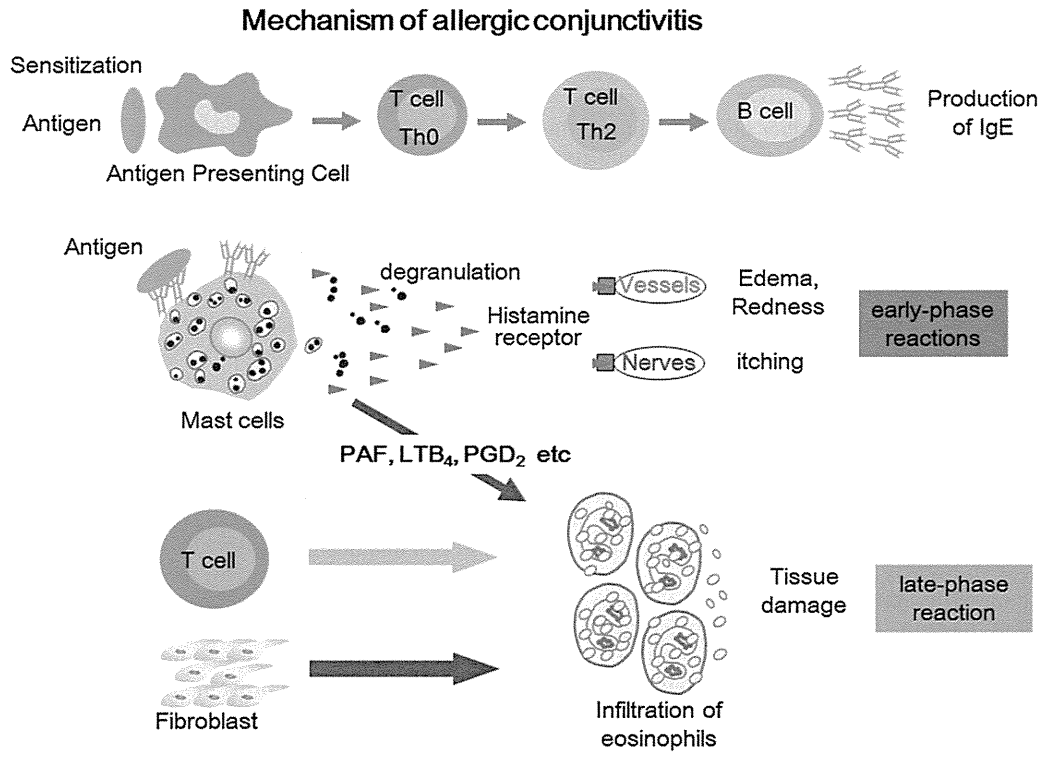

Fig. 10. Mechanism of allergic conjunctivitis. The allergic response is typically elicited to allergens that produce crosslinkage with membrane-bound IgE. This triggers mast cell degranulation and the release of a cascade of allergic and inflammatory mediators. The rapid release of histamine from mast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

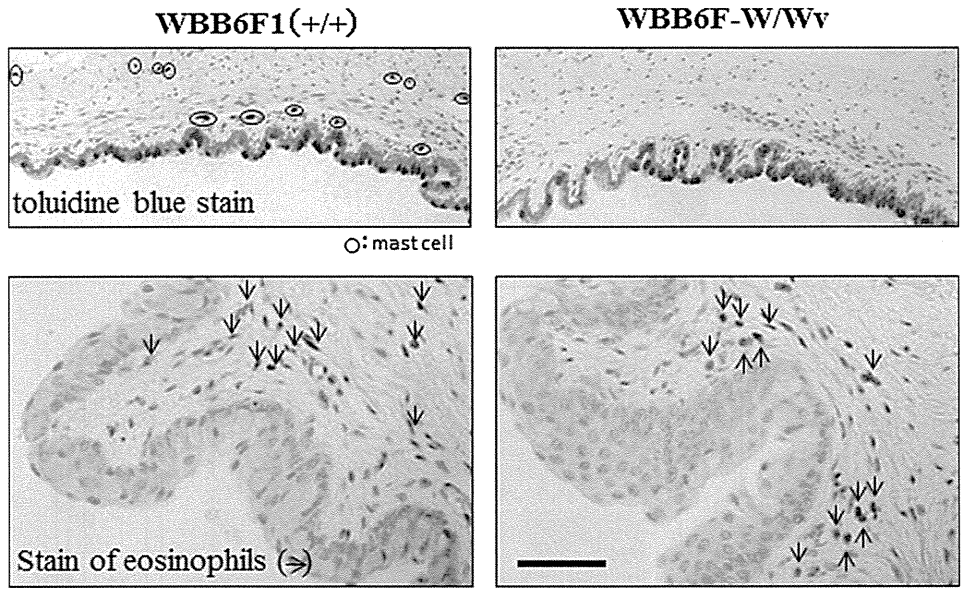

Fig. 11. Eosinophilic inflammation in the conjunctiva of mast cell-deficient mice. Mast cell-deficient mice (*WBB6F-W/Wv*) exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation similar to that seen in their congenic littermates (*WBB6F1* (+/+)). Bar = 50 μm.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

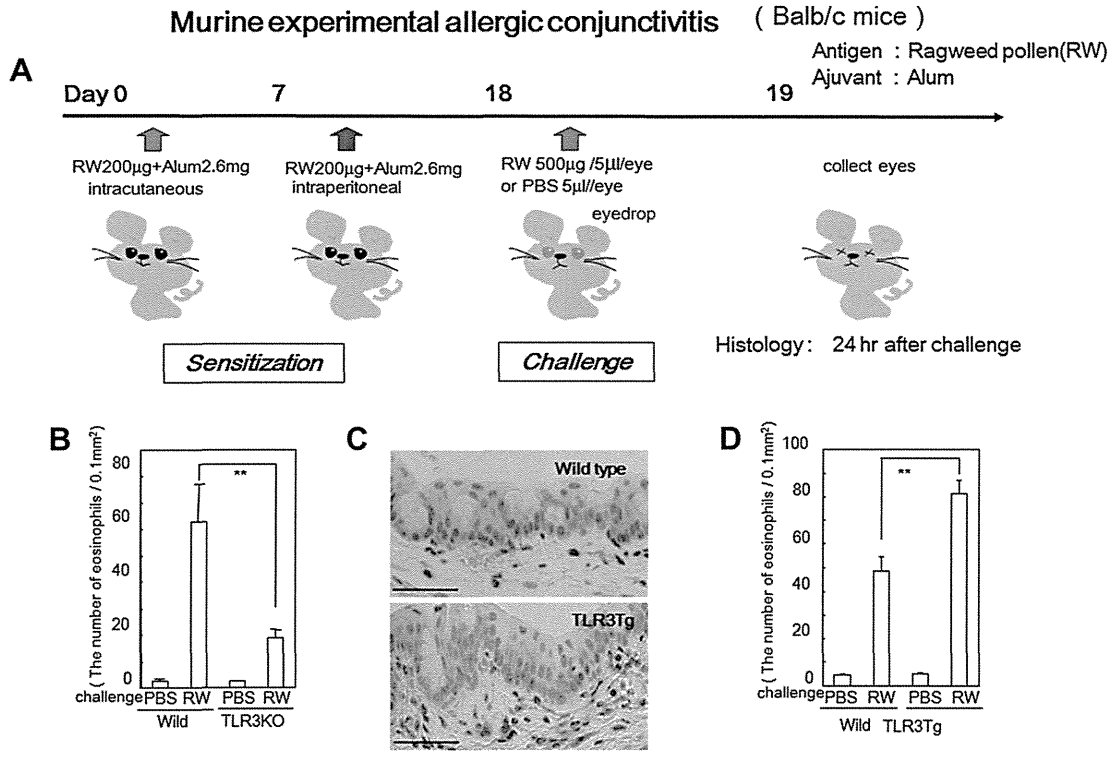

Fig. 12. A. Our balb/c mouse model of experimental allergic conjunctivitis (EAC). B. Reduced eosinophilic conjunctival inflammation in TLR3-KO mice. C. The infiltration of eosinophils into the conjunctiva of wild-type- and TLR3Tg-mice was detected with Luna's method. Scale bars, 50 μm. D. Pronounced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

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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

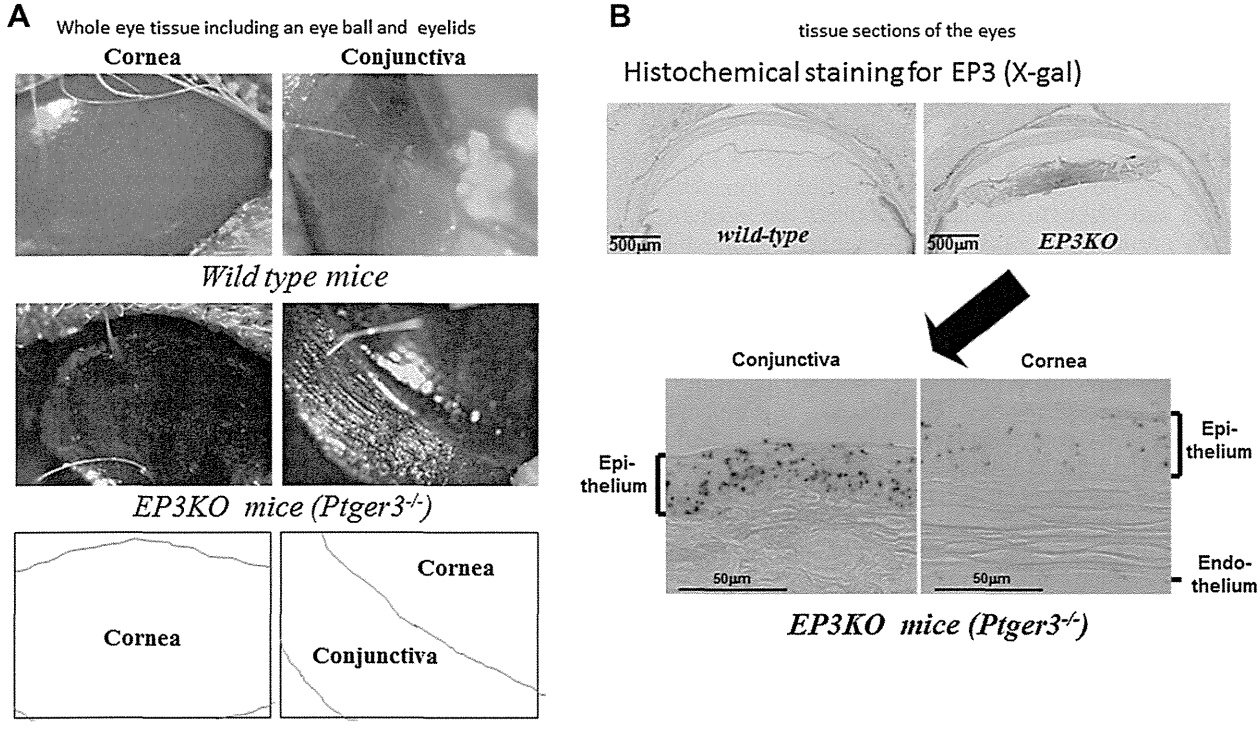

Fig. 13. Expression and localization of EP3 in the ocular surface (conjunctiva and cornea). A. X-gal staining of whole eye tissue (including the eye ball and eyelids). B. Histochemical staining for X-gal of tissues sections from the eyes. In EP3-KO mice (*Ptger3*$^{-/-}$) the β-galactosidase gene was '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).

