselwhite, 1975). When we used pulsed-field gel electrophoresis (PFGE) to examine the diverse genetic background of S. epidermidis isolated from the ocular surface of healthy donors we found that the bacterium was polyclonal (Ueta et al., 2007a) (Fig. 3A).

For analysis, DNA bands were compared by visual inspection and interpreted according to Tenover et al. (1995). Based on the number of DNA fragments that exhibited different pulse patterns, strains with identical pulse patterns, and those with 2–3 or 4–6 fragments of different patterns were considered indistinguishable, closely related, and possibly related, respectively. When more than 6 DNA fragments manifested a different migration pattern, the isolates were considered to be unrelated.

We analyzed commensal bacteria isolated from the conjunctival sac, upper and lower lid margins, and upper and lower meibomian glands of another 40 healthy volunteers. S. epidermidis was isolated from 12 individuals; 7 harbored these bacteria at multiple ocular surface sites. Interestingly, S. epidermidis organisms isolated from multiple sites in single subjects were polyclonal. When we analyzed S. epidermidis isolated from the conjunctival sac of the same donor at different time points, we also found that the organisms were polyclonal and yielded multiple colonies. At some sampling points there was a change in the dominant strain (Ueta et al., 2007a).

Although S. epidermidis is a common component of the normal ocular flora, it can lead to chronic blepharitis, conjunctivitis, and keratitis, especially in immunocompromised hosts (Baum, 1978; Pinna et al., 1999), suggesting that opportunistic infection with S. epidermidis is reflective of the status of the host. We encountered one instance in which monoclonal S. epidermidis was isolated from multiple sites in both eyes (Fig. 3B). The host was an immunocompromised patient who had undergone bone marrow transplantation. Disruption of the balance between S. epidermidis and the immune status of this host may have resulted in the monoclonality of these bacteria. Based on these observations we postulated that a balance between commensal bacteria and the host mucosal immunity maintains the polyclonality of S. epidermidis, which may contribute to homeostasis of the commensal organisms, and that a weakened host mucosal immune status may contribute to their pathogenicity. When the host mucosal immunity is normal, commensal bacteria are in a symbiotic relationship with the host, however, if the host's mucosal immunity is abnormal, commensal bacteria can become pathogenic (Ueta et al., 2007a) (Fig. 3C).

The finding of Seal et al. (1985) that a specific strain of S. epidermidis could increase in the lids of blepharitis patients and manifest pathogenicity on the ocular surface may indicate that the role of S. epidermidis on the ocular surface requires further investigation.

2.3. Hypersensitivity to bacteria

Ocular surface inflammations such as catarrhal ulcers (marginal keratitis) and phlyctenular keratitis are thought to reflect

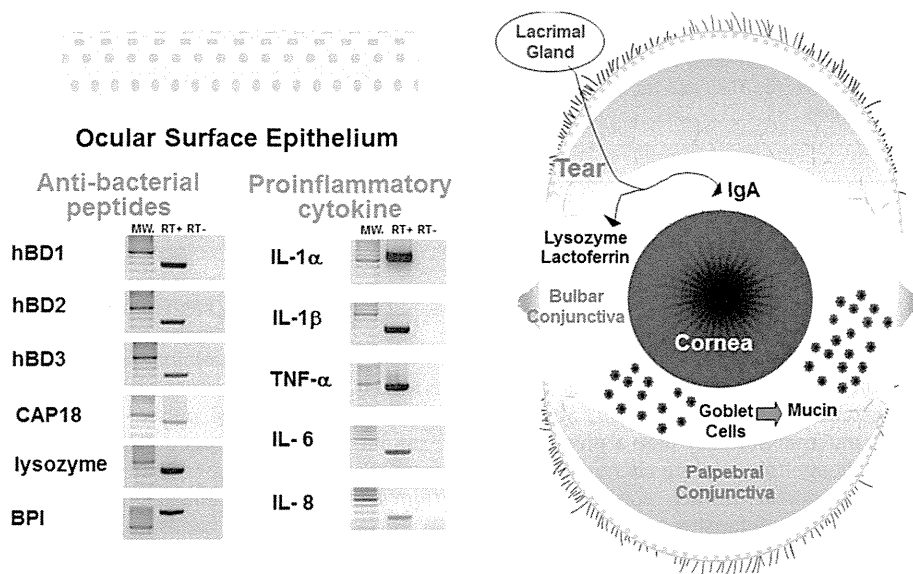## Non-specific defense mechanism against microbes on the ocular surface

Fig. 1. Non-specific defense mechanism against microbes on the ocular surface. The ocular surface epithelium harbors all isoforms of human beta defensins and can produce inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6, and IL-8 (left: RT-PCR, human corneal epithelial cells.). Tear fluids contain anti-microbial molecules such as IgA, lysozyme and lactoferrin. Goblet cells in conjunctival epithelium produce mucin (right).

a hypersensitivity to bacteria (Mondino et al., 1978, 1981; 1982; Seal et al., 1985).

In rabbits immunized with *Staphylococcus aureus* (*S. aureus*) cell walls, Mondino and Kowalski (1982) observed vascularized, elevated nodular infiltrates of the cornea; topical challenge with viable *S. aureus* produced peripheral corneal infiltrates separated from the limbus by al lucid interval. Patients with symptomatic marginal keratitis requiring treatment with steroids manifested enhanced delayed hypersensitivity to *S. aureus* cell wall antigens (Ficker et al., 1989). Catarrhal ulcers are usually a complication of long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis (Smolin and Okumoto, 1977; Thygeson, 1969) that may be

subclinical. Cultures from the lid margins of patients with long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis, usually yield colonies of *S. aureus* (Thygeson, 1969), although the lid margin of normal eyes does not usually harbor *S. aureus* (Hara et al., 1997; Doyle et al., 1995). Because corneal cultures tend to be negative for the organisms, it has been suggested that catarrhal ulcers are not the result of direct infection of the cornea but rather derive from an antigen–antibody reaction with complement activation and neutrophil infiltration in patients sensitized to staphylococcal antigens (Mondino et al., 1978, 1981; Smolin and Okumoto, 1977).

We used PFGE to analyze the relationship between catarrhal ulcers and the presence of *S. aureus*. The diagnosis of catarrhal ulcer
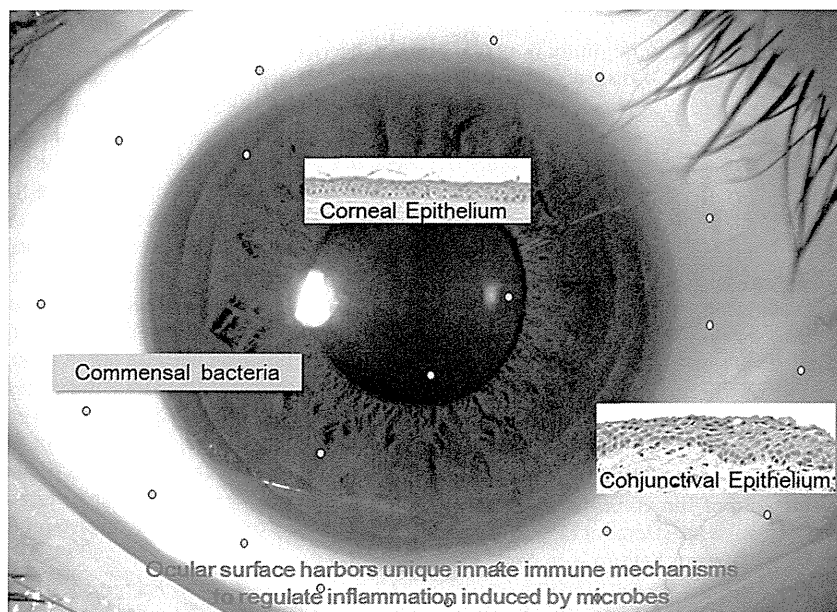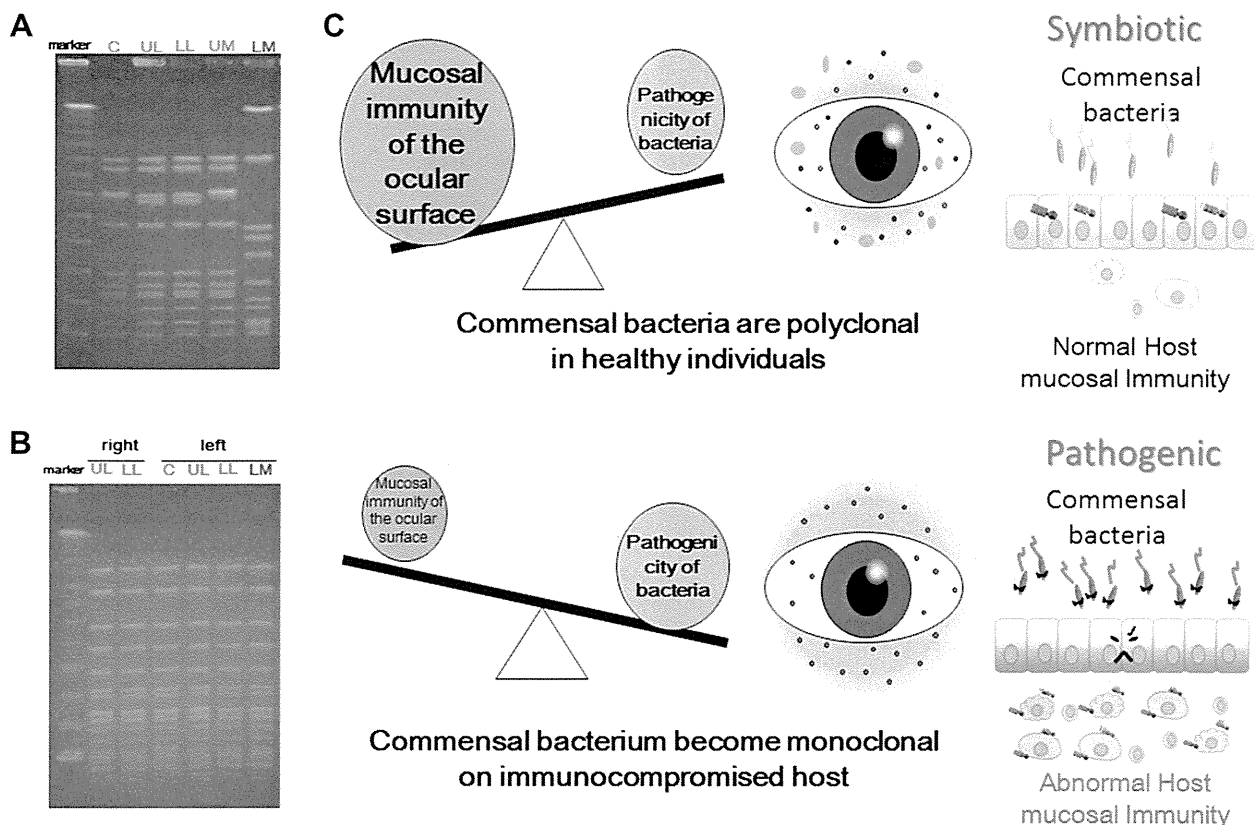

Fig. 2. Healthy ocular surface. The healthy ocular surface is not in an inflammatory state, although the ocular surface epithelium is in constant contact with bacteria and bacterial products. Ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes.

Fig. 3. Importance of the balance between mucosal immunity of the ocular surface and pathogenicity of bacteria. A. The PFGE patterns of *S. epidermidis* on healthy ocular surfaces were polyclonal. B. The PFGE patterns of *S. epidermidis* isolated from multiple sites in both eyes of an immunocompromised patient were monoclonal. (PFGE: pulsed-field gel electrophoresis, C: conjunctiva, UL: upper lid margin, LL: lower lid margin, UM: upper meibomian gland, LM: lower meibomian gland). A&B; Reprinted with permission from Ueta et al. (Ueta et al., 2007a). C. A good balance between commensal bacteria and host immunity maintains the polyclonality of *S. epidermidis*. A weakened host immune status may contribute to the bacterium's pathogenicity. When the host mucosal immunity is normal, commensal bacteria are in a symbiotic relationship with their host. However, if the host mucosal immunity is abnormal, commensal bacteria can be pathogenic.

was based on ocular surface manifestations. Clinical examinations revealed oval infiltrates, ulcers separated from the limbus by a distinct lucid border, and adjacent conjunctival inflammation (Fig. 4A). We examined 3 ocular sites (the conjunctival sac and the upper and lower lid margins) for the presence of bacteria and compared the *S. aureus* organisms isolated from 2 or more sites in each patient.

