A.

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

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

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

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

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

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REVIEW

Ocular Surface Inflammation Mediated by Innate Immunity

AQ: 1

AQ: 2

Mayumi Ueta, M.D., Ph.D., and Shigeru Kinoshita

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

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AQ: 4

rs.1801275 (Gln551Arg) were strongly associated ($P<0.0005$) with SJS/TEN with ocular surface complications, FasL rs.3830150 SNP was mildly associated ($P<0.005$), and IL13 rs.20541 (Arg110Gln) and IκBζ SNP rs.595788G/A exhibited a weak association ($P<0.05$). Genetic and environmental factors may play a role in an integrated cause of SJS, and there is the possibility of an association between SJS and a disordered innate immunity.

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

(*Eye & Contact Lens* 2010;5: 000–000)

INNATE IMMUNITY OF OCULAR SURFACE EPITHELIUM

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

Innate immunity, the early host defense against microbes, is primarily studied in host immune-competent cells such as macrophages. The ability of cells to recognize pathogen-associated molecular patterns (PAMPs) depends on the expression of a family of Toll-like receptors (TLRs).[2] Macrophages recognize and phagocytose microbes such as bacteria and produce inflammatory cytokines and chemokines, thus resulting in inflammation. These cells also activate adaptive immunity. However, it is now clear that the innate immunity of the mucosa in contact with commensal bacteria differs from conventional innate immunity (Fig. 1).[3] The ocular F1 surface is one of the mucosa that is in contact with commensal bacteria. The ocular surface epithelium neither usually respond to resident commensal bacteria nor induce inflammation under
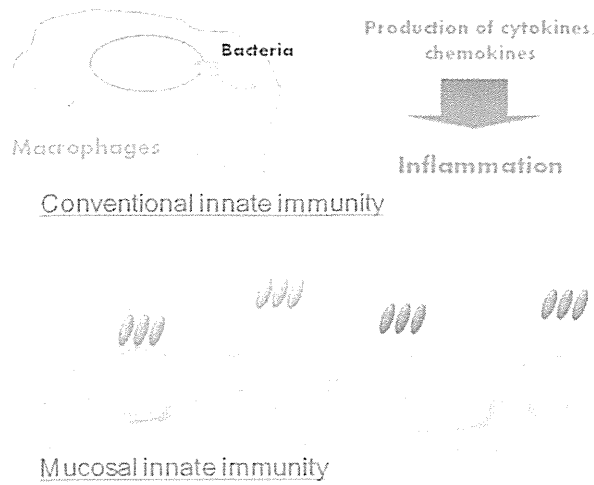
Conventional innate immunity

Mucosal innate immunity

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

AQ: 5 normal conditions. Therefore, we speculate that the ocular surface harbors unique innate immune mechanisms to regulate inflammation induced by microbes.[4–6]

The TLRs are important molecules associated with innate immunity, and the first line of defense against infection comprises evolutionarily conserved sets of TLR molecules. The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon (IFN)-α/β. For example, TLR2 recognizes lipoprotein or peptidoglycan, identifies components of the gram-positive bacterial cell wall, and forms a heterodimer with TLR1 or TLR6; TLR3 also recognizes viral double-strand (ds) RNA. The TLR4 recognizes lipopolysaccharide (LPS), a component of the gram-negative bacterial cell wall; TLR5 recognizes flagellin, a component of bacterial flagella; TLR7 and TLR8 recognize viral single-stranded RNA, and TLR9 recognizes bacterial or viral CpG DNA (Fig. 2). F2

Our study aimed to determine whether the human ocular surface epithelium expresses specific mRNA for TLRs 1 to 10. The results revealed that TLR1- to 10-specific mRNA expression was present in human conjunctival epithelium and TLR1- to 7- and TLR9- and TLR10-specific mRNA was found in human corneal epithelium (Fig. 3).[4,5,7,8] F3

The TLR3 recognizes the viral dsRNA synthesized by almost all viruses at the time of duplication. Because polyI:C mimics viral dsRNA, we used it in our experiments. We stimulated human peripheral mononuclear cells (HPMC), primary human corneal epithelial cells (PHCEC), and primary human conjunctival epithelial cells (PHCjEC) with polyI:C, the ligand of TLR3. In the primary human ocular surface epithelial cells, PHCEC and PHCjEC, but not in HPMC, stimulation with polyI:C significantly induced the secretion of IL-6 and IL-8 (Fig. 4A).[4,5,8] These F4
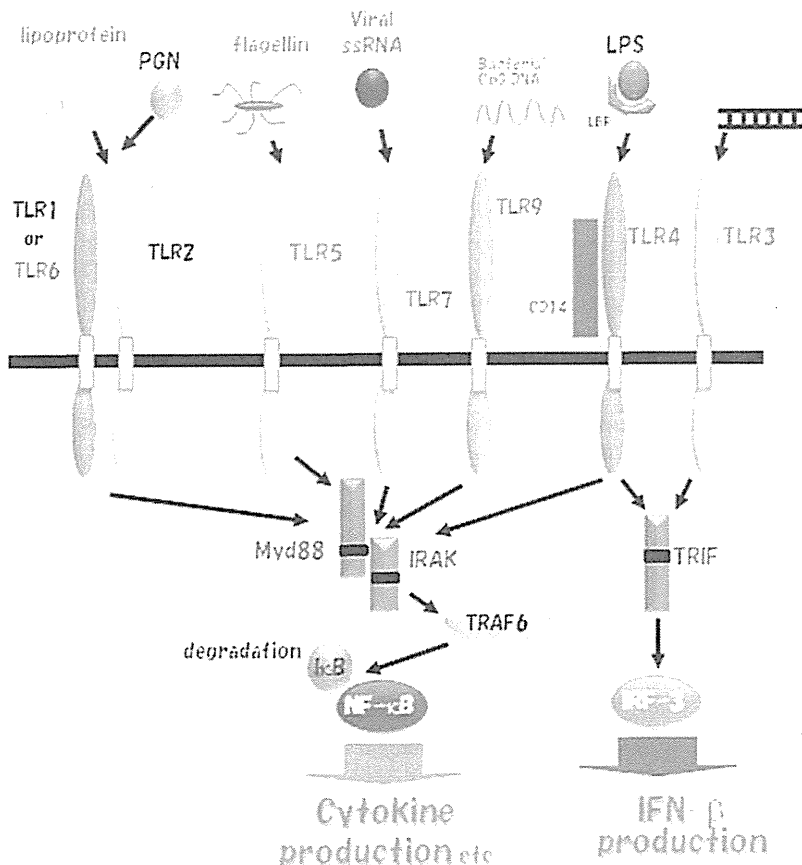
FIG. 2. Function of TLRs. The triggering of TLRs results in the secretion of proinflammatory cytokines and interferon α/β.
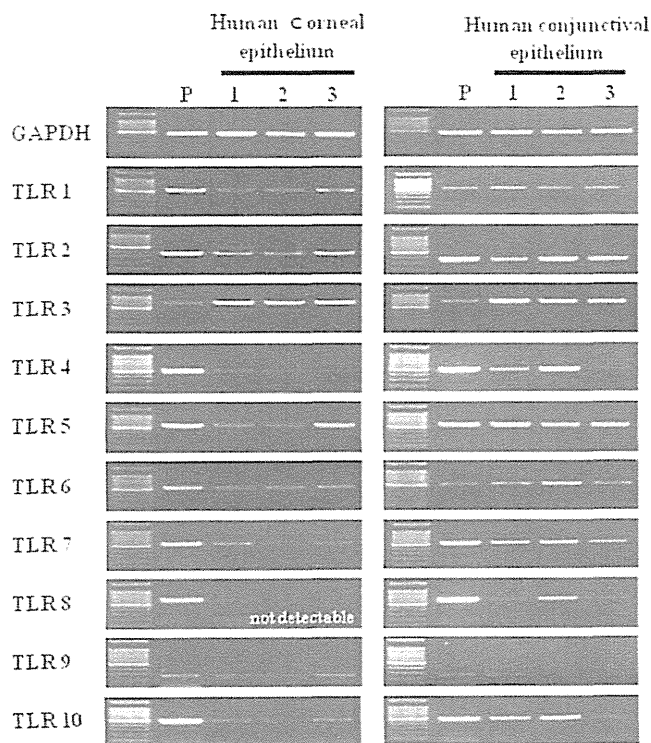
FIG. 3. Human ocular surface epithelium expresses TLR-specific mRNA. TLR1- to 10-specific mRNA expression was present in human conjunctival epithelium, and TLR1- to 7- and TLR9- and 10-specific mRNA was found in human corneal epithelium. The positive control (P) was human mononuclear cells. (Reprinted with permission from Ueta.[4])

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

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

The TLR5 recognizes flagellin, the bacterial flagella protein. Flagella are primarily present on gram-negative bacteria. Ocular surface-related bacteria with flagella include pathogenic *Pseudomonas aeruginosa* and nonpathogenic *Bacillus subtilis*. We stimulated HPMC, PHCEC, and PHCjEC with different flagellins and TLR5 ligands; we used flagellin from *P. aeruginosa* and from *B. subtilis*. We also used flagellin from *Salmonella typhimurium*, which is an intestinal but not an ocular pathogen. In HPMC, all

flagellin stimulation significantly increased the production of IL-6 and IL-8.[4,5,7,12] On the other hand, in PHCEC and PHCjEC, only flagellin derived from the ocular pathogen *P. aeruginosa* significantly induced the secretion of IL-6 and IL-8 and not flagellin derived from ocular nonpathogenic *B. subtilis* and intestinal pathogenic *S. typhimurium*.[4,5,7,12] We confirmed these findings at the mRNA expression level. In PHCEC and PHCjEC, only ocular pathogenic *P. aeruginosa*-derived flagellin resulted in a significant increase in the expression of IL-6- and IL-8-specific mRNA.[4,5,7,12] Interestingly, *P. aeruginosa*- and *S. typhimurium*-derived flagellin exhibit identical potency in inducing IL-8 protein production by cells from the human intestinal epithelial cell line HT29 (Fig. 5A).[12]