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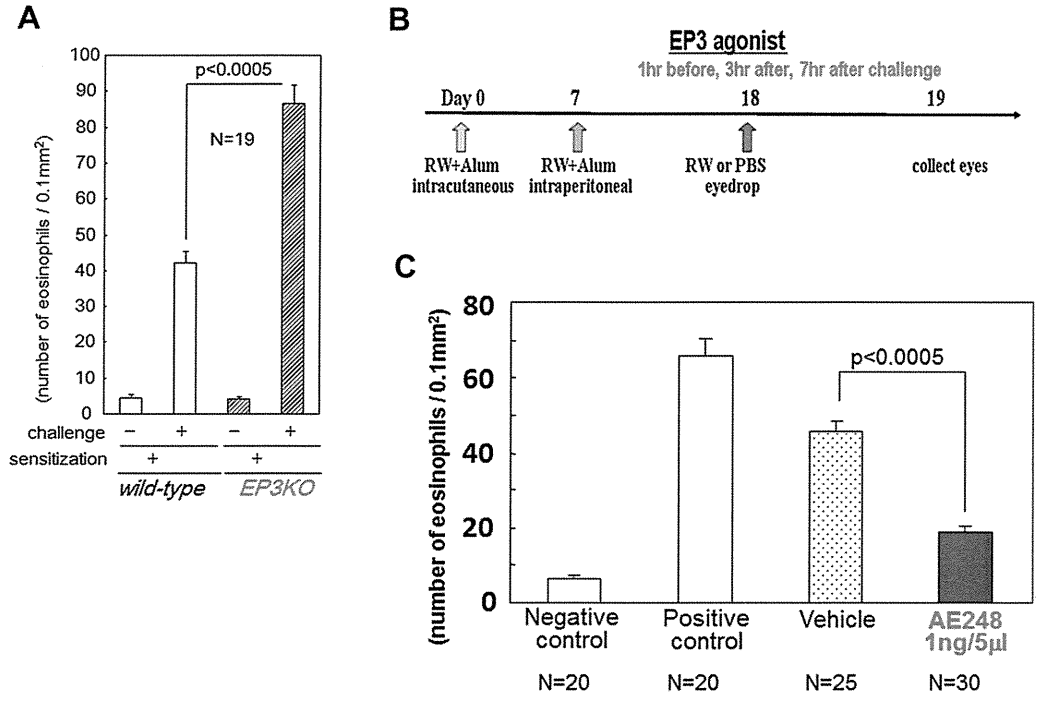

Fig. 14. A. Up-regulation of the number of eosinophils in the tarsal conjunctiva of EP3-KO mice (*Ptger3*$^{-/-}$). Eosinophils in the lamina propria mucosae of the tarsal conjunctiva were counted in wild-type- and EP3-KO-mice and the number of infiltrating eosinophils in sections containing the central 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).

PolyI: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

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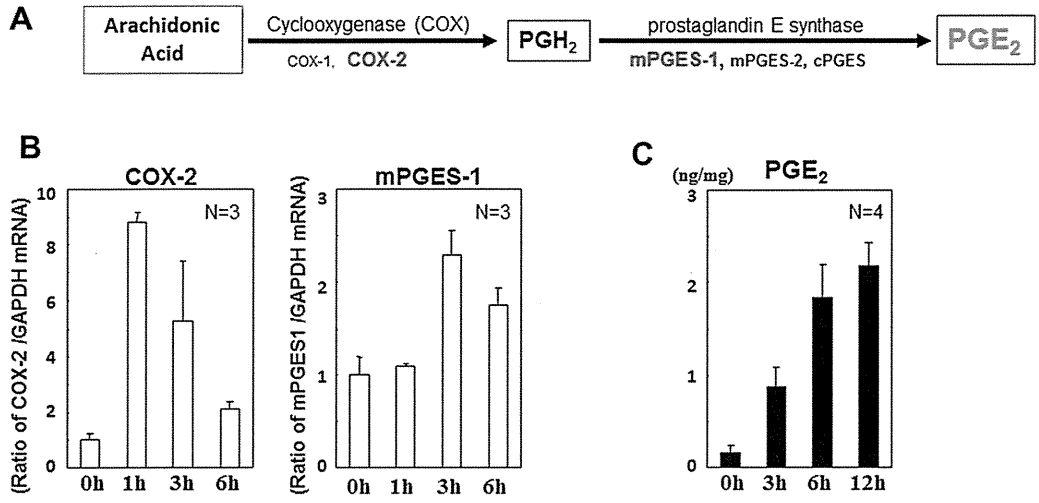

Fig. 15. RW challenge increased PGE_2 synthesis in mouse eyelids. A. PGE_2 synthesis pathway from arachidonic acid. B. Time-course of COX-2 and mPGES-1 mRNA expression in the eyelids during EAC. Relative mRNA levels of COX-2 and mPGES-1 in the eyelids after RW challenge are shown. The mRNA levels were 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 PGE_2 content in the eyelids during EAC. The PGE_2 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 IL-6 mRNA was dramatically increased, as was the 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).

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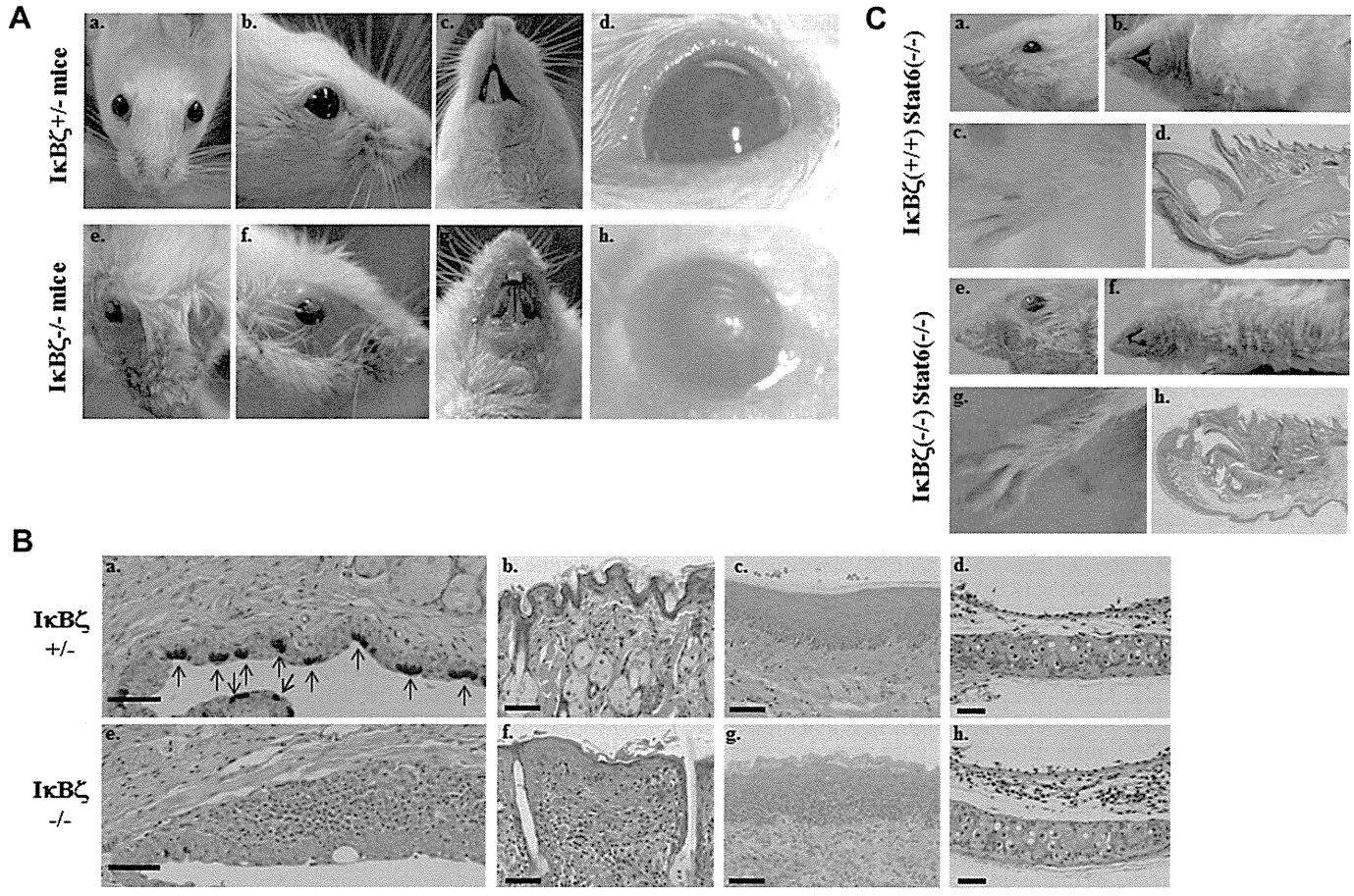

Fig. 16. Phenotype and histological findings in IκBζ-KO- and IκBζ/Stat6-DKO-mice. A. Phenotype of IκBζ KO mice. Photographs of the face and perioral skin of 32-week-old IκBζ+/− and IκBζ−/− mice taken 27 weeks after symptom onset. While IκBζ+/− mice were free of inflammation (a–d), IκBζ−/− mice exhibited 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).

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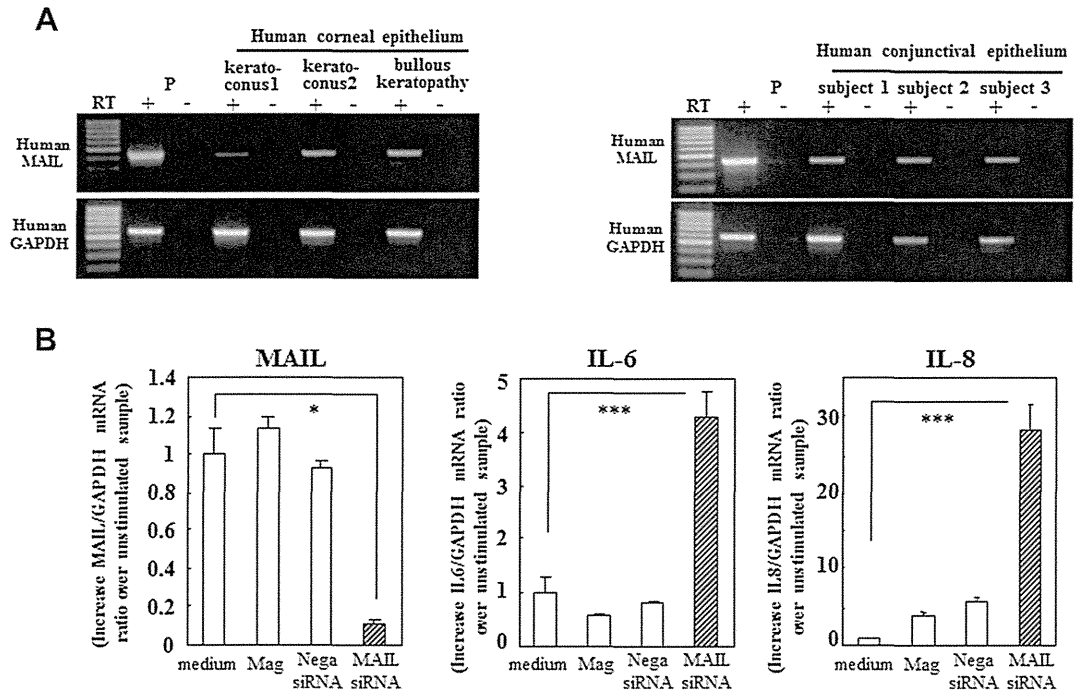

Fig. 17. Expression of MAIL (similar to mouse IκBζ)-mRNA on the human ocular surface. A. RT-PCR detected MAIL-specific mRNA in the human corneal and conjunctival epithelium. Human peripheral monocytes stimulated with 100 ng/ml LPS were the positive control. (P: adherent mononuclear cells stimulated with 100 ng/ml LPS). B. Knock down of MAIL enhanced the expression of IL-6- and IL-8-specific mRNA. For the transfection of small interfering RNA (siRNA), 1 μg/ml of the control- or targeting siRNA was transfected into primary human corneal epithelial cells using PolymagII (OZ BIOSCIENCES) according to the manufacturer's recommendations. Mag: only PolymagII, Nega siRNA: control siRNA. (*, $p < 0.05$; ***, $p < 0.005$). Reprinted with permission from Ueta et al. (Ueta et al., 2005b; Ueta and Kinoshita, 2010a).