The colonization by *S. aureus* is shown schematically in Fig. 4B. In case 1, *S. aureus* was detected in the lower lid margin of the affected- and the conjunctiva of the unaffected-eye; PFGE suggested that these *S. aureus* were the same clone. In case 2, *S. aureus* was detected in the upper lid margin and conjunctiva of the affected- and in the lower lid margin of the unaffected eye; PFGE again suggested that these organisms were the same clone. In case 3, *S. aureus* was detected in the lower lid margin of the affected eye (Ueta et al., 2009b).

Although our study included only a small number of patients, we found *S. aureus* in the lid margin of eyes affected by catarrhal ulcers. This suggests that its presence at that site rather than the conjunctival sac is important for the development of catarrhal ulcers. As we were able to detect all *S. aureus* organisms in enrichment cultures, it appears that the development of catarrhal ulcers does not require the presence of large amounts of the bacterium (Ueta et al., 2009b).

Interestingly, in case 2 we also found *S. aureus* in the lid margin of the unaffected eye. Thus, even if a patient sensitized to staphylococcal antigens harbors *S. aureus* on both eyes, catarrhal ulcers may develop on only one eye. Moreover, our PFGE analysis showed

that *S. aureus* detected in both eyes might be derived from the same clone, suggesting that the kind of the *S. aureus* clone is not necessarily important for the initiation of catarrhal ulcers. These findings raise the possibility that besides the presence of *S. aureus* on the lid margin and the patient's sensitivity to staphylococcal antigens, other factors may be necessary for the initiation of catarrhal ulcers (Ueta et al., 2009b).

It has been proposed that phlyctenular keratitis is reflective of hypersensitivity to tuberculin protein or *S. aureus* (Beauchamp et al., 1981; Neiberg and Sowka, 2008). Others (Suzuki et al., 2005) reported that phlyctenular keratitis in young patients might involve *P. acnes*, a commensal bacterium on the ocular surface, but not *S. aureus*.

3. Innate immunity of the ocular surface epithelium

3.1. Toll-like receptors (TLRs) of the ocular surface epithelium

The ability of cells to recognize microbial motifs is attributable to pattern recognition receptors, TLRs, important molecules associated with innate immunity (Kawai and Akira, 2007; Medzhitov et al., 1997). To date, 10 TLRs have been identified in humans; they are investigated primarily on mammalian host immune-competent cell types such as dendritic cells and macrophages. These cells are most likely to come into direct contact, via mucosal epithelia, with pathogens from the environment (Hornung et al., 2002).

The ability of cells to recognize pathogen-associated molecular patterns may depend on the expression of a family of TLRs whose
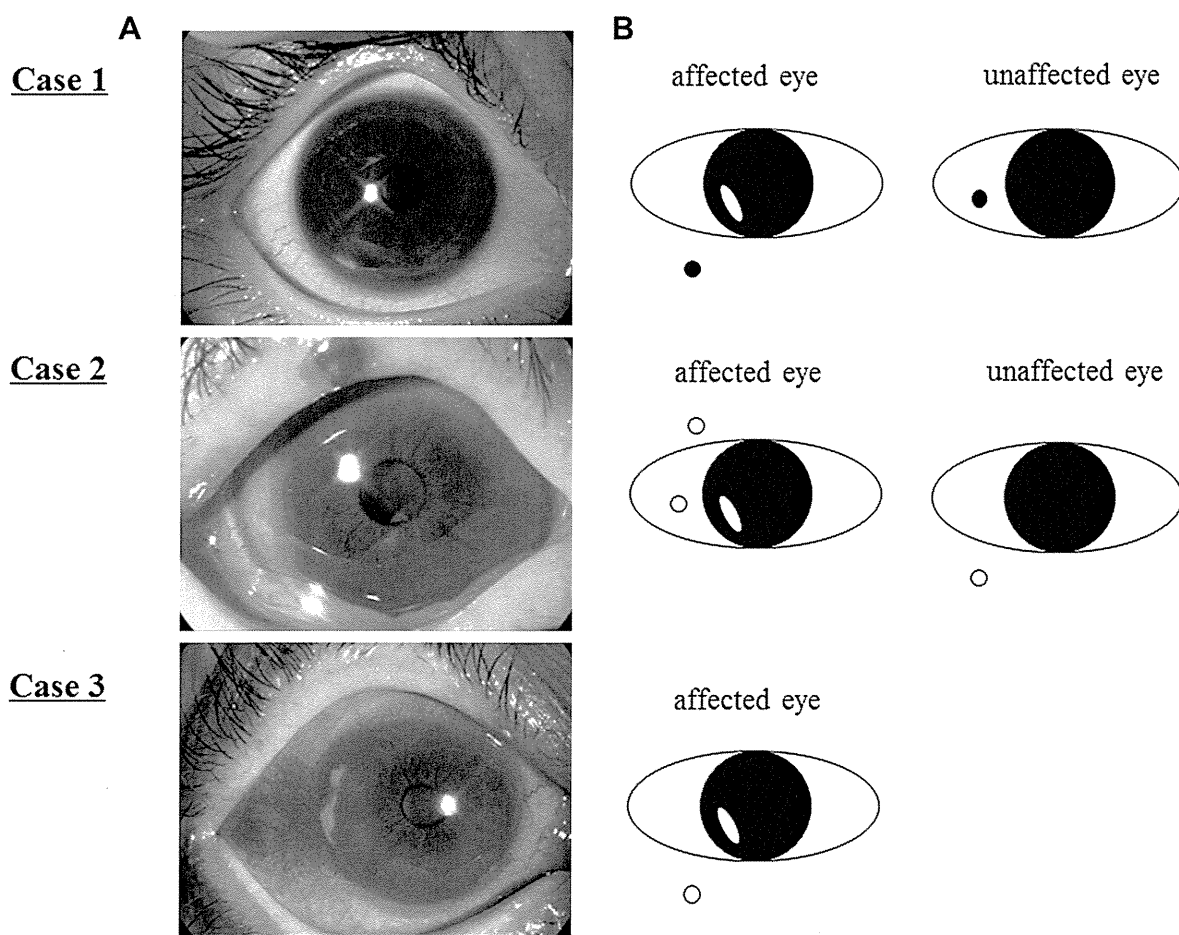
Fig. 4. Relationship between catarrhal ulcers and the presence of *S. aureus*. A. The diagnosis of catarrhal ulcer was based on ocular surface manifestations, i.e. oval infiltrates, ulcers separated from the limbus by a distinct lucid border, and adjacent conjunctival inflammation. B. The presence of *S. aureus* on the lid margin is important for the development of catarrhal ulcers. In case 1, *S. aureus* were detected on the lower lid margin of the affected- and the conjunctiva of the unaffected-eye. This suggested involvement of the same clone. In case 2, *S. aureus* were detected on the upper lid margin and the conjunctiva of the affected- and in the lower lid margin of the unaffected-eye, again suggesting involvement of the same clone. In case 3, *S. aureus* were detected on the lower lid margin of the affected eye. A&B; Reprinted with permission from Ueta et al. (Ueta et al., 2009b).

triggering results in the secretion of pro-inflammatory cytokines and interferon (IFN) α/β (Kawai and Akira, 2007; Medzhitov et al., 1997). For example, TLR2 recognizes peptidoglycan (PGN) or lipoprotein, components of the gram-positive bacterial cell wall, and forms a heterodimer with TLR1 or TLR6. TLR3 recognizes viral double-stranded (ds) RNA, which is mimicked by polyinosine-polycytidylic acid (polyI:C). Lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall, is recognized by TLR4 and flagellin, a component of bacterial flagellae, by TLR5. TLR7 or TLR8 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial and viral deoxy-cytidylate-phosphate-deoxy-guanylate (CpG) DNA. Bacterial and viral CpG DNA acts as a pathogen-associated molecular pattern by virtue of a 20-fold greater frequency of unmethylated CG dinucleotides in microbial-compared to vertebrate-DNA.

The function of TLR10 remains to be fully elucidated (Kawai and Akira, 2007) (Fig. 5).

TLR expression is not restricted to phagocytic cell types, rather, it appears that the majority of cells in the body including mucosal epithelial cells express TLRs (Zhang et al., 2004). Ocular surface epithelial cells are in constant contact with bacteria and bacterial products and they form a structural and functional barrier against numerous pathogenic and nonpathogenic bacteria.

Using reverse transcription-polymerase chain reaction (RT-PCR) assays we first examined whether the human ocular surface epithelium expresses mRNA specific for TLR1–TLR10. We found mRNA expression specific for TLR1–TLR10 in the human conjunctival epithelium; mRNA from all TLRs except TLR8 was present in human corneal epithelium (Ueta, 2008; Ueta and Kinoshita, 2010a) (Fig. 5B).

TLR3 recognizes viral dsRNA, which is synthesized by almost all viruses at the time of duplication. We used polyI:C in our experiments to stimulate both human peripheral mononuclear cells and primary human ocular surface epithelial cells (corneal and conjunctival epithelial cells), since viral dsRNA is mimicked by polyI:C, the ligand for TLR3. In human peripheral mononuclear cells polyI:C stimulation did not increase the production of IL-6 and IL-8. On the other hand, in human ocular surface epithelial cells, polyI:C stimulation significantly induced the secretion of IL-6 and IL-8. Since interferon (IFN)-β is controlled by TLR3 signaling, IFN-β-specific mRNA was significantly increased in polyI:C-stimulated cells. Quite surprisingly, IFN-β-specific mRNA expression was markedly higher in human corneal and conjunctival epithelial cells than peripheral mononuclear cells (Fig. 6) (Ueta et al., 2005a). Redfern et al. (2011) reported that the TLR3 agonist up-regulated the expression of the antimicrobial peptides, hBD-2 and hCAP-18, in primary human corneal epithelial cells.

