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Spontaneous Ocular Surface Inflammation and Goblet Cell Disappearance in IκBζ Gene-Disrupted Mice

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Purpose. The ocular surface epithelium is part of the mucosal defense system. Because transcription factor NF-κB in mucosal epithelial cells plays a central role in regulating the genes that govern the onset of mucosal inflammatory responses, we examined the role of a regulator of NF-κB, IκBζ, in murine ocular surface inflammation.

Methods. The eyes of IκBζ$^{-/-}$ mice were analyzed biomicroscopically and histologically. IκBζ expression in normal mouse cornea and conjunctiva was examined by RT-PCR. The results were compared with those obtained in other tissues by real-time PCR. IκBζ mRNA on the ocular surface and in other mucosal tissues was localized by in situ hybridization.

Results. IκBζ$^{-/-}$ mice manifested chronic inflammation, specifically in the ocular surface, but not in other tissues. In normal mice, IκBζ was expressed in a variety of mucosal tissues. The IκBζ transcript was predominantly distributed in the epithelia of these tissues. As inflammatory symptoms progressed on the ocular surface of IκBζ$^{-/-}$ mice, inflammatory cells, mainly CD45R/B220^{+} and CD4^{+} cells, intensely infiltrated the submucosa of the conjunctival epithelia. This infiltration was accompanied by an almost complete loss of goblet cells in the conjunctival epithelia.

Conclusions. The authors postulate that IκBζ in the ocular surface epithelia negatively regulates the pathologic progression of ocular surface inflammation. (*Invest Ophthalmol Vis Sci.* 2005;46:579-588) DOI:10.1167/iovs.04-1055

On the ocular surface, which consists of the conjunctiva and cornea, epithelial cell layers are the initial site of bacterial colonization. Protection of these cell layers against infection from a wide array of pathogens requires robust, innate defense mechanisms.[1-4]

Pathogens must battle nonspecific defense mechanisms, including blinking, tear flow, and mucin, which provide a physical barrier to prevent ocular surface infection under normal conditions.[5,6] In addition to these mechanical defenses, the human tear film contains innate defense molecules with antibacterial properties, such as lysozymes, lactoferrin, and defensins.[4,5] Thus, the ocular surface system presents an inhospitable environment for pathogens seeking to bind to the epithelial cell surface.

However, physiological destruction of the ocular surface by trauma, immunodeficiencies, or routine contact lens wear increases the incidence of sight-threatening corneal infection due to common pathogens, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[7,8] The conjunctival sac and eyelid edge are host to normal bacterial flora including coagulase-negative staphylococci (CNS), *Propionibacterium acnes,* and other commensal Gram-positive and -negative bacteria,[9,10] to which, under normal conditions, the ocular surface epithelia does not respond. Thus, we suggest that there may be a unique inflammation mechanism that prevents corneal opacity or neovascularization.[11]

The mammalian innate immune system plays a key role in recognizing pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs), and in stimulating the production of cytokines and other proinflammatory mediators, followed by the activation of acquired immune responses.[12-17] Members of the Toll-like receptor (TLR) family are essential components in the activation of innate immunity.[5,18-21] If epithelial cells are equipped with a highly sensitive pattern-recognition apparatus, they can be a constant source of proinflammatory mediators.[22]

IκBζ was originally reported as a regulator of transcription factor NF-κB, which is strongly induced by interleukin (IL)-1 and lipopolysaccharide (LPS), but not by tumor necrosis factor (TNF)-α.[23-26] IκBζ, induced by diverse PAMPs such as peptidoglycan (PGN), bacterial lipoprotein, flagellin, MALP-2, R-848, and CpG DNA,[27,28] regulates NF-κB activity, possibly to prevent excessive inflammation caused by bacterial components.[25,27] We used IκBζ gene-disrupted mice to study the role of IκBζ in ocular surface inflammation. These mice expressly exhibited severe, spontaneous ocular surface inflammation, suggesting that IκBζ participates in the negative regulation of ocular surface inflammation.

Materials and Methods

Mice and Reagents

C57BL6 mice were purchased from CLEA (Tokyo, Japan) and used at 8 weeks of age for RT-PCR, semiquantitative RT-PCR, and in situ hybridization of IκBζ. All studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

IκBζ gene-disrupted mice were produced by Shizuo Akira and Masahiro Yamamoto at the Department of Host Defense, Research Institute for Microbial Diseases at Osaka University. To genotype the 2- to 3-week-old mice from heterozygous parents, we used genomic DNA isolated from their tails (DNeasy kit; Qiagen, Valencia, CA). PCR amplification on a thermal cycler (GeneAmp; Applied Biosystems, Foster

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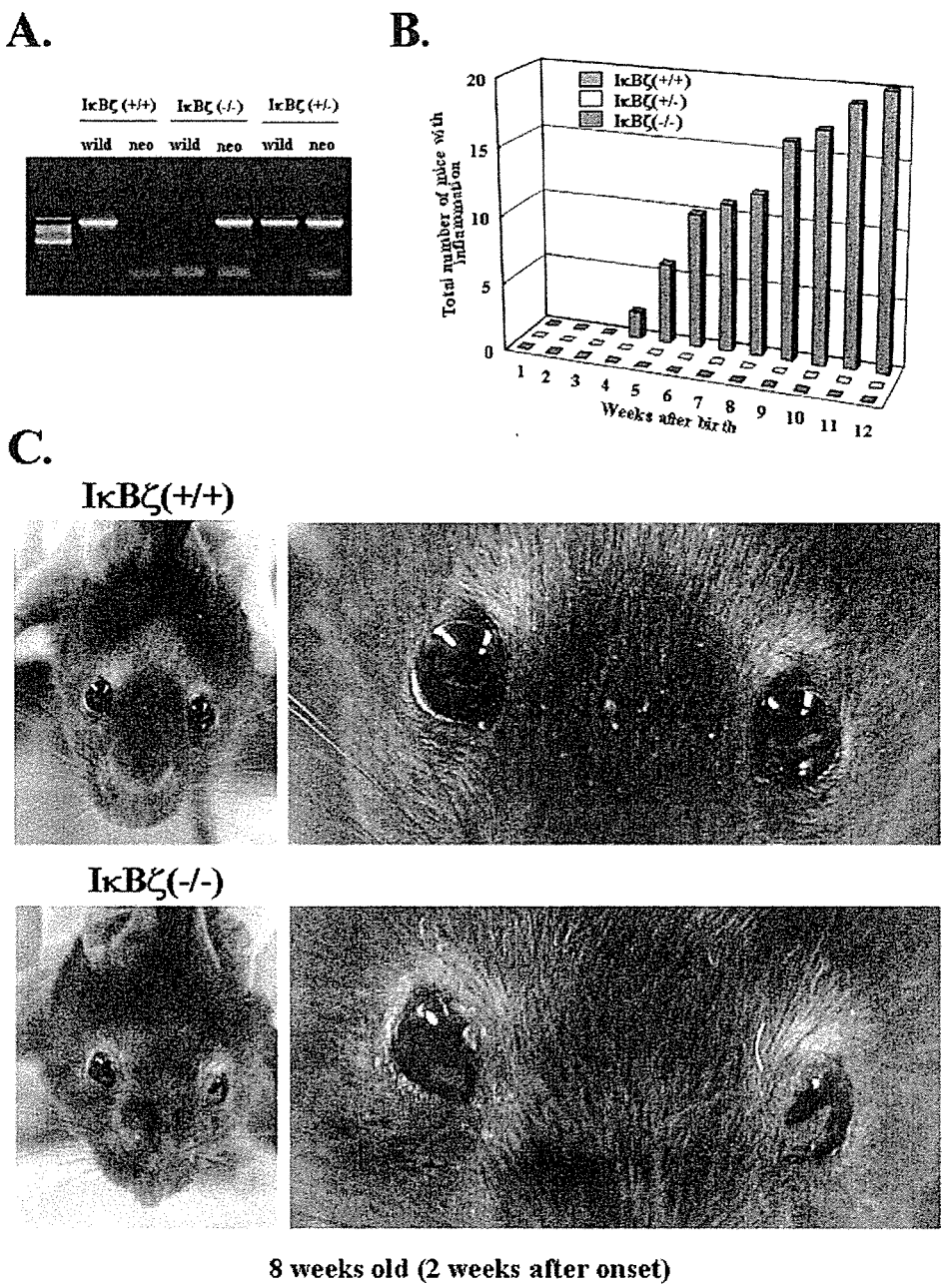