Many patients encountered by ophthalmologists present in the chronic stage of SJS/TEN; dermatologists tend to see patients with SJS/TEN in the acute stage. The differential diagnosis of SJS or TEN may be difficult in the chronic stage of SJS/TEN because at that point the vesiculobullous skin lesions present in the acute stage have healed. Thus, ophthalmologists tend to diagnose both SJS and TEN as SJS in the broad sense. Our diagnosis of SJS/TEN (SJS in the broad sense) was based on a confirmed history of acute-onset high fever, serious mucocutaneous illness with skin eruptions, and involvement of at least 2 mucosal sites including the ocular surface (Sotozono et al., 2007, 2009a; Ueta and Kinoshita, 2010a; Ueta et al., 2007c, 2008b,, 2007e, 2008d; c; Ueta et al., 2007d, 2010c, 2007e, 2008d).

The pathobiological mechanisms underlying the onset of SJS/TEN have not been fully established. The extreme rarity of cutaneous and ocular surface reactions to drug therapies led us to suspect individual susceptibility (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007c, 2008b,, 2012b, 2008d; c; Ueta et al., 2007d, 2010c, 2007e, 2012b, 2008d).

6.2. HLA analysis of SJS with ocular surface complications

In 1982, ophthalmologists first reported that the HLA-Bw44 antigen, a subgroup of HLA-B12, was significantly increased in Caucasian patients with SJS with ocular involvement compared with a control Caucasian population. In that study, the onset of SJS with ocular involvement was associated with putative viral syndromes or the administration of drugs (Mondino et al., 1982). Dermatologists also found that the frequency of the HLA-B12 antigen was significantly increased in French SJS/TEN patients whose disorder was clearly drug-induced compared with a French control population; the main causative agents were non-steroidal anti-inflammatory drugs (NSAIDs) (Roujeau et al., 1986).

We examined HLA-class I (HLA-A, -B, -C) antigens in Japanese SJS patients with severe ocular surface complications (Ueta et al., 2007e, 2008d) and found that the carrier frequency of the HLA-A*0206 antigen was significantly higher in 110 SJS patients compared to 220 Japanese controls (carrier frequency: 45.5% vs 13.6%, $p = 0.0000000002$, odds ratio (OR) = 5.3, gene frequency: 23.6% vs 6.8%, $p = 0.0000000007$, OR = 4.2). However, HLA-A*0206, strongly associated with SJS/TEN with ocular complications in Japanese individuals, is absent in Caucasians (Ueta et al., 2007e, 2008d).

On the other hand, in our study, as in earlier reports (Mondino et al., 1982; Yetiv et al., 1980), the onset of SJS with severe ocular surface complications was associated with putative viral syndromes and/or the administration of drugs (mainly NSAIDs) (Ueta et al., 2007c, 2008b, 2007d, 2010c, 2007e). We found no association with HLA-B12 in Japanese SJS patients (Kaniwa et al., 2008; Ueta et al., 2007e, 2008d) although this antigen was significantly increased in Caucasian SJS patients (Mondino et al., 1982; Roujeau et al., 1986), probably because in Caucasians the HLA-B12 antigen is primarily coded by HLA-B*4402 whereas in Japanese it is almost exclusively coded by HLA-B*4403 (Tokunaga et al., 1997).

Thus, our findings suggest strong ethnic differences in the association of SJS/TEN and HLA (Ueta et al., 2007e, 2008d). Specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype because SJS/TEN is rare and it probably has a complex genetic inheritance background (Ueta et al., 2007e, 2008d).

In Han Chinese (Chung et al., 2004) but not in Caucasian patients (Lonjou et al., 2008, 2006) there was a strong carbamazepine-specific association between HLA-B*1502 and carbamazepine-induced SJS/TEN. Because the allele frequency of HLA-B*1502 is very low in the Japanese, the carbamazepine-specific association between HLA and carbamazepine-induced SJS may be specific for certain ethnic groups (Kaniwa et al., 2008; Ueta et al., 2008d).

Although an allopurinol-specific association between HLA-B*5801 and allopurinol-induced severe cutaneous adverse reactions

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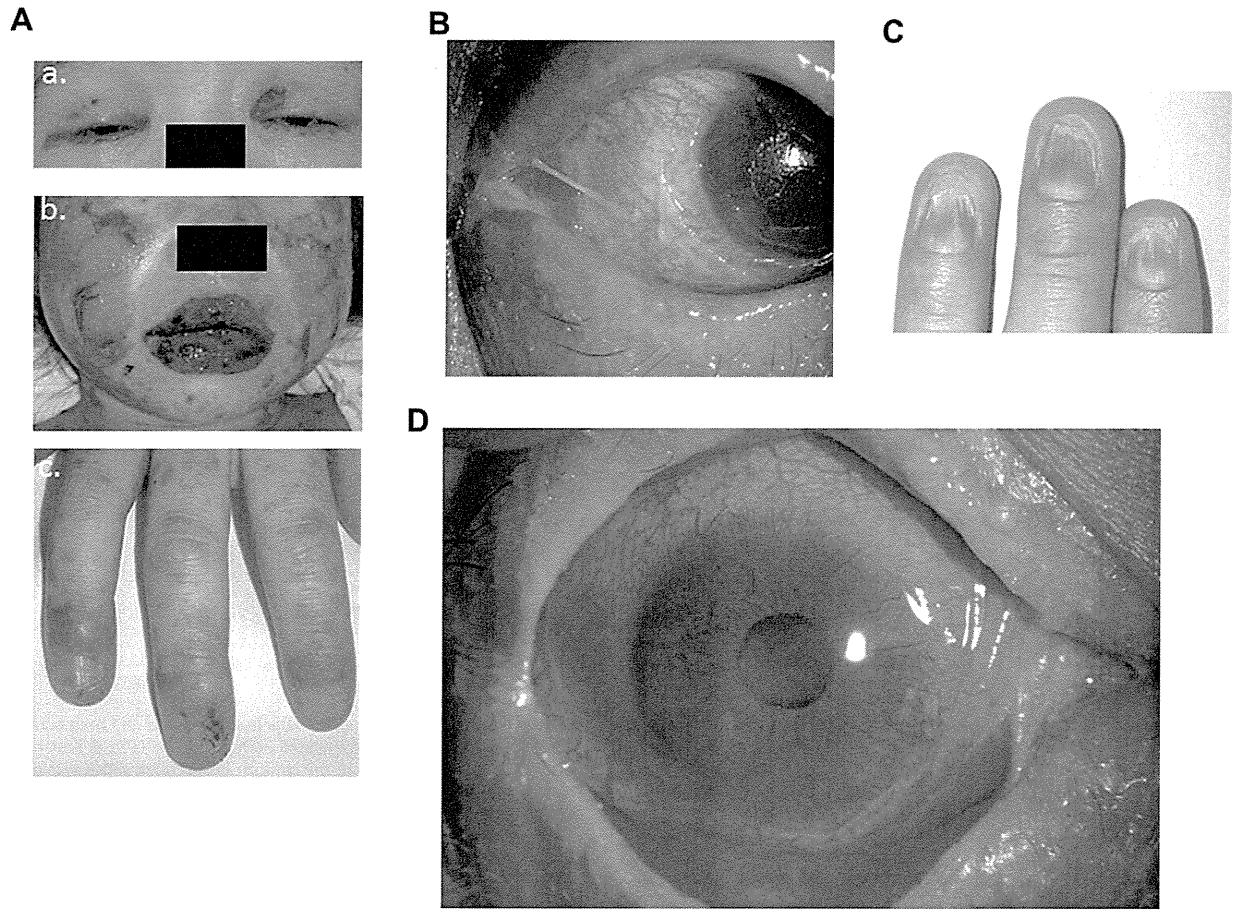

Fig. 18. Stevens-Johnson syndrome (SJS) with severe ocular surface complications. A. Typical features of SJS/TEN in the acute stage. a. Ocular surface inflammation with conjunctivitis and eyelids swelling. b. The face manifests swollen and crusted lips, blisters, and erosion of the skin. c. Paronychia. Reprinted with permission from Ueta et al. (Ueta and Kinoshita, 2010a). B. Ocular surface inflammation of SJS: severe conjunctivitis, pseudomembrane, epithelial defect, etc. C. Transformed fingernails in the chronic stage. D. Ocular surface complications in the chronic stage; conjunctival invasion into the cornea, symblepharon, trichiasis, and dry eye.

may be a universal phenomenon in all ethnic groups (Hung et al., 2005) allopurinol-induced severe adverse cutaneous reactions may not elicit serious sequelae on the ocular surface. In fact, few SJS patients with severe ocular surface complications manifested allopurinol-related SJS/TEN (Kaniwa et al., 2008).

Drugs are probably the most widely accepted etiologic factors in SJS/TEN (Levi et al., 2009; Mockenhaupt et al., 2008; Roujeau et al., 1995; Wolf et al., 2005). It is worth noting that our SJS/TEN patients with severe ocular surface complications often presented with prodromata including nonspecific fever, coryza, and sore throat that closely mimic upper respiratory tract infections commonly treated with antibiotics and NSAIDs (Ueta and Kinoshita, 2010a; Ueta et al., 2007d, 2010c). More than 80% of our SJS patients developed SJS after receiving treatment for the common cold with antibiotics, cold remedies, and/or NSAIDs; only about 5% progressed to SJS after drug treatment delivered to prevent the occurrence of convulsions (Ueta et al., 2010c).

6.3. Gene expression analysis of SJS with ocular surface complications

We have proposed the possibility of an association between a disordered innate immune response and SJS with severe ocular surface complication. Our hypothesis was based on the observation of an association between the onset of the SJS and microbial infections, because many SJS patients with severe ocular surface complications exhibited prodromata, including non-specific fever, coryza, and sore throat, ailments that closely mimic upper respiratory tract infections of viral or mycoplasma origin, which are commonly treated with antibiotics and NSAIDs (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007d, 2010c, 2012b). In addition, the SJS patients presented with opportunistic infection of the ocular surface by bacteria, especially methicillin-resistant *S. aureus* and *Staphylococcus epidermis* (MRSA and MRSE); the detection rate of MRSA and MRSE was higher on the ocular surface of SJS/TEN patients compared to individuals with other devastating ocular surface disorders (Sotozono et al., 2002). We posit that in SJS/TEN patients, opportunistic infections of the ocular surface by bacteria are ascribable to abnormalities in their innate immunity. Moreover, SJS/TEN patients presented with persistent inflammation of the ocular surfaces harboring commensal bacteria.