TLR4 recognizes LPS, a component of the cell wall of gram-negative bacteria. In human peripheral mononuclear cells, LPS stimulation significantly increased the production of IL-6 and IL-8;
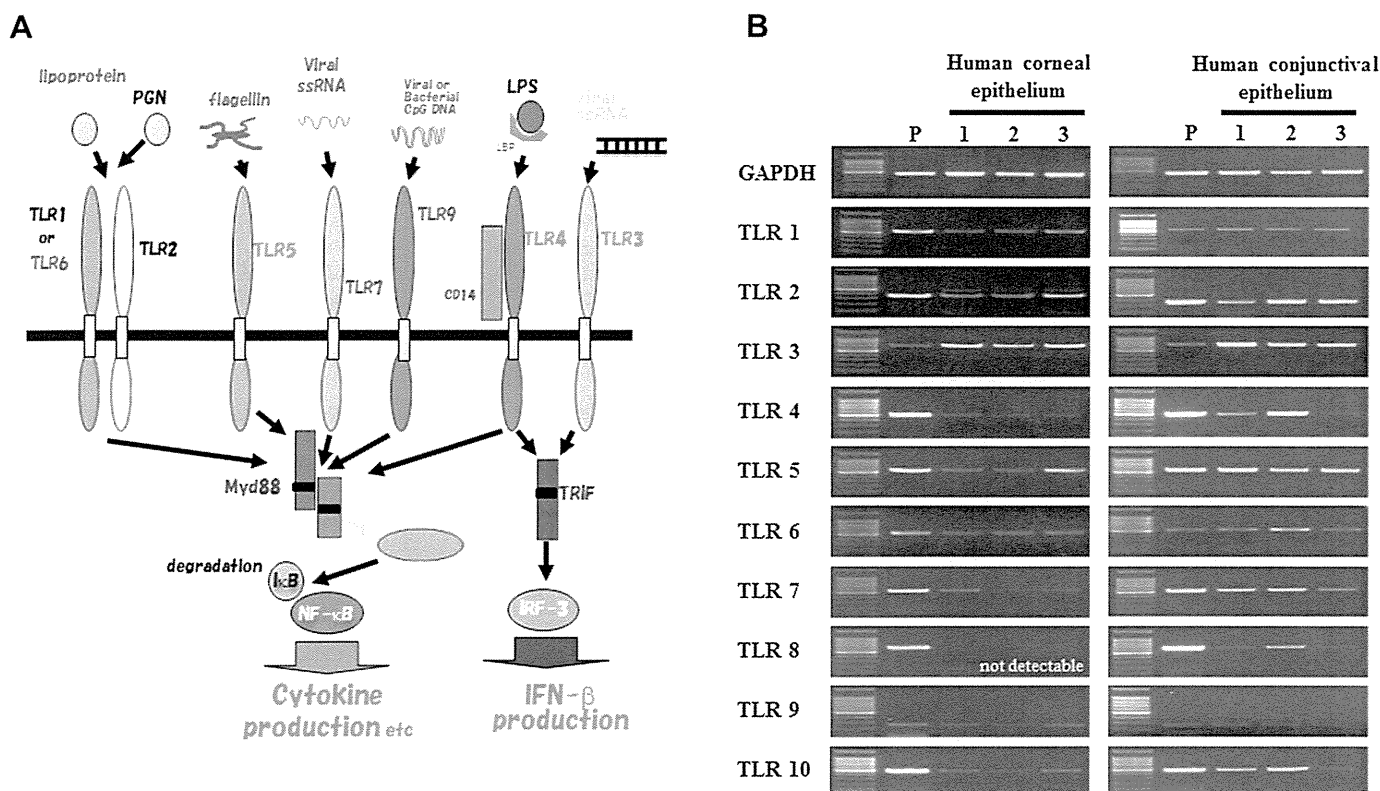
Fig. 5. A. Function of Toll-like receptors (TLRs) PGN: peptidoglycan LPS: lipopolysaccharides Myd88: myeloid differentiation factor 88 TRIF: TIR domain-containing adaptor-inducing IFN-β IRF-3: interferon regulatory factor 3. B. Human ocular surface epithelium expresses TLR-specific mRNA. The positive control (P) was human mononuclear cells. In human conjunctival epithelium we detected the expression of mRNA specific for TLR1–TLR10; mRNA from all TLRs except TLR8 was present in human corneal epithelium. (1,2,3 show samples from different individuals.). Reprinted with permission from Ueta et al. (Ueta, 2008).

in human ocular surface epithelial cells it did not induce the secretion of IL-6 and IL-8 (Fig. 6) (Ueta et al., 2004). Zhang et al. (2008) suggested that the LPS unresponsiveness of human corneal epithelial cells might be due to the deficient expression of MD-2, an essential component for LPS-TLR4 signaling. On the other hand, other groups reported that TLR4 of corneal epithelium could respond to their ligands (Johnson et al., 2005).

TLR5 recognizes flagellin, the protein of bacterial flagellae and Pseudomonas aeruginosa (P. aeruginosa) contributes to the inflammatory response of human corneal epithelium (Zhang et al., 2003). Flagellae are present mainly on gram-negative bacteria such as P. aeruginosa. Ocular surface-related bacteria with flagellae include pathogenic P. aeruginosa and non-pathogenic Bacillus subtilis (B. subtilis). We stimulated human peripheral mononuclear cells and primary human corneal and conjunctival epithelial cells with different kinds of flagellin as the ligand of TLR5. We used flagellin derived from the ocular surface pathogen P. aeruginosa, from the ocular surface non-pathogen B. subtilis, and from the intestinal pathogen Salmonella typhimurium (S. typhimurium). All flagellin stimulation of human peripheral mononuclear cells significantly increased the production of IL-6 and IL-8 (Fig. 7A). On the other hand, in human corneal and conjunctival epithelial cells only P. aeruginosa-derived flagellin significantly induced the secretion of IL-6 and IL-8; B. subtilis- and S. typhimurium-derived flagellin did not (Fig. 7A) (Hozono et al., 2006; Kojima et al., 2008; Ueta, 2008).

Our immunohistochemical studies showed that 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. 7B) (Hozono et al., 2006; Kojima et al., 2008; Ueta,

2008). Although ocular surface epithelial cells respond to flagellin derived from ocular pathogenic bacteria through TLR5 and produce inflammatory cytokines, superficial ocular surface epithelial cells do not express TLR5. Therefore, it is reasonable to speculate that TLR5 of the ocular surface epithelium cannot function on the healthy ocular surface without epithelial defects (Hozono et al., 2006; Kojima et al., 2008; Ueta, 2008).

According to Kumar et al. (2007), pre-exposure of human corneal epithelial cells to low-dose flagellin induced a state of tolerance characterized by the reduced production of IL-8 and TNF-α upon subsequent challenge with a high dose of flagellin; they noted Pseudomonas-induced up-regulation of antimicrobial genes such as hBD-2 and LL-37.

In summary, ocular surface (corneal and conjunctival) epithelial cells selectively respond to microbial components and induce limited inflammation. Immune-competent cells such as macrophages, on the other hand, recognize various microbial components through different TLRs, induce inflammation, and then exclude the microbes. The difference between macrophages and ocular surface epithelial cells may be ascribable to dissimilarities due to the latter's coexistence with commensal bacteria. The unique innate immune response of the ocular surface epithelium might contribute to its ability to coexist with commensal bacteria (Ueta, 2008; Ueta and Kinoshita, 2010a).

3.2. Function of TLR3 in the ocular surface epithelium

Stimulation with polyI:C, a TLR3 ligand, elicited an increase in the mRNA expression of IL-6, IL-8, and IFN-β in human ocular epithelial cells (corneal and conjunctival epithelial cells) (Ueta,

Human Peripheral Mononuclear Cells
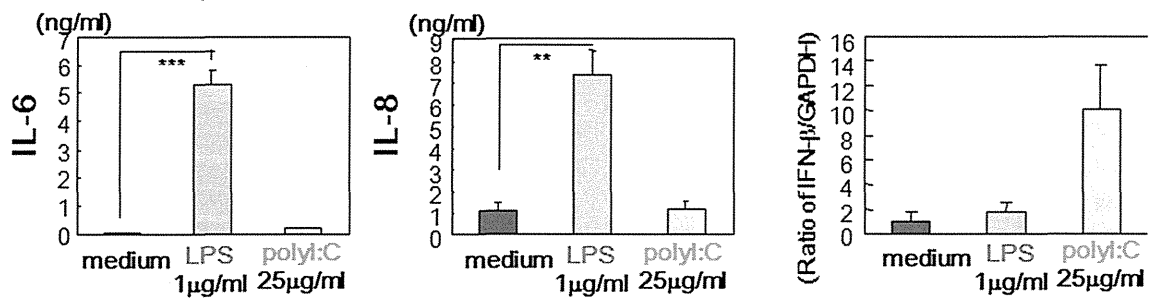
Human Corneal Epithelial Cells
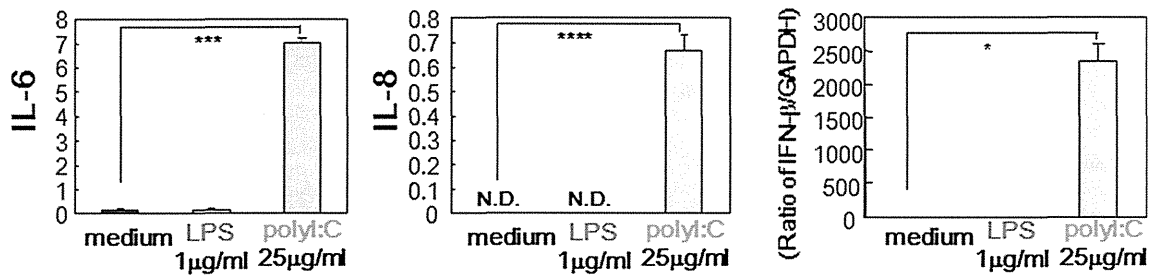
Human Conjunctival Epithelial Cells
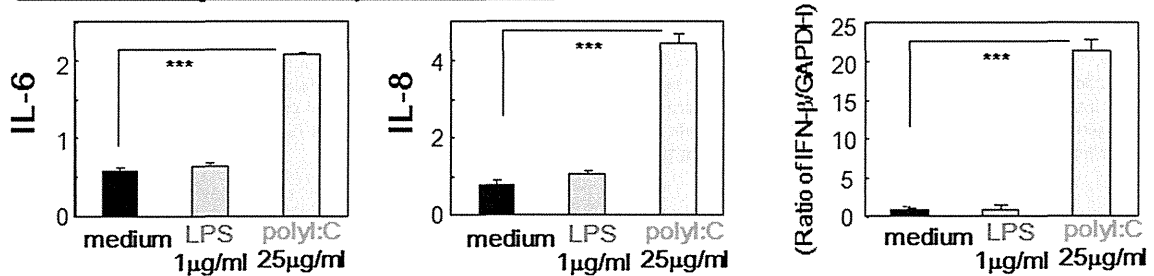
Fig. 6. Response of human peripheral mononuclear- and of primary human corneal- and -conjunctival epithelial cells to polyI:C, the TLR3 ligand, and LPS, the TLR4 ligand. Cultured cells were left untreated or exposed to polyI:C (25 μg/ml) or LPS from *P. aeruginosa* (1 μg/ml) for 24 h and assayed for the production of IL-6 and IL-8, or they were incubated for 3hr and assayed for the expression of IFN-β mRNA. For all cell types, the ratio of IFN-β/GAPDH mRNA (right-most column) shows an increase in specific mRNA over unstimulated cells. Data show the mean ± SEM (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$); evaluation was with Student's t-test using the Excel program. Modified with permission from Ueta et al. (Ueta et al., 2005a; Ueta and Kinoshita, 2010a).

2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). PolyI:C stimulation also up-regulated the mRNA expression of the antiviral chemokine IFN-γ inducible protein 10 (IP-10), myxovirus resistance gene A, and 2′,5′-oligoadenylate synthetase (Kumar et al., 2006).

To examine the comprehensive effects of polyI:C stimulation of primary human conjunctival epithelial cells we subjected cells that had, or had not been cultured with polyI:C to gene expression analysis. We found that polyI:C stimulation induced the up-regulation of many transcripts: 150 were up-regulated more than 3-fold and 47 were up-regulated more than 10-fold. Quantitative RT-PCR confirmed the up-regulation of 11 of these transcripts, i.e. CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I, and MDA-5 (Fig. 8) (Ueta et al., 2010b).

Although they are also innate-immune-response-related genes, CXCL11, CXCL10 (Klunker et al., 2003; Ying et al., 2008), IL28A (Bullens et al., 2008), CCL5, CCL4, and CCL20 (Gros et al., 2009) have been reported to be up-regulated in allergic diseases. TSLP (Soumelis et al., 2002; Ying et al., 2005), IL7R (Ziegler and Liu, 2006), and ICAM-1 (Hingorani et al., 1998) are allergy-related genes. At least 9 of the 47 transcripts that we found to be up-regulated more than 10-fold upon polyI:C stimulation of primary human conjunctival epithelial cells may be associated with allergy. Our results show that TLR3 of the human conjunctival epithelium

might not only induce anti-viral innate immune responses, but also regulate allergic reactions.

Among TLR1–TLR10, TLR3 is the most intensely expressed TLR in ocular surface epithelial cells (Ueta, 2008; Ueta and Kinoshita, 2010a). However, we found that RIG-I and MDA-5, reported to be implicated in viral dsRNA recognition (Kawai and Akira, 2009), were also remarkably up-regulated by polyI:C stimulation of primary human conjunctival epithelial cells.

