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Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines

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Abstract

Epithelial cells of the ocular surface are key in the first-line defense as a part of the mucosal immune system against pathogens. We investigated whether polyI:C induces the production by human corneal epithelial cells (HCEC) of pro-inflammatory cytokines and IFN-β, and whether Toll-like receptor (TLR)-3 expression is amplified by polyI:C. TLR3 was expressed on the surface of HCEC. Stimulation with polyI:C elicited the elevated production and mRNA expression of IL-6 and IL-8 in HCEC. While polyI:C induced IFN-β, far stronger than human fibroblasts, and TLR3 gene expression in HCEC, LPS stimulation did not. Similarly, polyI:C, but not LPS, induced the gene expression of IκBα and MAIL, members of the IκB family, in HCEC. The innate immune response of HCEC is distinct from that of immune-competent cells, and we suggest that this is indicative of the symbiotic relationship between corneal epithelium and microbes inhabiting the ocular surface.

Keywords: Human corneal epithelial cells; PolyI:C; TLRs; Inflammation; LPS

On the ocular surfaces as in the intestine, the surface epithelium serves a critical function in the front-line defense of the mucosal innate immune system [1–3]. Upon challenge, epithelial cells lining mucosal surfaces play a pivotal role in innate immunity by secreting chemokines and other immune mediators. The ability to detect microbes is arguably the most important task of the immune system. Exaggerated host defense reaction of the epithelium to endogenous bacteria may induce the initiation and perpetuation of inflammatory mucosal responses [4–6].

The ability of cells to recognize microbial motifs and pathogen associated molecular patterns (PAMPs) rests on pattern recognition receptors (PRRs) [7–9]. Signal transduction depends on the expression of a family of type I transmembrane receptors, Toll-like receptors (TLRs). To date, 11 TLRs have been identified in humans; they are expressed primarily on cell types that are mammalian host immune-competent cells such as dendritic cells and macrophages. These are the cells that are most likely to come into direct contact, via the mucosal epithelia, with pathogens from the environment [10]. TLR expression is not restricted to phagocytic cell types, rather, it appears that the majority of cells in the body including mucosal epithelial cells express at least a subset of TLRs [11]. Some PRRs are located on the cell membrane and respond to extracellular PAMPs; others exist in the cytosol and respond to PAMPs that cross the plasma membrane [12–14]. Signaling through TLRs results in the activation of IKK, NF-κB, and NF-κB target genes, and the coordinated activation of several transcription factors that regulate the expression of antimicrobial genes, cytokines, chemokines, and co-stimulatory molecules [7–9].

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Although the eye is relatively impermeable to microorganisms [1,3,15,16], if corneal integrity is compromised by trauma or contact lens wear, sight-threatening bacterial infection may occur [17,18]. Uniquely, human corneal epithelial cells (HCEC) are in constant contact with bacteria and bacterial products; they form a structural and functional barrier against numerous bacteria both pathogenic and nonpathogenic. Factors normally pro-inflammatory for other cell types do not induce epithelial cells to initiate a defensive response [19]. This is especially important with respect to the epithelial cells of the avascular and transparent cornea, where the formation of scar tissue in response to a host inflammatory reaction results in opacification and loss of vision. We previously reported that human corneal epithelial cells failed to respond functionally to PAMPs such as peptidoglycan (PGN) and lipopolysaccharide (LPS) because they lack TLR2 and TLR4 on their surface [20]. Despite the existence of TLR2 and TLR4 in the cytoplasm of HCEC, the experimental translocation of LPS to the cytoplasm did not elicit an immune response [20].

Among the TLRs, TLR4 which recognizes LPS, and TLR3 which recognizes the viral double-stranded RNA-mimic polyI:C have received the greatest attention [21–23]. TLR3-mediated responses are unique because TLR3 activation elicits lower levels of inflammatory cytokines than the activation of other TLR family members, although TLR3 activation induces the very robust secretion of IFN-β [21]. The remarkable similarities in the cellular responses to bacterial and viral infection after pathogen recognition are indicative of cross-talk between virus- and bacteria-induced signaling [24]. Although there were two reports by the same group describing the inhibitory effect of polyI:C against herpetic keratitis in rabbits, nothing is yet known on the reproducibility of their experiments or the effect on the innate immune response [25,26].