FIGURE 1. Genotype determination of mouse tail genomic DNA and phenotype of IκBζ gene-disrupted mice. (**A**) PCR products were analyzed on 1% agarose gels. A 1200-bp band was obtained from wild-type mice ($I\kappa B\zeta^{+/+}$) with the IκBζ gene primer pair IκBζ-wild and IκBζ-ex03, another 1200-bp band from homozygotes ($I\kappa B\zeta^{-/-}$) with the gene primer pair IκBζ-ex03 and PKG-rc2, and both fragments from heterozygotes ($I\kappa B\zeta^{+/-}$). (**B**) The incidence and onset time of ocular surface inflammation in $I\kappa B\zeta^{-/-}$, $I\kappa B\zeta^{+/-}$, and $I\kappa B\zeta^{+/+}$ mice, after birth. Ocular surface inflammation was clinically defined as redness of the eyelid and bulbar conjunctiva, accompanied by discharge. (**C**) Photographs of the face of a $I\kappa B\zeta^{+/+}$ mouse and an $I\kappa B\zeta^{-/-}$ mouse at 8 weeks of age and at 2 weeks after symptom onset. Whereas $I\kappa B\zeta^{+/+}$ mice were free of inflammation (*top*), $I\kappa B\zeta^{-/-}$ mice exhibited a severe inflammatory phenotype, involving the ocular surface and the eyelids (*bottom*).

City, CA) with the IκBζ gene primer pair for wild IκBζ, IκBζ-wild (GCTCATCCAGCTAACCTGAACAGTGTT) and IκBζ-ex03 (GTTTAAGGTGGCGGTTCTGCTCTTTG), resulted in approximately a 1200-bp fragment from wild-type ($I\kappa B\zeta^{+/+}$) mice. The gene primer pair for the inserted neomycin gene, IκBζ-ex03 and PKG-rc2 (CTAAAGCGCATGCTCCAGACTGCCTTG), yielded approximately a 1200-bp fragment from homozygotes ($I\kappa B\zeta^{-/-}$). Both fragments were obtained from heterozygotes ($I\kappa B\zeta^{+/-}$; Fig. 1A).

LPS was derived from *Escherichia coli* (0111:B4; Sigma-Aldrich, St. Louis, MO).

Histologic Analysis

The whole eyeball, together with the eyelids and conjunctiva, was fixed with 4% paraformaldehyde and embedded in optimal cutting temperature (OCT) compound (Sakura Finetek USA., Inc., Torrance, CA) and then snap frozen in liquid nitrogen. Sections (6 μm thick) were cut and stained with periodic acid-Schiff (PAS) reagent and hematoxylin.

RT-PCR

Using an extraction reagent (TRIzol Reagent; Invitrogen, Carlsbad, CA), we isolated total RNA from mouse tissue and human corneal- and conjunctival epithelial cells according to the manufacturer's instructions. RT was performed (SuperScript Preamplification system; Invitrogen) and then amplification was performed with DNA polymerase (Takara Shiga, Japan) for 30 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute (GeneAmp; Applied Biosystems). The primers for mouse IκBζ were (forward), 5′-GAAGCCCGATGAATACCACCCA-3′, and (reverse), 5′-CGCATTGTGAGCCACGACC-3′. For human molecules possessing ankyrin-repeats induced by LPS (MAIL) primers were (forward), 5′-AGGCGATTCAGAAGGAGCAGT-3′ and (reverse), 5′-TCATCAACAGGCGGACAGCAT-3′. For mouse or human