Quantitative RT-PCR assay showed that 11 transcripts (CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I and MDA-5) could be up-regulated upon polyI:C stimulation in not only primary human conjunctival epithelial cells but also primary human corneal epithelial cells (Ueta and Kinoshita, 2010b). As polyI:C stimulation up-regulated these 11 transcripts in human ocular surface epithelial cells, these cells can be induced by polyI:C stimulation to express many transcripts that include not only transcripts of anti-viral innate immune response-related but also of allergy-related genes.

3.3. RIG-I and MDA-5 of the ocular surface epithelium

The TLR family detects pathogen-associated molecular patterns on the surface of cells and in the lumina of intracellular vesicles

PA: Pseudomonas aeruginosa (Ocular pathogen)
ST: Salmonella typhimurium (Intestinal, but not ocular, pathogen)
BS: Bacillus subtilis (Ocular non-pathogen: Ocular commensal)
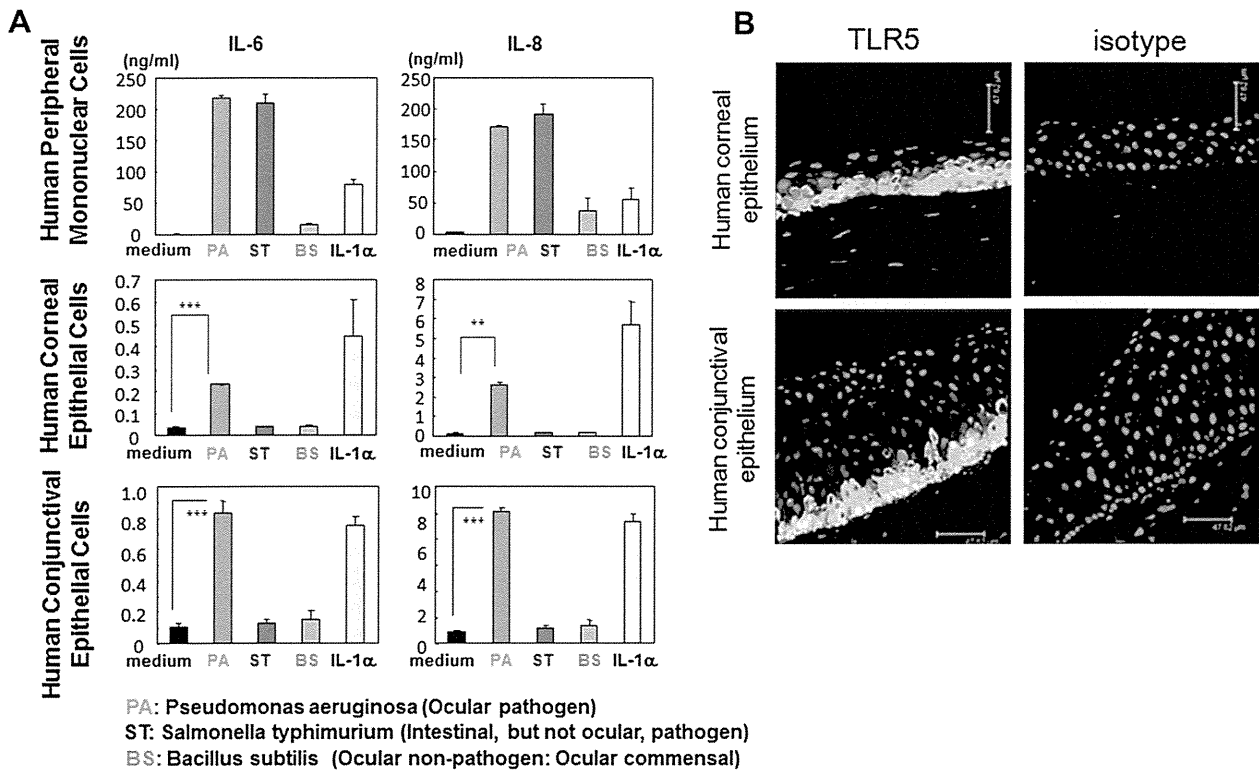
Fig. 7. Function of TLR5 in ocular surface. A. Responsiveness to various flagellins, which are TLR5 ligands, of human peripheral mononuclear cells and primary human corneal-and -conjunctival epithelial cells. Cultured cells were left untreated or exposed for 24 h to different flagellins (100 ng/ml). Data show the mean ± SEM (**, $p < 0.01$; ***, $p < 0.005$); evaluation was with Student's t-test using the Excel program. Modified with permission from Ueta et al. (Hozono et al., 2006; Kojima et al., 2008). B. Immuno-localization of TLR5 in human corneal and conjunctival tissue detected by immunofluorescence staining. Frozen cryostat sections were incubated with anti-TLR5 antibody or under isotype-control conditions. Bound antibodies were visualized after incubation with Alexa Fluor 488 goat anti-mouse IgG; nuclei were stained with propidium iodide.

such as endosomes or lysosomes. The existence of a cytosolic system for detecting intracellular pathogen-associated molecular patterns has also been confirmed. The cytosolic pattern recognition receptors include nucleotide-binding oligomerization domain

(NOD)-like receptors (NLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). Thus, the ability of cells to recognize pathogen-associated molecular patterns depends on the expression of a family of TLRs, NLRs, and RLRs.
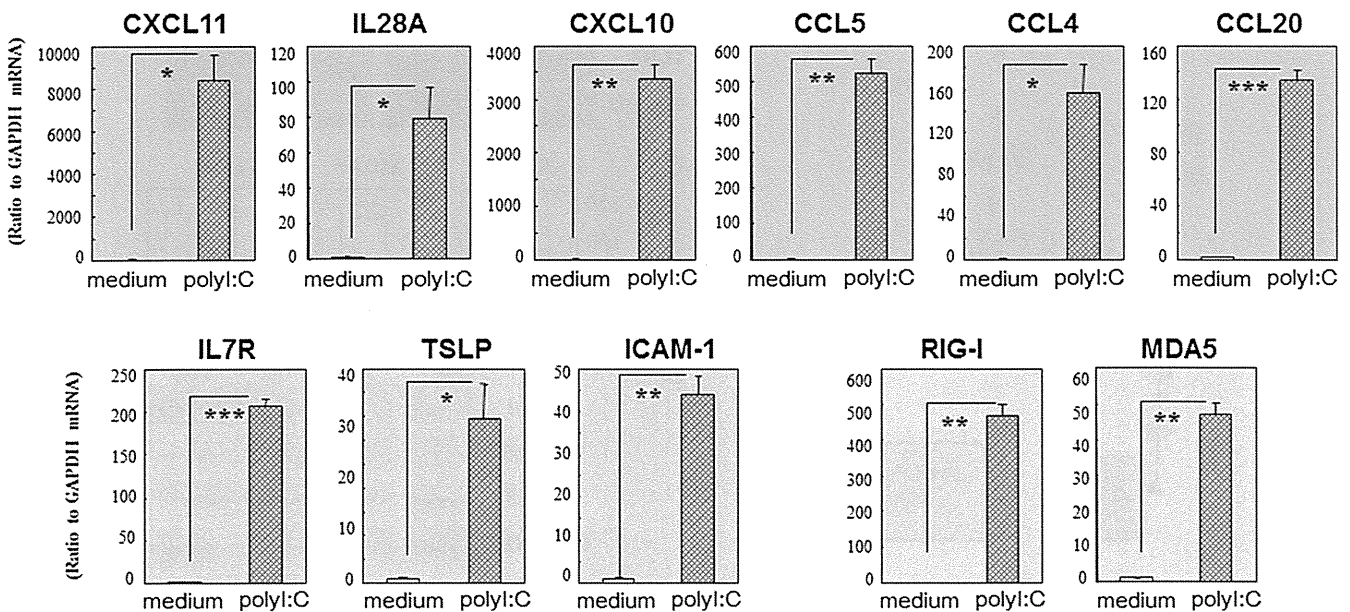
Fig. 8. mRNA expression of 11 transcripts in primary human conjunctival epithelial cells. The cells were exposed for 6 h to 25 μg/ml polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The Y axis shows the increase in specific mRNA over unstimulated samples (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$). Reprinted with permission from Ueta et al. (Ueta et al., 2010b).

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RLRs belong to the RNA helicase family that specifically detects virus-derived RNA species in the cytoplasm. They coordinate anti-viral responses via the induction of type I IFN. RIG-I and MDA-5, which were up-regulated in primary human conjunctival epithelial cells upon polyI:C stimulation, are RLRs (Kawai and Akira, 2009) (Fig. 9A).

The human ocular surface epithelium expresses TLR3, which recognizes dsRNA mimicking polyI:C, a synthetic dsRNA (Alexopoulou et al., 2001). PolyI:C stimulation induces the secretion of inflammatory cytokines such as IL-6 and IL-8, and type I IFN such as IFN-β (Ueta, 2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). Moreover, our gene expression analysis of primary human conjunctival epithelial cells using oligonucleotide microarrays to examine the comprehensive effects of polyI:C stimulation showed that transcripts including CXCL11, IL28A, CXCL10, CCL5, CCL4, IL7R, TSLP, CCL20, and ICAM-1 were up-regulated more than 10-fold (Ueta et al., 2010b). In addition, new receptors that recognize dsRNA and polyI:C, RIG-I and MDA-5, are also up-regulated upon polyI:C stimulation in primary human conjunctival epithelial cells (Ueta et al., 2010b).

We examined the expression of RIG-I and MDA-5 in human conjunctival epithelium because not only TLR3, but also RIG-I and MDA-5 detect viral dsRNA. Moreover, to determine whether RIG-I and/or MDA-5 contribute to polyI:C-inducible responses in conjunctival epithelium we investigated the function of IPS-1, an adaptor molecule common to RIG-I and MDA-5 (Kawai et al., 2005), using IPS-1-knock-out (KO) mice.

Human conjunctival epithelial cells express RIG-I-, MDA-5-, and TLR3 mRNA (Fig. 9B) and the expression of mRNA in RIG-I-, MDA-5-, and TLR3 genes was up-regulated by polyI:C stimulation. This up-regulation was particularly pronounced in RIG-I and MDA-5 (Fig. 9C) (Ueta et al., 2011a). The protein expression of RIG-I and MDA-5 but not of TLR3 was markedly up-regulated in polyI:C-

stimulated primary human conjunctival epithelial cells (Fig. 9D) (Ueta et al., 2011a).

We examined the function of IPS-1 and TLR3 in the conjunctival epithelium of IPS-1-KO- (Kawai et al., 2005) and TLR3-KO-mice. For the in vivo analysis of murine conjunctival epithelial cells we delivered a polyI:C solution subconjunctivally and as eyedrops, then we subjected these cells to gene expression analysis. Compared to control mice, Mx2 (myxovirus (influenza virus) resistance 2), Rsad2 (radical S-adenosyl methionine domain containing 2), Cmpk2 (cytidine monophosphate (UMP-CMP) kinase 2), Cxcl10 (chemokine (C-X-C motif) ligand 10), Mx1 (myxovirus (influenza virus) resistance 1), Ifi44 (interferon-induced protein 44), Ifi203 (interferon-activated gene 203), Iigp2 (interferon-inducible GTPase 2), and Rtp4 (receptor transporter protein 4) were significantly down-regulated in conjunctival epithelial cells of IPS-1-KO mice (Ueta et al., 2011a). Moreover, Mx2, Rsad2, Cmpk2, and Ccl5 (chemokine (C–C motif) ligand 5), but not Cxcl10, Mx1, Ifi44, Ifi203, Iigp2, and Rtp4 were significantly down-regulated in the conjunctival epithelium of TLR3-KO- compared to wild-type-mice (Ueta et al., 2011a). Thus, not only TLR3 but also RIG-I and/or MDA-5 contribute to polyI:C-inducible immune responses in the conjunctival epithelium (Ueta et al., 2011a).