Here we demonstrate that HCEC express TLR3 at the cell surface and thus respond to polyI:C to generate pro-inflammatory cytokines and IFN-β. We also show that the surface expression of TLR3 on HCEC was amplified in an autocrine/paracrine manner by polyI:C.

Materials and methods

All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants and their prior written informed consent was obtained.

Human corneal epithelial cells. For RT-PCR, human corneal epithelial cells (HCEC) were obtained from corneal buttons of patients undergoing corneal transplantation for early-stage bullous keratopathy (one eye) or keratoconus (two eyes) at the affiliated hospital of Kyoto Prefectural University of Medicine.

Primary HCEC, obtained from Kurabo (Osaka, Japan), were cultured at 37 °C under 95% humidity and 5% CO_2 in serum-free medium consisting of EpiLife (Kurabo) supplemented with HCEC growth supplement (HCGS) containing 1 ng/ml murine epidermal growth factor (mEGF), 5 μg/ml insulin from bovine pancreas, 0.18 μg/ml hydrocortisone, and 0.4% v/v bovine pituitary extract (Kurabo), 0.2% PSA solution, and antibiotic–antimycotic solution (5000 U/ml penicillin, 50 mg/ml streptomycin, and 12.5 μg/ml amphotericin B) (Kurabo) [20]. For assays, 2×10^6 primary HCEC were plated in 25 cm^2 flasks. After reaching sub-confluence, they were either left untreated, exposed to 1 μg/ml LPS from *Pseudomonas aeruginosa* (Sigma, St. Louis, MO), or exposed to 25 μg/ml polyI:C (Invivogen, San Diego, CA) for 1-, 3- or 6 h. The culture time of polyI:C-treated cells was adjusted to be optimal for the maximum induction of IL-6, IFN-β, IκBα, MAIL, and TLR3, it was 6 h for IL-6, IL-8, and TLR3, and 3 h for IFN-β, IκBα, and MAIL.

Purification of human peripheral mononuclear cells. Venous blood samples from healthy volunteers were anti-coagulated with 2Na-EDTA, placed in sterile 50-ml polypropylene tubes, mixed with 1 volume of Ca^{2+}-free PBS (PBS(−)), overlaid with Ficoll–Paque Plus (Amersham Biosciences AB, Uppsala, Sweden), and centrifuged for 20 min at 2000 rpm at 20 °C. Human peripheral mononuclear cells (HPMC) were gently aspirated from the interface and washed with PBS(−). For stimulation with LPS or polyI:C, isolated HPMC were cultured for 1-, 3- or 6 h in RPMI medium (Gibco-BRL Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco).

Human conjunctival fibroblasts. Human conjunctival fibroblasts (HCFB) were obtained from redundant subconjunctival tissues of patients undergoing cataract surgery at the affiliated hospital of Kyoto Prefectural University of Medicine. Primary HCFB were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco). For assays, all procedures were the same as those described above for HCEC.

MRC-5 and HeLa cells. MRC-5 and HeLa, expressing TLR3 on the cell surface and producing IFN-β upon polyI:C stimulation, were the gift of Dr. T. Seya (Hokkaido University). MRC-5, normal human lung fibroblasts, and HeLa cells were maintained in MEM (Gibco) supplemented with 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco), and 10% or 5% fetal calf serum (Gibco). For assays, all procedures were the same as those described above for HCEC.

RT-PCR. Total RNA was isolated from human corneal epithelium and HPMC using Trizol Reagent (Life Technologies, New York, NY) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript Preamplification kit (Invitrogen). PCR amplification was performed with DNA polymerase (Takara; Shiga, Japan) for 38 cycles at 94 °C for 1 min, annealing for 1 min, and 72 °C for 1 min on a commercial PCR machine (GeneAmp; PE Applied Biosystems). The primers we used are listed in Table 1. The integrity of the RNA was assessed by electrophoresis in ethidium bromide-stained 1.5% agarose gels.