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

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

INNATE IMMUNITY AND OCULAR SURFACE INFLAMMATION

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

TLR3 and Allergy

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

Although a relationship between viral infection and allergic inflammation has been reported, the function of TLR3 in allergic inflammation remains to be defined. Allergic conjunctivitis is an ocular surface inflammation associated with type I hypersensitivity reactions; the degree of eosinophil infiltration in the conjunctiva reflects the degree of its late-phase reaction. Using our murine model of experimental allergic conjunctivitis (EAC) (Fig. 7A)[14] and TLR3 knockout (KO) and TLR3 transgenic (Tg) mice (TLR3KO and TLR3Tg, respectively), we have directly assessed the role of TLR3 in conjunctival eosinophil infiltration.
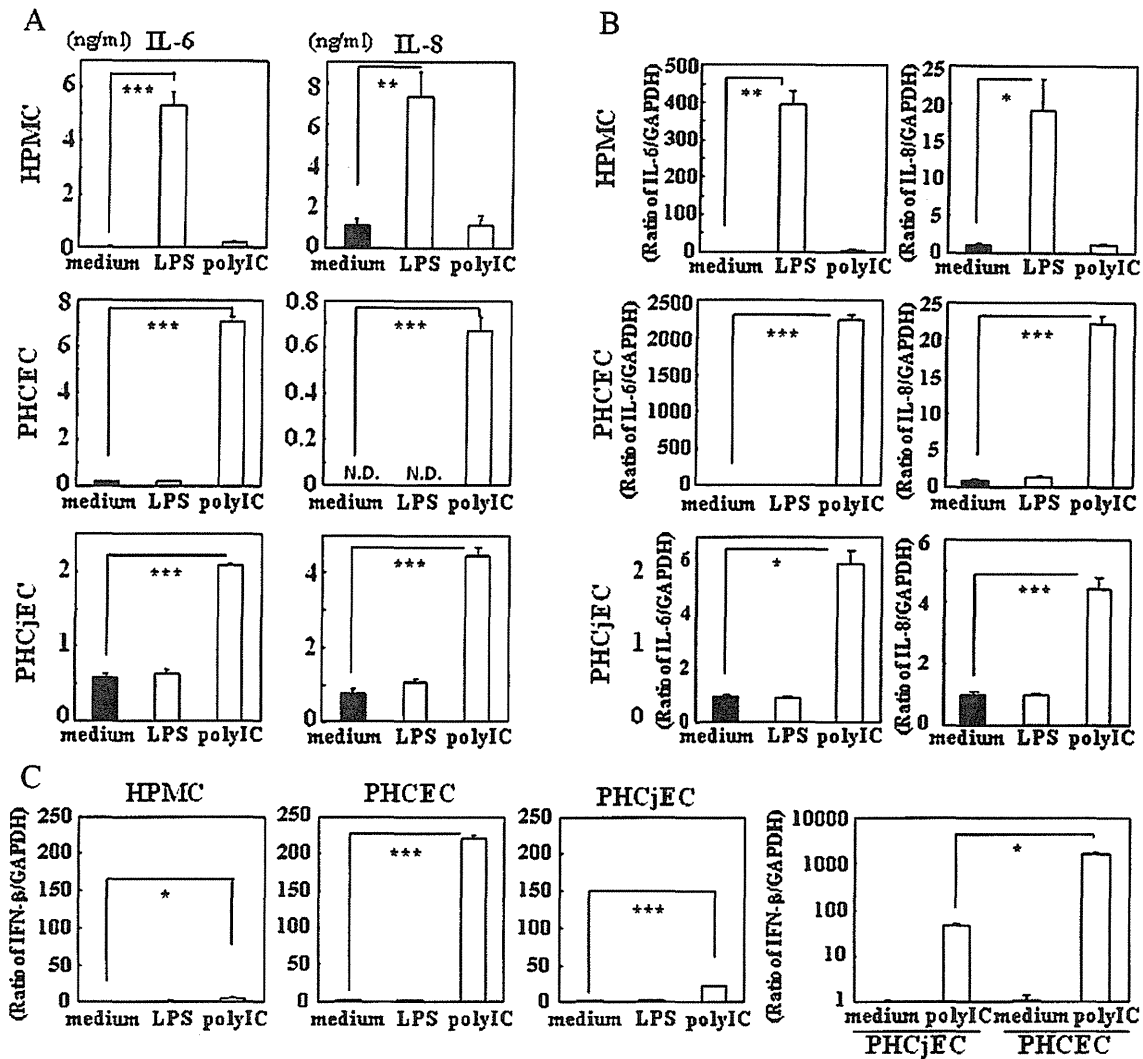
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FIG. 4. Responsiveness against polyI:C, the TLR3 ligand, of PHCEC and PHCjEC. (A), Production of IL-6 and IL-8. The HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 24 hours to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). (B), Expression of IL-6 and IL-8 specific mRNA. HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 6 hours (HPMC, PHCEC) or 3 hours (PHCjEC) to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). The y-axis for the ratio of IL-6 or IL-8/GAPDH mRNA shows an increase in specific mRNA over unstimulated cell samples. (C), Expression of IFN-β-specific mRNA. HPMC, PHCEC, and PHCjEC were cultured, then left untreated or exposed for 3 hours to polyI:C (25 μg/mL) or LPS from *P. aeruginosa* (1 μg/mL). The y-axis for the ratio of IFN-β/GAPDH mRNA shows an increase in specific mRNA over unstimulated samples of each cells or PHCjEC. (Reprinted with permission from Ueta and Kinoshita.[5])

In our model of murine EAC, the number of eosinophils in the lamina propria mucosae of the conjunctiva was significantly increased in mice after sensitization and challenge, although sensitization without challenge had no effect. Sensitization with RW induced RW-specific immune responses equally in wild-type, TLR3KO, and TLR3Tg mice; sensitization also produced an increase in IgE and IgG₁ antigen-specific antibody responses. This effect was similar in magnitude in all three groups of mice. Comparing the number of eosinophils in the lamina propria mucosae of the conjunctiva in TLR3KO and wild-type mice revealed significantly lower levels in TLR3KO than in wild-type mice (Fig. 7B).[14] Moreover, comparing eosinophil infiltration in TLR3Tg and wild-type mice revealed that the numbers of eosinophils in TLR3Tg mice after sensitization and challenge were significantly larger than in wild-type mice (Fig. 7B).[14] These results

suggest that TLR3 positively regulates the late-phase reaction of EAC, which causes reduced eosinophilic conjunctival inflammation in TLR3KO mice and pronounced eosinophilic conjunctival inflammation in TLR3Tg mice.[14]

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

AQ: 8

F8

FIG. 5. Responsiveness against various flagellins, the TLR5 ligands, of PHCECs and PHCjEC. (A), In HPMC, all flagellin stimulation significantly increased the production of proinflammatory cytokine such as IL-6 and IL-8. On the other hand, in PHCEC and PHCjEC, only flagellin derived from the ocular pathogen *P. aeruginosa* significantly induced the secretion of proinflammatory cytokine and not flagellin derived from ocular nonpathogenic *B. subtilis* and intestinal pathogenic *S. typhimurium*. In human intestinal epithelial cell line HT29, *P. aeruginosa*- and *S. typhimurium*-derived flagellin induced the secretion of proinflammatory cytokine. (B), Expression of TLR5 protein in ocular surface epithelium. TLR5 proteins were present only at basal and wing sites. Human corneal tissues were obtained from corneal buttons of a patient undergoing corneal transplantation for early-stage bullous keratopathy; human conjunctival tissues were obtained at the time of conjunctivochalasis surgery. Isotype control incubation was the negative control. Bound antibodies were visualized by Alexa Fluor 488 donkey anti-mouse IgG−, nuclei by propidium iodide staining. Each bar represents a length of 50 μm in corneal epithelium or 75 μm in conjunctival epithelium. (Modified with permission from Ueta.[4]) (C) Function of TLR5 on an ocular surface. Ocular surface epithelial cells respond to the flagellin derived from ocular pathogenic bacteria through TLR5 to produce inflammatory cytokines. However, superficial ocular surface epithelial cells do not express TLR5. Therefore, TLR5 of ocular surface epithelium might be not functional on a healthy ocular surface free of epithelial defects.
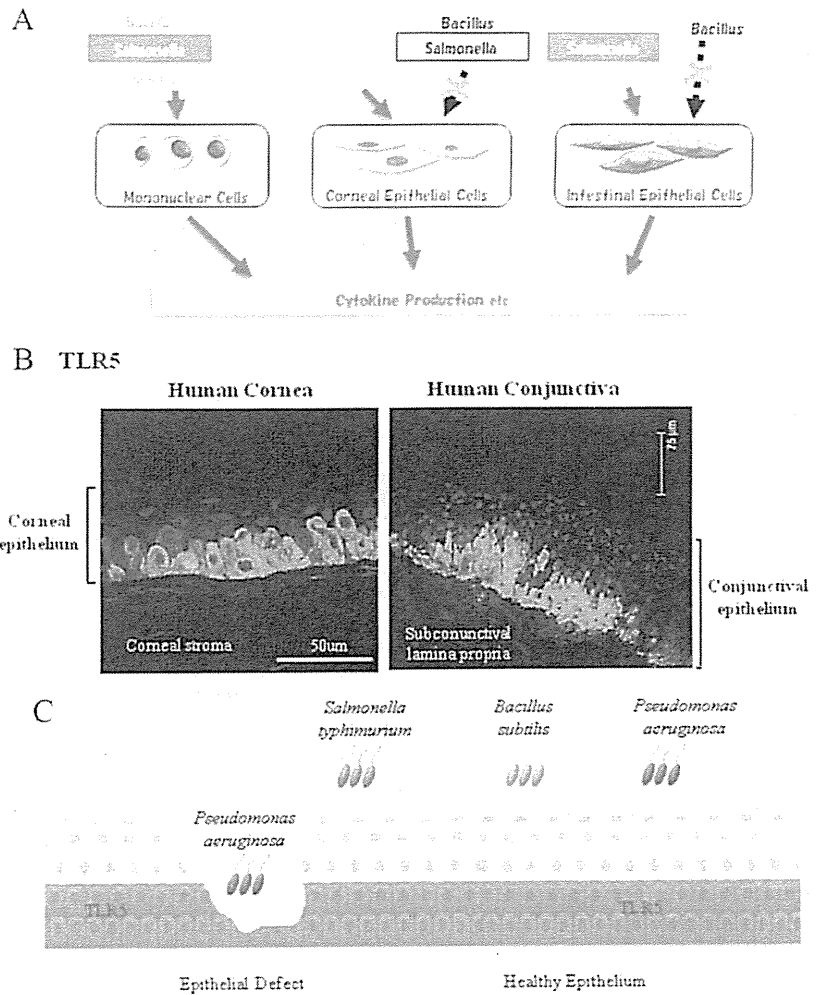
AQ: 12

Elsewhere, we showed that EP3 is expressed in the ocular surface epithelium (Fig. 9A),[14] and that the PGE$_2$-EP3 pathway in conjunctival epithelium works as a negative regulator for allergic conjunctivitis; *Ptger3$^{-/-}$* mice demonstrated significantly increased eosinophil infiltration in conjunctiva after RW-challenge compared to wild-type mice (Fig. 9B).[14] It is evident that ocular surface epithelial cells regulate the inflammation of allergic conjunctivitis.[14]

F9

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

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

Thus, although CXCL11,[17–19] CXCL10,[17–19] IL28A,[20,21] CCL5,[22,23] CCL4,[22,23] and CCL20[22,23] are innate immune re-

sponse-related genes, they have also been reported to be upregulated in allergic diseases. IL7R,[24] TSLP,[24–26] and ICAM-1[27] are allergy-related genes. At least 9 of the 47 transcripts that were found to be upregulated more than 10-fold on polyI:C stimulation of the PHCjECs from two individuals might be associated with allergy.