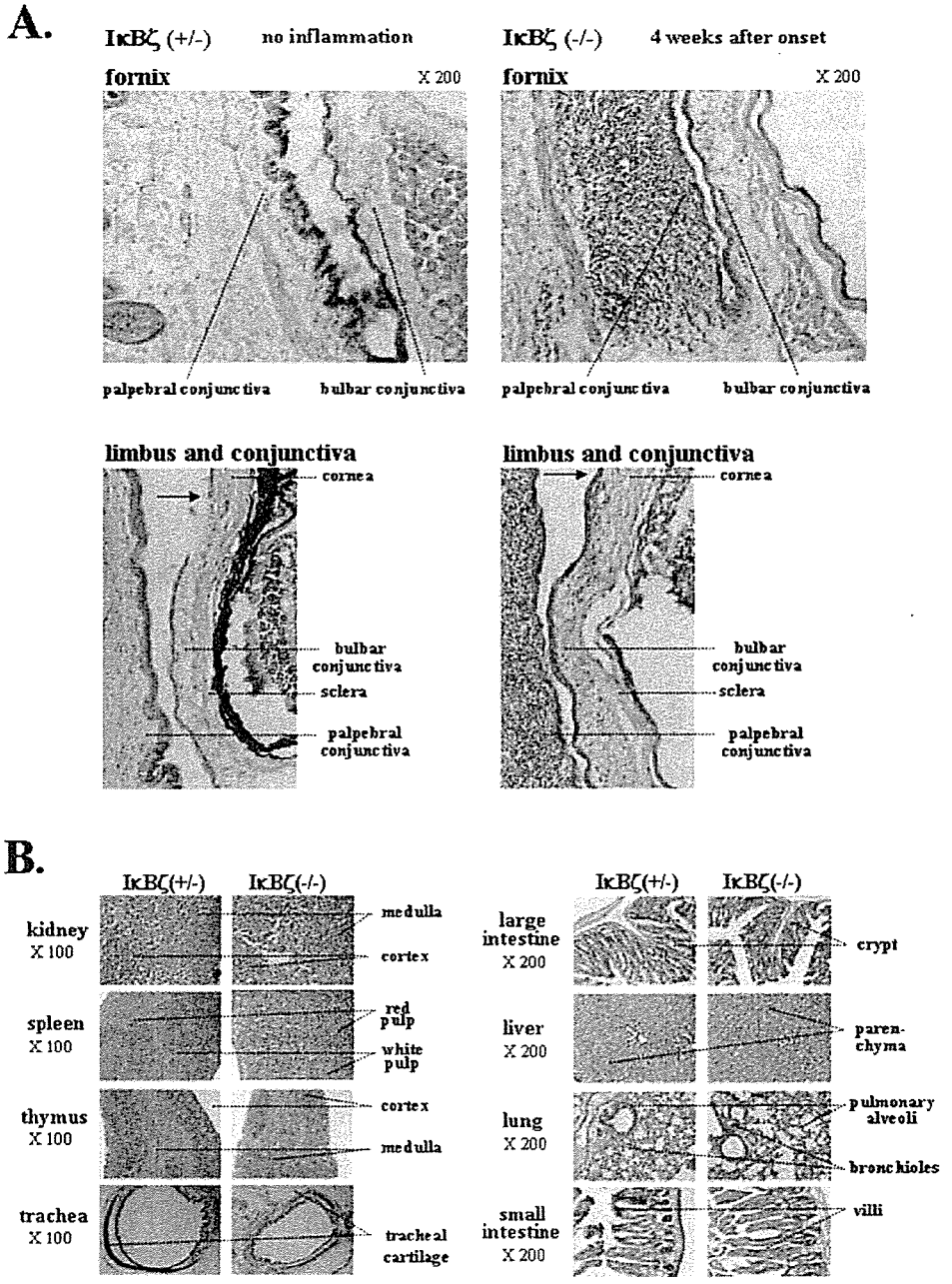


FIGURE 2. Histologic analyses of the eyes of IκBζ$^{-/-}$ mice (PAS stain). (**A**) Histologic analyses of the eyes of IκBζ$^{-/-}$ mice aged 14 weeks (at 4 weeks after the onset of inflammatory symptoms), revealed heavy infiltration of inflammatory cells into the submucosa under the conjunctival epithelia of the palpebral conjunctiva. There was moderate infiltration in the limbus. In IκBζ$^{-/-}$ mice, there was degeneration and loss of goblet cells in the conjunctival epithelia of both the palpebral and bulbar conjunctiva. The eyes of IκBζ$^{+/-}$ mice of the same age demonstrated neither noticeable pathologic changes nor infiltrating inflammatory cells. *Arrows*: the limbal–corneal junction. (**B**) In both IκBζ$^{+/-}$ and IκBζ$^{-/-}$ mice, there were no pathologic changes, such as inflammatory phenotypes, in other tissues: kidney, spleen, thymus, trachea, large intestine, liver, lung, and small intestine.

GAPDH they were (forward), 5′-CCATCACCATCTTCCAGGAG-3′, and (reverse), 5′-CCTGCTTCACCACCTTCTTG-3′. The integrity of RNA was electrophoretically confirmed on ethidium bromide–stained 1.5% agarose gels.

Real-Time Semiquantitative PCR

We used a PCR system (Prism 7700; Applied Biosystems), according to the manufacturer's instructions and a previously described protocol.[29] Total cellular RNA extractions and the first cDNA synthesis were as described for RT-PCR. Primer and probes (*Taq*Man; Applied Biosystems) for mouse IκBζ and mouse GAPDH were ready-made assay-on-demand. For IκBζ and mouse GAPDH cDNA amplification, real-time PCR was performed in a 25-μL total volume containing a 1-μL cDNA template in 2× PCR master mix (*Taq*Man Universal PCR Master Mix; Applied Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed on computer (Sequence Detection Software: Applied Biosystems), and the level of IκBζ mRNA expression was normalized to the expression of the housekeeping gene GAPDH.

In Situ Hybridization of Mouse Tissues and Human Conjunctiva

Mouse IκBζ cDNA fragments cloned from male mouse kidney cDNA with PCR were used for probes. For PCR cloning, the primers for mouse IκBζ were TGGCCTGACTCCCCTACATT (1663-1682) and CGGGCTGTTCATTCTCCAAG (2078-2059). In situ hybridization was performed as previously described.[30] Briefly, anesthetized C57BL/6 mice were perfusion-fixed with 4% paraformaldehyde, and dissected tissues were sectioned after paraffin embedding.

Human MAIL cDNA fragments cloned with PCR from human kidney cDNA (BD-Clontech, Palo Alto, CA) were used for probes. For PCR cloning, the primers for human MAIL were GCCAACCATTCCAAGTCAGG (854-873) and GCTCCACCTGCCACTGAAAA (1318-1299). Hu-

man conjunctival tissues obtained from patients who had given prior informed consent were fixed with 4% paraformaldehyde and sectioned after paraffin embedding. nitroblue tetrazolium/5-bromo-4-chloro-3-indoyl phosphate (NBT-BCIP) substrate (Roche, Basel, Switzerland) was used to visualize the signal, followed by counterstaining with nuclear fast red. Throughout, the corresponding sense probes did not give rise to staining. The DNA templates used for preparation of the digoxigenin-labeled riboprobes were as described earlier.

Immunohistological Analysis

The whole eyeball, together with the eyelids and conjunctiva, was embedded in OCT compound (Sakura Finetek) and then flash frozen in liquid nitrogen. Sections (6 μm thick) were cut and fixed with 100% acetone at 4°C for 10 minutes and blocked (30 minutes) with 10% normal donkey serum in phosphate-buffered saline (PBS). The primary antibody was applied for 1 hour at room temperature. The rat monoclonal antibody was reactive with mouse CD45R/B220 or mouse CD4 (BD Biosciences, San Diego, CA). The rat IgG2a isotype (BD Biosciences) acted as the negative control. After specimens were washed with PBS, the secondary antibody (Biotin-SP-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-rat IgG(H+L); 1:500 dilution; Jackson ImmunoResearch, West Grove, PA) was applied for 30 minutes. After sections were washed with PBS, they were incubated for 30 minutes (Vectastain ABC Reagent; Vector Laboratories, Inc., Burlingame, CA), washed with PBS, and incubated for 2 minutes with peroxidase substrate solution (3,3'-diaminobenzidine [DAB] substrate kit: Vector), and the slides were then covered with coverslips. Sections were examined under a microscope and photographed with a digital camera.