Mx2 is an interferon-regulated gene that selectively inhibits hanta virus replication (Jin et al., 2001). Rsad2 is an interferon-inducible protein that inhibits many DNA and RNA viruses (Shaveta et al., 2010). Cmpk2, a pyrimidine nucleoside monophosphate kinase, is thought to be involved in macrophage activation and inflammatory responses (Xu et al., 2008). In conjunctival epithelial cells, Mx2 and Rsad2, which exert anti-viral actions, and Cmpk2, which is involved in inflammatory responses, were regulated by TLR3 and IPS-1 (RIG-I or/and MDA-5) (Ueta et al., 2011a).

Ifi44 is associated with hepatitis C virus infection although its function is unknown (Hallen et al., 2007). Iigp2 plays a cell-
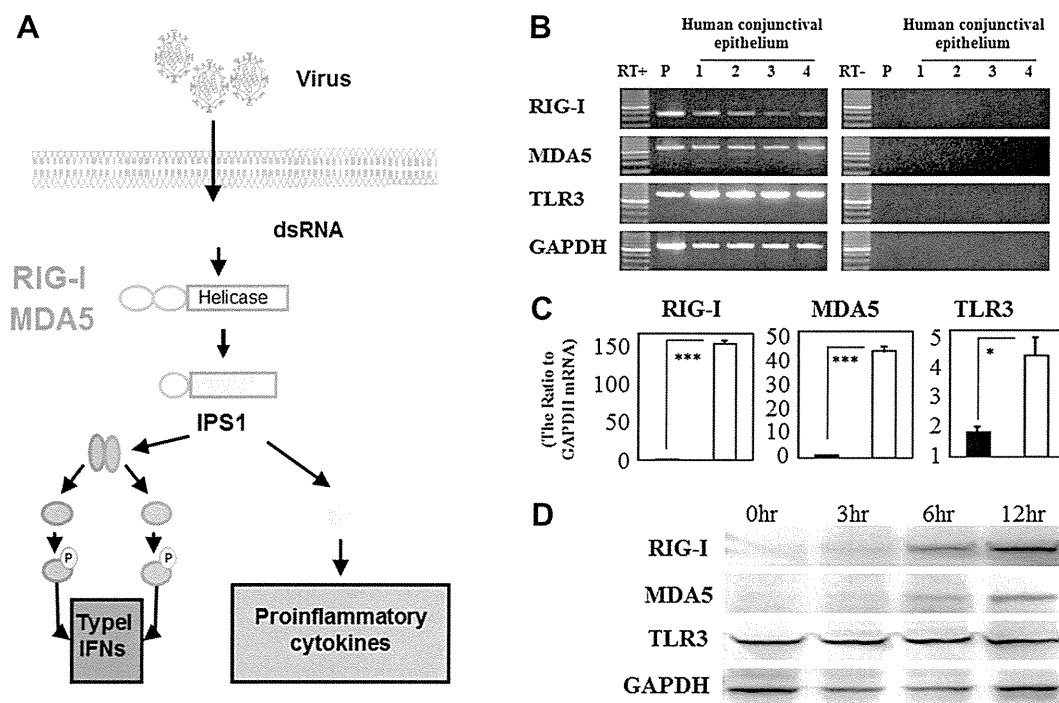
Fig. 9. A. RIG-I and MDA-5 are retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). B. Expression of RIG-I, MDA-5, and TLR3 mRNA in human conjunctival epithelial cells. The positive control (P) was mRNA isolated from human mononuclear cells (*, p < 0.05; ***, p < 0.0005). C. Up-regulation of RIG-I, MDA-5, and TLR3 mRNA expression in human conjunctival epithelial cells stimulated with polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The Y axis shows the increase in specific mRNA over unstimulated samples. D. Up-regulation of the protein expression of RIG-I and MDA-5 in primary human conjunctival epithelial cells by polyI:C stimulation. Reprinted with permission from Ueta et al. (Ueta et al., 2011a).

autonomous role in IFN-γ-mediated chlamydia inhibition (Miyairi et al., 2007). *Mx1* is an interferon-regulated gene that selectively interferes with the multiplication of influenza viruses (Horisberger, 1995). *Cxcl10* is expressed primarily in response to a wide range of DNA and RNA viruses and plays a role in the recruitment of leukocytes during inflammation (Farber, 1997). The expression of *Cxcl10* is also increased in allergic diseases; it was elevated in the epidermis of patients with atopic dermatitis (Klunker et al., 2003) and in the bronchoalveolar lavage fluid of patients with severe asthma (Ying et al., 2008). *Ifi203,* a member of the *Ifi-200* gene family, is induced by type I and II interferons; it has been reported as a regulator of cell proliferation and differentiation, and plays a role in apoptotic and inflammatory processes (Mondini et al., 2010). *Rtp4* is a member of the receptor transport protein (RTP) family and participates in the export of odorant and taste receptors (Saito et al., 2004). As these 6 transcripts are dominantly regulated by IPS-1 (RIG-I or/and MDA-5), it is evident that not only TLR3 but also RIG-I and MDA-5 contribute to the polyI:C-induced innate immune response (Ueta et al., 2011a).

Ccl5 is up-regulated in the presence of viral infection (Prehaud et al., 2005) and in the skin lesions of patients with chronic atopic dermatitis (Gros et al., 2009). *Ccl5,* which plays a role in inflammation and allergy, was dominantly regulated by TLR3 in conjunctival epithelial cells (Ueta et al., 2011a). We reported that TLR3 regulated the late-phase reaction of experimental allergic conjunctivitis (EAC) in a mouse model; eosinophilic conjunctival inflammation was reduced in TLR3-KO- and exacerbated in TLR3 transgenic-mice (Ueta et al., 2009c). These findings suggest that TLR3 in conjunctival epithelial cells can induce anti-viral innate immune responses and that it exerts other functions such as the regulation of allergic reactions. It has been reported that in the absence of viral infection TLR3 amplified immune responses during acute inflammatory processes, a phenomenon that may involve TLR3 stimulation by endogenous RNA from necrotic cells (Cavassani et al., 2008). Thus, innate immunity can respond to endogenous molecules released by host cells as a result of necrosis, pathogen infection, damage, injury, and certain pathological conditions that are directly or indirectly recognized by TLRs, NLRs, and RLRs and by yet to be identified sensors (Kawai and Akira, 2009). Endogenous RNA from tissues or cells might stimulate not only TLR3 but also RIG-I or/and MDA-5.

4. Allergic conjunctivitis may be regulated by epithelial cells

4.1. Allergic conjunctivitis

Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions. It is accompanied by characteristic symptoms (itching, conjunctival edema, redness, and tearing) during the early phase; eosinophils infiltrate the conjunctivae during the late phase. The signs and symptoms of allergic conjunctivitis have a significant deleterious effect on the patients' health, comfort, and quality of life. Current treatments are not curative and may elicit side-effects; corticosteroids place patients at increased risk for the development of glaucoma and cataracts (Ono and Abelson, 2005). Continuing efforts are needed to better understand allergic responses and to develop effective and safer drugs.

The allergic response in conjunctivitis is typically elicited by ocular exposure to allergens such as grass or tree pollen that leads to the crosslinkage of membrane-bound IgE. This in turn triggers mast cell degranulation and a release of a cascade of allergic and inflammatory mediators. The rapid release of histamine from mast cells within minutes of exposure to allergens is important in early-phase reactions. In addition, mediators released by mast cells

during this phase may contribute to the development of late-phase reactions in which eosinophils are recruited to tissue sites affected by allergic inflammation (Broide, 2007). T cells (Fukushima, 2007) and fibroblasts (Fukuda et al., 2006) have been reported to contribute to the development of late-phase reactions (Fig. 10).

4.2. Development of eosinophilic conjunctival inflammation during the late-phase reaction in mast cell-deficient mice

Mast cells and the mediators they release are thought to contribute to the development of allergic conjunctivitis which is triggered by IgE cross-linking on mast cells; their mediators produce early-phase reactions in the conjunctiva (Graziano et al., 2001). Preformed or newly synthesized mediators, including histamine, are released from mast cells in the acute phase of allergic reaction. This results in clinical manifestations such as conjunctival redness, eye itching, and increased tearing.

Although mast cells play a central role in immediate allergic reactions and in the early phase of allergic conjunctivitis (Graziano et al., 2001), their role in the late-phase response is not clearly defined. The magnitude of eosinophil infiltration into the conjunctiva reflects the severity of the late-phase reaction. Using genetically mast cell-deficient (W/W^v) mice and our C57BL/6 mouse model of allergic conjunctivitis (Ueta et al., 2007b) we directly assessed the role of mast cells in conjunctival eosinophil infiltration.

We compared eosinophil infiltration in congenic WBB6F1-normal- (+/+) and mast cell-deficient ([WB-W/+ × C57BL/6-W^v/+]F1; W/W^v) mice. In mice sensitized and challenged by ragweed (RW), the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in both mast cell-deficient mice and their congenic littermates, although no sensitization and sensitization without challenge did not affect the number of eosinophils. There was no difference between mast cell-deficient and -sufficient mice (Fig. 11) (Ueta et al., 2007b).

We next compared the expression of eotaxin-specific mRNA in the eyelids of WBB6F1-normal- (+/+) and mast cell-deficient ($W/$ W^v)-mice because chemokines such as eotaxin recruit eosinophils. Sensitization and challenge by RW significantly increased the expression of eotaxin-specific mRNA compared with sensitization alone in both mast cell-deficient and -sufficient mice (Ueta et al., 2007b). After RW sensitization, the level of serum total IgE, anti-RW IgE, and anti-RW IgG$_1$ was comparable in mast cell-deficient mice and their congenic littermates (Ueta et al., 2007b).

Sensitization with challenge of mast cell-deficient mice produced an increase in IgE and IgG$_1$ antigen-specific antibody responses, conjunctival eosinophils, and eotaxin-specific mRNA in the eyelids. In this respect, these mice were indistinguishable from their congenic littermates. However, mast cells were identified histologically in the submucosa of WBB6F1-normal- (+/+) but not of W/W^v-mice, suggesting that the development of eosinophilic conjunctival inflammation in the late phase of allergic conjunctivitis is not dependent on the presence of functional mast cells (Ueta et al., 2007b).

Our findings indicate that mast cells do not play an essential role in the development of eosinophilic conjunctival inflammation in mice sensitized and challenged. However, this does not exclude the contribution of mast cells to other aspects of late-phase allergic conjunctivitis (Ueta et al., 2007b).

4.3. TLR3 and allergy

TLR3 recognizes dsRNA, a component of the life-cycle of most viruses, mimicking polyI:C (Alexopoulou et al., 2001). Among TLR1–TLR10, TLR3 is expressed most intensely in the ocular surface

Mechanism of allergic conjunctivitis
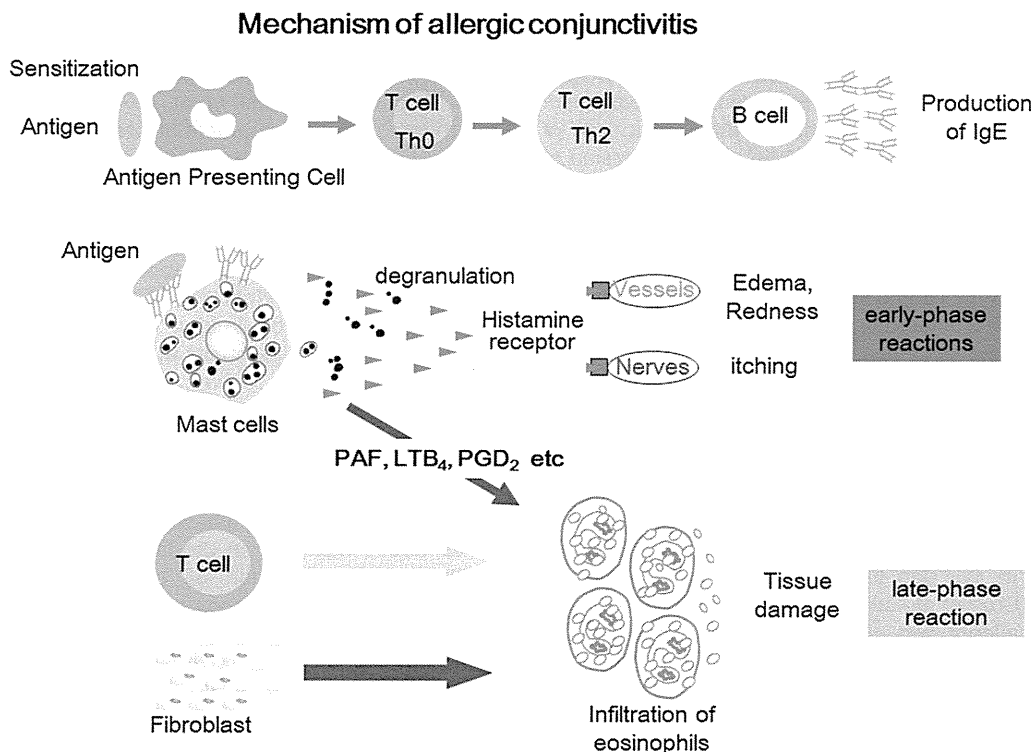
Fig. 10. Mechanism of allergic conjunctivitis. The allergic response is typically elicited to allergens that produce crosslinkage with membrane-bound IgE. This triggers mast cell degranulation and the release of a cascade of allergic and inflammatory mediators. The rapid release of histamine from mast cells within minutes of exposure to allergens is important in early-phase reactions. In addition, mediators released by mast cells during the early-phase reaction may contribute to the development of the late-phase reaction, in which eosinophils are recruited to tissue sites of allergic inflammation. T cells and fibroblasts are now known to contribute to the development of the late-phase reaction such as eosinophils infiltration.

epithelium and more intensely than in mononuclear cells (Ueta, 2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a).