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

On the other hand, our results showed that RIG-I and MDA5, which are reportedly implicated in viral dsRNA recognition, are also remarkably upregulated by polyI:C stimulation of PHCjECs. We previously reported that TLR3 was the most intensely expressed among TLR1 to 10 in ocular surface epithelial cells and speculated that TLR3 mainly contributes to polyI:C inducible responses in ocular surface epithelial cells. However, in this study, we found that new receptors that recognize dsRNA and polyI:C, RIG-I and MDA5 are also expressed in PHCjECs and are upregulated by polyI:C stimulation. Although the TLR family detects PAMPs either on the cell surface or in the lumina of intracellular vesicles such as endosomes or lysosomes, recent studies have
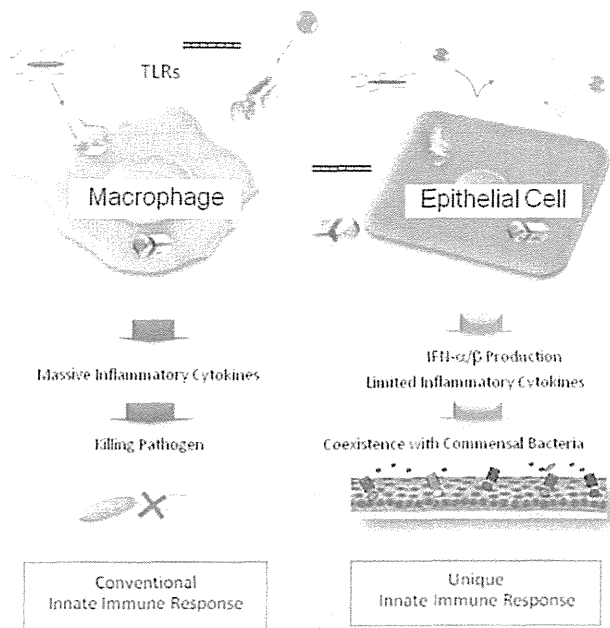
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FIG. 6. Unique innate immune response of the ocular surface epithelium. Immune-competent cells such as macrophages do recognize various microbial components via TLRs, induce inflammation, and then exclude microbes, whereas ocular surface epithelial cells selectively respond to microbial components and induce limited inflammation. This difference in the action of macrophages and ocular surface epithelial cells may be caused by dissimilarities in their coexistence with commensal bacteria.

confirmed the existence of a cytosolic system for detecting intracellular PAMPs. These cytosolic PRRs include RIG-I-like receptors (RLRs) and nucleotide-binding oligomerization domain–like

receptors. RLRs belong to the RNA helicase family that specifi- AQ: 9
cally detects RNA species derived from viruses in the cytoplasm and coordinates antiviral programs via type I IFN induction. RIG-I and MDA5 are RLRs. Further investigation is required to resolve how these receptors contribute to polyI:C-inducible responses.

We also examined whether these 11 transcripts could be upregulated on polyI:C stimulation in PHCEC to perform a quantitative RT-PCR assay. Our results showed that polyI:C stimulation upregulated these 11 transcripts (CXCL11, CXCL10, IL28A, CCL5, CCL4, CCL20, IL7R, TSLP, ICAM-1, RIG-I, and MDA5) in PHCEC (Fig. 10). The actual role of TLR3 in ocular surface F10 inflammation must be further investigated.

In summary, we demonstrated that human ocular surface epithelial cells can be induced by polyI:C stimulation to express many transcripts, including not only antiviral innate immune response-related genes but also allergy-related genes.

IKBZ AND OCULAR SURFACE INFLAMMATION WITH THE DISAPPEARANCE OF GOBLET CELLS

IκBζ is induced by diverse PAMPs and regulates nuclear factor (NF)-κB activity.[28,29] Thus, IκBζ is important for TLR/IL-1 receptor signaling, which is essential for an innate immune response. We previously reported that IκBζ KO mice exhibit severe, spontaneous ocular surface inflammation accompanied by the eventual loss of almost all goblet cells.[30] Moreover, balb/c background IκBζ KO mice exhibited not only spontaneous ocular surface inflammation but also spontaneous perioral inflammation (Fig. 11A).[31] Some IκBζ KO mice manifested ocular surface inflam- F11 mation with corneal opacity (Fig. 11A).[5] We considered IκBζ KO mice a suitable model for Stevens-Johnson syndrome (SJS), a severe, human ocular surface inflammatory disease, because these
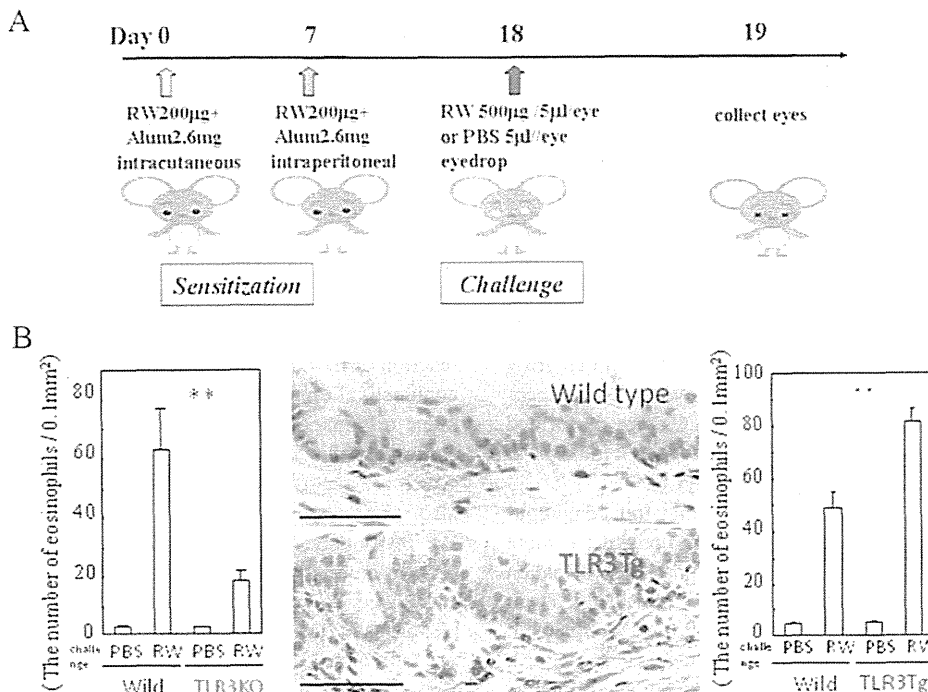

AQ: 13

FIG. 7. Our murine model of EAC. (A) Eosinophilic conjunctival inflammation in TLR3KO and TLR3Tg mice. The infiltration of eosinophils into the conjunctiva of wild-type and TLR3Tg mice was detected with Luna's method. Scale bars, 50 μm. (Reprinted with permission from Ueta et al.[51])

FIG. 8. Eosinophilic conjunctival inflammation in mast cell-deficient mice. Mast cell-deficient mice exposed to sensitization and eye drop challenge developed eosinophilic conjunctival inflammation similar to that seen in their congenic littermates. (Modified with permission from Ueta et al.[15])
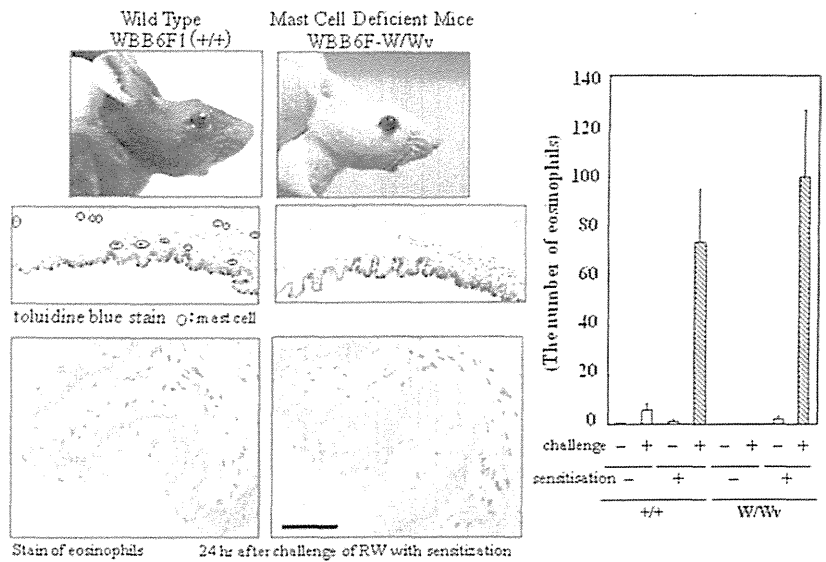
animals presented with ocular surface inflammation accompanied by a loss of goblet cells, and perioral inflammation is seen in patients with SJS (Fig. 11B).[5] IκBζ KO mice also manifested the airway inflammation and oral mucositis seen in human SJS (Fig. 11B).[5] Furthermore, IκBζ/Stat6 double-KO mice presented with severe dermatitis not only of the facial area but also of the abdominal skin; these animals also exhibited paronychia (Fig.

11C).[5,31] Our findings provide convincing evidence that IκBζ KO mice are a suitable model for SJS with ocular surface complications because patients with SJS present with ocular surface inflammation, perioral inflammation, and paronychia in the acute stage (Fig. 11D).[5]

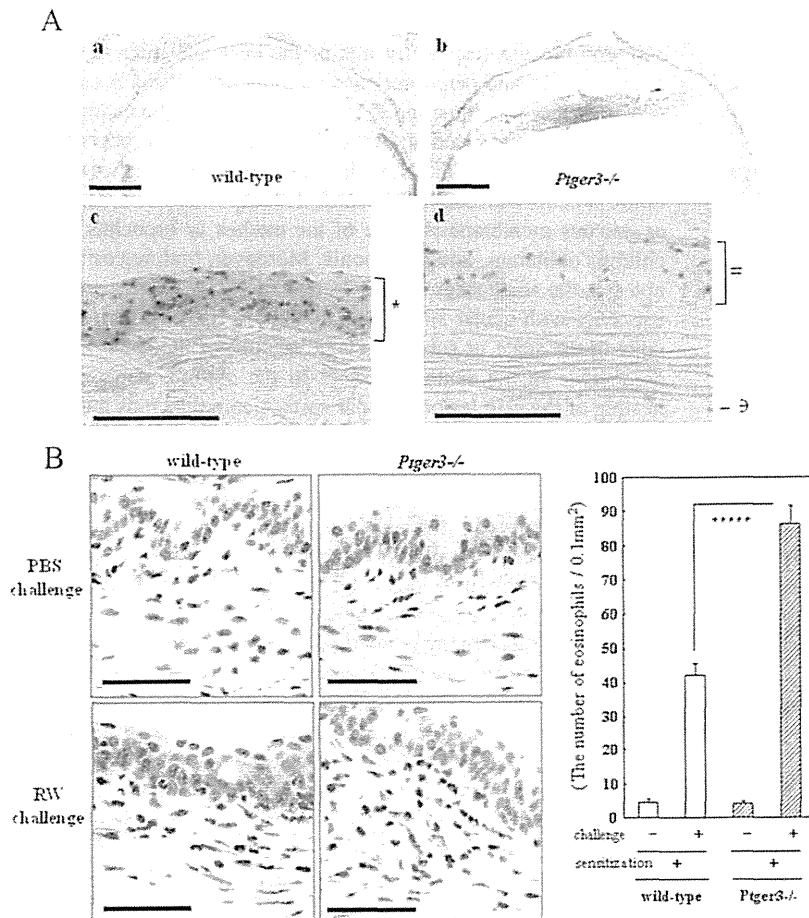IκBζ induced by diverse PAMPs regulates NF-κB activity, possibly to prevent excessive inflammation in the presence of