Under the hypothesis of a disordered innate immune response in SJS with ocular complications, we performed gene expression analysis of monocytes, which are essential in innate immunity. We found differences in IL4R gene expression; it was down-regulated in SJS/TEN patients upon LPS stimulation and slightly up-regulated in the controls (Fig. 19A) (Ueta, 2008). We also found that in human ocular surface (corneal and conjunctival) epithelial cells IL4R-specific mRNA was down-regulated upon stimulation with PolyI:C which mimics viral components (data not shown). This observation suggests that IL4R is linked with innate immunity (Ueta, 2008). We also found that after 1-hr culture without LPS, the expression of IκBζ- and IL-1α-specific mRNA was lower in monocytes from SJS/TEN patients than normal controls (Fig. 19B, C) (Ueta,

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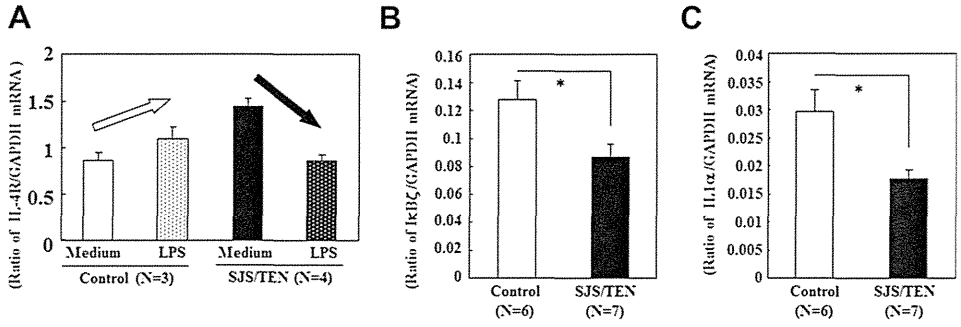

Fig. 19. Gene expression analysis of monocytes ($CD14^+$ cells) from SJS with ocular complications. A: Difference in IL4R gene expression between SJS patients and normal volunteers. $CD14^+$ cells from peripheral blood were subjected to gene expression analysis. The cells were cultured for 1 h with or without LPS. B, C. Low expression of IκBζ and IL-1α by isolated monocytes from SJS patients after 1-hr culture. Quantitative RT-PCR assay confirmed that IκBζ (B) and IL-1α (C) gene expression was significantly lower in cultured monocytes from 7 SJS/TEN patients than the 6 controls. Data show the mean ± SEM. (*, $p < 0.05$; ***, $p < 0.005$); evaluation was with Student's *t*-test using the Excel program. Reprinted with permission from Ueta et al. (Ueta, 2008).

2008). This suggests that the reduced expression of IκBζ and IL-1α genes may play an important role in the pathophysiology of SJS/TEN.

Possibly to prevent excessive inflammation in the presence of bacterial components, IκBζ induced by diverse pathogen-associated molecular patterns regulates NF-κB activity (Yamazaki et al., 2001). Elsewhere we documented that IκBζ gene-disrupted mice manifested ocular surface inflammation (Ueta et al., 2008a, 2005b) and that IκBζ in the ocular surface epithelium can suppress the production of pro-inflammatory cytokines such as IL-6 and IL-8 (Ueta and Kinoshita, 2010a). This suggests that the ocular surface epithelium suppresses inflammation via the expression of IκBζ (Ueta et al., 2008a, 2005b; Ueta and Kinoshita, 2010a).

We also reported that the TLR3 ligand, elicited the elevated expression of human IκBζ-specific mRNA in ocular surface epithelial cells (Ueta et al., 2005a). Because TLRs could induce the expression of IκBζ (Ueta et al., 2005a), the ocular surface inflammation seen in SJS/TEN patients may be related to innate pathogen-associated molecular pattern-amplified immune responses to microbes.

IL-1α was significantly lower and sIL-2R significantly higher in the blister fluid of TEN- than burn patients (Correia et al., 2002). We also detected a significant difference between SJS/TEN patients and the controls with respect to the expression of IL-1α by $CD14^+$ monocytes (Ueta, 2008; Ueta and Kinoshita, 2010a).

6.4. Single nucleotide polymorphism (SNP) analysis of SJS with severe ocular surface complications

6.4.1. The candidate gene approach

While the administration of some drugs may result in the development of SJS/TEN, not all patients taking these drugs develop SJS/TEN. As the incidence of SJS/TEN is very low, we suspected a genetic predisposition (Ueta, 2008; Ueta and Kinoshita, 2010a). We therefore performed SNP association analysis using candidate genes associated with innate immunity (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007d), allergy (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007c, 2008b), or apoptosis (Ueta et al., 2008c).

We first examined candidate genes associated with innate immunity. We investigated the IκBζ gene, which yielded different findings for SJS/TEN patients and controls in our gene expression analysis, and whose disruption results in ocular surface and skin inflammation. Another was the IL1α gene which, based on gene expression analysis, is also different in SJS/TEN patients and the controls. Other candidate genes were the TLR2 gene which is closely related to *S. aureus* and *S. epidermidis*, including MRSA and MRSE, and the TLR3 gene which is the gene most highly expressed on ocular surface epithelium among 1–10 TLRs and which responds to the virus dsRNA-mimic polyI:C to generate pro-inflammatory cytokines and IFN-β (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007d).

To investigate IκBζ we analyzed 7 polymorphisms (rs.2305991, rs.622122, rs.14134, rs.3217713, rs.595788, rs.677011, rs.3821727) in the Japanese Single Nucleotide Polymorphisms (JSNP) database (Ueta, 2008). We found that in 110 SJS patients and 220 controls there was no significant association among these SNPs. Regarding IL1α, we analyzed 5 SNPs (rs.1609682, rs.1894399, rs.2071373, rs.2071375, rs.2071376) reported in JSNP. Again, we found no significant association among these SNPs (Ueta, 2008). For TLR2 we analyzed 3 SNPs (rs.3840100, rs.3840099, rs.3840097) in JSNP and found no significant association among these SNPs (Ueta, 2008). However, our analysis of 7 SNPs for TLR3 (rs.3775290, rs.3775291, rs.3775292, rs.3775293, rs.3775294, rs.3775295, rs.3775296) (Ueta et al., 2007d) revealed that in 110 SJS patients and 220 controls, SNP rs.3775296 showed a significant association under a recessive model (rs.3775296T/T vs T/G + G/G, p value = 0.00004, OR = 4.3) and a weak association with allele frequency (T vs G, p value = 0.006, OR = 1.6) and SNP rs.3775290 also showed a significant association under a recessive model (rs.3775290 A/A vs A/G + G/G, p value = 0.007, OR = 2.3) (Fig. 20). Thus, our findings suggested that polymorphisms in the TLR3 gene are associated with SJS with severe ocular surface complications in the Japanese population (Ueta et al., 2007d).

We have reported that human ocular surface epithelial cells strongly expressed TLR3 and that its ligand, polyI:C could induce various molecules such as pro-inflammatory cytokines and anti-viral- and allergy-related-molecules (Ueta et al., 2005a, 2010b; Ueta and Kinoshita, 2010a, 2010b). Elsewhere we offered the hypothesis that viral infection and/or drugs may trigger a disorder in the host innate immune response and that this event is followed by aggravated inflammation of the mucosa, ocular surface, and skin (Ueta, 2008; Ueta and Kinoshita, 2010a; Ueta et al., 2007d, 2012b).

Next we examined candidate genes associated with allergy. Our gene expression analysis had shown that with respect to the IL4R gene there are differences between SJS patients and the controls (Ueta, 2008). This gene is essential for both IL-4 and IL-13 signaling because it is a component of IL-4 and IL-13 receptors. We analyzed Gln551Arg (rs.1801275), Ile50Val (rs.1805010), and Ser478Pro (rs.1805015) polymorphisms of IL4R as they are associated with allergic diseases such as asthma (Ueta et al., 2007c, 2008b). We found no significant association between Ile50Val (rs.1805010), and Ser478Pro (rs.1805015) (Ueta et al., 2007c, 2008b). On the other hand, Gln551Arg was significantly associated with allele frequency

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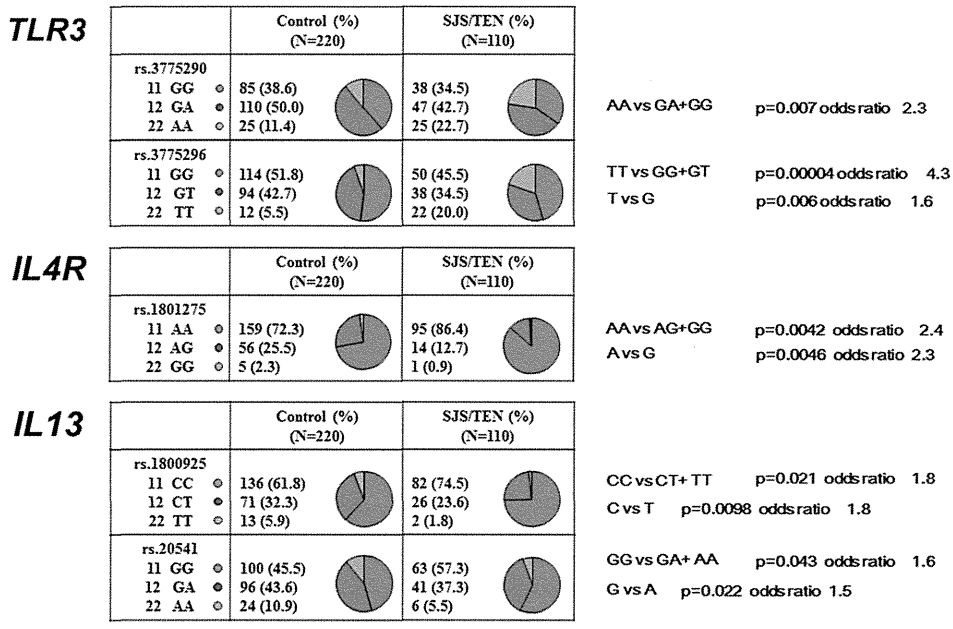

Fig. 20. Association between TLR3, IL4R, and IL13 SNPs and SJS with severe ocular surface complications. Using the candidate gene approach we identified SNPs of TLR3, IL4R and IL13 genes that were associated with SJS with severe ocular surface complications.

(A vs G, p value = 0.0046, OR = 2.3) and the dominant model (A/A vs A/G + G/G, p value = 0.0042, OR = 2.4) in the 110 SJS patients and the 220 controls (Fig. 20)(Ueta et al., 2007c, 2008b).

We also investigated IL13 and IL4, ligands of IL4R. With respect to the IL13 gene we analyzed polymorphisms of the promoter −1111C/T SNP (rs.1800925) and the Gln110Arg SNP (rs.20541); they are associated with allergic diseases such as asthma. There was a significant association of the promoter −1111C/T SNP with allele frequency (C vs T, p value = 0.0098, OR = 1.8) in all 110 SJS patients and the 220 controls; the Gln110Arg SNP exhibited a significant association with allele frequency (G vs A, p value = 0.022, OR = 1.5)(Fig. 20). We detected a significant increase in Arg110 in our SJS/TEN patients (Ueta et al., 2008b), although Gln110 was significantly increased in patients with asthma (Heinzmann et al., 2000).

With respect to the IL4 gene we analyzed polymorphisms of the promoter −590C/T (rs.2243250) related to higher IgE levels. We found no significant association between the SJS patients and the controls (Ueta et al., 2008b).

