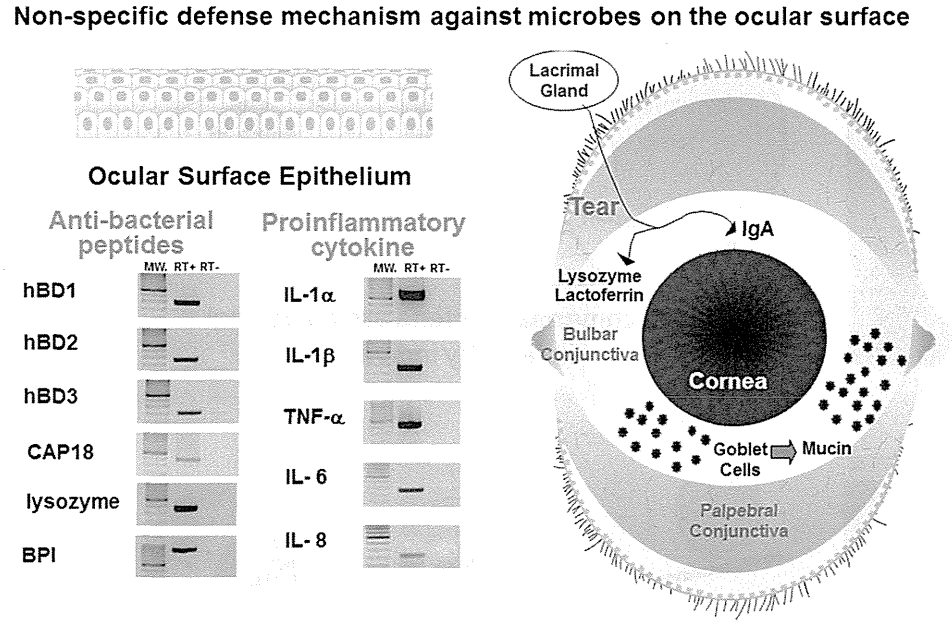

Fig. 1. Non-specific defense mechanism against microbes on the ocular surface. The ocular surface epithelium harbors all isoforms of human beta defensins and can produce inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6, and IL-8 (left: RT-PCR, human corneal epithelial cells.). Tear fluids contain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

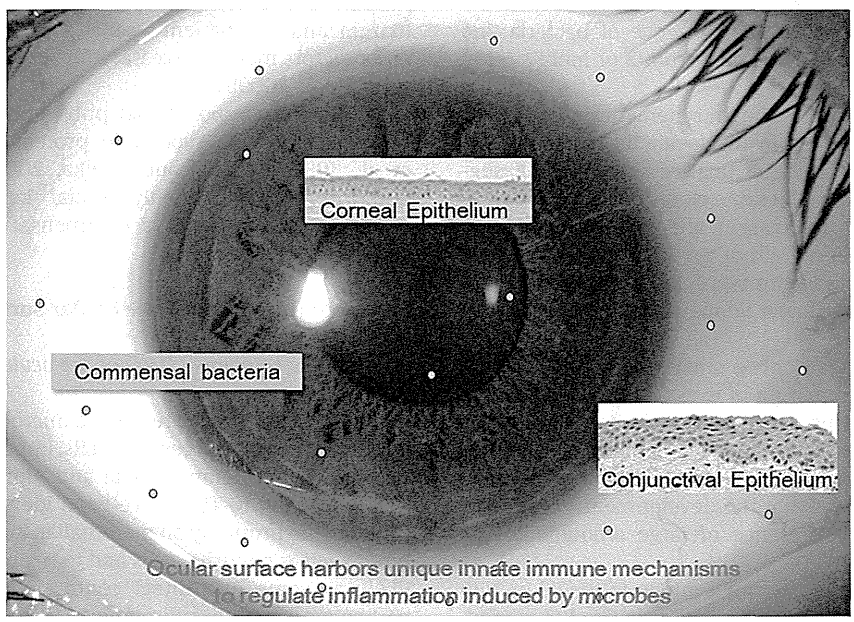

Fig. 2. Healthy ocular surface. The healthy ocular surface is not in an inflammatory state, although the ocular surface epithelium is in constant contact with bacteria and bacterial products. Ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes.