Flow cytometric analysis. Human primary corneal epithelial cells were treated with 0.02% EDTA. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated with mouse anti-human TLR3 monoclonal antibody (mAb; Imgenex, San Diego, CA) or isotype control mouse IgG1 (DakoCytomation, Kyoto, Japan) for 30 min at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR) was used as the secondary antibody. For TLR2 and TLR4 expression, cells were incubated for 30 min at 4 °C with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody (eBioscience, San Diego, CA), or isotype control mouse IgG2a (BD PharMingen). Stained cells were analyzed with a FACSCalibur (Becton–Dickinson, San Jose, CA); data were analyzed using Cellquest software (Becton–Dickinson). Moreover, HCFB, MRC-5, and HeLa were examined for their cell-surface expression of TLR3.

Table 1
The primer list of TLRs

Gene	Accession No.		Primers	Bases	Product size (bp)	Annealing
TLR1	NM003263	Sense	5′-TGCCCTGCCTATATGCAA-3′	(381–398)	555	54
		Anti-sense	5′-GAACACATCGCTGACAACT-3′	(918-936)		
TLR2	XM003304	Sense	5′-GCCAAAGTCTTGATTGATTGG-3′	(1783–1803)	346	52
		Anti-sense	5′-TTGAAGTTCTCCAGCTCCTG-3′	(2110–2129)		
TLR3	NM003265	Sense	5′-CGCCAACTTCACAAGGTA-3′	(277–294)	689	54
		Anti-sense	5′-GGAAGCCAAGCAAAGGAA-3′	(949–966)		
TLR4	XM005336	Sense	5′-TGGATACGTTTCCTTATAAG-3′	(1768–1787)	506	52
		Anti-sense	5′-GAAATGGAGGCACCCCTTC-3′	(2256–2274)		
TLR5	NM003268	Sense	5′-ATCTGACTGCATTAAGGGGAC-3′	(2274–2294)	567	52
		Anti-sense	5′-TTGAGCAAAGCATTCTGCAC-3′	(2822–2841)		
TLR6	NM006068	Sense	5′-CCTCAACCACATAGAAACGAC-3′	(832–852)	531	50
		Anti-sense	5′-CACCACTATACTCTCAACCCAA-3′	(1342–1363)		
TLR7	NM016562	Sense	5′-AGTGTCTAAAGAACCTGG-3′	(2222–2239)	544	50
		Anti-sense	5′-CCTGGCCTTACAGAAATG-3′	(2749–2766)		
TLR8	NM016610	Sense	5′-CAGAATAGCAGGCGTAACACATCA-3′	(1909–1932)	639	56
		Anti-sense	5′-AATGTCACAGGTGCATTCAAAGGG-3′	(2522–2545)		
TLR9	NM017442	Sense	5′-GTGCCCCACTTCTCCATG-3′	(791–808)	259	50
		Anti-sense	5′-GGCACAGTCATGATGTTGTTG-3′	(1030–1050)		
TLR10	NM030956	Sense	5′-CTTTGATCTGCCCTGGTATCTC-3′	(2286–2307)	497	52
		Anti-sense	5′-AGCCCACATTTACGCCTATCCT-3′	(2783–2286)		
GAPDH	XM033263	Sense	5′-CCATCACCATCTTCCAGGAG-3′	(293–312)	575	60
		Anti-sense	5′-CCTGCTTCACCACCTTCTTG-3′	(849–868)		

ELISA. To quantify cytokine secretion, culture supernatants were harvested and the level of IL-6 and IL-8 was assayed by human cytokine-specific ELISA (Biosource, Camarillo, CA).