Lastly we examined FasL genes, the candidate genes associated with apoptosis; they have been reported to be increased in the serum of SJS/TEN patients in the acute stage (Abe et al., 2003). We examined 4 SNPs (rs.929087, rs.2639614, rs.2859247, rs.3830150) and found that rs.3830150 A/G (intron) showed a weak association with the dominant model (A/G + G/G vs A/A, p value = 0.015, OR = 1.8) in 110 SJS patients and 220 controls (Ueta et al., 2008c).

In summary, we found that TLR3 rs.3775296 SNP, IL4R SNP rs.1801275 (Gln551Arg), and IL13 rs.20541 (Arg110Gln) were significantly associated with SJS/TEN with ocular surface complications (Ueta, 2008; Ueta and Kinoshita, 2010a).

6.4.2. Genome-wide association study (GWAS)

To elucidate the pathophysiology of SJS with severe ocular surface complications in more detail we performed GWAS of more than 10^5 SNPs. GWAS permits the identification of genetic loci and genes associated with complex human traits without bias or *a priori* knowledge of the function or involvement of genes in the disease pathway. GWAS detected 3 SNPs (rs1325975: chr6, rs17131450: chr1, rs11238074: chr11) that were significantly associated with SJS with severe ocular surface complications. Because 2 of the SNPs (rs1325975 and rs11238074) were from the "gene desert" region, we focused on a SNP (rs17131450) that mapped close to the *PTGER3* gene, which is the gene of *EP3* protein of human, located in the 1p31 region of the human genome (Ueta et al., 2010c).

Based on our GWAS results we performed fine-mapping analysis of the *PTGER3* region using a custom DNA array to analyze the SNPs in and near *PTGER3* gene through the two major linkage disequilibrium (LD) blocks of the HapMap Japanese (JPT) plus the Han Chinese (CHB) population. The rs17131450 SNP showing a significant association with SJS in the GWAS also showed a significant association ($p < 0.01$) in our fine-mapping analysis. We also identified 5 other significantly associated ($p < 0.01$) SNPs in *PTGER3* gene (rs5702, rs1325949, rs7543182, rs7555874, and rs4147114) (Ueta et al., 2010c). One of the 6 SNPs in *PTGER3* gene (rs5702) was in an exon as a silent SNP (sSNP), four (rs1325949, rs7543182, rs7555874, rs4147114) were in introns (iSNPs), and the remaining SNP (rs17131450) was a genome SNP (Ueta et al., 2010c). Lastly we assessed the association of the 6 SNPs by direct sequencing (Ueta et al., 2010c). A summary of our case–control analysis based on sequence data from 110 SJS patients and 220 control subjects is shown in Fig. 21. Based on our GWAS and direct sequencing analysis we identified 6 SNPs associated with SJS/TEN, 5 of these were located within the *PTGER3* gene (Ueta et al., 2010c).

Because EP3, which is the protein of PTGER3 gene, is constitutively expressed in mouse conjunctival epithelial cells (Ueta et al., 2009a) we examined its expression in normal human conjunctival epithelial cells. RT-PCR assay showed that normal human conjunctival epithelial cells expressed *PTGER3* mRNA and immunohistochemistry disclosed the presence of EP3 protein (Ueta et al., 2010c, 2011d). When we looked for the expression of EP3 in the conjunctival epithelium of SJS/TEN patients with severe ocular surface complications we did not find EP3 protein. On the other hand, the protein was present in the control conjunctival epithelium from patients with conjunctivochalasis or pterygium (Ueta et al., 2010c, 2011d).

In support of the genetic association of *PTGER3* gene polymorphisms and SJS with severe ocular surface complications, we found that compared to the controls, the expression of EP3 protein

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	Control (%) (N=220)	SJS/TEN (%) (N=110)	
rs17131450 11 CC 12 CT 22 TT	193 (87.7) 26 (11.8) 1 (0.5)	84 (76.4) 20 (18.2) 6 (5.5)	CC vs CT+TT p=0.008 odds ratio 0.4 TT vs CT+CC p=0.003 odds ratio 12.6 T vs C p=0.00057 odds ratio 2.5
rs5702 11 CC 12 CT 22 TT	108 (49.1) 95 (43.2) 17 (7.7)	72 (65.5) 28 (25.5) 10 (9.1)	CC vs CT+TT p=0.005 odds ratio 2.0 C vs T p=0.04 odds ratio 1.5
rs1325949 11 AA 12 AG 22 GG	104 (47.3) 98 (44.5) 18 (8.2)	76 (69.1) 25 (22.7) 9 (8.2)	AA vs AG+GG p=0.0002 odds ratio 2.5 A vs G p=0.003 odds ratio 1.8
rs7543182 11 GG 12 GT 22 TT	111 (50.5) 94 (42.7) 15 (6.8)	78 (70.9) 23 (20.9) 9 (8.2)	GG vs GT+TT p=0.0004 odds ratio 2.4 G vs T p=0.0075 odds ratio 1.7
rs7555874 11 GG 12 GA 22 AA	111 (50.5) 94 (42.7) 15 (6.8)	77 (70.0) 24 (21.8) 9 (8.2)	GG vs GA+AA p=0.0007 odds ratio 2.3 G vs A p=0.01 odds ratio 1.7
rs4147114 11 CC 12 CG 22 GG	53 (24.1) 118 (53.6) 49 (22.3)	48 (43.6) 46 (41.8) 16 (14.5)	CC vs CG+GG p=0.0003 odds ratio 2.4 C vs G p=0.0009 odds ratio 1.8

Fig. 21. Association between PTGER3 SNPs and SJS with severe ocular surface complications. Using the genome wide association study (GWAS) we found that 6 SNPs of the *PTGER3* gene were associated with SJS with severe ocular surface complications.

was greatly reduced in the conjunctival epithelium of these patients. This suggests that EP3 contributes functionally to the pathogenesis of SJS/TEN (Ueta et al., 2010c, 2011d).

Based on the finding that more than 75% of our SJS patients had used cold medications, possibly including NSAIDs, before the onset of their disease we posited that the observed *PTGER3* polymorphisms are associated with a NSAID-related susceptibility to SJS with severe ocular surface complications (Ueta et al., 2010c). Drugs are probably the most widely accepted etiologic factor for SJS (Roujeau et al., 1995); in fact, many patients who develop SJS with severe ocular involvement do so after taking remedies for the common cold or NSAIDs, drugs that inhibit the production of the EP3 ligand, PGE_2. This observation supports the hypothesis that EP3 is involved in the development of SJS with severe ocular surface involvement.

6.4.3. *Interaction between the TLR3 and the EP3*

We reported that polymorphisms in *PTGER3*, the gene of EP3, were significantly associated with SJS with severe ocular surface complications (Ueta et al., 2010c), that PGE_2 is a ligand for EP3 in

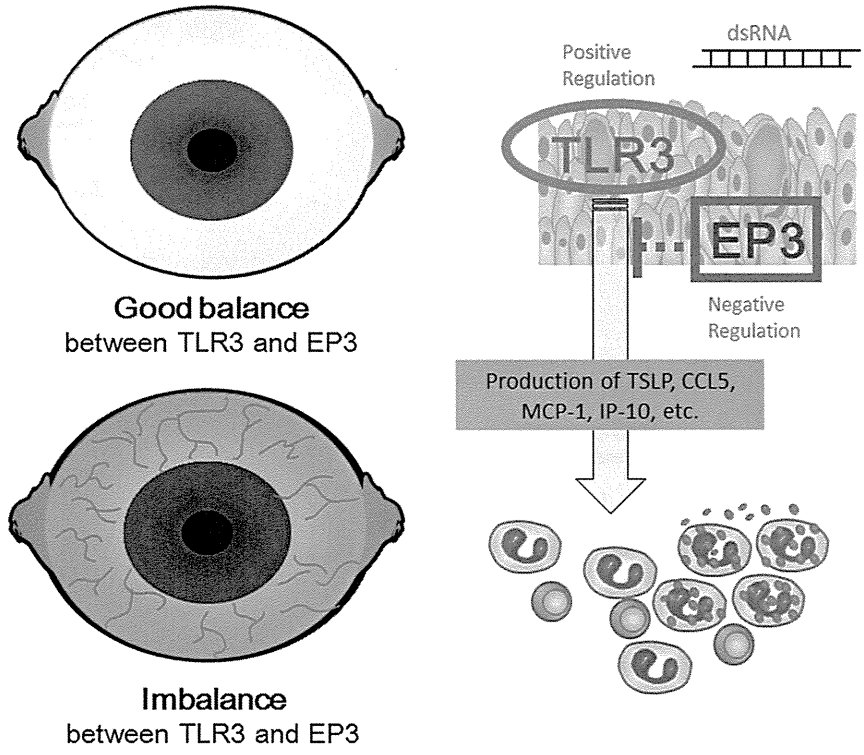

Fig. 22. Lack of balance between TLR3 and EP3 might trigger ocular surface inflammation.

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the conjunctival epithelium, and that the PGE_2-EP3 pathway down-regulates the progression of murine EAC (Ueta et al., 2009a). We also documented that TLR3 polymorphisms are associated with SJS (Ueta et al., 2007d), that the human ocular surface epithelium strongly expresses TLR3, and that cytokine production is up-regulated by polyI:C, a TLR3 ligand (Ueta, 2008; Ueta et al., 2005a; Ueta and Kinoshita, 2010a). Based on these findings we examined the function of EP3 in polyI:C-stimulated primary human conjunctival epithelial cells using an EP3 agonist. We found that the agonist significantly suppressed the production and mRNA expression of CCL5, CXCL10, CXCL11, IL-6, TSLP, and MCP-1 in polyI:C-stimulated primary human conjunctival epithelial cells, suggesting that cytokine production by conjunctival epithelial cells in response to polyI:C stimulation can be suppressed through the activation of EP3 (Ueta et al., 2011b, c; Ueta et al., 2012a).

In the past decade, SNPs were widely used as genetic markers for identifying human disease-susceptibility genes. It is now apparent that gene–gene interactions should be considered in addition to major single-locus effects (Cordell, 2009). In particular, non-additive (epistatic) models for some complex diseases fit with actual observations, suggesting interactions involving multiple loci (Ritchie et al., 2001). We performed a statistical search for interactions between all possible pairs of loci by applying high-dimensional variable selection methods to the comprehensive dataset obtained from our previous studies that involved a total of 14 immune-related genes including *PTGER3 and TLR3*. We found a variable with susceptible effects on SJS; these effects were involved in locus-pairs of *PTGER3-TLR3*. The *PTGER3* rs.4147114G/C SNP and the *TLR3* rs.3775296T/T SNP exhibited a higher odds ratio (OR: 25.3, $p = 0.0000527$) than only *TLR3* rs.3775296T/T SNP (OR: 5.35, $p = 0.00025$) or only *PTGER3* rs.4147114G/C SNP (OR: 2.66, $p = 0.0023$) (Ueta et al., 2012b).

Next we focused on the epistatic interaction between *PTGER3* and *TLR3* and analyzed an additional 32 SNPs of *PTGER3* and 10 SNPs of *TLR3* (a total of 38 SNPs of *PTGER3* and 17 SNPs of *TLR3*). We found that besides the previously reported 6 *PTGER3*- and 2 *TLR3*-SNPs, 14 additional *PTGER3* SNPs and 5 additional *TLR3* SNPs were associated with SJS with severe ocular surface complications (Ueta et al., 2012b).