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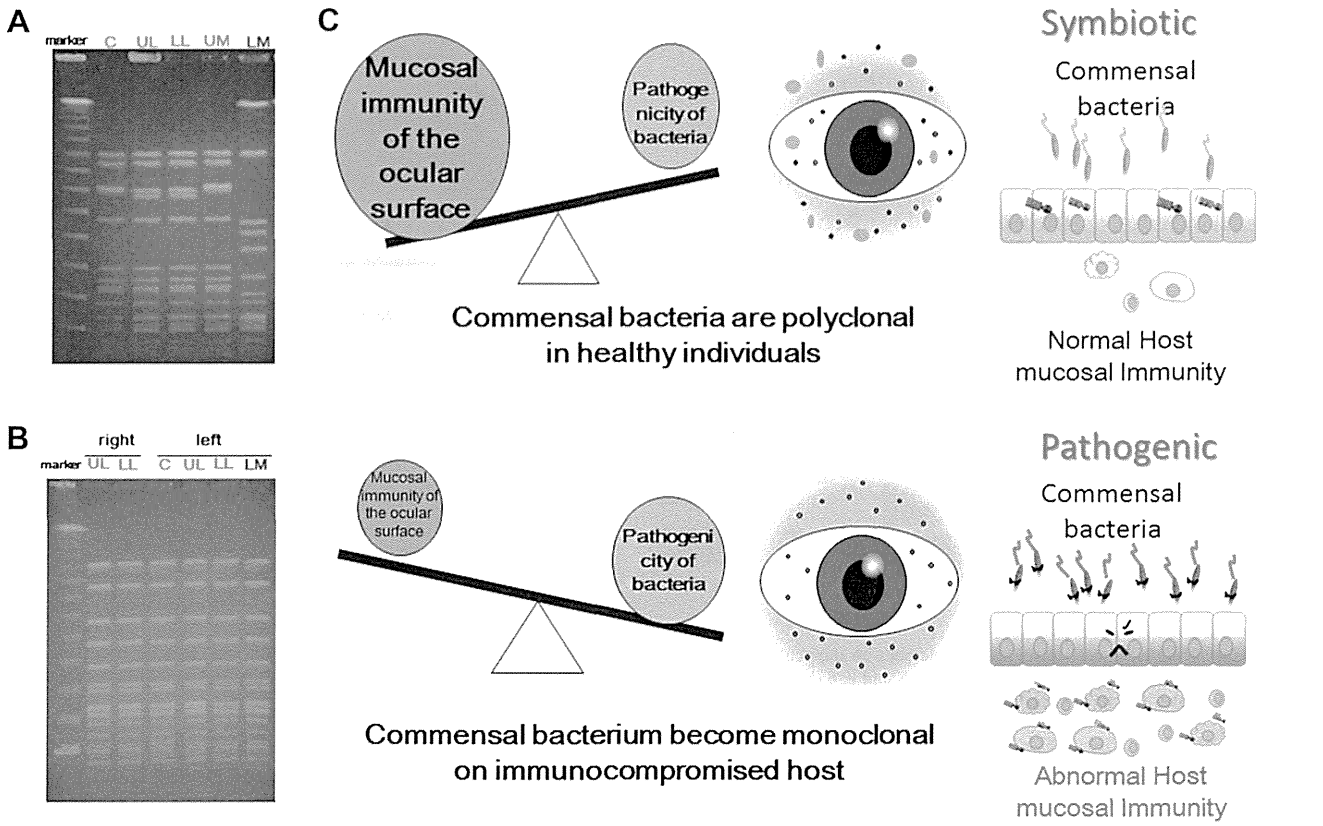

Fig. 3. Importance of the balance between mucosal immunity of the ocular surface and pathogenicity of bacteria. A. The PFGE patterns of *S. epidermidis* on healthy ocular surfaces were polyclonal. B. The PFGE patterns of *S. epidermidis* isolated from multiple sites in both eyes of an immunocompromised 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