Human Corneal and Conjunctival Epithelial Cells

For RT-PCR, human corneal epithelial cells were obtained from corneal buttons at the time of corneal transplantation for bullous keratopathy (one eye) and keratoconus (two eyes). Human conjunctival epithelial cells were also obtained by conjunctival brush cytology from three volunteers at the University Hospital of Kyoto Prefectural University of Medicine. The purpose of the research and the experimental protocol were explained to all participants, and their prior informed consent was obtained. All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration.

Results

Ocular Surface of IκBζ Gene-Disrupted Mice

For genotyping of the 2- to 3-week-old mice from heterozygous parents, we used genomic DNA isolated from their tails. PCR amplification was performed with the IκBζ gene primer pair (Fig. 1A). We compared the results obtained in IκBζ gene-disrupted ($I\kappa B\zeta^{-/-}$) mice with those in wild-type ($I\kappa B\zeta^{+/+}$) mice and their heterologous ($I\kappa B\zeta^{+/-}$) littermates. Whereas no $I\kappa B\zeta^{+/+}$ and $I\kappa B\zeta^{+/-}$ mice exhibited symptoms of ocular surface inflammation throughout the experimental period (until they reached the age of 24 weeks), $I\kappa B\zeta^{-/-}$ mice became spontaneously symptomatic by 12 weeks (Fig. 1B). Figure 1C shows the face of an $I\kappa B\zeta^{-/-}$ mouse at 8 weeks of age and at 2 weeks after symptom onset. Although $I\kappa B\zeta^{+/+}$ mice had no inflammation, $I\kappa B\zeta^{-/-}$ mice exhibited a severe inflammatory phenotype on the ocular surface, especially along the eyelids. The inflammatory phenotype was absent at the time of their birth. It became evident when they were between 4 and 12 weeks of age. There were no gender differences with respect to the inflammatory phenotype and the age at symptom onset. In other eye compartments such as the lens, retina, uvea, and sclera, there were no pathologic changes in $I\kappa B\zeta^{-/-}$ mice, and they were not different from $I\kappa B\zeta^{+/+}$ mice (data not shown). No special behavioral abnormalities, including the amount of food and water consumed, were observed in $I\kappa B\zeta^{-/-}$ mice. When these mice were kept under conventional conditions, ocular surface inflammation became more prominent. In some animals, this was accompanied by dermatitis-like skin lesions (data not shown).

Histologic analysis of the eyes of $I\kappa B\zeta^{-/-}$ mice aged 14 weeks (4 weeks after the onset of inflammatory symptoms), showed heavy infiltration by inflammatory cells of the submucosal area of the whole conjunctiva and moderate infiltration of the corneal limbus, the junction of the cornea and conjunctiva. Moreover, we noted degeneration and loss of goblet cells in the conjunctival epithelia of both the palpebral and bulbar conjunctiva. Neither obvious pathologic changes nor infiltrating inflammatory cells were detected in the eyes of $I\kappa B\zeta^{+/-}$ mice of the same age (Fig. 2A). No pathologic changes, such as inflammatory phenotypes, were evident in other tissues such

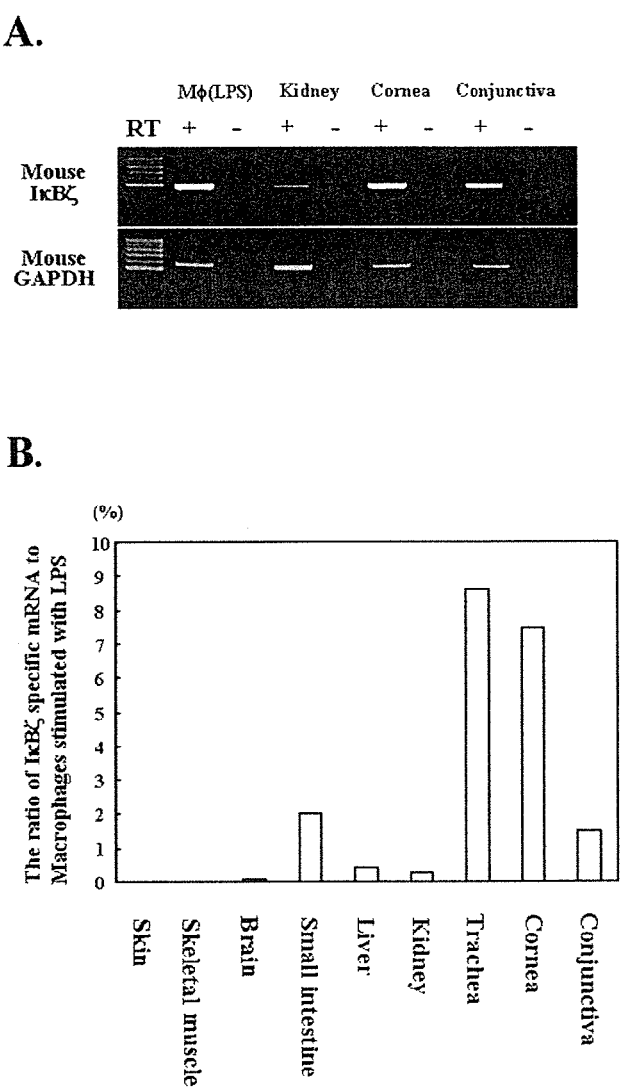


FIGURE 3. Expression of IκBζ mRNA detected by RT-PCR (A) and real-time PCR (B). (A) IκBζ mRNA was expressed in both corneal and conjunctival tissues of normal C57BL/6 mice. We confirmed the specificity of the PCR product for IκBζ by examining peritoneal macrophages stimulated with 100 ng/mL LPS and kidney tissue. (B) IκBζ mRNA was scarcely detected in the skin, skeletal muscle, and brain tissues, slightly expressed in liver and kidney tissues, and intensely expressed in mucosal tissues such as those of the small intestine, trachea, cornea, and conjunctiva.

as those of the kidney, spleen, thymus, trachea, large intestine, liver, lung, and small intestine (Fig. 2B).

IκBζ mRNA Expression

Next, we examined whether ocular surface tissues of mice express IκBζ-specific mRNA. Indeed, IκBζ mRNA was expressed in both corneal and conjunctival tissues of normal C57BL/6 mice (Fig. 3A). We isolated, subcloned, and sequenced the PCR products to ensure the expression of specific IκBζ. The sequences of these PCR products were identical with those of mouse IκBζ. As IκBζ-specific mRNA has been detected in the murine peritoneal macrophages stimulated with LPS, kidney, liver, lung, and heart by Northern blot analysis,[25] we confirmed the specificity of the PCR product for IκBζ by using peritoneal macrophages stimulated with 100 ng/mL LPS and kidney as a positive control.

We then compared the level of IκBζ expression in a variety of murine tissues by using real-time PCR. IκBζ mRNA was scarcely detected in the skin, skeletal muscle, and brain tissue. It was slightly expressed in liver and kidney tissue, and intensely expressed in mucosal tissues, such as the small intestine, trachea, cornea, and conjunctiva (Fig. 3B). Unexpectedly, it was more intensely expressed in the cornea than the conjunctiva, although severe pathologic changes were most evident in conjunctival lesions. This observation suggests interplay between corneal and conjunctival tissues.