Real-time semi-quantitative PCR. This was performed on an ABI-prism 7700 (Applied Biosystems, Foster City, CA) according to a previously described protocol [27] and the manufacturer's instructions. Total cellular RNA extraction and the first cDNA synthesis were as described above. The primers and probes for human IFN-β, human molecules possessing ankyrin-repeats induced by LPS (MAIL), and human GAPDH were from Perkin-Elmer Applied Biosystems. Previously reported primer and TaqMan probes for human IL-6 and IL-8 were used. The primers for IL-6 were 5′-TGACAAACAAATT CGGTACATCCT-3′ and 5′- AGTGCCTCTTTGCTGCTTTCAC-3′; the TaqMan probe for human IL-6 was 5′-TTACTCTTGTTACA TGTCTCCTTTCTCAGGGCTG-3′ [28]. The primers for human IL-8 were 5′-GCGCCAACACAGAAATTATTGTAA-3′ and 5′-TTATGA ATTCTCAGCCCTCTTCAA-3′; the TaqMan probe for IL-8 was 5′-TTCTCCACAACCCTCTGCACCCAGTT-3′ [27]. The probes were synthesized by Perkin-Elmer Applied Biosystems. To amplify human IL-6, IL-8, IFN-β, MAIL, and GAPDH cDNA, PCR was performed in a 25-μl total volume that contained a 1 μl cDNA template in 2× TaqMan universal PCR master mix (Applied Biosystems) at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The results were analyzed with sequence detection software (Applied Biosystems); the expression level of each mRNA was normalized to the expression of the human housekeeping gene GAPDH.

Data analysis. Data were expressed as means ± SE and evaluated by Student's *t* test using the Excel program.

Results

Expression of TLR3-specific mRNA in human corneal epithelium

We first examined whether human corneal epithelium expresses specific mRNA for TLRs 1–10. TLR-specific RT-PCR showed that mRNA from all but TLR8 was present in normal human corneal epithelium (Fig. 1). Among TLR-specific mRNA tested, TLR3 was expressed most intensely. When, as a positive control, we also subjected mRNA isolated from HPMC to RT-PCR, we found that these cells expressed TLRs 1–10. We then isolated, subcloned, and sequenced the PCR products. The obtained sequences were >95% identical with the known nucleotide sequences of human TLRs. Our findings suggest that while human corneal epithelium harbors messages for most TLRs, TLR3 is the one with the highest expression level. The expression of TLR3 was higher, while that of the other TLRs was lower, in human corneal epithelium than HPMC.

Primary HCEC express TLR3, but not TLR2 and TLR4, on the cell surface

Next we examined the cell-surface expression of TLR2, TLR3, and TLR4 on primary HCEC. While TLR3 was expressed on the surface of primary HCEC, TLR2 and TLR4 were not (Fig. 2). In positive controls, TLR2 and TLR4 were expressed on the cell surface of human peripheral blood monocytes and TLR3 was expressed on the cell surface of MRC-5 [29].

Primary HCEC respond to polyI:C but not LPS

Next we determined whether HCEC respond to polyI:C, a mimic of TLR3 ligand dsRNA. We first examined the production of inflammatory cytokines by primary HCEC stimulated with polyI:C and LPS. As