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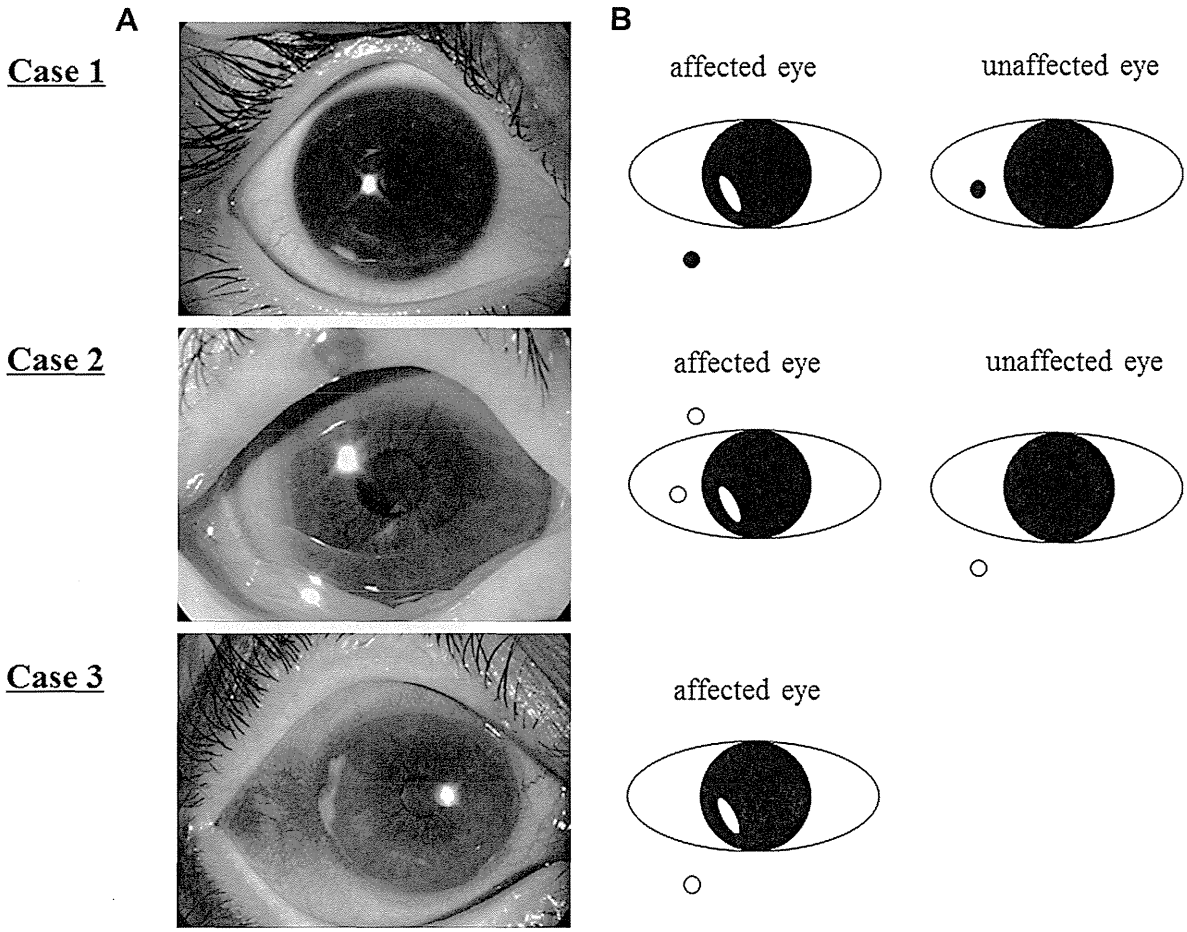

Fig. 4. Relationship between catarrhal ulcers and the presence of *S. aureus*. A. The diagnosis of catarrhal ulcer was based on ocular surface manifestations, i.e. oval infiltrates, ulcers separated from the limbus by a distinct lucid border, and adjacent conjunctival inflammation. B. The presence of *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;

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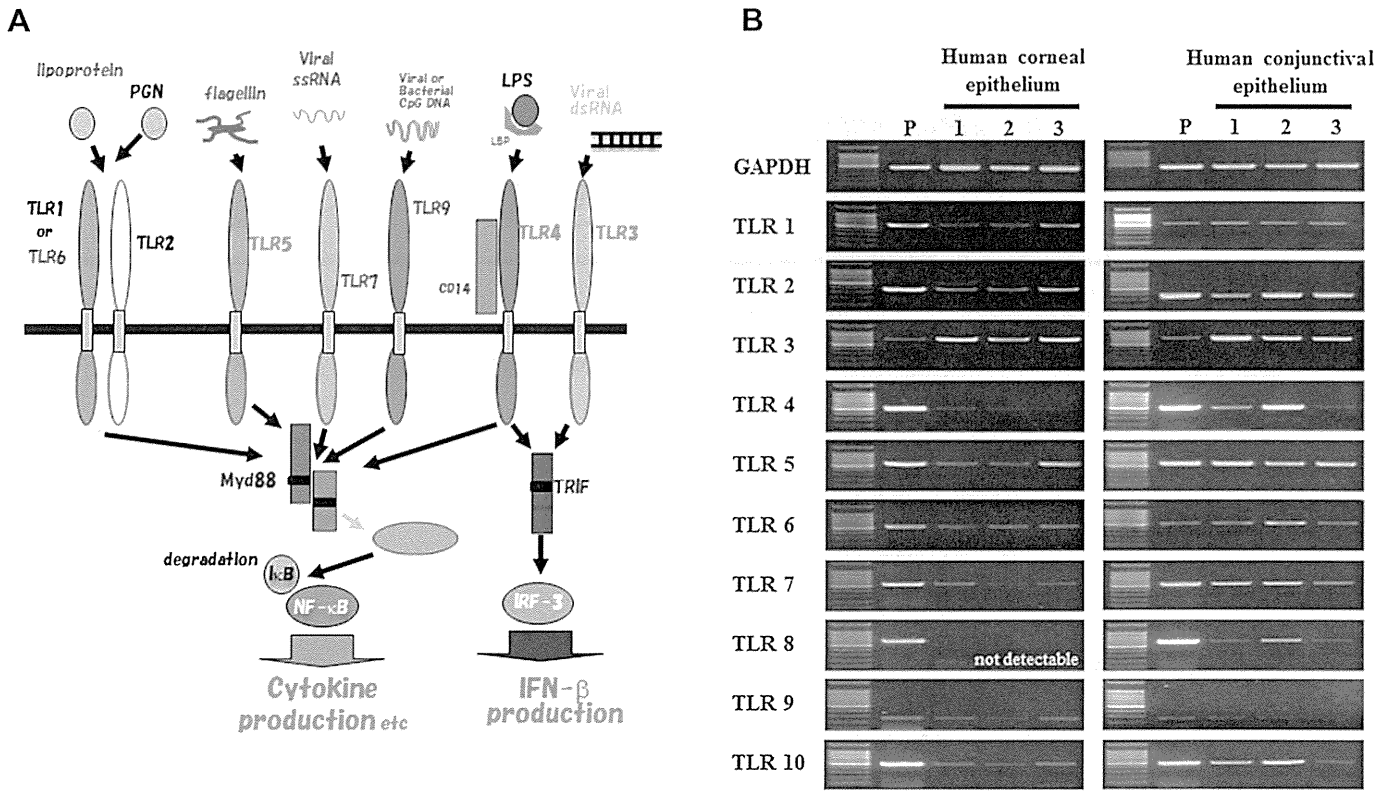