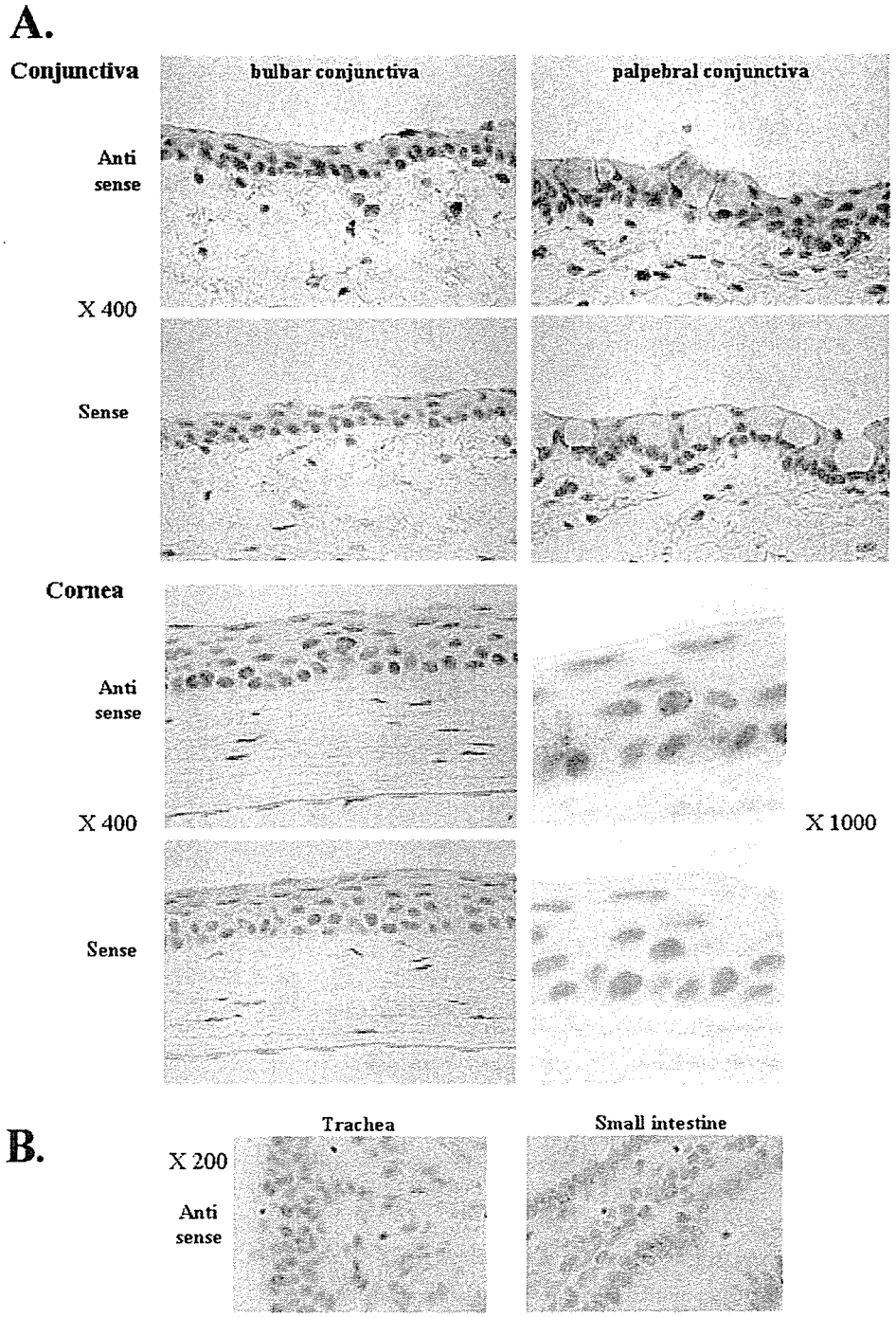


FIGURE 4. In situ hybridization of IκBζ-specific mRNA in ocular surface tissue and other mucosal tissues. The spatial-anatomic distribution of IκBζ transcripts was investigated by in situ hybridization of C57BL/6 conjunctival and corneal tissues and mucosal tissues such as those of the trachea and small intestine. (**A**) IκBζ was expressed in corneal epithelial cells, conjunctival epithelial cells and in some subconjunctival cells. (**B**) The expression of IκBζ transcript was primarily localized in the epithelia of the trachea and small intestine.