Elsewhere we showed that conjunctival eosinophilic infiltration in EAC was significantly more marked in EP3-KO mice (Ueta et al., 2009a) and significantly less marked in TLR3-KO mice than in wild-type mice (Ueta et al., 2009c). We also reported that in EP3/TLR3-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; it was significantly lower than in EP3-KO- and wild-type-mice (Ueta et al., 2012b). These findings suggest that in EAC, EP3 negatively regulates the eosinophilic infiltration induced by TLR3 (Ueta et al., 2012b).

Thus, we provide evidence that there are functional interactions between TLR3 and EP3 that exert susceptibility effects with respect to SJS with severe ocular surface complications and that the

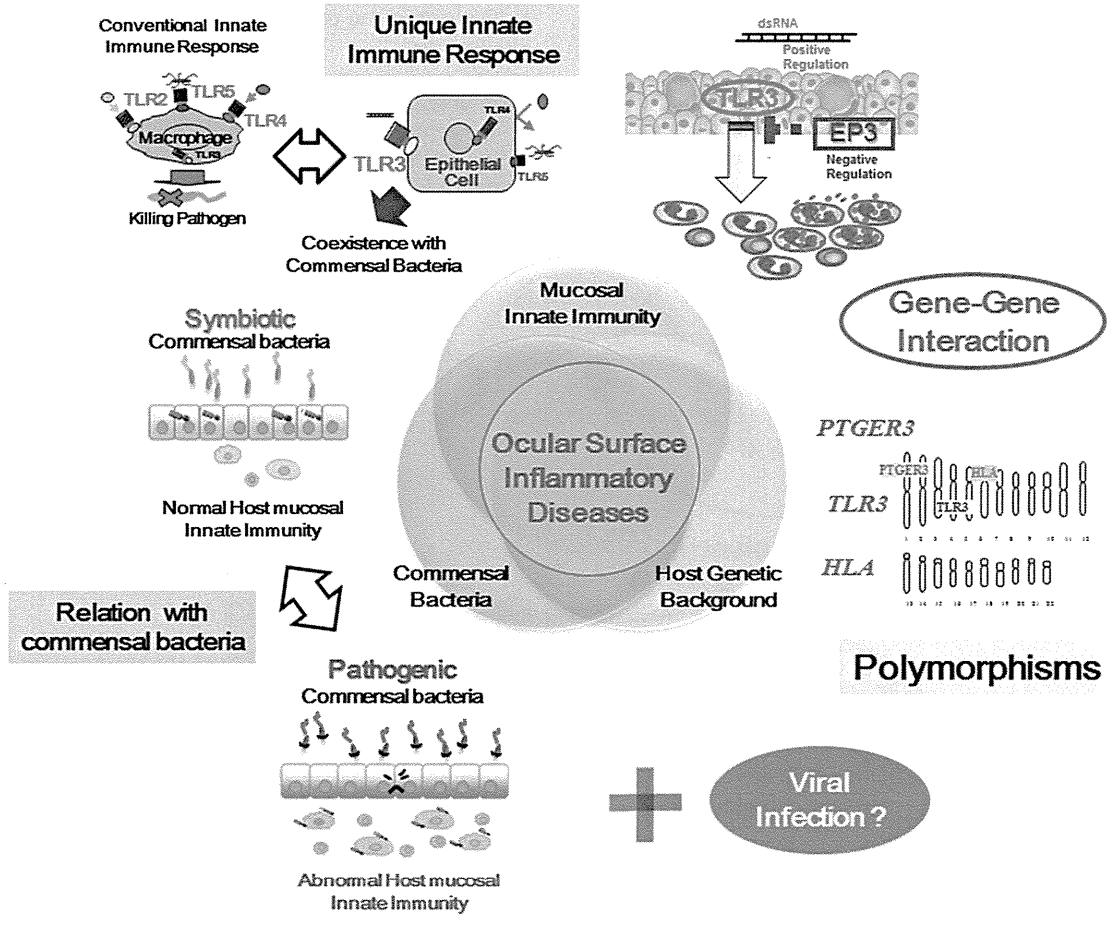

Fig. 23. The presumed pathophysiological mechanism of the ocular surface inflammatory diseases. Ocular surface inflammatory diseases are involved with mucosal innate immunity, commensal bacteria, and host genetic background. Unique innate immune response of epithelial cells contributes to coexistence with commensal bacteria. The pathogenicity of commensal bacteria is influenced by the abnormal condition of host mucosal innate immunity. Host genetic background such as polymorphisms is involved with host mucosal innate immunity. Gene–gene interactions also contribute to pathobiological mechanisms of human ocular surface inflammatory diseases.

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interactions are epistatic (Ueta et al., 2012b). Based on the findings discussed here we strongly suspect that the lack of balance between TLR3 and EP3 can trigger ocular surface inflammation (Fig. 22).

7. Conclusions and future directions

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

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 balance between the mucosal immunity of the ocular surface and the pathogenicity of bacteria is very important. We suspect that 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 may become pathogenic. Some ocular surface inflammatory diseases such as catarrhal ulcers and phlyctenular keratitis are considered to be hypersensitivity to bacteria.

We also showed that although immune-competent cells such as macrophages could recognize various microbial components through various TLRs, induce inflammation and then exclude the microbes, ocular surface epithelial cells can selectively respond to microbial components and induce limited inflammation. We suspect that the difference between ocular surface epithelial cells and macrophages lies in their dissimilarity with respect to their coexistence with commensal bacteria. The unique innate immune response machinery of the ocular surface epithelium may explain the permissive coexistence with commensal bacteria. We also document that human ocular surface epithelial cells can be induced upon stimulation with polyI:C, a ligand of TLR3, RIG-I and MDA-5, to express many transcripts including not only anti-viral innate immune response-related- but also allergy-related-genes.

We provided evidence that allergic eosinophilic infiltration of the conjunctiva can be regulated by conjunctival epithelial cells through EP3 and TLR3.

Our findings indicate that disordered innate immunity can induce ocular surface inflammation because mice in which IκBζ was knocked out, expressly exhibited severe, spontaneous ocular surface inflammation with the eventual loss of almost all goblet cells.

Lastly we suggest that the pathogenesis of SJS with severe ocular surface complications, a devastating severe ocular surface inflammatory disease, is associated with innate immune reaction abnomalies, especially those related with the epistatic interactions between TLR3 and EP3. Thus, the lack of balance between TLR3 and EP3 might trigger ocular surface inflammation.

Focusing on the innate immunity of the ocular surface might help to elucidate the pathogenesis of various ocular surface diseases (Fig. 23).

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Abbreviation

PFGE: pulsed-field gel electrophoresis
TLRs: Toll-like receptors
IFN: interferon
IL: interleukin
TNF: tumor necrosis factor
PGN: peptidoglycan
ds: double-stranded
polyI:C: polyinosine-polycytidylic acid
LPS: Lipopolysaccharide
CpG: deoxy-cytidylate-phosphate-deoxy-guanylate
RT-PCR: reverse transcription-polymerase chain reaction
IP-10: IFN-gamma inducible protein 10
NLRs: nucleotide-binding oligomerization domain (NOD)-like receptors
RLRs: retinoic acid-inducible gene-I (RIG-I)-like receptors
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
Rtp4: receptor transporter protein 4
TSLP: thymic stromal lymphopoietin
PG: prostaglandin
TX: thromboxane
SJS: Stevens-Johnson syndrome
TEN: toxic epidermal necrolysis
NSAIDs: non-steroidal anti-inflammatory drugs

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Stem Cells, Tissue Engineering, and Hematopoietic Elements

ROCK Inhibitor Converts Corneal Endothelial Cells into a Phenotype Capable of Regenerating *In Vivo* Endothelial Tissue

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Corneal endothelial dysfunction accompanied by visual disturbance is a primary indication for corneal transplantation. We previously reported that the adhesion of corneal endothelial cells (CECs) to a substrate was enhanced by the selective ROCK inhibitor Y-27632. It is hypothesized that the inhibition of ROCK signaling may manipulate cell adhesion properties, thus enabling the transplantation of cultivated CECs as a form of regenerative medicine. In the present study, using a rabbit corneal endothelial dysfunction model, the transplantation of CECs in combination with Y-27632 successfully achieved the recovery of corneal transparency. Complications related to cell injection therapy, such as the abnormal deposition of the injected cells as well as the elevation of intraocular pressure, were not observed. Reconstructed corneal endothelium with Y-27632 exhibited a monolayer hexagonal cell shape with a normal expression of function-related markers, such as ZO-1, and Na^+/K^+-ATPase, whereas reconstruction without Y-27632 exhibited a stratified fibroblastic phenotype without the expression of markers. Moreover, transplantation of CECs in primates in the presence of the ROCK inhibitor also achieved the recovery of long-term corneal transparency with a monolayer hexagonal cell phenotype at a high cell density. Taken together, these results suggest that the selective ROCK inhibitor Y-27632 enables cultivated CEC-based therapy and that the modulation of Rho-ROCK signaling activity serves to enhance cell engraftment for cell-based regenerative medicine. ***(Am J Pathol 2012, 181:268–277; http://dx.doi.org/10.1016/j.ajpath.2012.03.033)***

Corneal endothelial dysfunction is a major cause of severe visual impairment, because corneal endothelial cells maintain corneal transparency through their barrier and Na^+-K^+ transport system. Highly effective surgical techniques to replace corneal endothelium (eg, Descemet's stripping endothelial keratoplasty) have been developed,[1,2] aimed at replacing penetrating keratoplasty for overcoming pathological dysfunctions of corneal endothelial tissue. Several research groups, including ours, have devoted an intensive amount of effort in an attempt to establish new treatment methods suitable for a practical clinical intervention to repair corneal endothelial dysfunctions.[3–6] Because corneal endothelium is composed of a monolayer and is technically difficult to transplant into the anterior chamber as a structurally flexible cell sheet, those research teams cultured corneal endothelial cells (CECs) on substrates such as collagen sheets and amniotic membrane.

The injection of cultivated cells has been reported for the treatment of a number of organs associated with degenerative diseases such as the heart,[7] vessels,[8] pancreas,[9] and cartilage.[10] In regard to corneal endothelium, it is known that injected cultured CECs appear to be washed off by aqueous humor flow, thus resulting in the poor adhesion of those injected cells onto the corneal tissue. To develop an effective method for delivering cultivated CECs to the posterior cornea, the magnetic attachment of iron powder or superparamagnetic microspheres incorporated in the cultivated CECs has been attempted. This method has been shown to work in a

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rabbit transplantation model[11,12] and in an organ culture model of the human eye[13]; however, these methods have yet to be applied in the clinical setting.

Cell adhesion is known to be mediated through transmembrane adhesion molecules linked to the intracellular cytoskeleton. In addition to the structural function, these adhesion molecules reportedly serve as a platform for the interplay with the surrounding environments.[14,15] Rho GTPase proteins are key modulators of cytoskeletal dynamics that occur after cell adhesion.[16–18] It has been reported that Rho GTPases induce a specific type of actin cytoskeleton through mediating downstream effectors mDia and Rho-associated kinase (ROCK), and that they regulate a variety of cellular functions.[19] Cell adhesion, motility, and cell morphogenesis are thought to be determined by the balance between mDia and ROCK activities.[19] We recently reported that the adhesion of CECs to a substrate was enhanced by inhibiting Rho/ROCK signaling.[20] This finding coincides well with those of other studies that demonstrated that Rho-ROCK signaling negatively regulates the integrin-mediated adhesion of monocytes, and that the inhibition of ROCK by a selective ROCK inhibitor upregulates adhesion.[17,18] These features have led us to hypothesize that the inhibition of ROCK signaling may provide a way to manipulate the cell adhesion property of cultivated corneal endothelium to the extent practical for regenerative medicine.