Fig. 5. A. Function of Toll-like receptors (TLRs) PGN: peptidoglycan LPS: lipopolysaccharides Myd88: myeloid differentiation factor 88 TRIF: TIR domain-containing adaptor-inducing IFN-β IRF-3: interferon regulatory factor 3. B. Human ocular surface epithelium expresses TLR-specific mRNA. The positive 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,

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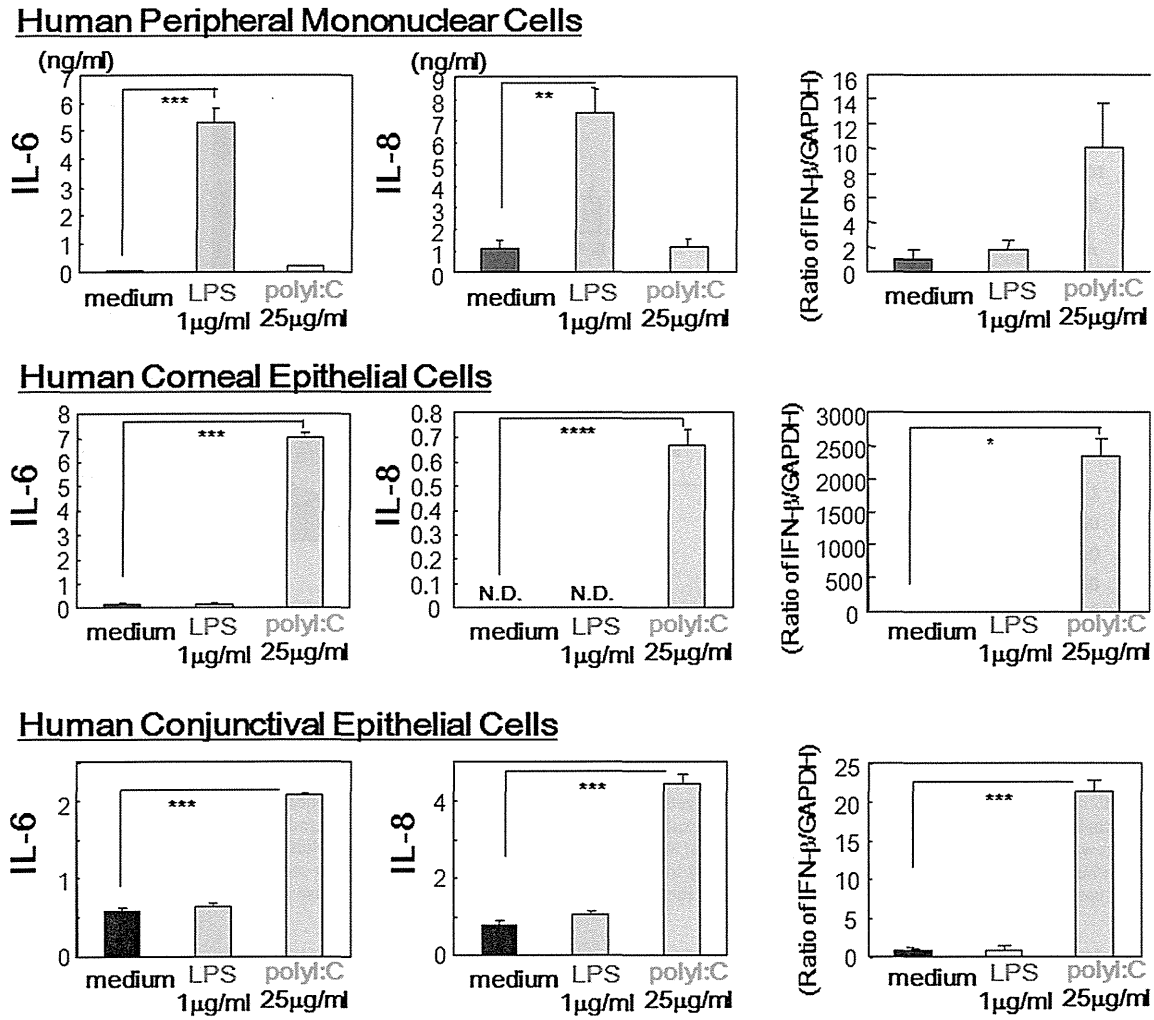