FIG. 9. Expression and localization of EP3 in conjunctiva and cornea. Histochemical staining for EP3 (X-gal). Ocular surface tissues from *Ptger3*^{−/−} mice expressing the β-galactosidase gene at the *Ptger3* locus was stained for β-galactosidase activity with the substrate X-gal. Sections of ocular surface tissues from *Ptger3*^{−/−} mice (b, c, d) and from wild type mice (a) were counterstained with hematoxylin (*purple*) (a, b) or eosin (*red*) (c, d). Scale bars, 500 μm (a, b) or 50 μm (c, d). Positive signals (*blue*) were shown on *conjunctival epithelium (c), #corneal epithelium (d), and ∂corneal endothelium(d). Data are representative of three experiments. (A), Infiltration of eosinophils into the conjunctiva of wild-type and *Ptger3*^{−/−} mice were detected by using Luna's method, which stained eosinophil granules with a distinctive red. Pronounced eosinophil infiltration was observed in *Ptger3*^{−/−} mice compared with wild-type mice. Scale bars, 50 μm. The number of eosinophils in the lamina propria mucosae of the tarsal conjunctiva was quantified in wild-type and *Ptger3*^{−/−} mice. The data are shown as mean ± SEM of samples from 19 animals, all the mice examined. *****$P<0.0005$. (Reprinted with permission from Ueta et al.[14])
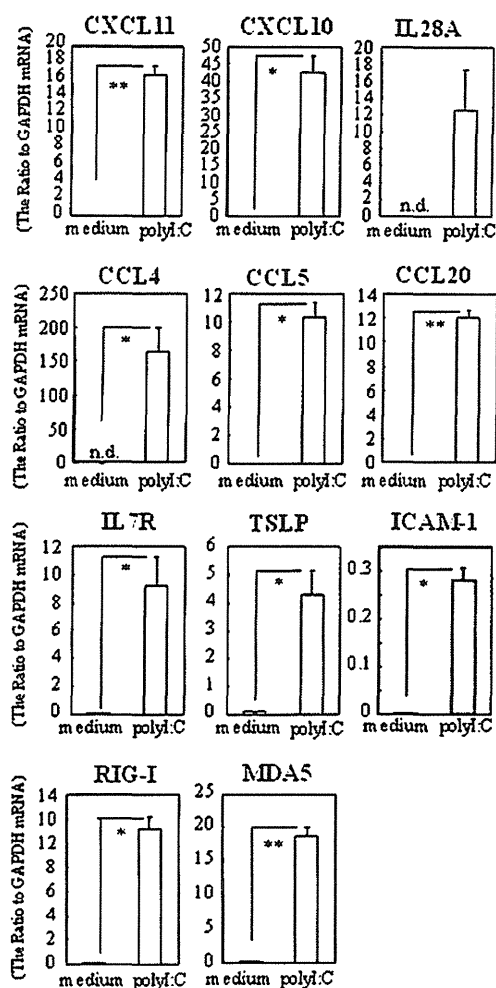
FIG. 10. The mRNA expression of the 11 transcripts in PHCEC exposed to 25 μg/mL polyI:C for 6 hours. The quantification data were normalized to the expression of the housekeeping gene GAPDH. Data are representative of three separate experiments and are given as the mean ± SEM from one experiment. (*$P<0.05$; **$P<0.005$; ***$P<0.0005$.)

bacterial components.[28] The spontaneous ocular surface inflammation observed in IκBζ KO mice suggested that dysfunction/abnormality of innate immunity can play a role in ocular surface inflammation.[5]

IκBζ mRNA was expressed in both corneal and conjunctival tissues from normal C57BL/6 mice.[30] When we compared the levels of IκBζ expression in murine tissues using RT-PCR, we found that IκBζ mRNA was intensely expressed in mucosal tissues such as the small intestine, trachea, cornea, and conjunctiva and slightly expressed in liver and kidney tissue.[30] Moreover, the predominant expression of IκBζ transcripts in the ocular surface tissue of the mice was localized spatially to corneal and conjunctival epithelia.[30] Human corneal and conjunctival epithelia also expressed human MAIL (similar to mouse IκBζ)-specific mRNA.[30]

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To examine whether MAIL (similar to mouse IκBζ) can suppress the production of proinflammatory cytokines, we performed siRNA experiments to knockdown mRNA levels of MAIL. PHCECs were transfected with control- or MAIL-targeting siRNA and cultured for 24 hours. The knockdown of MAIL mRNA was

confirmed by quantitative RT-PCR. The expression of IL-6 and IL-8 mRNA was enhanced in MAIL-knockdown PHCECs.[5] These results suggested that MAIL in the ocular surface epithelium may suppress the production of proinflammatory cytokines such as IL-6 and IL-8 and that the ocular surface epithelium might suppress inflammation via the expression of IκBζ.[5]

We also suggested that IκBζ exerts regulatory effects selectively not only on cytokines through NF-κB but also in a tissue- or cell type-specific manner (spatially orchestrated regulation).[31]

Furthermore, IκB$\zeta^{-/-}$ mice may provide further insight into the interplay between microorganisms and innate immune responses in the presence of ocular surface disorders because they may be a suitable model for SJS. Although the role of acquired immunity in the pathogenicity of SJS/toxic epidermal necrolysis (TEN) has been reported, it was not previously recognized that innate immunity plays a critical role in bridging the acute response to invading nonself molecules and chronic local immune inflammation in the pathogenicity of SJS/TEN.

SJS AND ABNORMALITY OF INNATE IMMUNITY

The 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 TEN. Both SJS and TEN are commonly associated with infectious agents or inciting drugs or both,[32–34] and the pathophysiologic mechanisms of this disease have yet to be fully elucidated. In the acute stage, patients manifest vesiculobullous lesions of the skin and mucosa (especially that of the eyes and mouth), severe conjunctivitis, and persistent corneal epithelial defects because of ocular surface inflammation.[35,36] Oral involvement, including blisters, erosions, and bleeding of the mouth and lips, was observed in all patients with SJS/TEN with ocular surface complications.[35] Some patients with SJS/TEN manifested respiratory disorders such as mucous membrane damage of the trachea or bronchus, bronchiolitis obliterans, and pneumonia. Moreover, oral mucositis was noted in the acute stage of SJS/TEN, and almost all patients with SJS/TEN with ocular surface complications had lost their fingernails in the acute or subacute stage because of the occurrence of paronychia in the acute stage.[32,35] In the chronic stage, despite healing of the skin lesions, ocular surface complications including conjunctival invasion into the cornea, dry eye, symblepharon, ankyloblepharon, and in some instances, keratinization of the ocular surface, persist. Alopecia and trichiasis of the eyelashes were also observed.[37]

We considered the possibility of an association between SJS/TEN and a disordered innate immune response. Our reflections were based on an association between the onset of SJS/TEN and infections because many patients with SJS/TEN exhibited prodromata, including nonspecific fever, coryza, and sore throat—ailments that closely mimic upper respiratory tract infections commonly treated with antibiotics.[32] In addition, patients with SJS/TEN presented with opportunistic bacterial infections of the ocular surfaces, in particular methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *S. epidermidis* (MRSE). In fact, compared with individuals with other devastating ocular surface disorders, the detection rate of MRSA and MRSE was higher with respect to the ocular surfaces of patients with SJS/TEN.[38] More-

FIG. 11. (A) Phenotype of IκBζ KO mice. Photographs of the face including the perioral skin of 32-week-old IκBζ$^{+/-}$ and an IκBζ$^{-/-}$ mice taken 27 weeks after symptom onset. Although IκBζ$^{+/-}$ mice were free of inflammation (a–d), IκBζ$^{-/-}$ mice exhibited a severe inflammatory phenotype; the inflammation involved the ocular surface, the eyelids and the perioral skin (e–g). The IκBζ$^{-/-}$ mouse also manifested corneal opacity with ocular surface inflammation (h). (B) Histologic findings on various tissues of IκBζ$^{-/-}$ mice. Histologic findings on the palpebral conjunctiva, perioral skin, oral mucosa, and trachea of IκBζ$^{+/-}$ and IκBζ$^{-/-}$ mice. Histologic analysis of 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 under the conjunctival epithelia and loss of goblet cells in the conjunctival epithelia (a). Histologic analysis of the perioral skin of an IκBζ$^{-/-}$ mouse (at 2 weeks after the onset of inflammatory symptoms) revealed 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 (b). Histologic analysis of the oral mucosa of an 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 oral mucosal epithelia (c). Histologic analysis of the trachea of an IκBζ$^{-/-}$ mouse (at 8 weeks after the onset of inflammatory symptoms) revealed infiltration of inflammatory cells into the submucosa under the tracheal epithelia (d). We observed no pathologic changes such as inflammatory phenotypes in IκBζ$^{+/-}$ mice (e–h). Each bar represents a length of 50 μm. (C) Phenotype and histologic findings in an IκBζ/Stat6 double-KO mouse. In the IκBζ/Stat6 WKO mouse, severe inflammatory symptoms were elicited on the ocular surface and not only on the facial but also on the abdominal skin (c, d). The IκBζ/Stat6 WKO mouse also manifested paronychia (g, h). No obvious dermatitis or paronychia was observed in Stat6 single-KO mice (a, b, e, f). (D), Typical features of SJS/TEN in acute stage. Ocular surface inflammation with conjunctivitis and eyelids swelling (*left*). The face manifests swollen and crusted lips, blisters, and erosions of skin (*middle*). Paronychia (*right*). (Reprinted with permission from Ueta and Kinoshita.[5])
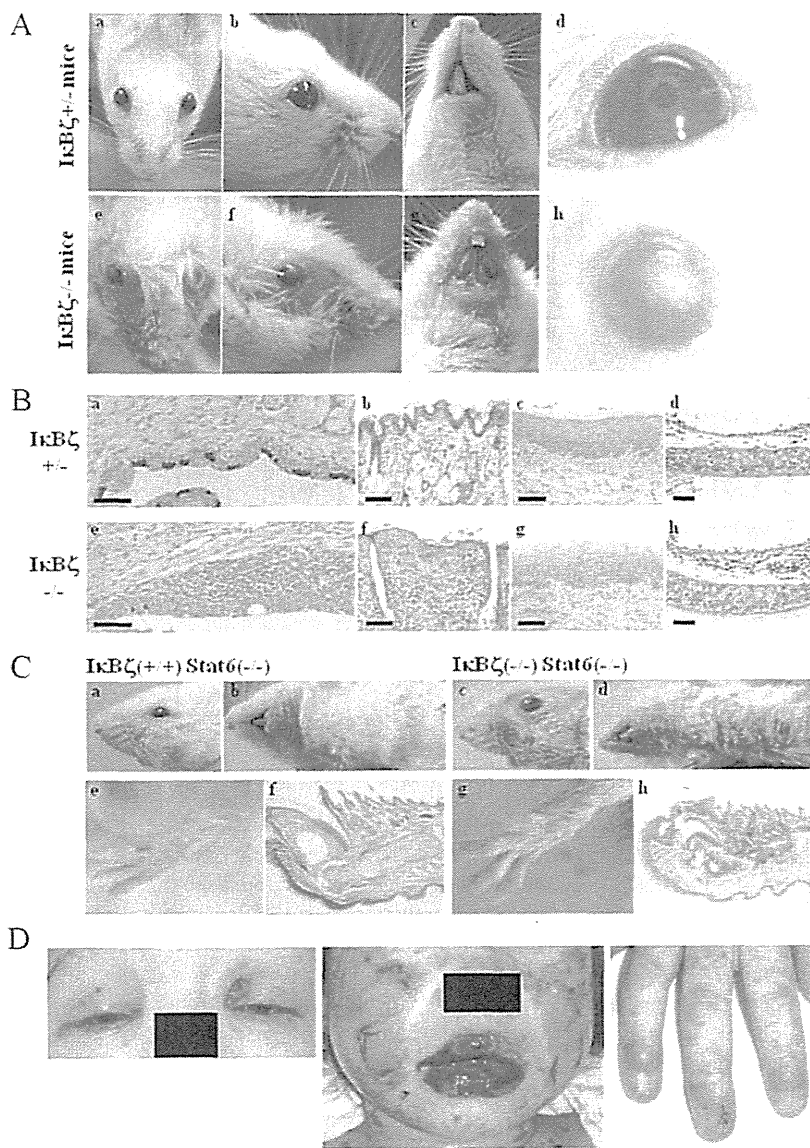
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over, patients with SJS often have severe ocular surface inflammation when MRSA or MRSE reside on the ocular surface, although there is no inflammation on the ocular surface in normal contexts, despite resident commensal bacterium such as *S. epidermidis* and *P. acnes*. Notably, elderly people who are hospitalized have no ocular surface inflammation even when MRSA or MRSE reside on the ocular surface. The ocular surface inflammation of patients with SJS is also greatly reduced after treatment with antibiotics against MRSA or MRSE.[4,5] Finally, patients with SJS/TEN presented with persistent inflammation of the ocular surfaces harboring commensal bacteria.