Although a relationship between viral infection and allergic inflammation has been reported (Peebles, 2004), the function of

TLR3 in allergic inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions and the degree of eosinophil infiltration into the conjunctiva reflects the severity of the

Fig. 11. Eosinophilic inflammation in the conjunctiva of mast cell-deficient mice. Mast cell-deficient mice (*WBB6F-W/Wv*) exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation similar to that seen in their congenic littermates (*WBB6F1* (+/+)). Bar = 50 μm. Modified with permission from (Ueta et al., 2007b).

late-phase reaction. Using our Balb/c mouse model of EAC (Fig. 12A)(Ueta et al., 2009a) and TLR3-KO- and TLR3 transgenic (TLR3Tg)-mice, we 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 after sensitization and challenge, although sensitization without challenge had no effect (Ueta et al., 2009a). Sensitization with RW induced RW-specific immune responses (IgE- and IgG$_1$-antigen-specific antibody responses) equally in wild-type-, TLR3Tg-, and TLR3-KO mice. (Ueta et al., 2009a). Comparison of the number of eosinophils in the lamina propria mucosae of the conjunctiva in wild-type- and TLR3-KO-mice revealed significantly lower numbers in TLR3-KO- than wild-type-mice (Fig. 12B)(Ueta et al., 2009a). Moreover, the number of eosinophils was significantly larger in sensitized and challenged TLR3Tg- than wild-type-mice (Fig. 12C, D) (Ueta et al., 2009a). Our findings suggest that TLR3 positively regulates the late-phase reaction in EAC, resulting in reduced eosinophilic conjunctival inflammation in TLR3-KO mice and in pronounced eosinophilic conjunctival inflammation in TLR3Tg mice (Ueta et al., 2009a).

In human conjunctival epithelial cells, the significant up-regulation of CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, which are increased in allergic diseases (Ueta et al., 2010b), might be consistent with our finding that TLR3 positively regulates the late-phase reaction in our mouse EAC model.

Mast cells do not play an essential role in the development of eosinophilic conjunctival inflammation during the late-phase reaction because mast cell-deficient mice exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation whose severity was similar to that seen in their congenic littermates (Ueta et al., 2007b). Conjunctival epithelial cells may be implicated in the eosinophilic conjunctival inflammation

seen in allergic conjunctivitis. Our findings raise the possibility that ocular surface epithelial cells regulate inflammation in allergic conjunctivitis (Ueta et al., 2009a, 2009c).

Although the function of TLR3 in allergy remains to be defined, in airway epithelial cells (Kato et al., 2007) and keratinocytes (Kinoshita et al., 2008) the expression of thymic stromal lympho-poietin (TSLP), which plays a key role in allergic inflammation, is reportedly induced by stimulation with the TLR3 ligand polyI:C. TSLP is highly expressed by airway epithelial cells of asthma patients (Ying et al., 2005) and by keratinocytes in the skin lesions of patients with atopic dermatitis (Soumelis et al., 2002). The human ocular surface epithelium expressed TLR3 and cytokine production was up-regulated by polyI:C (Ueta, 2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). TSLP is also induced by stimulation with polyI:C in human conjuctival and corneal epithelial cells (Ueta and Kinoshita, 2010a, b; Ueta et al., 2011c). It is possible that TLR3 positively regulates the late-phase reaction in EAC via the induction of TSLP.

4.4. EP3 and allergy

Prostanoids, which include prostaglandin (PG)D$_2$, PGE$_2$, PGF$_{2\alpha}$, PGI$_2$, and thromboxane (TX)A$_2$, are a group of lipid mediators that form in response to various stimuli. In mammals ranging from mice to humans, 8 types of prostanoid receptors are conserved: the PGD receptor (DP), 4 subtypes of the PGE receptor (EP1, EP2, EP3, and EP4), the PGF receptor (FP), the PGI receptor (IP), and the TXA receptor (TP) (Narumiya et al., 1999). It has been reported that the PGE receptor subtype EP3 inhibits keratinocyte activation and exerts anti-inflammatory actions in mouse contact hypersensitivity (Honda et al., 2009) and that the PGE$_2$-EP3 pathway negatively regulates allergic reactions in a murine allergic asthma model (Kunikata et al., 2005). PGE$_2$ acts on one of its 4 receptor subtypes,

Fig. 12. A. Our balb/c mouse model of experimental allergic conjunctivitis (EAC). B. Reduced eosinophilic conjunctival inflammation in TLR3-KO mice. C. The infiltration of eosinophils into the conjunctiva of wild-type- and TLR3Tg-mice was detected with Luna's method. Scale bars, 50 μm. D. Pronounced eosinophilic conjunctival inflammation in TLR3Tg mice. B and D.Data are shown as the mean ± SEM of samples from all 12 mice examined. **, $p < 0.01$. Reprinted with permission from Ueta et al. (Ueta et al., 2009c).

EP3, and negatively regulates allergic reactions: allergic inflammation was significantly more pronounced in EP3-KO (EP3-KO)-than wild-type-mice, and the EP3-selective agonist suppressed the inflammation (Honda et al., 2009; Kunikata et al., 2005). Intriguingly, EP3 is expressed in airway epithelial- but not in infiltrating-cells (Kunikata et al., 2005).

We also raised the possibility that ocular surface epithelial cells regulate inflammation in allergic conjunctivitis. We tested the hypothesis that ocular surface epithelial cells express EP3 and regulate the inflammation of allergic conjunctivitis through the PGE_2-EP3 pathway by examining ocular-surface EP3 expression and analyzing its role in our Balb/c EAC model. In these experiments we used EP3-KO mice and a selective EP3 agonist, ONO-AE-248.

To examine the mRNA expression of EP3 in the murine ocular surface we performed RT-PCR assays. Ocular surface tissues, both conjunctival and corneal, express EP3 mRNA (Ueta et al., 2009a). To examine the localization of EP3 we used EP3-KO mice; in these animals the β-galactosidase gene was 'knocked-in' at the EP3 gene locus. X-gal stains of eye tissues also showed that the conjunctiva, cornea, and eyelids were stained densely positive (Fig. 13A) (Ueta, 2010). In EP3-KO mice, conjunctival and corneal epithelia manifested dense positive- and positive signals for X-gal staining (Fig. 13B) (Ueta et al., 2009a). Thus, EP3 was constitutively expressed in the murine ocular surface epithelium (Ueta, 2010; Ueta et al., 2009a).

We also examined the number of eosinophils in the lamina propria of the conjunctiva from wild-type- and EP3-KO mice to investigate whether EP3 plays a role in the late-phase reaction in our Balb/c EAC model. In EP3-KO mice the number of eosinophils was significantly larger than in wild-type mice, although both wild-type- and EP3-KO mice manifested eosinophil-dominant infiltration into the lamina propria after challenge (Fig. 14A) (Ueta et al., 2009a). We also investigated the eotaxin-1 mRNA expression in

the eyelids by quantitative RT-PCR assay. Although RW challenge significantly increased its expression in both genotypes, its level was significantly larger in EP3-KO- than wild-type-mice (Ueta et al., 2009a). Thus, after RW challenge, EP3-KO mice demonstrated significantly greater eosinophil infiltration into the conjunctiva than wild-type mice (Ueta et al., 2009a). We consistently found significantly higher eotaxin-1 mRNA expression in EP3-KO mice (Ueta et al., 2009a).

Next we assessed the effects of the EP3-selective agonist ONO-AE-248 to determine whether allergic inflammation can be suppressed by stimulating the PGE_2-EP3 pathway in EAC. We topically administered the EP3 agonist to the eyes of RW-sensitized mice, three times, as shown in Fig. 14B. We found that this significantly inhibited the infiltration of eosinophils compared with vehicle-treated wild-type mice (Fig. 14C) and that the inhibition was mediated by EP3 because the inhibitory effect of the EP3 agonist was absent in EP3-KO mice (Ueta et al., 2009a). Thus, the treatment of wild-type mice with EP3-selective agonist eyedrops resulted in a significant decrease in eosinophil infiltration.

We also studied the expression of cyclooxygenase-2 (COX-2) and mPGES (prostaglandin E synthase) -1 mRNA in the eyelids during EAC, because COX and PGESs are necessary for PGE_2 synthesis (Fig. 15A) (Vane et al., 1998). The relative expression levels of COX-2 mRNA increased and peaked at 1 h after RW challenge, and the relative expression levels of mPGES-1 mRNA also increased and peaked at 3 h (Fig. 15B). We found that during EAC the PGE_2 contents in the eyelids increased time-dependently up to 12 h after RW challenge (Fig. 15C) (Ueta et al., 2009a).