Fig. 6. Response of human peripheral mononuclear- and of primary human corneal- and -conjunctival epithelial cells to polyI:C, the TLR3 ligand, and LPS, the TLR4 ligand. Cultured cells were left untreated or exposed to polyI:C (25 μg/ml) or LPS from *P. aeruginosa* (1 μg/ml) for 24 h and assayed for 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

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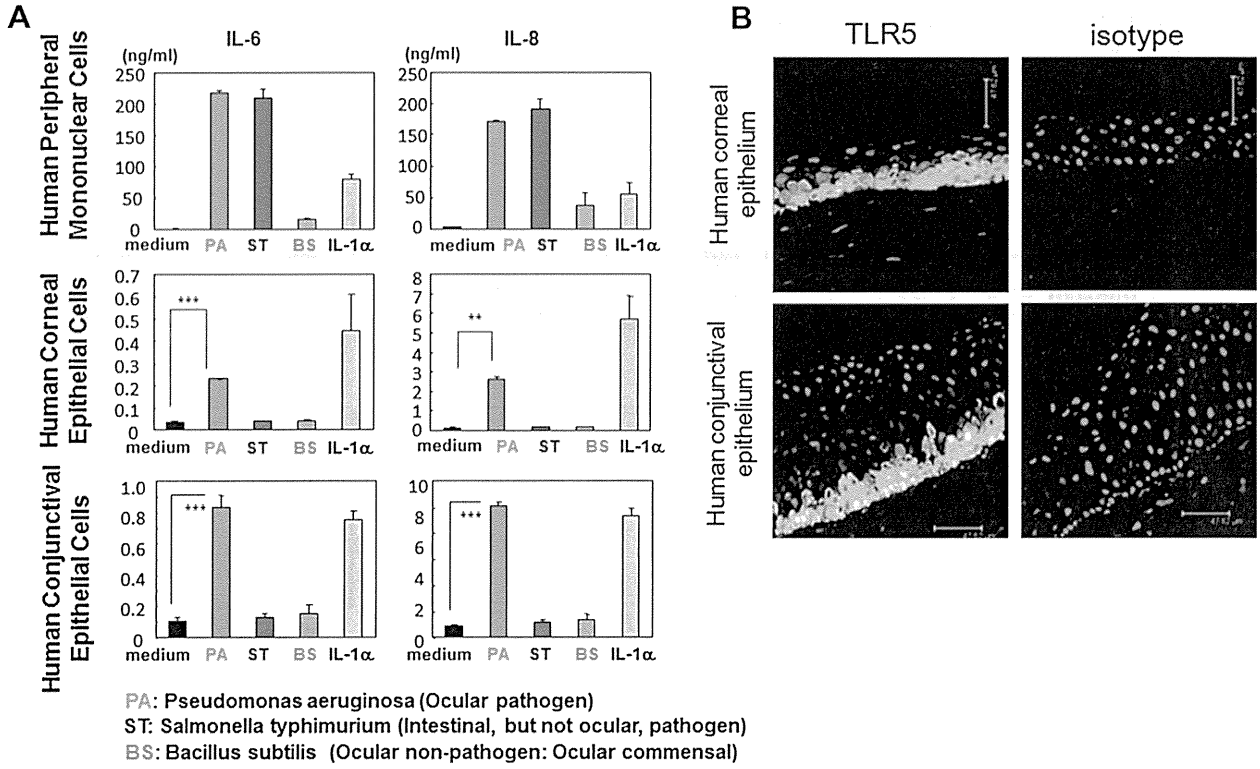

Fig. 7. Function of TLR5 in ocular surface. A. Responsiveness to various flagellins, which are TLR5 ligands, of human peripheral mononuclear cells and primary human corneal- and -conjunctival epithelial cells. Cultured cells were left untreated or exposed for 24 h to different flagellins (100 ng/ml). 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. Immunolocalization 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.