Under the hypothesis of a disordered innate immune response in SJS/TEN, we performed gene expression analysis of monocytes, cells that are essential in innate immunity. First, we found differences in *IL4R* gene expression; on LPS stimulation, *IL4R* gene expression was downregulated in patients with SJS/TEN and slightly upregulated in the controls (Fig. 12A).[4,39] Second, after a 1-hour culture without LPS, the expression of IL-1α (Fig. 12B) and IκBζ-specific mRNA (Fig. 12C) was

lower in monocytes from patients with SJS/TEN than in the normal controls, suggesting that the reduced expression of IL-1α and IκBζ genes may play an important role in the pathophysiology of SJS/TEN.[4] According to Correia et al.,[40] IL-1α was significantly lower and sIL-2R was significantly higher in the blister fluid of patients with TEN than in that of patients with burn injury. Our study detected a significant difference between patients with SJS/TEN and controls with respect to the expression of IL-1α by CD14$^+$ monocytes. IκBζ is induced by diverse PAMPs regulates NF-κB activity, possibly to prevent excessive inflammation in the presence of bacterial components.[28] Our preliminary report pointed to the presence of ocular surface inflammation in IκBζ gene-disrupted mice.[5,30,31] We previously reported that virus dsRNA-mimic polyI:C, a TLR3 ligand, elicited increased expression of human IκBζ-specific mRNA in primary corneal epithelial cells.[32] Considering the induction of IκBζ by TLRs, the ocular surface inflammation seen in patients with SJS/TEN may be related to an innate PAMP-amplified immune response to microbes.
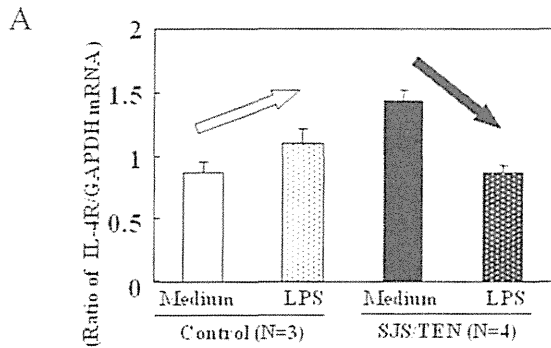
A

B

C

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FIG. 12. (A), Difference of *IL4R* gene expression between patients with SJS/TEN and normal volunteers. CD14+ cells from peripheral blood were subjected to gene expression analysis; the cells were cultured for 1 hour with or without LPS. SJS/TEN patients *N* = 4, normal volunteers *N* = 3. Low expression of IL-1α (B) and IκBζ (C) by isolated monocytes from patients with SJS/TEN after 1-hour culture. Quantitative RT-PCR assay confirmed that IL-1α (B) and IκBζ (C) gene expression was significantly lower in cultured monocytes from seven patients with SJS/TENthan the six controls. Data show the mean ± SEM. (*P<0.05; ***P<0.005); evaluation was performed with Student *t* test using the Excel program. (Reprinted with permission from Ueta.[4])
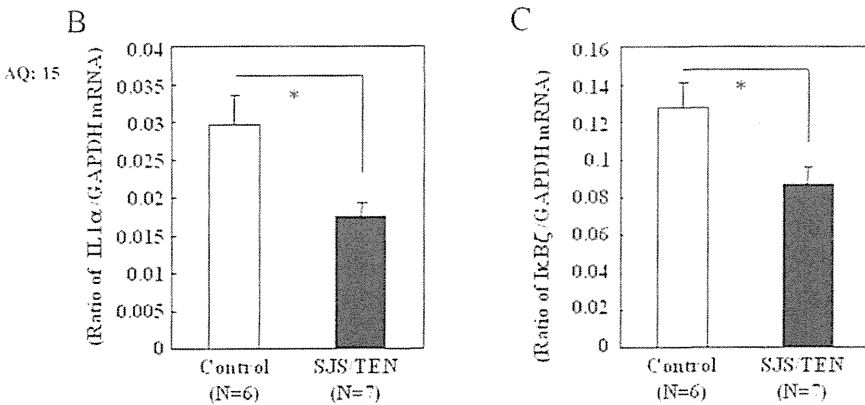
While SJS/TEN can be induced by drugs, not all individuals treated with these drugs developed SJS/TEN. Because the incidence of SJS/TEN is very low, we suspected a genetic predisposition and performed single-nucleotide polymorphism (SNP) association analysis using candidate genes associated with innate immunity,[4,32] allergy,[39,41] or apoptosis.[42]

For the SNP analysis, we enrolled 80 patients with SJS/TEN in the chronic or subacute phase; all presented with ocular surface complications. The controls were 160 healthy volunteers. All participants and volunteers were Japanese residing in Japan. The average age (mean ± SD) of the patients and controls was 45.3 ± 16.9 years and 36.2 ± 11.5 years, respectively. The male:female ratios in the patient and control groups were 35:45 and 57:103, respectively. SNP analysis was performed by direct sequencing.[4]

First, we examined the candidate genes associated with innate immunity, such as the *IL1α* genes (which differed between SJS/TEN and controls in our gene expression analysis), the *IκBζ* genes (which also differed between SJS/TEN and controls in our gene expression analysis and which lead to ocular surface and skin inflammation when disrupted), the *TLR2* genes (which are closely related to *S. aureus* and *S. epidermidis*, including MRSA and MRSE), and the *TLR3* gene (which is the one most highly expressed on ocular surface epithelium and responds to virus dsRNA-mimic polyI:C to generate proinflammatory cytokines and IFN-β).[4,32]

To investigate IκBζ, we analyzed seven polymorphisms (rs2305991, rs622122, rs14134, rs3217713, rs595788, rs677011, and rs3821727) in JSNP (the Japanese Single Nucleotide Polymorphisms database). The SNP rs595788 showed a weak inverse association under a dominant model (rs595788G/G vs. G/A+A/A: *P* value (χ^2) = 0.04, odds ratio [OR] = 0.55) (Fig. 13), although the results ceased to be significant when we corrected the

P value for the number of alleles tested (*n* = 7).[4] There was no significant association in the other six polymorphisms.[4]

Regarding *IL1α*, we analyzed five SNPs (rs1609682, rs1894399, rs2071373, rs2071375, and rs2071376) reported in JSNP. There was no significant association among these five SNPs.[4]

With regard to TLR2, we analyzed three SNPs (rs3840100, rs3840099, and rs3840097) reported in JSNP. There was no significant association among these three SNPs.[4]