In this current study, in two animal models (rabbit and primate) of corneal endothelial dysfunctions, the transplantation of cultivated CECs in combination with ROCK inhibitor Y-27632 successfully achieved the recovery of corneal transparency. Inhibition of the ROCK signaling manipulated the adhesion property of the cultivated CECs. Moreover, the injected CECs functioned sufficiently well to reconstruct the corneal endothelium with an appropriate cell density, morphology, and expression of function-related markers. This novel treatment strategy may provide a new therapeutic modality for corneal-endothelium–associated pathological dysfunctions.

Materials and Methods

Materials

Rabbit eyes were purchased from Funakoshi Corporation (Tokyo, Japan). Alizarin red S stain and selective ROCK inhibitor Y-27632 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium supplemented with penicillin, streptomycin, and basic fibroblast growth factor (bFGF), Vybrant DiI cell-labeling solution, Alexa Fluor 594-conjugated phalloidin, Alexa Fluor 488-conjugated phalloidin, Alexa Fluor 488-conjugated goat anti-mouse IgG, anti-vinculin antibody, ROCK1 Stealth RNAi (HSS109291, HSS109292, and HSS109293), ROCK2 Stealth RNAi (HSS114106, HSS114107, and HSS114108), Stealth RNAi negative control medium GC #2, and Lipofectamine RNAiMAX were purchased from Life Technologies (Carlsbad, CA). Dispase II was purchased from Roche Applied Science (Penzberg, Germany). FNC Coating Mix was purchased from Athena Environmental Sciences, Inc. (Baltimore, MD). Ki-67 monoclonal antibody, propidium iodide (PI), and Cytochalasin D were purchased from Sigma-Aldrich Co. (St. Louis, MO). ZO-1 polyclonal antibody was purchased from Zymed Laboratories (South San Francisco, CA). α–Smooth muscle actin (α-SMA) monoclonal antibody was purchased from Thermo Fisher Scientific (Kalamazoo, MI). Na^+/K^+-ATPase monoclonal antibody was purchased from Upstate Biotech (Lake Placid, NY). DAPI was purchased from Vector Laboratories (Burlingame, CA). CellTiter-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI).

Animal Experiment Approval

In all experiments, animals were housed and treated in accordance with The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The rabbit experiments were performed at Doshisha University (Kyoto, Japan) according to the protocol approved by that university's Animal Care and Use Committee (approval no. 0831). The monkey experiments were performed at the Research Center for Animal Life Science at Shiga University of Medical Science (Otsu, Japan) according to the protocol approved by that university's Animal Care and Use Committee (approval no. 2008-10-5).

Cell Culture of Rabbit and Monkey CECs

Ten rabbit eyes were used for the rabbit CECs (RCECs) culture. Eight corneas from four cynomolgus monkeys (3 to 5 years of age; estimated equivalent human age: 5 to 20 years) housed at the Nissei Bilis Co. (Otsu, Japan) and the Keari Co. (Wakayama, Japan), respectively, were used for the monkey CECs (MCECs) culture. The RCECs and MCECs were cultivated as described previously.[3,20] Briefly, Descemet's membrane with CECs was stripped and incubated in 0.6 U/mL of Dispase II to release the CECs. After a 60-minute incubation at 37°C, the CECs obtained from individual corneas were resuspended in culture medium and plated in one well of a six-well plate coated with cell attachment reagent (FNC Coating Mix). All primary cell cultures and serial passages of CECs were performed in growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, and 2 ng/mL bFGF. CECs were cultured in a humidified atmosphere at 37°C in 5% CO_2. The culture medium was changed every 2 days. When cells reached confluency in 10 to 14 days, they were rinsed in Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline (PBS), trypsinized with 0.05% Trypsin-EDTA (Life Technologies, Carlsbad, CA) for 5 minutes at 37°C, and passaged at ratios of 1:2 to 1:4. Cultivated CECs derived from both rabbit and monkey corneas at passages 3 through 5 were used for all experiments. To confirm the cultivation of the CECs, the morphology and density of the cultivated cells were compared with normal *in vivo* rabbit CECs examined using a noncontact specular microscope (Noncon Robo, SP-8800; Konan Medical, Nishinomiya,

Japan) and stained with Alizarin red. In some experiments, to investigate the fate of the injected CECs *in vivo*, the CECs were labeled with fluorescein by use of the Vybrant DiI cell-labeling solution according to the manufacturer's protocol.

Rabbit Corneal Endothelial Dysfunction Model

To create rabbit corneal endothelial pathological dysfunction models, the lenses of both eyes of 12 Japanese white rabbits were removed under general anesthesia by use of the Alcon Series 20000 Legacy Surgical System (Alcon, Fort Worth, TX) to deepen the anterior chamber. Next, the corneal endothelium of each of those eyes was mechanically scraped with a 20-gauge silicone needle (Soft Tapered Needle; Inami, Tokyo, Japan) from Descemet's membrane as described previously.[3,4] The scraped area was then confirmed by 0.04% trypan blue staining during surgery. In the preliminary experiments, we confirmed that Descemet's membrane was intact, the mechanically scraped area had no cells on Descemet's membrane, and that residual CECs were detected in only a 500- to 600-μm area at the edge of Descemet's membrane.

Injection of Cultivated CECs into the Rabbit Eyes

To evaluate the injection of cultivated CECs with ROCK inhibitor, RCECs at a density of 2.0×10^5 cells were suspended in 200 μl DMEM supplemented with 100 μmol/L of Y-27632 and then injected into the anterior chamber of the eyes of the above-described corneal endothelial dysfunction rabbit model. RCECs with Y-27632 were injected into the right eyes of six rabbits, and RCECs without Y-27632 were injected into the right eyes of the other six rabbits. After the injection, the eyes of those 12 rabbits were kept in the face-down position for 3 hours under general anesthesia. The left eyes of those 12 rabbits in which the corneal endothelium was removed mechanically were used as a control. One rabbit injected with RCECs with Y-27632 and one rabbit injected with RCECs without Y-27632 were euthanized 3 hours after injection for histological examination. The corneal appearance of the other 10 rabbits was examined daily by use of a slit-lamp microscope for the first week, and then once every 2 days for the following 2 weeks. Those 10 rabbits were then euthanized for histological examination. Corneal thickness was determined by use of an ultrasound pachymeter (SP-2000; Tomey, Nagoya, Japan), and the mean of 10 measured values was then calculated (up to a maximum thickness of 1200 μm, the instrument's maximum reading). Intraocular pressure was measured by use of a pneumatonometer (30 Classic; Reichert, NY).

Histological Examination of Rabbit Eyes After CEC Injection

Sections (6-μm) of corneal specimens obtained from the 10 rabbits euthanized 2 weeks after injection were embedded in OCT compound and then fixed in 4% formaldehyde. Differential interference contrast (DIC) images and fluorescence images of DiI-labeled cells were obtained by use of a fluorescence microscope (TCS SP2 AOBS; Leica Microsystems, Welzlar, Germany). For flat-mount examinations, whole corneal specimens were fixed in 4% formaldehyde and incubated in 1% bovine serum albumin (BSA) to block any nonspecific binding. To evaluate the effect of Y-27632 on the adhesion property of the cells, corneas obtained from the 2 rabbits euthanized 3 hours after injection were examined by actin staining performed with a 1:400 dilution of Alexa Fluor 488–conjugated phalloidin. Actin staining was used to evaluate the cellular morphology. The cell nuclei were then stained with PI. To investigate the phenotype of the reconstructed corneal endothelium obtained from the 10 rabbits euthanized 2 weeks after injection, immunohistochemical analyses of actin, α-SMA, ZO-1, Na^+/K^+-ATPase, DiI, and Ki-67 were performed. α-SMA was used to evaluate the fibroblastic change. ZO-1, a tight-junction–associated protein, and Na^+/K^+-ATPase, the protein associated with pump function, were used for function related markers of CECs. The α-SMA, ZO-1, and Na^+/K^+-ATPase staining were performed with a 1:200 dilution of α-SMA monoclonal antibody, ZO-1 polyclonal antibody, and Na^+/K^+-ATPase monoclonal antibody, respectively. Ki-67 (a cell-proliferation–related maker) staining was performed using a 1:400 dilution of anti-mouse Ki-67 antibody. For the secondary antibody, a 1:2000 dilution of Alexa Fluor 488–conjugated goat anti-mouse IgG was used. Cell nuclei were then stained with DAPI, and the slides were inspected by fluorescence microscopy.

Effect of Y-27632 on MCECs in Culture

MCECs were cultured at a density of 2.5×10^4 cells/cm^2 on Lab-Tek Chamber Slides (NUNC A/S, Roskilde, Denmark). Actin staining was performed with 1:400-diluted Alexa Fluor, as described above, after 24 hours of seeding, and vinculin staining was performed using 1:200-diluted vinculin after 3 hours of seeding. The number of attached MCECs was evaluated by use of CellTiter-Glo Luminescent Cell Viability Assay performed according to the manufacturer's protocol. The MCECs were seeded with a different concentration of Y-27632 at the density of 1.0×10^3 cells onto 96-well plates, and the number of adhered MCECs at 24 hours after seeding was then measured by use of a Veritas Microplate Luminometer (Promega). In addition to ROCK signaling inhibition, to evaluate the effect of inhibition of actin polymerization on CECs adhesion, MCECs were seeded with a different concentration of cytochalasin D at the density of 1.0×10^3 cells onto 96-well plates, and the number of adhered MCECs at 24 hours after seeding was then measured. Five samples were prepared for each group.

To determine the adhesion property of the MCECs onto the basement membrane, the cells were seeded onto rabbit corneas in which the corneal endothelium was mechanically denuded and the basement membranes were exposed. The cells were seeded at the density of 2.5×10^4 cells/cm^2 suspended in culture medium supplemented with or without 10 μmol/L Y-27632. Actin staining was performed at 3 hours after seeding in the same

manner as with the Alexa Fluor 488–conjugated phalloidin staining described above. Cell nuclei were then stained with PI. MCECs at the density of 2.0×10^5 cells were also seeded, with or without Y-27632, onto Descemet's membrane of four rabbits from each group, and the membrane was then mechanically peeled off at 3 hours after seeding. The adhered MCECs were recovered by trypsin digestion, and the cell numbers were then counted.

Inhibition of ROCK Signaling by siRNA on MCECs in Culture

MCECs seeded at the density of 2.5×10^4 cells/cm^2 onto a 24-well plate were incubated with RNAi duplex (ROCK1 Stealth RNAi and ROCK2 Stealth RNAi) and Lipofectamine RNAiMAX according to the manufacturer's protocol. Briefly, 1 day before transfection, the culture medium was replaced with fresh medium without antibiotics. RNAi duplex at the final concentration of 10 nmol/L and Lipofectamine RNAiMAX complexes were added to each well. The MCECs were incubated for 12 hours at 37°C in a CO_2 incubator. Random RNAi was used as a control. The MCECs were then seeded at the density of 1.0×10^3 cells onto 96-well plates, and the number of attached MCECs was evaluated by use of CellTiter-Glo Luminescent Cell Viability Assay. Knockdown of both ROCK1 and ROCK2, two ROCK isoforms that were identified in the mammalian system,[21] was confirmed by quantitative PCR analysis (data not shown). Representative data were from six independent experiments using three kinds of ROCK1 Stealth RNAi and ROCK2 Stealth RNAi, respectively.