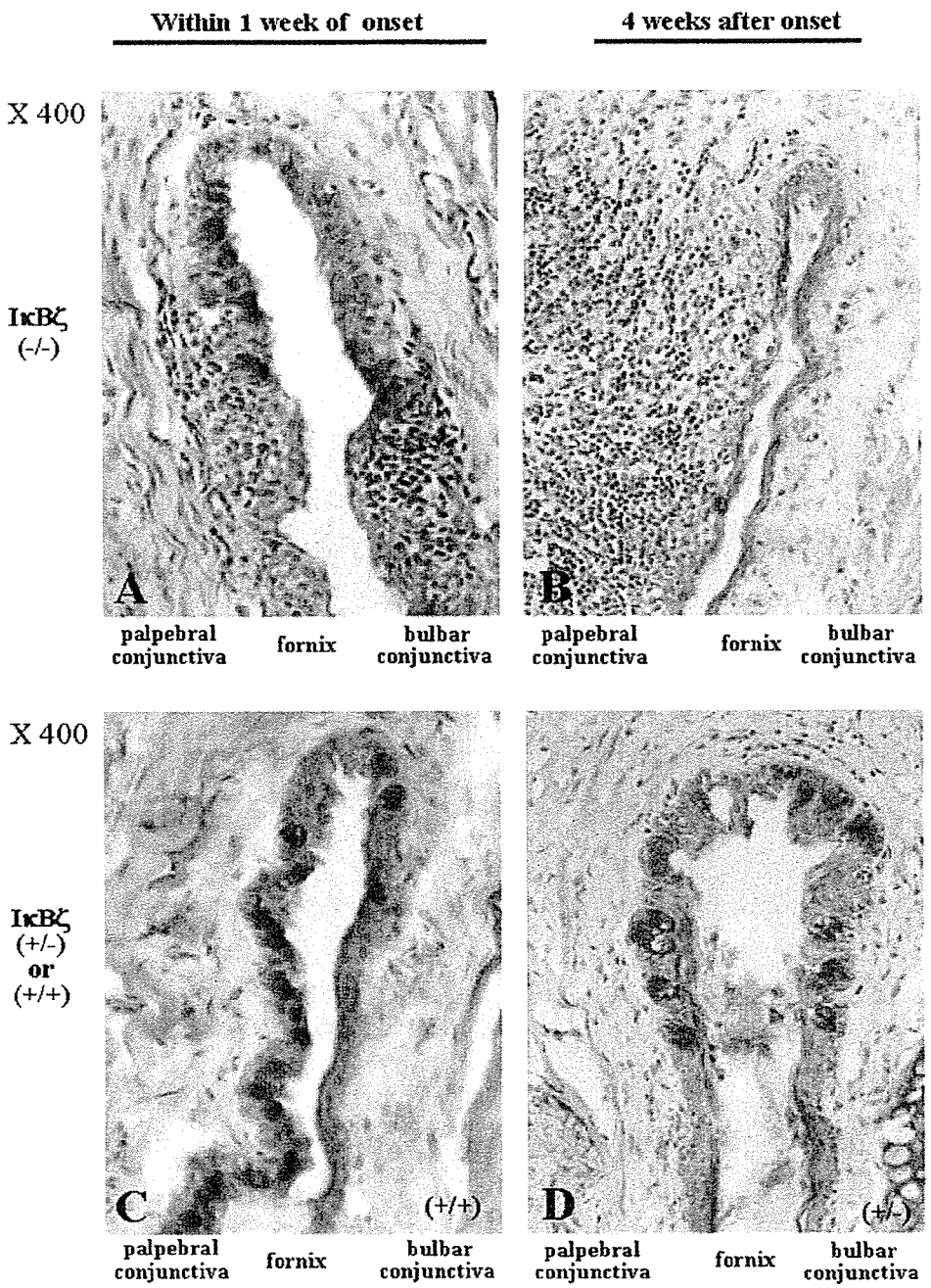


FIGURE 5. Inflammatory infiltrates and loss of goblet cells on the ocular surface of IκBζ$^{-/-}$ mice. Examination of the eyes of 4-week-old IκBζ$^{-/-}$ mice within 1 week after inflammatory symptom onset, showed infiltration of inflammatory cells into the conjunctival epithelia accompanied by simultaneous moderate degeneration of goblet cells (A). The eyes of 14-week-old mice, examined 4 weeks after symptom onset, clearly exhibited the pathologic progression of mucosal inflammation. Note the almost complete loss of goblet cells in the epithelia of both the palpebral and bulbar conjunctiva at this stage (B). In contrast, the eyes of IκBζ$^{+/-}$ or $^{+/+}$ mice did not show any loss of goblet cells or infiltration of inflammatory cells into the conjunctiva (C, D).

Expression of IκBζ in the Ocular Surface Epithelia

We investigated the spatial-anatomic distribution of IκBζ transcript by in situ hybridization of C57BL/6 in conjunctival and corneal tissues and other mucosal tissues, such as those of the trachea and small intestine. On the ocular surface, IκBζ was expressed in corneal epithelial cells, conjunctival epithelial cells, and some subconjunctival cells (Fig. 4A). Furthermore, the predominant expression of IκBζ transcript was localized spatially to the epithelia in the trachea and small intestine (Fig. 4B).

Pathology on the Ocular Surface of IκBζ$^{-/-}$ Mice

We kinetically monitored the pathologic changes in the eyes of IκBζ$^{-/-}$ mice as they grew older. First, we analyzed the eyes of IκBζ$^{-/-}$ mice at 4 weeks of age, within 1 week of the onset of spontaneous inflammatory symptoms. The infiltration of inflammatory cells into the conjunctival epithelia was evident, together with a moderate loss of goblet cells in the conjunctival epithelia (Fig. 5A). The eyes of 14-week-old IκBζ$^{-/-}$ mice (4 weeks after symptom onset), exhibited clear signs of progressive mucosal inflammation. In contrast to the normal spatial distribution of goblet cells in the conjunctival epithelia, which were free from inflammatory infiltrates in IκBζ$^{+/-}$ mice, IκBζ$^{-/-}$ mice manifested heavy infiltration by inflammatory cells under the conjunctival epithelia of the palpebral conjunctiva. There was an almost complete loss of goblet cells in the epithelia of both the palpebral and bulbar conjunctiva in IκBζ$^{-/-}$ mice at this stage (Fig. 5B). Before the manifestation of ocular surface inflammation, the eyes of IκBζ$^{-/-}$ mice exhib-

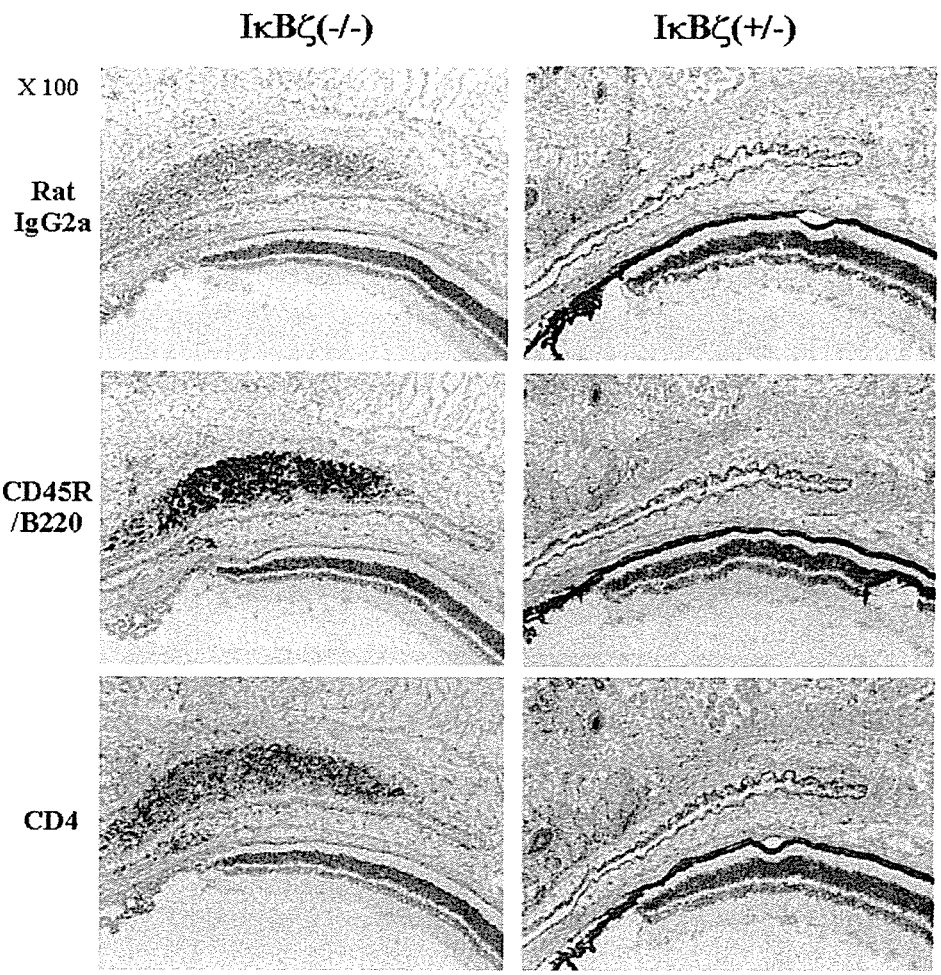


FIGURE 6. Inflammatory infiltrates comprised B- and helper T-cells. Immunohistological analysis of the eyes of IκBζ$^{-/-}$ mice (14 weeks old, 4 weeks after symptom onset) revealed that inflammatory infiltrates in subconjunctival tissues of the eyelid consisted of CD45R/B220^{+} and CD4^{+} cells. Similarly, CD45R/B220^{+} and CD4^{+} cells were detected in the corneal stroma and subconjunctival tissues of the limbus. These cells were not detected immunohistologically in the subconjunctival tissues of IκBζ$^{+/-}$ mice. There was a color difference in the ciliary pigment of epithelial cells. In IκBζ$^{-/-}$ mice the color was brown, and in IκBζ$^{+/-}$ mice it was black.