Regarding TLR3, we analyzed seven SNPs (rs3775290, rs3775291, rs3775292, rs3775293, rs3775294, rs3775295, and rs3775296) reported in the JSNP database. The SNP rs3775296 showed a significant association under a recessive model (rs3775296 T/G + G/G vs. T/T, raw *P* value = 0.0001, corrected *P* value = 0.0007, OR = 0.20) and a weak inverse association with allele frequency (G vs. T, raw *P* value = 0.01, corrected *P* value = 0.07, OR = 0.6) (Fig. 13).[4,32] However, when we corrected the *P* value for the number of alleles tested (*n* = 7), the results ceased to be significant; SNP rs3775290 also showed a significant association under a recessive model (rs3775290 A/G + G/G vs. A/A, raw *P* value = 0.0094, corrected *P* value = 0.0658, OR = 0.40) (Fig. 13).[4,32] We also analyzed the genotype pattern of SNPs rs3775296T/G and rs3775290A/G and found that it (rs3775290A/A~rs3775296T/T) too strongly associated with SJS/TEN in Japanese patients (χ^2 test, *P*=0.00028, OR = 5.4, 95% CI, 2.0–14.8).[4,32] This association was stronger than observed for the single locus (rs3775296). There was no significant association among other five SNPs. Our results suggest that polymorphisms in the *TLR3* gene may be associated with SJS/TEN in the Japanese population.[32] We hypothesized that viral infection or drugs or both may trigger a disorder in the host innate immune response and that
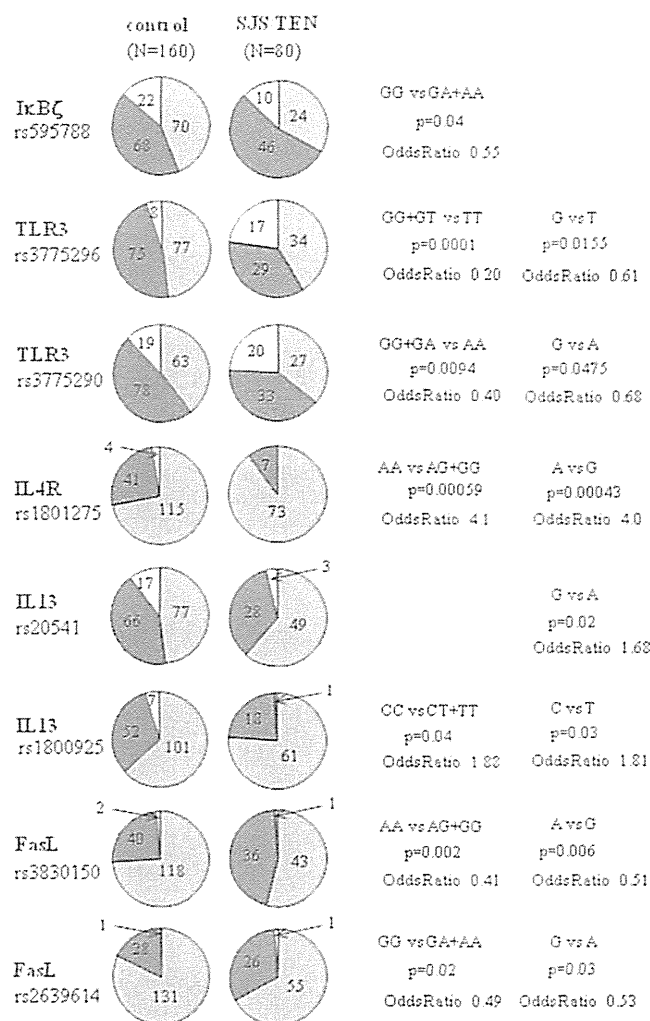
FIG. 13. The SNP association analysis using the candidate genes.

this event is followed by aggravated inflammation of the mucosa, ocular surface, and skin.[4,32]

Next, we examined the candidate genes associated with allergy. We examined *IL4R* genes (in which there are differences between patients with SJS/TEN and controls as determined by our gene expression analysis). Notably, *IL4R* is essential for both IL-4 and IL-13 signaling because it is a component of IL-4 and IL-13 receptors. Regarding the *IL4R* gene, we analyzed polymorphisms of Ile50Val (rs.1805010), Ser478Pro (rs.1805015), and Gln551Arg (rs.1801275) in the *IL4R* gene to compare Japanese patients with SJS/TEN and Japanese healthy volunteers. Among three SNPs in *IL4R*, Gln551Arg showed a significant association with allele frequency (A vs. G, raw P value = 0.00043, corrected P value = 0.00129, OR = 3.95) and the dominant model (A/A vs. A/G + G/G, raw P value = 0.00059, corrected P value = 0.00177, OR = 4.1) (Fig. 13).[4,39,41] We also investigated IL4 and IL13 (which are ligands of IL4R). Regarding the *IL4* gene, we analyzed polymorphisms of promoter −590C/T (rs.2243250) related to higher IgE levels, and we found no significant association between SJS/TEN and controls.[41] With regard to the *IL13* gene, we analyzed polymorphisms of the promoter −1111C/T SNP (rs.1800925) and Gln110Arg SNPs (rs.20541) in the *IL13* gene among Japanese patients with SJS/TEN and Japanese healthy volunteers. There was

a weak association of the promoter −1111C/T SNP in the *IL13* gene related to asthma with allele frequency (C vs. T, raw P value = 0.029, corrected $P(Pc)$ value = 0.057, OR = 1.8); correction of the P value for the number of alleles detected (n = 2) showed that the results were not significant (Fig. 13).[41] Gln110Arg SNPs in *IL13* exhibited a significant association with allele frequency (G vs. A, raw P value = 0.021, corrected P value = 0.042, OR = 1.7) even when we corrected the P value for the number of alleles detected of *IL13* SNPs (n = 2) (Fig. 13).[41] These findings contrast with those of Heinzmann et al.[43] who reported that Gln110 was significantly increased in human asthma. We detected a significant increase in Arg110 in our patients with SJS/TEN.

Finally, we examined the candidate genes associated with apoptosis, the *FasL* genes (which reported to manifest increased serum levels in patients with SJS/TEN in the acute stage). We examined four SNPs of FasL (rs.929087, rs.2639614, rs.2859247, and rs.3830150) reported in JSNP and found that rs.3830150 A/G (intron) showed a significant inverse association with allele frequency (A vs. G, raw P value = 0.006, corrected P value = 0.024, OR = 0.5) and the dominant model (A/A vs. A/G + G/G, raw P value = 0.0019, corrected P value = 0.0075, OR = 0.4) (Fig. 13). Analysis of the genotype pattern of SNPs rs.3830150 and rs.2639614 (rs.3830150 A/A - rs.2639614 G/G) also manifested a strong inverse association with SJS/TEN in Japanese patients (P value = 0.0016, OR = 0.4) (Fig. 13). There was no significant association among other two SNPs.

In summary, we found that TLR3 rs.3775296 SNP and *IL4R* SNP rs.1801275 (Gln551Arg) were strongly associated (P<0.0005), FasL rs.3830150 SNP was mildly associated (P<0.005), and IL13 rs.20541 (Arg110Gln) and IκBζ SNP rs.595788G/A exhibited a weak association (P<0.05) with SJS/TEN with ocular surface complications (Fig. 14A).

Furthermore, we examined human leukocyte antigen (HLA)-class I (HLA-A, HLA-B, and HLA-C) and HLA II (DRB1 and DQB1) antigens in 71 Japanese patients with SJS/TEN with ocular complications and 113 healthy volunteers. We found that HLA-A*0206 was strongly associated with SJS/TEN with ocular complications (carrier frequency: P<0.00005, corrected P value <0.0005, OR = 4.1; gene frequency: P<0.0005, corrected P value <0.005, OR = 3.2) and that HLA-A*1101 was inversely associated (carrier frequency: P<0.01, corrected P value = 0.078, OR = 0.23; gene frequency: P<0.005, corrected P value <0.05, OR = 0.22), although there was no association with HLA-class II.[44,45] The onset of SJS with ocular complications was associated with putative viral syndromes or the administration of drugs, a finding that coincided with that of Mondino.[46] Although the HLA-B12 antigen was significantly increased in white patients with SJS,[46–48] we found no association with HLA-B12 in Japanese patients with SJS. This result is likely because in whites, the HLA-B12 antigen is primarily coded for by HLA-B*4402, whereas in Japanese, it is almost exclusively coded for by HLA-B*4403.[49] On the other hand, HLA-A*0206 is strongly associated with SJS/TEN with ocular complications in Japanese individuals. This association is absent in whites. We detected no significant association between SJS/TEN and HLA-DQB1*0601, although HLA-DQB1*0601 was associated with ocular complications in white patients with SJS.[50]

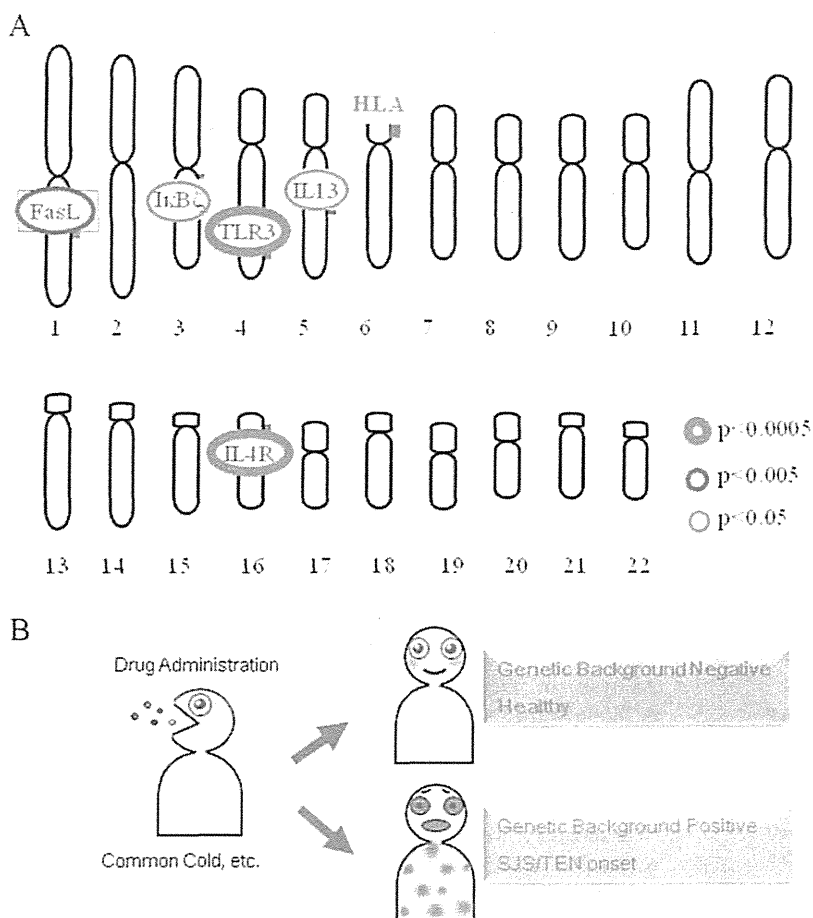Thus, our findings suggest strong ethnic differences in the association of SJS/TEN with HLA. Because SJS/TEN is rare and

FIG. 14. Conclusions of SNP association analysis. TLR3 rs.3775296 SNP and IL4R SNP rs.1801275 (Gln551Arg) were strongly associated ($P<0.0005$), FasL rs.3830150 SNP was mildly associated ($P<0.005$), and IL13 rs.20541 (Arg110Gln) and IκBζ SNP rs.595788G/A exhibited a weak association ($P<0.05$) with SJS/TEN with ocular surface complications. (A) Not only environmental but also genetic factors may play a role in an integrated etiology of SJS/TEN.

probably has a complex genetic inheritance background, specific combinations of genes and certain environmental factors may be required for the manifestation of this rare phenotype. Interestingly, HLA class I has been reported to be related to immune responses to virus.

Not only environmental but also genetic factors may play a role in an integrated cause of SJS/TEN (Fig. 14B). A possible association exists between SJS/TEN and disordered innate immunity. Agendas for future research are further examination of the involvement of disordered innate immunity in SJS and investigation of the mechanism of ocular surface inflammation in SJS, especially the correlation with ocular surface involvement.