Injection of Cultivated CECs into Monkey Eyes with Corneal Endothelial Dysfunction

To create monkey corneal endothelial pathological dysfunction models, the corneal endothelium of the left eyes of four monkeys was mechanically scraped with a 20-gauge silicone needle under general anesthesia, as described above for the rabbit model. Next, a 2.0×10^5 density of cultivated MCECs suspended in 200 μl DMEM supplemented with 100 μmol/L Y-27632 were injected into the anterior chamber of two of the four monkeys. Cultivated MCECs suspended in 200 μl DMEM without Y-27632 were injected into the anterior chamber of the other 2 monkeys. The eyes of all four monkeys were kept in the face-down position for 3 hours under general anesthesia. The MCECs were labeled with DiI before transplantation.[3,4] The corneal appearance of all four monkeys was examined daily by use of a slit-lamp microscope for the first week, and then once per week for the following 3 months. Two monkeys from each group (the MCEC-injection with Y-27632 group, and the MCEC-injection without Y-27632 group) were euthanized at 14 days after the injection, and the other 2 monkeys were euthanized at 3 months after the injection. For flat-mount examinations, whole corneal specimens were fixed in 4% formaldehyde, incubated in 1% BSA to block nonspecific binding, and then prepared for histological examination. To investigate the phenotype of the reconstructed corneal endothelium, immunohistochemical analyses of actin, ZO-1, and Na^+/K^+-ATPase were performed in the same manner as that of the above-described rabbit experiments. After the actin immunostaining, the corneal endothelium of the four monkeys was evaluated by KSS-400EB software version 2.71 (Konan Medical, Hyogo, Japan).

Statistical Analysis

The statistical significance (*P* value) in mean values of the two-sample comparison was determined by Student's *t*-test. Values shown on the graphs represent the mean ± SEM.

Results

Injection of Cultivated RCECs with ROCK Inhibitor Enables Regeneration of Cornea in Rabbit Corneal Endothelial Dysfunction Model

The third-passaged RCECs exhibited a monolayer of hexagonal shaped cells, similar to *in vivo* RCECs with a cell density of approximately 2600 cells/mm^2 as previously reported[5,6] (Figure 1A). Cultivated RCECs injected together with Y-27632 were successful in recovering complete transparency of the corneas with pathological dysfunctions. In contrast, RCECs injected without Y-27632 induced hazy and severely edematous corneas, thus indicating that the corneal endothelial dysfunctions were sustained, comparable with those of the control corneas. Slit-lamp microscopy performed at 48 hours after injection revealed complete corneal transparency with the iris and the pupil clearly observed in the eyes injected with RCECs with Y-27632, whereas the iris and pupil could not be observed in the eyes injected with RCECs without Y-27632 and in the control eyes in which the corneal endothelium was mechanically scraped (Figure 1B). Consistent with the slit-lamp microscopy findings, histological analysis performed at 14 days after injection also revealed that the eyes injected with RCECs with Y-27632 had a normal range of corneal thickness, whereas those without Y-27632 exhibited a thick cornea with severe stromal edema. The corneal thicknesses of those specimens were 409 μm and 730 μm, respectively (Figure 1C). In the eyes injected with RCECs with Y-27632, the corneal edema was moderate (<800 μm) at day 1, yet gradually recovered to the normal level. In contrast, in both the control eyes and the eyes injected with RCECs without Y-27632, prominent corneal edema (>1200 μm) was observed at day 1, and corneal edema persisted throughout the observation period (Figure 1D). Next, possible complications associated with cell injection into the anterior chamber were investigated, as the injected cells might possibly interfere with normal aqueous humor outflow and produce an increase in intraocular pressure. No abnormal deposition of the injected DiI-positive RCECs onto the trabecular meshwork or onto the iris and no anatomical abnormality such as mechanical angle closure or peripheral anterior synechia were de-

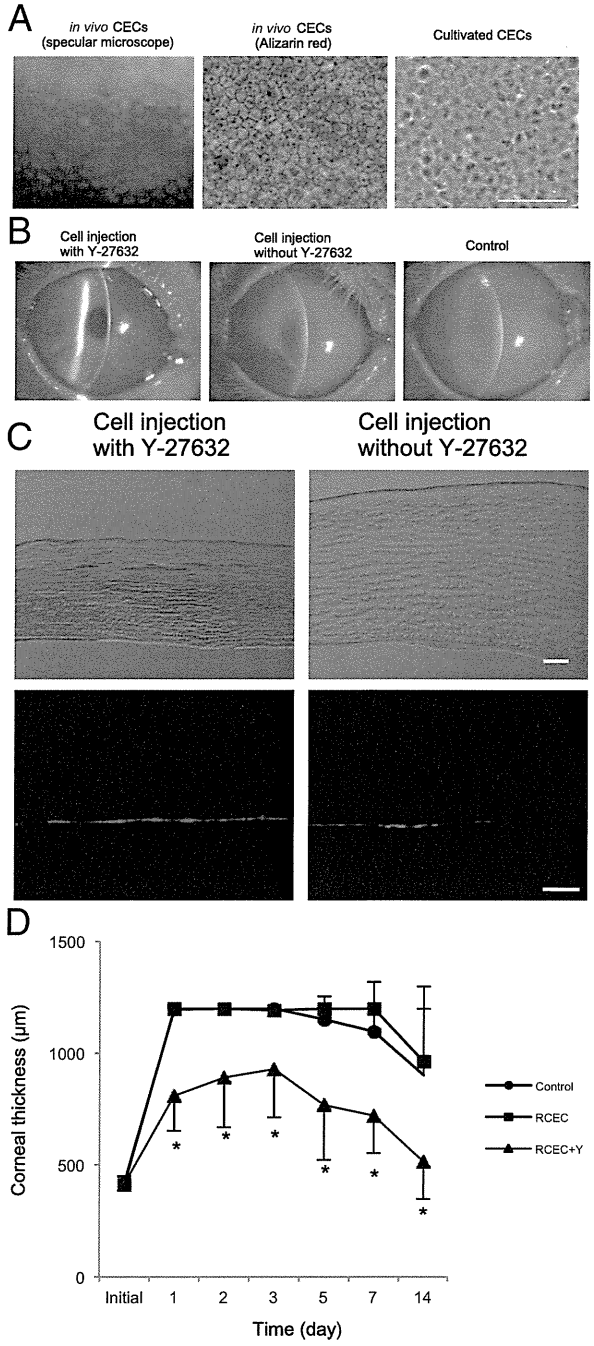

Figure 1. Cell regeneration in a rabbit corneal endothelial dysfunction model. **A:** *In vivo* normal corneal endothelium (**left panel:** specular microscope, **middle panel:** Alizarin red staining) and cultivated rabbit corneal endothelial cells (RCECs) (**right panel:** phase contrast image). The cultivated RCECs exhibit a homogeneous monolayer of hexagonal cells with a cell density of approximately 2600 cells/mm^2. The morphology and density of the cultivated RCECs are similar to that of *in vivo* corneal endothelium. Scale bar = 100 μm. **B:** Slit-lamp photographs of rabbit eyes injected with cultivated RCECs with Y-27632, cultivated RCECs without Y-27632, and control corneal endothelial dysfunction model after 48 hours. **C:** Histological analysis of rabbit corneas injected with cultivated RCECs with (**left column**) or without (**right column**) Y-27632 (**top row:** DIC; **bottom row:** DiI). Injection of RCECs with Y-27632 induces a normal-range thickness (409 μm) of the cornea, whereas injection of RCECs without Y-27632 exhibits a thick (730 μm) cornea with severe corneal stromal edema at 14 days after injection. Scale bars: 100 μm. **D:** Time course of corneal thickness measured by ultrasound pachymeter. In control eyes and in the eyes injected with RCECs without Y-27632, the corneal edema is prominent (>1200 μm) at day 1 and persists throughout the observation period. In contrast, in the eyes injected with RCECs with Y-27632, the corneal edema is moderate (<800 μm) at day 1 and gradually recovers to the normal level.

tected (Figure 2A). Intraocular pressures were found to be in the normal range in all groups (Figure 2B). To evaluate the injected CECs proliferation status *in vivo*, a flat-mount cornea was examined at 14 days after injection. Immunofluorescence analysis using the Ki-67 monoclonal antibody (a marker of cell proliferation) revealed that the cell cycle of the nearly all of the injected cells was arrested 2 weeks after injection (Figure 2C). These results

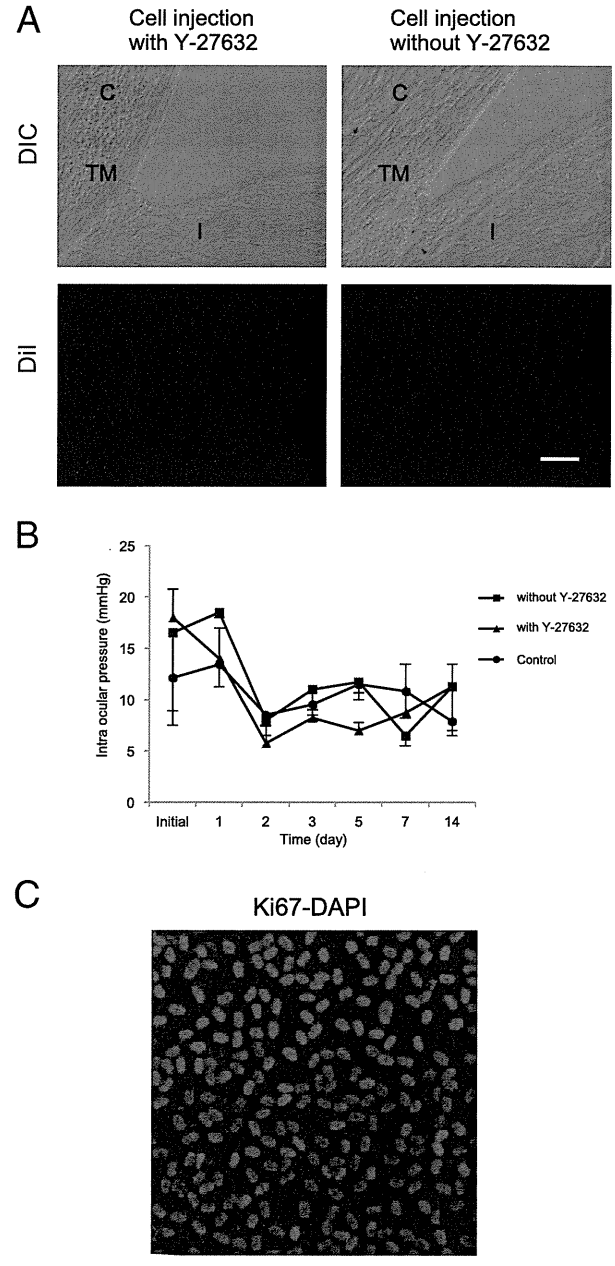

Figure 2. Evaluation of the possible adverse effects of cultivated RCEC injection. **A:** Histological examination of the iris and the angle tissue. **Top row:** representative images of the DIC section taken from the rabbit eye injected with cultivated RCECs with or without Y-27632 after 14 days. **Bottom row:** DiI images of the same sections shown in the **top row**. C, cornea; I, iris; TM, trabecular meshwork. Scale bar = 100 μm. **B:** Intraocular pressures after the injection of RCECs. **C:** Immunohistochemical staining for cell proliferation marker Ki-67 in the reconstructed RCECs in the eye injected with RCECs with Y-27632 on day 14.