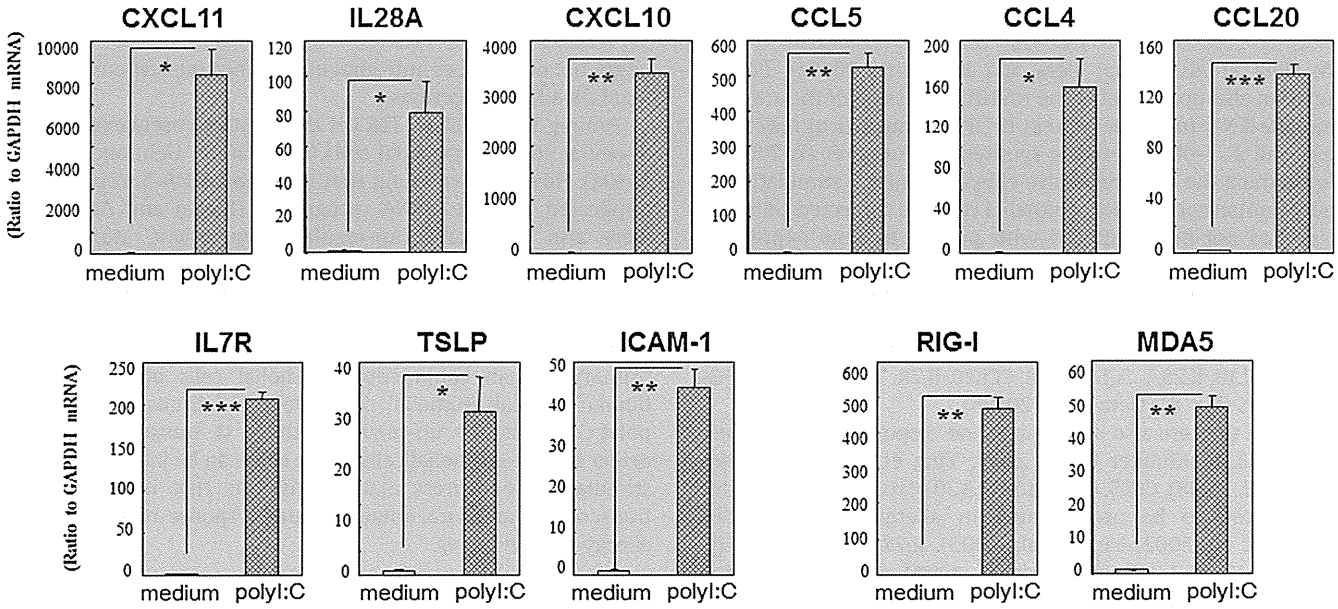

Fig. 8. mRNA expression of 11 transcripts in primary human conjunctival epithelial cells. The cells were exposed for 6 h to 25 μg/ml polyI:C. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The *Y* axis shows the increase in specific mRNA over unstimulated samples (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$). 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 antiviral 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-