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第113回　日本眼科学会総会　特別講演 I

角膜疾患の未来医療

木下　茂

京都府立医科大学医学部視覚機能再生外科学

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要　約

種々の角膜疾患に対する新規治療方法を開発するためには，臨床現場から得たヒントと自由な発想を基礎的研究成果と結びつけ，トランスレーショナルリサーチとして立ち上げることが大切である．そこで，我々のグループが角膜の未来医療を見据えて行っている7つの研究プロジェクトを概説する．

1．膠様滴状角膜ジストロフィの病態解明

Tumor-associated calcium signal transducer 2(TACSTD 2)が原因遺伝子として同定されている本疾患では，loss of function型の遺伝子変異によりタイトジャンクション関連蛋白質が機能障害を起こし，角膜上皮バリアー機能の高度な障害が生じ，その結果，涙液成分の角膜内への浸透が生じ，これがアミロイド沈着を促していると考えられた．

2．培養粘膜上皮移植の開発と臨床応用

上皮細胞の in vitro から in vivo への橋渡しは，第2世代の眼表面再建術と位置づけることができる．1999年から同種培養角膜上皮移植を，2002年から自家培養角膜上皮移植と自家培養口腔粘膜上皮移植を開始した．このシート移植は難治性眼表面疾患のみならず結膜上皮幹細胞疲弊にも有効であった．

3．Stevens-Johnson 症候群(SJS)の病態解明と新規治療法の開発

角膜上皮幹細胞の喪失が視力低下に相関することから，急性期のステロイドパルス療法で角膜上皮幹細胞の喪失を最小限にすることが良好な視力予後につながると考えられた．急性期に生じているサイトカイン・ストームの抑制が治療として必須であることを示唆している．本疾患では自然免疫応答の異常が発症に大きく関係していた．

4．アレルギー性眼表面疾患への新しい取り組み

上皮細胞に発現する EP 3 の眼表面炎症抑制作用をアレルギー性結膜炎モデルで明らかにした．EP 3 はヒト上皮細胞にも発現し，その遺伝子多型が関与する炎症性眼表面疾患が存在することから，アレルギー性炎症が上皮細胞を介して制御されている可能性がある．一方，上皮細胞に発現する toll like receptor 3 は眼表面炎症の増悪に関与していた．

5．眼表面上皮細胞の機能制御

上皮細胞内のグルタチオン(GSH)含量は細胞内レドックス状態を制御しており，例えば，ドライアイでは GSH 量の低下がみられ，涙点プラグで回復した．アミノ酸も細胞機能の制御に関与しているが，涙液のアミノ酸プロファイルは血漿とはまったく異なっていた．炎症

眼において発現が亢進したアミノ酸は酸化型のレドックス応答と考えられた.

　6．水疱性角膜症への新しい治療方法の開発

　培養角膜内皮細胞を用いた再生医学的治療法の開発に取り組んだ．サル培養角膜内皮シート移植は，4年が経過しても透明性を維持した．Rho キナーゼ阻害剤の添加によりヒト角膜内皮を良好な形態を維持したまま効率的に培養できた．Rho キナーゼ阻害剤を併用した前房内細胞注入療法や Rho キナーゼ阻害剤点眼の開発にも可能性がある.

　7．新しい涙液検査法の開発

　涙液油層の上方伸展は，開瞼直後に生じた油層の表面張力の勾配に基づいて引き起こされ，レオロジーの Voigt モデルに近似されることが明らかになった．このため，油層動態解析は，角膜上の涙液の量と質の情報を低侵襲，定量的に得ることのできる近未来のドライアイ検査法になると考えられた．(日眼会誌 114：161—201，2010)

キーワード：膠様滴状角膜ジストロフィ，培養粘膜上皮移植，Stevens-Johnson 症候群，一塩基遺伝子多型，プロスタノイド受容体，EP 3，細胞内レドックス，グルタチオン，培養角膜内皮細胞，Rho キナーゼ阻害剤，涙液油層，レオロジー

A Review

Research and Development for Treating Devastating Corneal Diseases

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Abstract

In order to develop new therapeutic modalities for corneal diseases, it is essential to combine cutting-edge translational research based upon liberal original ideas obtained from clinical experience with state-of-the-art basic science and technology. Here, I describe seven important research projects on which our group has been working.

1. Elucidation of the pathogenesis in gelatinous drop-like corneal dystrophy (GDLD). Due to loss of function of the tumor-associated calcium signal transducer 2 (TACSTD2), a responsible gene for this dystrophy, tight-junction-related proteins cease to function, resulting in severe corneal epithelial barrier impairment. As a result, various proteins contained in tear fluid continuously penetrate into the corneal stroma, promoting the development of massive amyloid deposits.

2. The development of cultivated mucosal epithelial transplantation : A landmark surgery, involving the transplantation of cultivated mucosal epithelial cells from *in vitro* to *in vivo*, now recognized as the next generation of ocular surface reconstruction. We began performing cultivated allo-corneal epithelial transplantations in 1999, and cultivated auto-corneal and auto-oral mucosal epithelial transplantations in 2002. These proved to be very effective in the reconstruction of both the corneal surface and the conjunctival fornix.

3. Elucidation of the pathogenesis of Stevens-Johnson syndrome : Studies have shown that there is a close relationship between corneal epithelial stem cell loss and the associated degree of visual impairment. We discovered that a steroid pulse therapy at the acute phase aimed at minimizing stem cell loss is very effective in restoring visual acuity. This implies that inhibition of the cytokine storm is essential for the treatment of acute-phase Stevens-Johnson syndrome. The innate immunity abnormality seems to be heavily involved at the onset of this devastating disease.

4. Elucidation of the involvement of EP3 and toll like receptor 3 (TLR3) in inflammatory ocular surface reactions : We discovered that EP3, one of the prostanoid receptors expressed by ocular surface epithelium, has a dramatic inhibitory effect on ocular surface inflammation in a mouse model. Since EP3 is also expressed in human ocular surface epithelium, and since abnormality of its single nucleotide polymorphisms (SNPs) is involved in some ocular surface inflammatory diseases, we theorized that an allergic reaction may be negatively regulated by EP3 which is predominantly expressed by the ocular surface epithelium. Our findings show that this is similarly true for TLR3, which, conversely, up-regulates ocular surface inflammation.

5. Functional regulation of the ocular surface epithelium : Our findings show that intracellular

glutathione (GSH) content in the ocular surface epithelium regulates its intracellular redox state. For instance, the GSH content of the conjunctival epithelium decreases in dry eye diseases, yet recovers after the surgical insertion of a punctal plug. Since various amino acids are also heavily involved in the regulation of cellular functions, we investigated the profile of amino acids contained in tear fluids. Our results indicate that there is a marked difference in amino acid profiles between tear fluids and plasma. Furthermore, we found that several amino acids are up-regulated in inflamed eyes, probably due to an oxidative redox response.

6. The development of new therapeutic modalities for corneal edema : We are developing a new therapeutic modality of cultivated corneal endothelial transplantation using methods based on regenerative medicine. For instance, our findings show that cultivated corneal endothelial sheet transplantation in monkeys maintains corneal transparency for at least four years after transplantation. The supplementation of a Rho kinase (ROCK) inhibitor in the culture media produces an excellent result in culturing human corneal endothelium, maintaining a normal-looking endothelial cell morphology. The use of a ROCK inhibitor, both for cultivated endothelial cell injection into the anterior chamber and for use as a topical application, may prove to be a potential tool for the treatment of corneal endothelial dysfunction.

7. The development of a new type of tear function test : The results of our investigations show that the time-dependent changes of tear film lipid layer (TFLL) spread are compatible with the Voigt model of viscoelasticity, and that the initial velocity of the TFLL spread after a blink decreases in proportion to the decrease in tear volume. Thus, a lipid-layer analysis will become an important tear analysis tool.

The above are projects representing the way we believe new treatments for severe corneal diseases are heading.

I 緒 言

角膜疾患の治療方法は，いくつかのパラダイムシフトを経て，現在の治療法に到達した．しかし，今なお難治な角膜疾患に対する新規治療方法を開発するためには，臨床現場から得たヒントと自由な発想を基礎的研究成果と結びつけ，トランスレーショナルリサーチとして立ち上げることが大切である．そこで，本総説では，我々のグループが角膜の未来医療を見据えて現在行っているいくつかの研究プロジェクトへの考え方と，その成果の一部を要約する．いくつかの研究プロジェクトとは，膠様滴状角膜ジストロフィの病態解明，培養粘膜上皮移植の開発と臨床応用，Stevens-Johnson症候群の病態解明と新規治療法の開発，アレルギー性眼表面疾患への新しい取り組み，眼表面上皮細胞の機能制御，水疱性角膜症への新しい治療方法の開発，そして新しい涙液検査法の開発である．

II 膠様滴状角膜ジストロフィの病態解明

膠様滴状角膜ジストロフィ (gelatinous drop-like corneal dystrophy : GDLD) は，角膜上皮下と角膜実質へのアミロイド沈着を特徴とする，きわめて重症かつ稀少な角膜ジストロフィであり[1]~[3]，この疾患の新しい治療法を考案するためには病態の解明が必須である．この疾患は，常染色体劣性遺伝を示し，1998年にTsujikawaらによって原因遺伝子がM1S1と同定されたが[4][5]，世界的にみて日本人に特徴的に発症するため founder effect が関与しているとも想像されている．原因遺伝子である tumor-associated calcium signal transducer 2 (以下 TACSTD 2，かつては M1S1 と呼称されていた) に関しては，いくつかの種類の癌細胞で発現が亢進すること[6]~[8]，TACSTD 2抗体の刺激により細胞内カルシウム濃度が変化すること[9]などが報告されているが，この遺伝子変異が GDLD の病態に結びつくプロセスについては未だ不明である．そこで，我々は長年にわたって，この病態解明について研究を進めてきた．