ited no distinct histologic changes. They were similar to the eyes of IκBζ$^{+/+}$ mice (data not shown). We posit that the observed loss of goblet cells may be a consequence of inflammatory cell infiltration into the conjunctival epithelia of IκBζ$^{-/-}$ mice.

Inflammatory Infiltrates

Using immunohistological analysis, we found that the inflammatory infiltrates in the subconjunctival tissue of the eyelids of 14-week-old IκBζ$^{-/-}$ mice (4 weeks after symptom onset) were CD45R/B220- and CD4-positive cells. These cells were also present in the limbal tissue (Fig. 6). They were not detected in the subconjunctival tissue of IκBζ$^{+/-}$ mice.

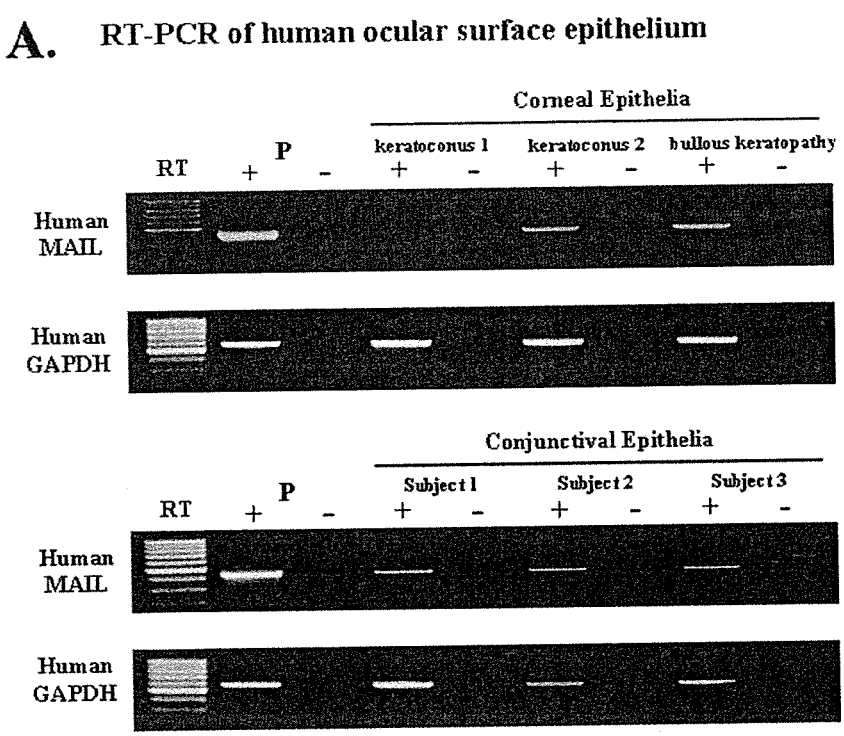
Expression of MAIL mRNA on Human Ocular Surface

Human MAIL is reportedly similar to mouse IκBζ.[31] RT-PCR showed that human corneal and conjunctival epithelia expressed MAIL-specific mRNA (Fig. 7A). Isolating, subcloning, and sequencing the PCR products confirmed that they were identical with human MAIL. We further confirmed the specificity of the PCR products for human MAIL with human peripheral monocytes stimulated with 100 ng/mL LPS. In situ hybridization of human conjunctival tissues revealed the restricted spatial distribution of human MAIL transcripts on the ocular surface. Human MAIL was dominantly expressed in conjunctival epithelial cells and moderately expressed in subconjunctival cells (Fig. 7B). Based on these findings, we conclude that a regulator of transcription factor NF-κB, MAIL, is also expressed on the human ocular surface.

DISCUSSION

In IκBζ$^{-/-}$ mice, spontaneous chronic inflammation is selectively elicited in ocular surface tissue but not in other tissues. The IκBζ transcript was predominantly distributed in the epithelia of a variety of mucosal tissues. As the inflammatory symptoms on the ocular surface progressed, inflammatory cells, mainly consisting of CD45R/B220^{+} and CD4^{+} cells, intensely infiltrated the submucosa of the conjunctival epithelia. There was a concurrent loss of goblet cells in the conjunctival epithelia. Our findings suggest that the presence of IκBζ in the ocular surface epithelia inhibits the pathologic progression of ocular surface inflammation. Our observation that a MAIL transcript (a human homologue of mouse IκBζ) was expressed in human corneal and conjunctival epithelia, promotes the new concept that a regulator of NF-κB, MAIL, plays a pivotal role in the pathogenesis of infections via PRRs on the human ocular surface. Studies are under way in our laboratory to determine how IκBζ selectively regulates the pathogenesis of ocular surface inflammation.

The ocular surface is particularly vulnerable to debilitating infections.[4,5,18–21] Although the conjunctival sac and eyelid edge host commensal bacterial flora, under normal conditions there is no inflammatory response by the corneal and conjunctival epithelia. Protection of the eyes from microbial attack is of



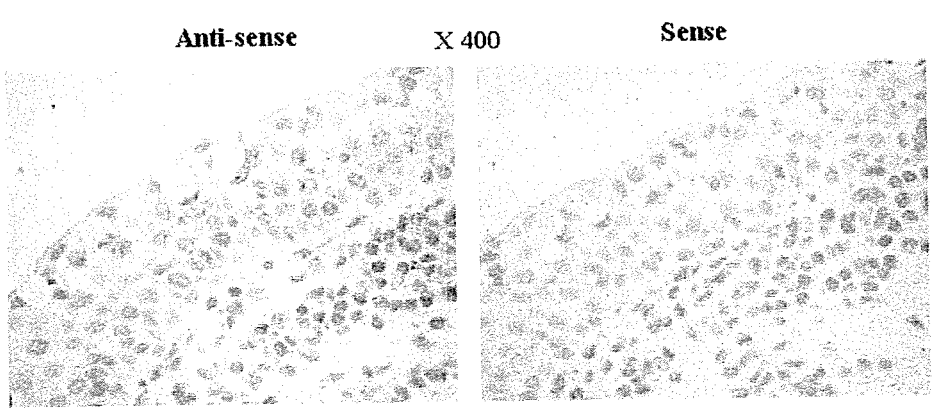


FIGURE 7. Human corneal and conjunctival epithelial tissues expressed MAIL mRNA. (**A**) RT-PCR detected MAIL-specific mRNA in both the corneal and conjunctival epithelia. The specificity of the PCR product for human MAIL was confirmed by using human peripheral monocytes stimulated with 100 ng/mL LPS. (**B**) In situ hybridization of human conjunctival tissues revealed the restricted spatial distribution of a human MAIL transcript on the ocular surface. Human MAIL was dominantly expressed in conjunctival epithelial cells and moderately expressed in subconjunctival cells.