Immunohistologic studies of mPGES-1 to determine the localization of PGE_2 synthesis showed that conjunctival epithelial cells expressed mPGES-1 protein, suggesting that PGE_2 synthesis through mPGES-1 occurs in conjunctival epithelium. Thus, in the eyelids after RW challenge, the expression of COX-2 and mPGES-1 was up-regulated and the PGE_2 content was increased. Our
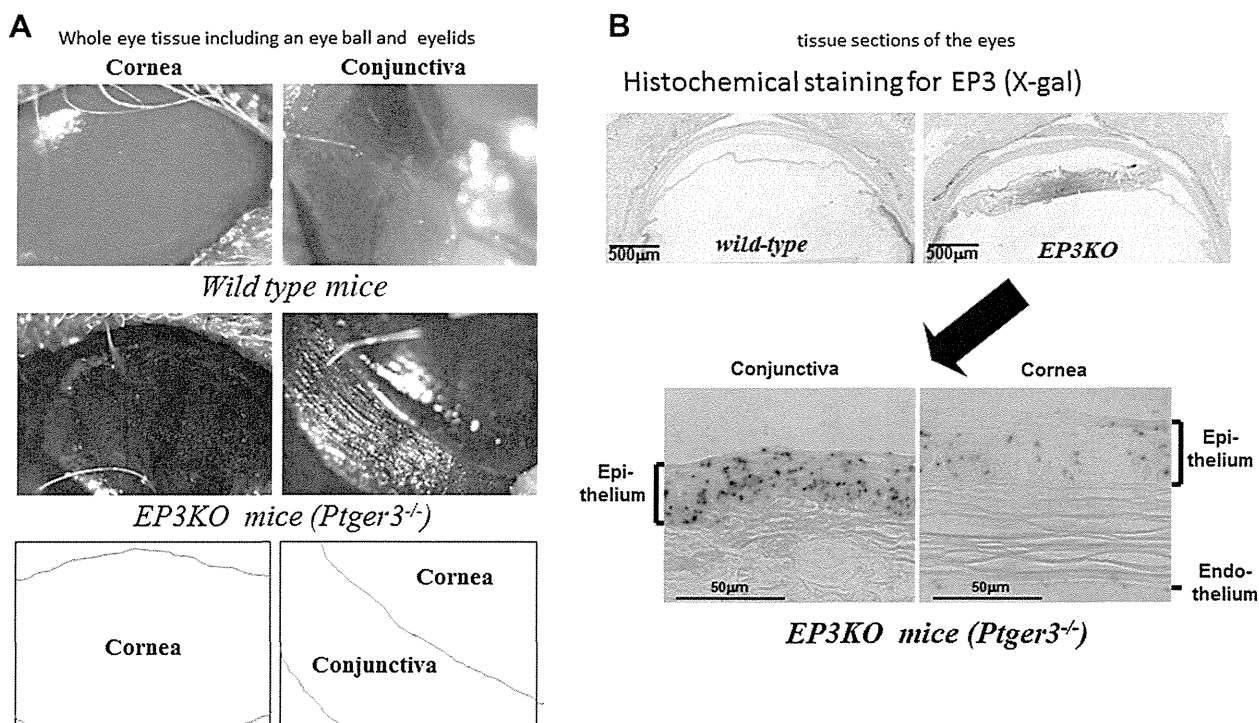
Fig. 13. Expression and localization of EP3 in the ocular surface (conjunctiva and cornea). A. X-gal staining of whole eye tissue (including the eye ball and eyelids). B. Histochemical staining for X-gal of tissues sections from the eyes. In EP3-KO mice (Ptger3−/−) the β-galactosidase gene was 'knocked-in' at the EP3 gene locus. Blue (X-gal) staining in EP3-KO mice shows the localization of EP3. Modified with permission from (Ueta, 2010; Ueta et al., 2009a).
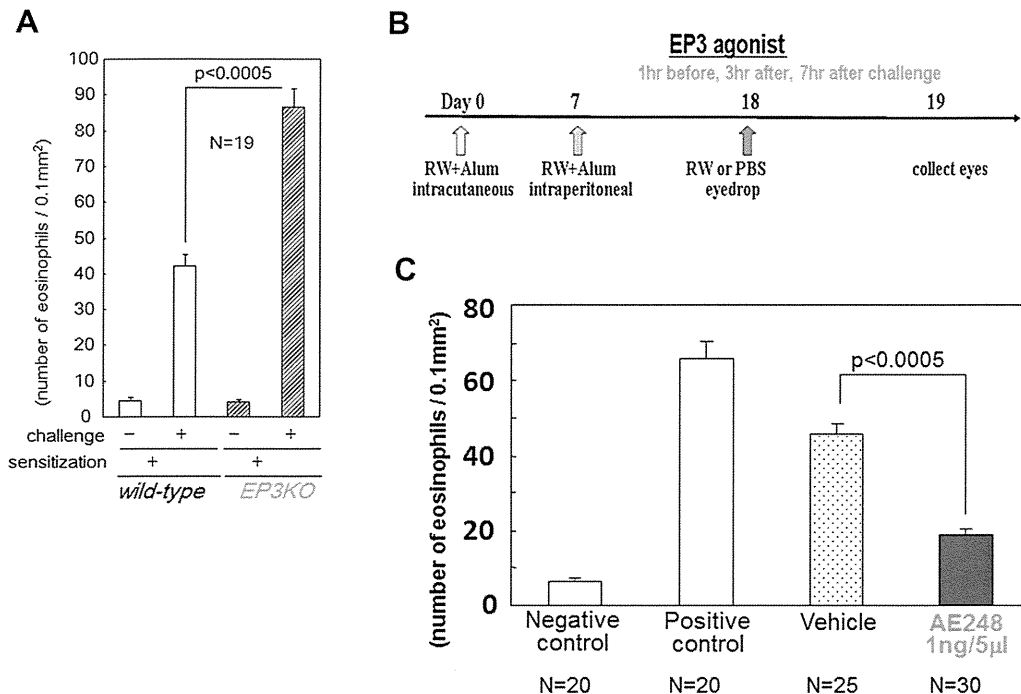
Fig. 14. A. Up-regulation of the number of eosinophils in the tarsal conjunctiva of EP3-KO mice (*Ptger3*$^{-/-}$). Eosinophils in the lamina propria mucosae of the tarsal conjunctiva were counted in wild-type- and EP3-KO-mice and the number of infiltrating eosinophils in sections containing the central portion of the eye. Cell counts are expressed as the number of infiltrating eosinophils divided by the area (mm^2). Data are shown as the mean ± SEM of samples from 19 examined mice. B. Protocol for the topical administration of the EP3-selective agonist ONO-AE-248. Either ONO-AE-248 (1 ng in 5 μl PBS) or vehicle was topically administered to the eyes of RW-sensitized wild-type mice at 1-, 3-, and 7 h after RW challenge. C. Effect of the EP3-selective agonist AE-248 on eosinophil infiltration in the conjunctiva of wild-type mice. The number of eosinophils in the conjunctiva of wild-type mice is shown. Ragweed-sensitized mice were the negative control, mice subjected to RW sensitization and challenge were the positive control. Data are the mean ± SEM of samples from all examined mice. Modified with permission from (Ueta et al., 2009a).

findings suggest that PGE$_2$ acts on EP3 in conjunctival epithelium and down-regulates the progression of EAC (Ueta et al., 2009a).

4.5. Interaction between TLR3 and EP3 in allergy

We reported that conjunctival eosinophilic infiltration in murine EAC was significantly more marked in EP3-KO-, and significantly less marked in TLR3-KO- than wild-type-mice. Considering the opposite roles of the EP3 and the *TLR3* in allergic conjunctivitis, we posited an unknown functional interaction between EP3 and TLR3. To test this hypothesis we examined whether EP3 negatively regulates TLR3-dependent eosinophilic infiltration in allergic conjunctivitis. Using our EAC model we compared conjunctival eosinophil infiltration in wild-type-, TLR3-KO-, EP3-KO-, and TLR3/EP3-double-knock-out (DKO) mice. We found that although RW sensitization without challenge (RW eyedrops) did not affect the number of eosinophils, after sensitization and challenge the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in all of these mice. However, their number was significantly larger in RW sensitized and challenged EP3-KO mice (Ueta et al., 2009a) and significantly smaller in TLR3-KO than wild-type mice (Ueta et al., 2009c). Furthermore, in TLR3/EP3-DKO mice the number of eosinophils in the lamina propria mucosae of the conjunctiva was decreased to a level similar to that in TLR3-KO mice and significantly lower than in EP3-KO- and wild-type-mice (Ueta et al., 2012b).

Poly I:C is a TLR3 ligand and elsewhere we reported that in conjunctival epithelial cells an EP3 agonist could suppress polyI:C-induced cytokine production and the mRNA expression of TSLP (Ueta et al., 2011c) and RANTES (Ueta et al., 2011b) that are important for eosinophil recruitment. Considering that the EP3

agonist suppressed the production and mRNA expression of TSLP and RANTES, our results suggest that EP3 might suppress TLR3-induced cytokine production, resulting in the negative regulation of eosinophilic infiltration induced by TLR3. This also explains our observation that eosinophilic conjunctival inflammation was decreased in TLR3/EP3-DKO mice although it was pronounced in EP3-KO mice (Ueta et al., 2012b).

5. IκBζ and ocular surface inflammation

5.1. Ocular surface inflammation and the disappearance of goblet cells in IκBζ-KO mice

Inflammatory bowel disease is thought to result from an abnormal response to gut microbiota (Cho, 2008). We hypothesized that an abnormality in the proper innate immunity of the ocular surface may result in ocular surface inflammation.

IκBζ is important for the TLR/IL-1 receptor signaling that is essential for an innate immune response (Yamamoto et al., 2004). IκBζ-KO mice expressly exhibit severe, spontaneous ocular surface inflammation and the eventual loss of almost all goblet cells (Ueta et al., 2005b). Moreover, Balb/c background IκBζ-KO mice developed not only spontaneous ocular surface- but also spontaneous perioral inflammation (Ueta et al., 2008a) and in some IκBζ-KO mice ocular surface inflammation was accompanied by corneal opacity (Fig. 16A) (Ueta and Kinoshita, 2010a).

Stevens-Johnson syndrome (SJS) with severe ocular surface complications is a human ocular surface inflammatory disease. We considered IκBζ-KO mice to be a suitable model for human SJS with severe ocular surface complications because their ocular surface inflammation is accompanied by a loss of goblet cells as is seen in SJS patients (Ohji et al., 1987) and because, like these patients, they
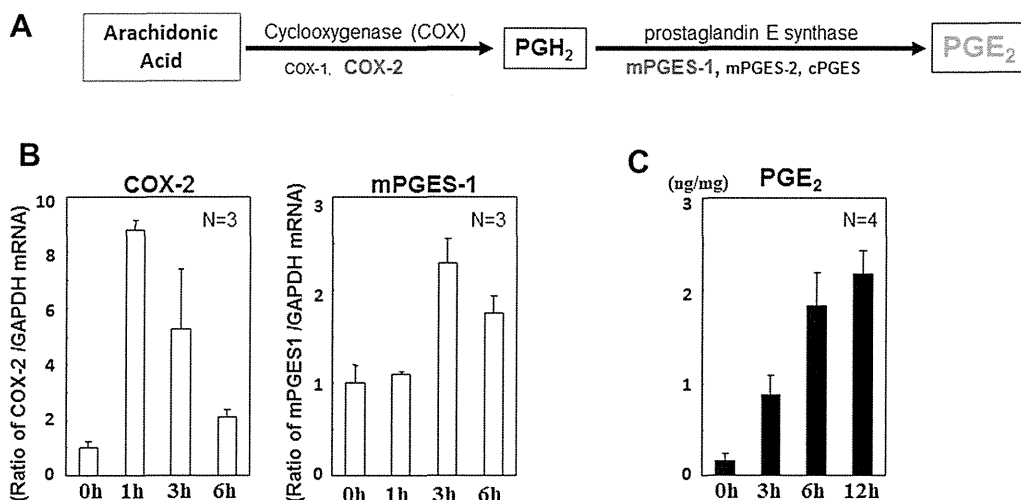
Fig. 15. RW challenge increased PGE₂ synthesis in mouse eyelids. A. PGE₂ synthesis pathway from arachidonic acid. B. Time-course of COX-2 and mPGES-1 mRNA expression in the eyelids during EAC. Relative mRNA levels of COX-2 and mPGES-1 in the eyelids after RW challenge are shown. The mRNA levels were normalized by the level of GAPDH measured in the same sample. The Y axis shows the increase in specific mRNA over the 0-hr samples, the X axis the time after RW challenge. Data are the mean ± SEM of 3 samples. C. Time-course of PGE₂ content in the eyelids during EAC. The PGE₂ content in the eyelids after RW challenge is shown. X axis: time after RW challenge. Data are the mean ± SEM of 4 samples. Reprinted with permission from Ueta et al. (Ueta et al., 2009a).

developed perioral inflammation (Sotozono et al., 2009a) In addition, IκBζ-KO mice also manifested the oral mucositis and airway inflammation as is seen in human SJS (Fig. 16B) (Ueta and Kinoshita, 2010a). IκBζ/Stat6-DKO mice presented with not only severe dermatitis of the facial- but also the abdominal skin; these animals also exhibited paronychia (Fig. 16C) as is seen in human SJS (Ueta and Kinoshita, 2010a).

Possibly to prevent excessive inflammation in the presence of bacterial components, IκBζ, which is induced by diverse pathogen-associated molecular patterns, regulates NF-κB activity (Yamazaki et al., 2001). The spontaneous ocular surface inflammation observed in IκBζ-KO mice suggests that dysfunction/abnormality of innate immunity plays a role in ocular surface inflammation (Ueta et al., 2008a, 2005b; Ueta and Kinoshita, 2010a).