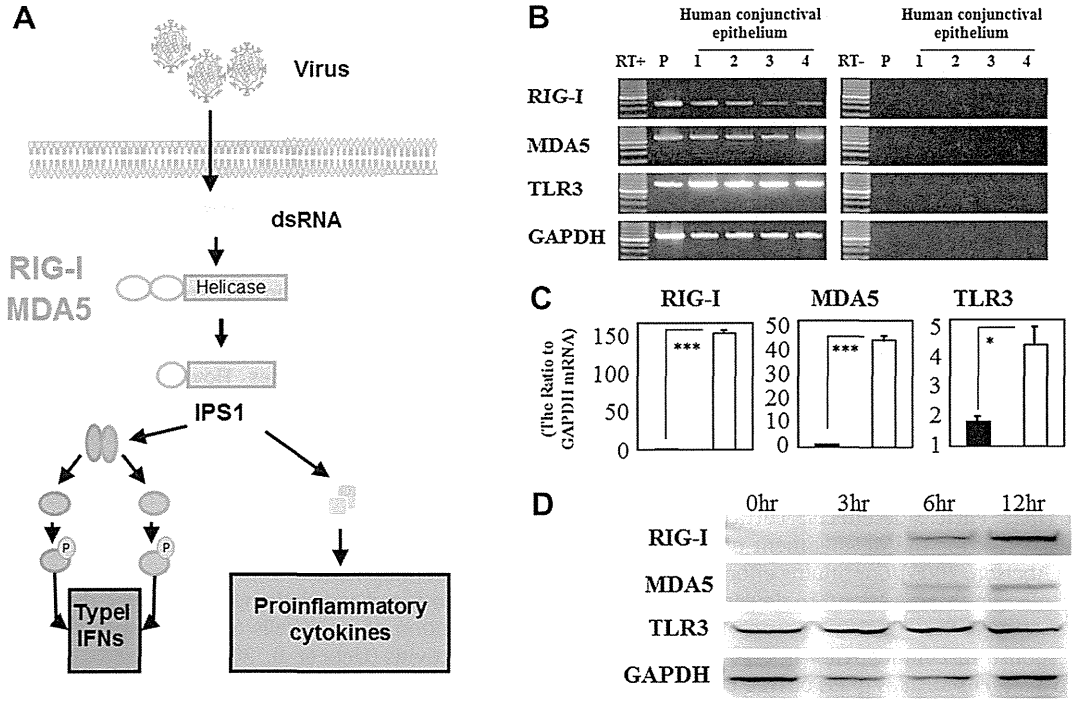

Fig. 9. A. RIG-I and MDA-5 are retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). B. Expression of RIG-I, MDA-5, and TLR3 mRNA in human conjunctival epithelial cells. The positive control (P) was mRNA isolated from human mononuclear cells (*, $p < 0.05$; ***, $p < 0.0005$). C. Up-regulation 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).

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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

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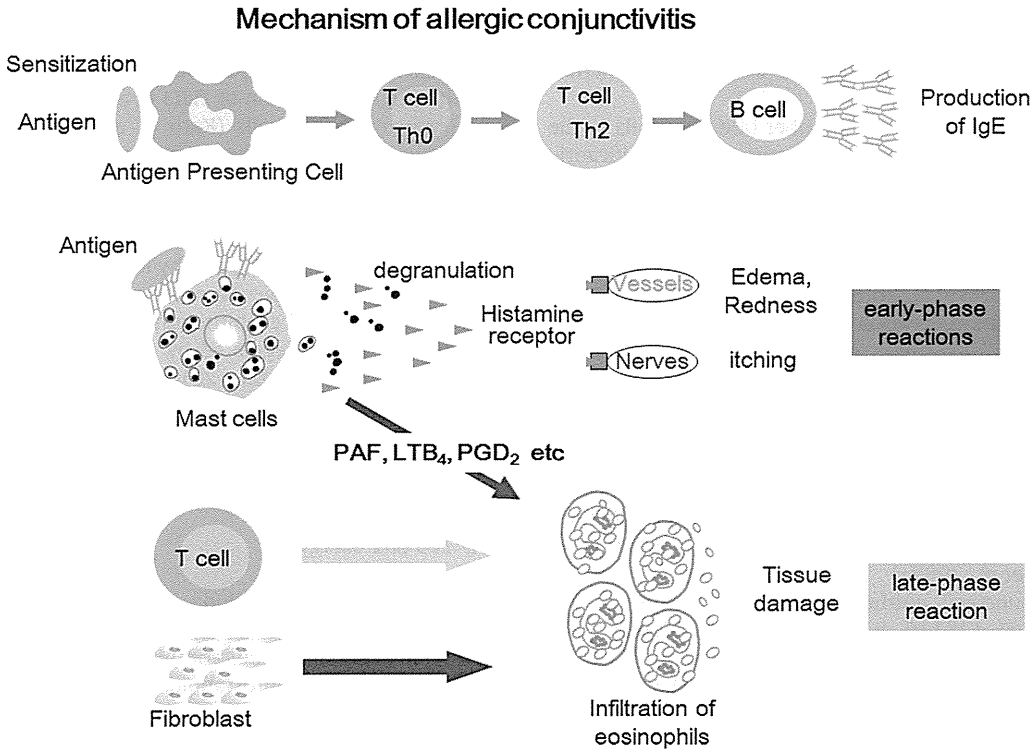

Fig. 10. Mechanism of allergic conjunctivitis. The allergic response is typically elicited to allergens that produce crosslinkage with membrane-bound IgE. This triggers mast cell degranulation and the release of a cascade of allergic and inflammatory mediators. The rapid release of histamine from mast 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