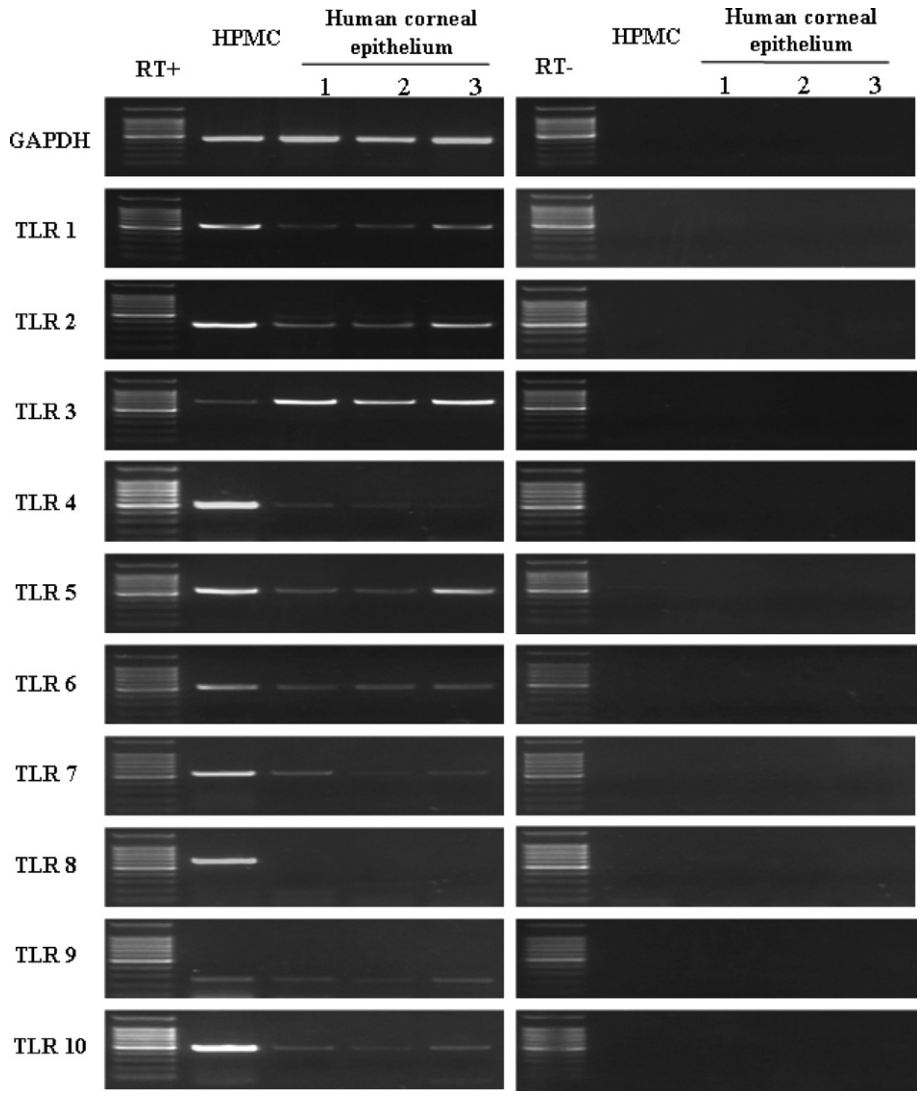


Fig. 1. The level of TLR3 expression is higher in human corneal epithelium than HPMC. Normal human corneal epithelium expresses mRNAs for TLRs 1–7 and 9–10 but not TLR8. As a positive control, mRNA isolated from human peripheral mononuclear cells (HPMC) was subjected to RT-PCR (left column). RT− indicates data were obtained without reverse transcription (controls).

shown in Fig. 3A, polyI:C stimulation induced the secretion of IL-6 and IL-8 while LPS treatment did not; in LPS-treated primary HCEC the level of IL-6 and IL-8 was similar to that seen in unstimulated cells. On the other hand, LPS stimulation significantly increased the production of IL-6 and IL-8 by HPMC and HCFB.

These findings were confirmed at the mRNA expression level. In primary HCEC, stimulation with polyI:C, but not LPS, resulted in the increased expression of IL-6- and IL-8-specific mRNA. Conversely, HPMC responded to LPS- but not to polyI:C stimulation and HCFB responded to both LPS and polyI:C (Fig. 3B).

IFN-β is controlled with TLR3/IRF-3 signaling. Thus, IFN-β-specific mRNA was significantly elevated in polyI:C- but not LPS-stimulated primary HCEC. Similarly, polyI:C but not LPS stimulated the induction of IFN-β in HPMC and HCFB. Surprisingly, IFN-β-specific mRNA expression was markedly higher in primary HCEC than HPMC and HCFB (Figs. 4A and B). Although primary human fibroblasts such as HCFB and MRC-5 expressed TLR3 on their cell surface, the cell-surface expression of TLR3 was more notable in primary HCEC (Fig. 4C).

Induction of IκBα- and MAIL-specific mRNA by polyI:C, but not LPS, in primary HCEC

In epithelial cells, the transcription factor NF-κB plays a central role in regulating genes that govern the onset of mucosal inflammatory responses. The primary consequences of TLR activation are NF-κB activation, cytokine secretion, and the expression of co-stimulatory molecules [9,30]. These responses help to promote and shape the critical immunological processes that facilitate