paramount importance, as diseases of the ocular surface, in particular corneal infection, can have devastating effects on vision,[1,2] due to the resultant corneal opacity, neovascularization of the cornea, or invasion of the conjunctival epithelia, covering the cornea. Besides possessing several defense modalities in common with other mucosal tissues, the eye has a unique array of protective mechanisms that maintain visual acuity.[1-4]

We have reported[11] that human corneal epithelial cells fail to respond functionally to PAMPs, such as PGN and LPS, due to the lack of TLR2 and TLR4 on their surfaces. Despite the presence of these receptors in the cytoplasm of human corneal epithelial cells, the translocation of LPS to the cytoplasm did not elicit a response by those cells. This suggests that human corneal epithelial cells possess a unique regulatory mechanism to promote the inhibition of TLR2- and TLR4-mediated inflammatory responses. Although the stimulation of macrophages with PGN, LPS, or CpG DNA reportedly results in IκBζ induction,[25,27] we found that stimulation with LPS or CpG DNA did not induce IκBζ in human corneal epithelial cells (data not shown). This suggests that ocular surface epithelial cells possess a unique regulatory mechanism that inhibits TLR-mediated inflammatory responses and contributes to the immunostable environment of the ocular surface epithelia.

To prevent excessive inflammation, the production of pro- and anti-inflammatory mediators must be strictly regulated during the inflammatory course. NF-κB regulates the expression of a wide range of genes,[32-34] including those that encode proinflammatory cytokines and chemokines (e.g., IL-1, IL-6, IL-12,

TNF-α, IL-8, MCP1, RANTES, and eotaxin), adhesion molecules, and inducible effector enzymes (e.g., iNOS and COX-2).[35-37] The production of antimicrobial peptides such as β-defensins, constitutively produced by mucous epithelial cells, is also regulated by NF-κB.[35,36] NF-κB activation also induces the migration and maturation of leukocytes.[37] Furthermore, NF-κB plays a key role in the regulation of apoptosis.[37]

A novel IκB protein, IκBζ/MAIL, induced by IL-1 and PAMPs, but not by TNF-α, regulates NF-κB in the nucleus. The induction of IκBζ is controlled by NF-κB, which, in turn, is regulated by IκBζ. Therefore, NF-κB and IκBζ may comprise an autonomous negative-feedback loop.[27] In contrast, Yamamoto et al.[28] recently reported that IκBζ was indispensable for IL-6 production in response to TLR ligands and IL-1. They proposed a new role for IκBζ as a positive regulator of NF-κB in the two-step process of IL-6 gene activation. IL-6 is a multifunctional cytokine with both pro- and anti-inflammatory effects.[38-41] The former plays an essential role in tissue inflammation and the control of infection. IL-6 can augment or inhibit Th2 tissue inflammation,[42-46] and its anti-inflammatory, counter-regulatory, and healing activities have been documented.[47]

We considered whether the pathologic changes on the ocular surface of IκBζ$^{-/-}$ mice were attributable to an overly robust Th1 or Th2 response due to impaired IL-6 production. However, IL-6$^{-/-}$ mice did not manifest the ocular surface inflammation in IκBζ$^{-/-}$ mice (Akira S, et al., unpublished data, 1995). As IL-6 induces pro- and anti-inflammatory effects that could be tissue or stimulus specific,[48,49] impaired IL-6 production may be the underlying factor in the pathogenesis of ocular surface inflammation in IκBζ$^{-/-}$ mice. Alternatively, the prolonged production of TNF-α in these mice[28] may account for the discrepancies observed in IκBζ$^{-/-}$ and IL-6$^{-/-}$ mice.

Although IκBζ was expressed not only on the ocular surface but also in mucosal tissues such as the trachea and small intestine, in IκBζ$^{-/-}$ mice the inflammatory changes were limited to the ocular surface. The reason for this finding necessitates further investigation. Although regulatory T-cells contribute to the regulation of inflammation in the small intestine,[50] it is not clear whether they play a role in ocular surface inflammation. There is some evidence that the regulation of NF-κB, which dictates inflammatory responses, varies among distinct mucous tissues.[51]

Conjunctival mucosal cells consist of epithelial- and goblet cells that secrete mucin into the tear film. The marked loss of goblet cells in IκBζ$^{-/-}$ mice implies a dramatic decrease of mucin in their tears and a weakening of the innate host defense mechanisms in the ocular surface.[6] Although this weakening may render the ocular surface more highly susceptible to pathogenic and commensal microbes, we currently do not have unequivocal clinical or histologic evidence of bacterial infection in the ocular surfaces of IκBζ$^{-/-}$ mice.

To the best of our knowledge, there have been no rodent models showing the spontaneous loss of goblet cells in their conjunctiva. Rodent models of allergic conjunctivitis displayed no change in these cells,[52-54] and NC/Nga mice with spontaneous atopic dermatitis manifested an increase in goblet cell density.[55] Therefore, IκBζ$^{-/-}$ mice are different from mice with allergy-related conjunctivitis. Considering the regulation of TLRs by NF-κB[12-15] and the induction of IκBζ by TLRs,[25,27] the ocular surface inflammation we observed in the IκBζ$^{-/-}$ mice may be closely related to innate PAMPs/PRRs-amplified immune responses to microbes on the NF-κB axis.

Of particular interest is our observation that IκBζ$^{-/-}$ mice eventually lost almost all goblet cells in the course of persistent inflammation. Humans with devastating ocular surface disorders such as Stevens-Johnson syndrome and ocular cicatricial pemphigoid may experience a similar loss of goblet cells. In a patient with conjunctival inflammation due to Stevens-Johnson syndrome, Kawasaki et al.[56] identified CD4$^+$ T-cells in the cell population infiltrating the conjunctival tissues over the cornea. Our findings suggest that IκBζ$^{-/-}$ mice may be a suitable model for Stevens-Johnson syndrome and that these mice may be useful for mimicking the secondary conjunctival inflammation that often occurs in patients with Stevens-Johnson syndrome and/or cicatricial ocular pemphigoid. Moreover, IκBζ$^{-/-}$ mice may provide further insight into the interplay between microorganisms and innate immune responses in the presence of ocular surface disorders.

Acknowledgments

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