その結果，GDLD では，角膜実質のアミロイド沈着部位にラクトフェリン[10]やクラスタリン[11]などが沈着することから，我々は涙液成分の角膜実質への浸透が何らかのかたちでアミロイド沈着に関与しているとの仮説を立ててきた．このような涙液蛋白質の実質沈着には角膜上皮バリアー機能の持続的な障害が必須なはずであり，
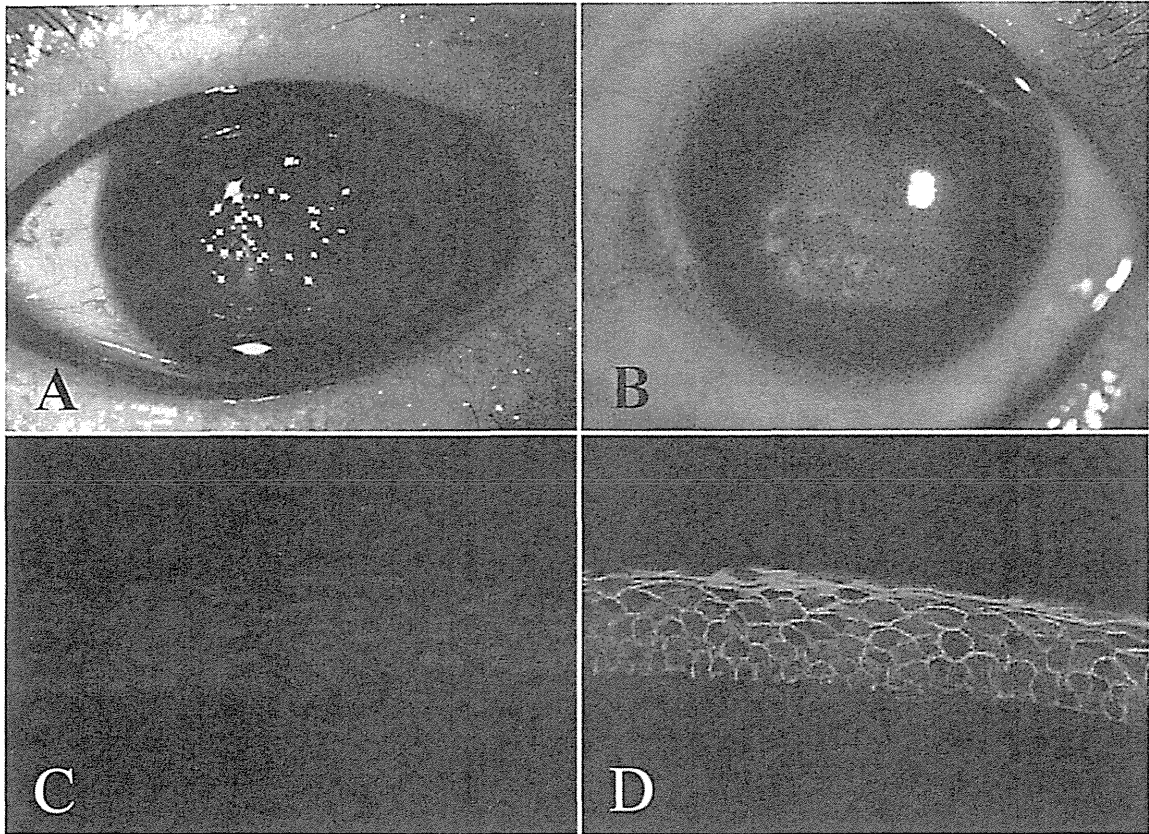
図 1　膠様滴状角膜ジストロフィ(GDLD)角膜の臨床および免疫染色の所見.
GDLD 患者角膜所見(A：Mulberry type，B：Band keratopathy type)．Tumor-associated calcium signal transducer 2(TACSTD 2)の発現(緑色)は GDLD 患者の角膜(C)では認められないが，正常角膜(D)では全層の細胞膜に認められる．赤色は核染色を示す．

我々は GDLD におけるこの現象を電子顕微鏡的にも生理学的にも証明した[12][13]．さらに，GDLD の角膜上皮では，タイトジャンクション関連遺伝子である ZO-1(zonula occludens 1)やオクルディンの発現が認められないことから[14]，このバリアー機能の障害とタイトジャンクション機能低下との密接な関係を予想してきた．さらに，我々の免疫組織化学的検討によると，TACSTD 2 蛋白質の発現は，GDLD ではほぼ完全に消失していたが，正常角膜上皮では全層の細胞の細胞膜に明瞭に認められた(図 1)．他の組織についても検討を行ったところ，TACSTD 2 は我々が検討したすべての重層扁平上皮で発現しており，その局在も角膜上皮細胞と同様であった．

次に我々は TACSTD 2 蛋白質と機能的に関連する蛋白質を同定することを試みた．方法としては免疫沈降法と proximity ligation assay(以下 PLA)を用いた．免疫沈降は二つの蛋白質の結合を，PLA は二つの蛋白質が近距離にあること(50 nm 以下とされている)を示す実験法である．結果として，我々が候補蛋白質としたもののなかで，少なくとも 3 つの蛋白質(X はその 1 つ)が免疫沈降実験や PLA で陽性シグナルを示した(図 2)．これらの蛋白質は，正常角膜上皮では全層の細胞に発現し

ているにもかかわらず，GDLD 角膜では発現量が著しく減弱しており，また細胞内局在も変化していた．しかし，レーザーマイクロキャプチャーにより得た上皮細胞を検討してみると，3 つの蛋白質の mRNA 発現は，GDLD と正常角膜でほぼ同レベルであった．次に我々は TACSTD 2 遺伝子をノックダウンするため shRNA 発現ベクターを構築した．shRNA とは short hairpin RNA のことで，細胞内で発現させると RISC 複合体に取り込まれて small interfering RNA(siRNA)として機能しターゲット遺伝子の mRNA を分解する働きをもつ．ベクターとしては pLKO.1 というレンチウイルス発現系のものを用いた．ノックダウンする細胞としては，ヒト不死化角膜上皮細胞のなかで限界希釈法により高い上皮バリアー機能をもつものをクローニングして用いた．TACSTD 2 遺伝子をノックダウンするとヒト不死化角膜上皮細胞の上皮バリアー機能は著しく低下し，GDLD において認められる角膜上皮バリアー機能の障害を反映しているものと考えられた．次にノックダウンした細胞における上述の 3 つの蛋白質の発現を検討したところ，発現量が減弱し，またその細胞内局在が GDLD におけるものと同様に変化していた．以上の結果より，GDLD においては TACSTD 2 遺伝子の loss of
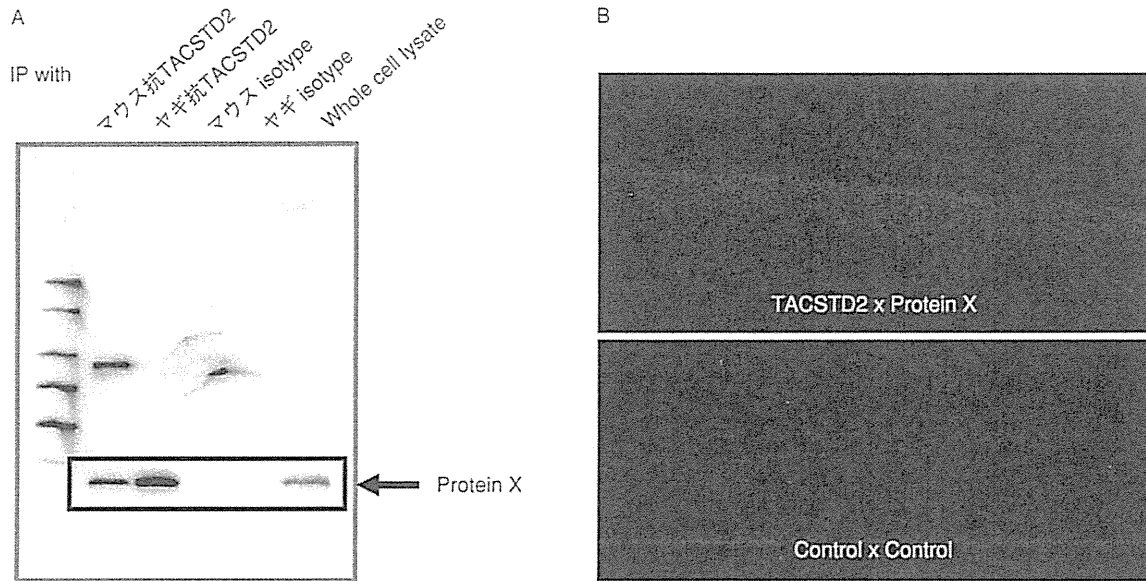
図 2　TACSTD 2 蛋白質は Protein X と結合する.
A：Protein X は TACSTD 2 抗体にて免疫沈降(immunoprecipitation：IP)する．B：TACSTD 2 は Protein X と近距離に存在する．赤色が proximity ligation assay のシグナル．対照(下写真)ではシグナルは検出されない．

function 型の遺伝子変異により，3 つの蛋白質の発現量，細胞内局在の変化が生じ，この結果，上皮バリアー機能の障害が起こるのではないかと考えられた．今後は，TACSTD 2 遺伝子と 3 つの蛋白質の結合ドメインの同定，蛋白質発現量の低下，細胞内局在の変化に関する詳細について検討する予定である．

　臨床的には，角膜上皮バリアー機能低下を代償させるような試み，例えばソフトコンタクトレンズの連続装着などが有効性を示しているが，最終的には，TACSTD 2 遺伝子の導入などにより，角膜上皮バリアー機能を正常化させる試みがなされるかもしれない．これには，遺伝子導入の倫理性，そして費用対効果における有用性が検討されるであろうが，角膜疾患として遺伝子治療が最も奏功する可能性のある疾患であることには違いない．

Ⅲ　培養粘膜上皮移植の開発

1．眼表面再建術の開発の歴史

　Stevens-Johnson 症候群，眼類天疱瘡，熱化学外傷などの重症角結膜疾患では，角膜上皮幹細胞を含む角膜輪部領域が広範囲に障害されるため，正常な角膜上皮は供給不可能となる．その結果，周辺の結膜上皮が炎症，瘢痕，血管新生などを伴って眼表面を覆い，著しい視機能障害を来す．慢性瘢痕期には眼表面の非角化粘膜上皮は病的角化を来し，不可逆的となる．このように，角膜上皮幹細胞が機能不全を来す疾患は "難治性眼表面疾患" あるいは "角膜上皮幹細胞疲弊症" と呼ばれており，その病態・病理の解明，治療法の開発についてこれまでさまざまな基礎および臨床研究がなされてきた．従来これらの疾患に対する治療戦略としては，炎症を消退させて

慢性瘢痕期まで待ち外科的再建をするというのが世界的にコンセンサスを得られている考え方であった．

　これまで，難治性眼表面疾患に対する外科的再建法としては，結膜移植術[15]，角膜上皮形成術(keratoepithelioplasty)[16][17]そして角膜輪部移植術(limbal transplantation)[18][19]などが開発されてきた．概念的には，喪失した角膜上皮細胞を結膜上皮からの再生上皮で補充する手法が結膜移植術であり，幹細胞を含む角膜輪部や周辺部角膜からの再生上皮で再建する移植が角膜輪部移植術や角膜上皮形成術である．いずれの移植法も，in vivo における移植片からの再生上皮により眼表面の再建(in vivo expansion 法)を目的とする手術法であり，第一世代の眼表面再建術といえる．

　上述の移植法で，自家移植の行える片眼性疾患で健眼から移植片を採取可能な症例数には限りがあり，移植片の採取領域にも限界がある．両眼性の疾患ではドナー角膜を利用する同種移植を行うことになる．しかし我が国におけるドナー不足は深刻であり，再生医療学的見地から，必要とする移植組織が少量でよければ理想的である．そこで難治性眼表面疾患に対する眼表面再建術として近年注目を集めているのが培養粘膜上皮移植術である．必要とする細胞を少量採取して生体外で培養粘膜上皮シートを作製したのち移植するというコンセプトは，これまでの角膜移植の長い歴史の中で細胞移植(cellular surgery)の分野を確立したといえる．その先駆けとなったのは，1997 年 Pellegrini らによる臨床報告である[20]．彼女らは，自家培養角膜上皮シート移植による 2 例の成功例を報告し，これが角膜再生医療の幕開けとなった．当時，我々のグループも同様な研究を行っていたが，こ