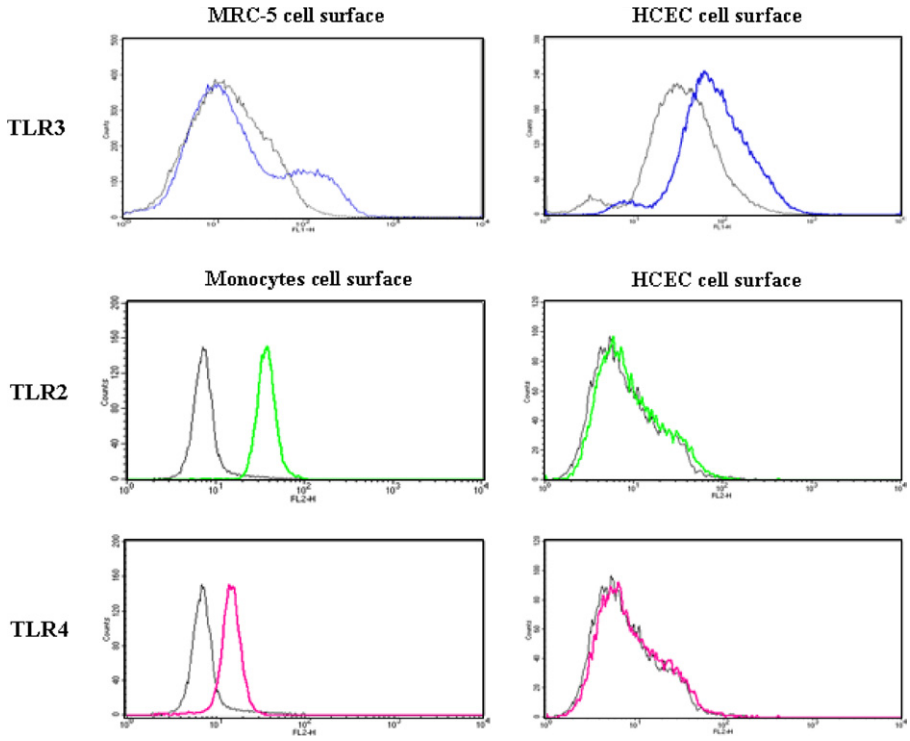


Fig. 2. Primary HCEC express TLR3, but not TLR2 and TLR4, on the cell surface. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. For TLR2 and TLR4 expression, cells were incubated (30 min, 4 °C) with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody, or isotype control mouse IgG2a. The histogram data are representative of three separate experiments.

the control and clearance of pathogens. We examined whether polyI:C stimulation of primary HCEC induced mRNA specific for the IκB family, regulators of NF-κB, such as IκBα and MAIL. We found that the expression of IκBα- and MAIL-specific mRNA was in fact elevated by polyI:C but not LPS (Fig. 5). These mRNAs were not up-regulated in polyI:C-stimulated HPMC, but their expression was significantly up-regulated upon stimulation with LPS as described previously [31]. It is of note that MAIL-specific mRNA was elevated by both polyI:C and LPS in HCFB. Taken together, these findings show that polyI:C could up-regulate IκBα and MAIL expression in primary HCEC via TLR3 (Fig. 5).

PolyI:C stimulates the gene expression and surface expression of TLR3 in primary HCEC

In macrophages, high TLR3- but not TLR4 gene expression levels are induced by TLR3 and TLR4 agonists [32]. In the context of this autocrine loop of TLR3 expression, we examined whether TLR-specific mRNA was inducible in primary HCEC by the TLR3 agonist polyI:C. As shown in Fig. 6A, TLR3-specific mRNA was highly elevated in primary HCEC stimulated with polyI:C. Interestingly, increased TLR2 and TLR4 gene expression was also observed in polyI:C- but not LPS-stimulated primary HCEC. Furthermore, as shown in Fig. 6B, the cell-surface expression of TLR3, but not of TLR2 and TLR4, was increased. These observations raise interesting questions regarding the role of TLR3 in the host defense mounted by corneal epithelium.