5.2. Function of IκBζ in ocular surface epithelial cells

IκBζ mRNA was expressed in both conjunctival and corneal tissues from normal mice and the predominant expression of IκBζ transcripts in the murine ocular surface was localized spatially to conjunctival and corneal epithelia (Ueta et al., 2005b). Human MAIL is similar to mouse IκBζ and human conjunctival and corneal epithelia also expressed MAIL-specific mRNA (Fig. 17A) (Ueta et al., 2005b). Unlike typical IκB proteins, IκBζ is stably accumulated in the nucleus. There is no consensus on the function of IκBζ. Like other IκBs it has been reported as a negative regulator of NF-κB (Yamazaki et al., 2001) and it has been also reported as a positive regulator of NF-κB (Yamamoto et al., 2004). Yamazaki et al. (2001) who investigated its function using fibroblasts concluded that IκBζ was a negative regulator of NF-κB. On the other hand, Yamamoto et al. (2004) studied its function in macrophages and reported IκBζ as a positive regulator of NF-κB. These observations suggest that IκBζ exerts regulatory effects selectively in a cell-type-specific manner. Compared to IκBζ+/− and IκBζ+/+ mice, in the eyelids of IκBζ−/− mice, the mRNA expression of TNF-α, IL-4, IL-17α, and IFN-γ. These observations suggest that IκBζ exerts regulatory effects selectively not only on cytokines through NF-κB, but also in a tissue- or cell-type-specific-manner (Ueta et al., 2010a, 2008a). To investigate whether MAIL can suppress the production of pro-inflammatory cytokines, we performed siRNA experiments to knock down the mRNA levels of MAIL. The expression of IL-6 and IL-8 mRNA was enhanced in MAIL-knock-down primary human corneal epithelial cells (Fig. 17B), suggesting that MAIL in the ocular surface epithelium can suppress the production of pro-inflammatory cytokines such as IL-6 and IL-8 and that the ocular surface epithelium suppresses inflammation via the expression of IκBζ (Ueta and Kinoshita, 2010a).

6. Abnormality of innate immunity and Stevens-Johnson syndrome (SJS) with ocular surface complications

6.1. SJS with severe ocular surface complications

SJS is an acute inflammatory vesiculobullous reaction of the skin and mucosa including the ocular surface. In individuals with extensive skin detachment and a poor prognosis, the condition is called toxic epidermal necrolysis (TEN). Both SJS and TEN are commonly associated with inciting drugs or infectious agents (Yetiv et al., 1980). In the acute stage, SJS/TEN patients manifest vesiculobullous lesions of the skin and mucosa, especially of the eyes and mouth, paronychia, severe conjunctivitis, alopecia of the eyelashes, and persistent corneal epithelial defects due to ocular surface inflammation (Fig. 18A, B). We observed oral involvement including blisters, bleeding, and erosions of the lips and mouth in all SJS/TEN patients with ocular surface complications (Sotozono et al., 2009b; Ueta and Kinoshita, 2010a). Some SJS/TEN patients also manifested respiratory disorders such as mucous membrane damage of the bronchus or trachea, bronchiolitis obliterans, and pneumonia (Yamane et al., 2007). Moreover, due to the occurrence of paronychia in the acute stage, almost all SJS/TEN patients with ocular surface complications had lost their fingernails in the acute or subacute stage; in some patients the fingernails were transformed even in the chronic stage (Fig. 18C) (Sotozono et al., 2009b; Ueta and Kinoshita, 2010a; Ueta et al., 2007d). In the chronic stage, ocular surface complications including conjunctival invasion into the cornea, symblepharon, ankyloblepharon, dry eye, and in some instances, keratinization of the ocular surface, persist despite the healing of the skin lesions. Trichiasis and alopecia of the eyelashes was also observed (Fig. 18D) (Sotozono et al., 2007; Ueta and Kinoshita, 2010a).
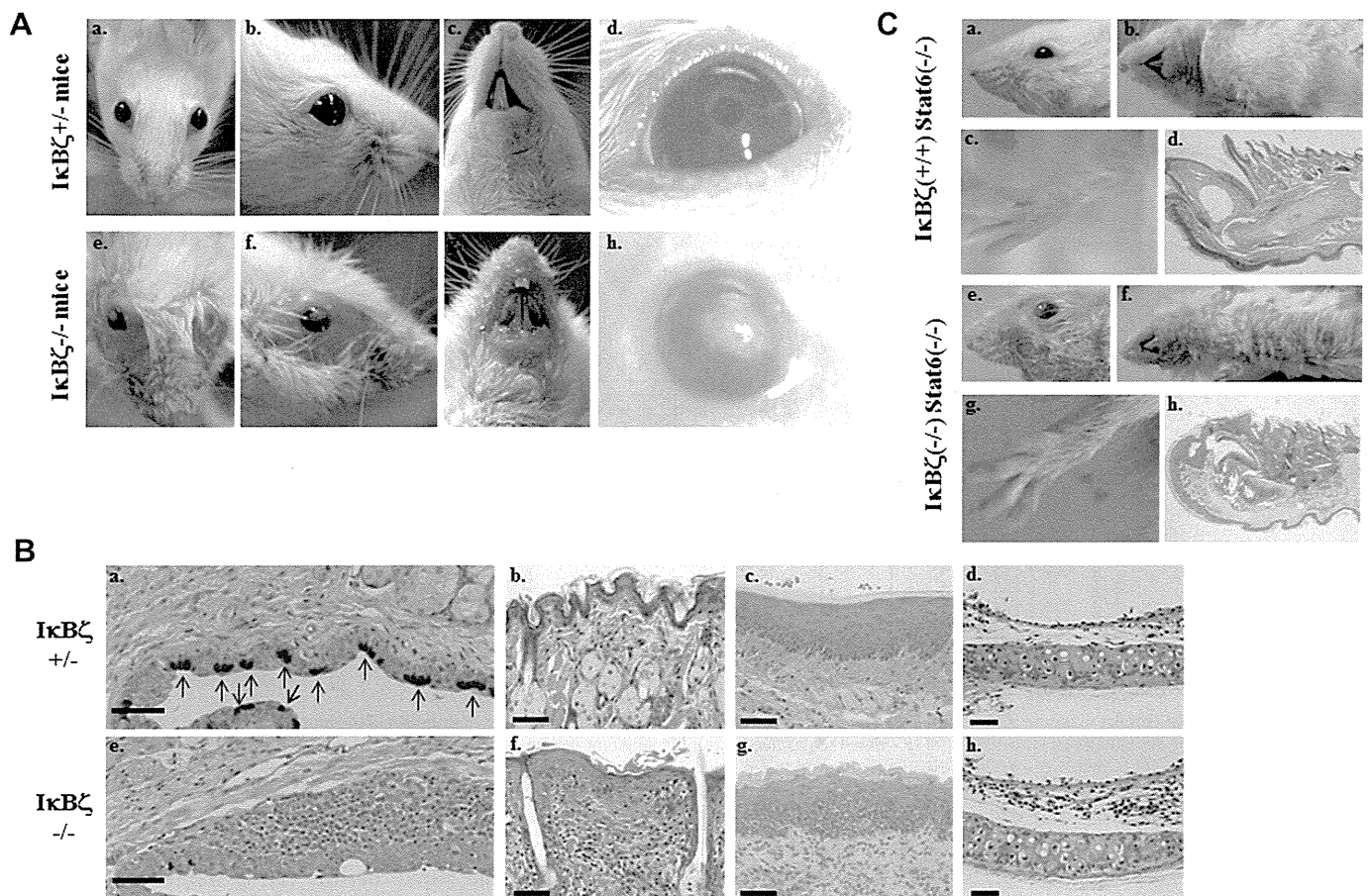
Fig. 16. Phenotype and histological findings in IκBζ-KO- and IκBζ/Stat6-DKO-mice. A. Phenotype of IκBζ KO mice. Photographs of the face and perioral skin of 32-week-old IκBζ+/− and IκBζ−/− mice taken 27 weeks after symptom onset. While IκBζ+/− mice were free of inflammation (a–d), IκBζ−/− mice exhibited a severe inflammatory phenotype. Their inflammation involved the ocular surface, eyelids and perioral skin and some of these mice manifested corneal opacity with ocular surface inflammation (e–h). B. Histological findings on the palpebral conjunctiva, perioral skin, oral mucosa, and trachea of IκBζ+/− and IκBζ−/− mice. We observed no pathological changes such as inflammatory phenotypes in IκBζ+/− mice (a–d). However, the palpebral conjunctiva of an IκBζ−/− mouse (at 2 weeks after the onset of inflammatory symptoms) revealed heavy infiltration by inflammatory cells into the submucosa and loss of goblet cells (showing by arrows in a) in the conjunctival epithelia (e). The perioral skin of the IκBζ−/− mouse showed hyperplasia and spongiosis in the epidermis including the hair follicles, inter- and intracellular edema in the epidermis, and heavy infiltration of the dermis by inflammatory cells (f). The oral mucosa of another IκBζ−/− mouse (at 9 weeks after the onset of inflammatory symptoms) revealed spongiosis in the epithelium and infiltration by inflammatory cells into the submucosa under the oral mucosal epithelia (g). In the trachea of another IκBζ−/− mouse (at 8 weeks after the onset of inflammatory symptoms) we found infiltration of inflammatory cells into the submucosa under the tracheal epithelia (h). Each bar represents a length of 50 μm. C. Phenotype and histological findings in an IκBζ/Stat6 double-KO mouse. No obvious dermatitis or paronychia was observed in Stat6 single-KO mice (a, b, c, d). However, in the IκBζ/Stat6-DKO mouse severe inflammatory symptoms were elicited on the ocular surface and not only the facial- but also the abdominal skin (e, f). The IκBζ/Stat6 WKO mouse also manifested paronychia (g, h). Reprinted with permission from Ueta et al. (Ueta and Kinoshita, 2010a).

Although the role of acquired immunity in the pathogenicity of SJS/TEN has been reported, it was not recognized that innate immunity plays a critical role in the bridging between the acute response to invading non-self molecules and chronic local immune inflammation. We considered the possibility of an association between a disordered innate immune response and SJS/TEN with severe ocular surface complications because like Yetiv et al. (1980) we found an association between infection and the onset of SJS/TEN. In fact, many SJS/TEN patients with severe ocular surface complications exhibited prodromata including non-specific fever, sore throat, coryza, and ailments that closely mimic upper respiratory tract infections commonly treated with antibiotics (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007d, 2010c, 2012b).

SJS was first described in 1922 by two pediatricians, Stevens and Johnson, who encountered 2 boys aged 8 and 7 years who manifested extraordinary, generalized skin eruptions, persistent fever, inflamed buccal mucosa, and severe purulent conjunctivitis resulting in severe visual disturbance. They carefully ruled out drug ingestion as a causative factor of their patients' skin eruptions

(Stevens and Johnson, 1922). Subsequently, pediatricians reported that SJS was associated with infectious agents such as *Mycoplasma pneumoniae*, (Leaute-Labreze et al., 2000) and a viral etiology involving herpes simplex-, Epstein-Barr-, varicella zoster-, and cytomegalo-virus (Forman et al., 2002). On the other hand, dermatologists claimed that more than 100 different drugs were involved in eliciting SJS and its severe variant, TEN. Others cited life-threatening severe adverse drug reactions characterized by high fever, rapidly developing blistering exanthema of macules, and target-like lesions accompanied by mucosal involvement and skin detachment (Roujeau et al., 1995; Wolf et al., 2005).

The reported estimated annual incidence of SJS and TEN is 0.4–1.0 and 1–6 per million persons, respectively (Auquier-Dunant et al., 2002; Yetiv et al., 1980); the mortality rate is 3% and 27%, respectively (Power et al., 1995). Although rare, these reactions carry high morbidity and mortality rates and often result in severe and definitive sequelae such as vision loss; the incidence of ocular complications in SJS/TEN was reported to be 50–68% (Power et al., 1995; Yetiv et al., 1980).