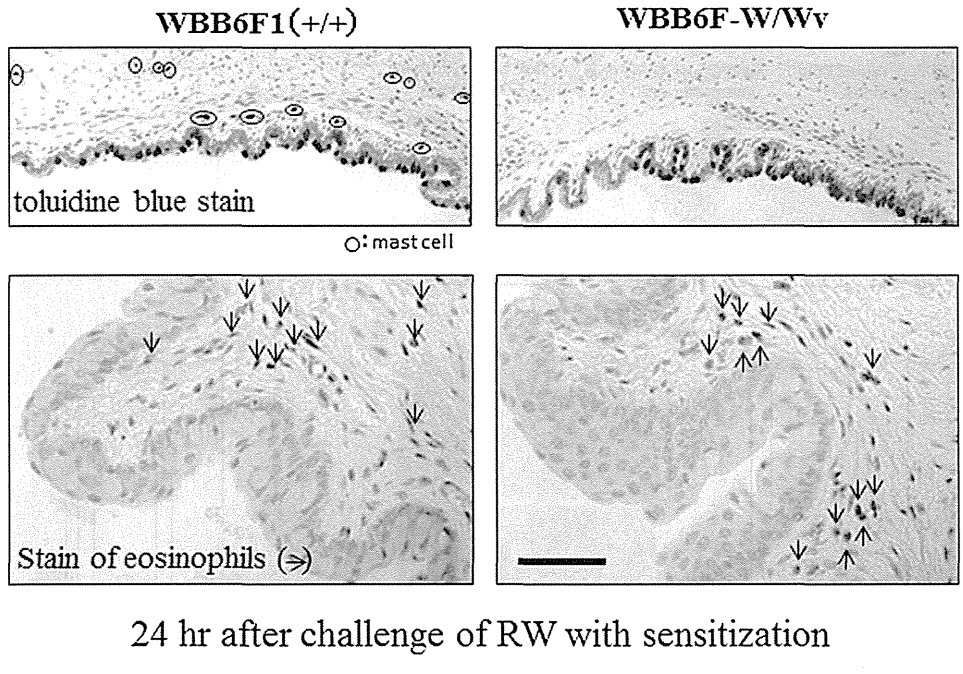

Fig. 11. Eosinophilic inflammation in the conjunctiva of mast cell-deficient mice. Mast cell-deficient mice (*WBB6F-W/Wv*) exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation similar to that seen in their congenic littermates (*WBB6F1* (+/+)). Bar = 50 μm.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

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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 lymphopoietin (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 conjunctival 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,

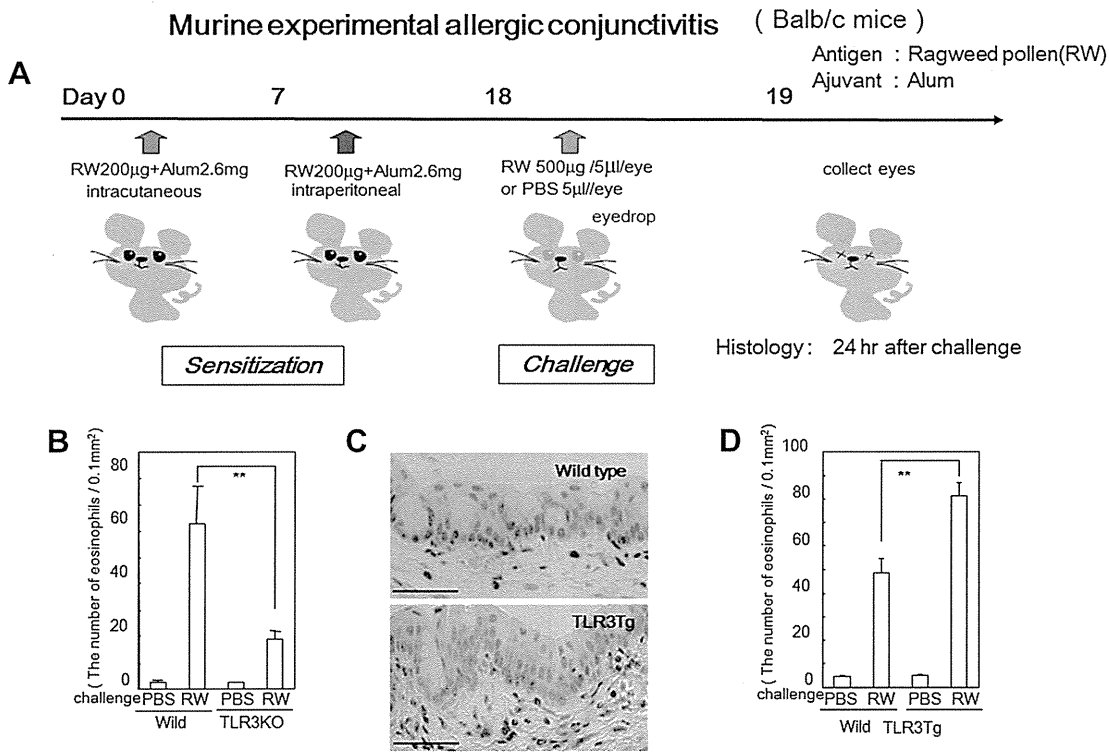

Fig. 12. A. Our balb/c mouse model of experimental allergic conjunctivitis (EAC). B. Reduced eosinophilic conjunctival inflammation in TLR3-KO mice. C. The infiltration of eosinophils into the conjunctiva of wild-type- and TLR3Tg-mice was detected with Luna's method. Scale bars, 50 μm. D. Pronounced 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).

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