Discussion

We provide evidence for the gene and surface expression of TLR3 in human corneal epithelium and suggest that expressed TLR3 is functionally active in the secretion of the inflammatory mediators IL-6, IL-8, and IFN-β. We thus documented that polyI:C can induce the secretion of inflammatory mediators by primary HCEC. The ability of IFN-β to prevent the death of anergic cells, in addition to its anti-proliferative effect, may be one way in which the immune system regains a quiescent state after activation [33]. Further studies are necessary to elucidate the pathological role of IFN-β produced by HCEC. It is noteworthy that TLR3 expression was up-regulated by the TLR3 agonist polyI:C. Furthermore, the up-regulation in primary HCEC of IκB-α and MAIL (a human homologue of IκBζ, a NF-κB regulator in the nucleus) by polyI:C suggests a

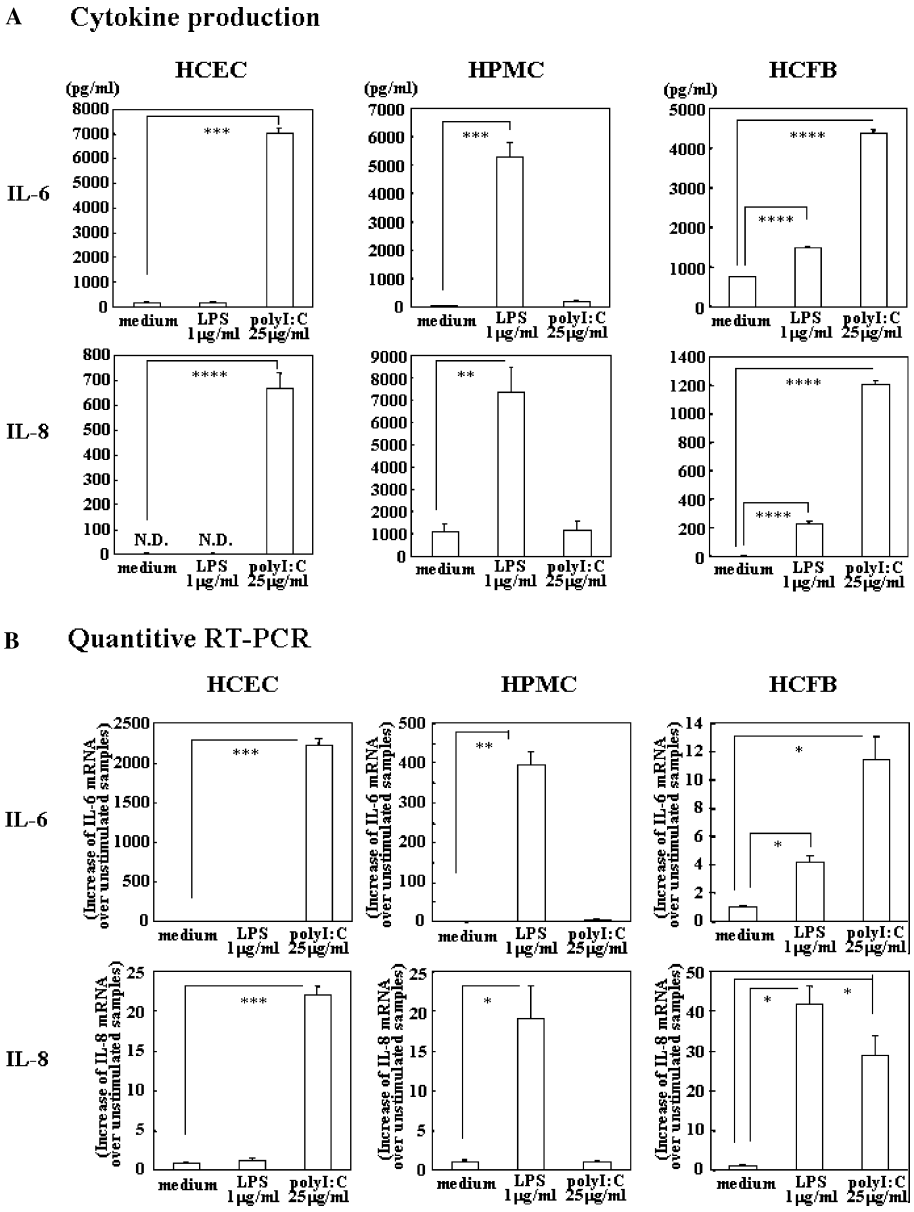
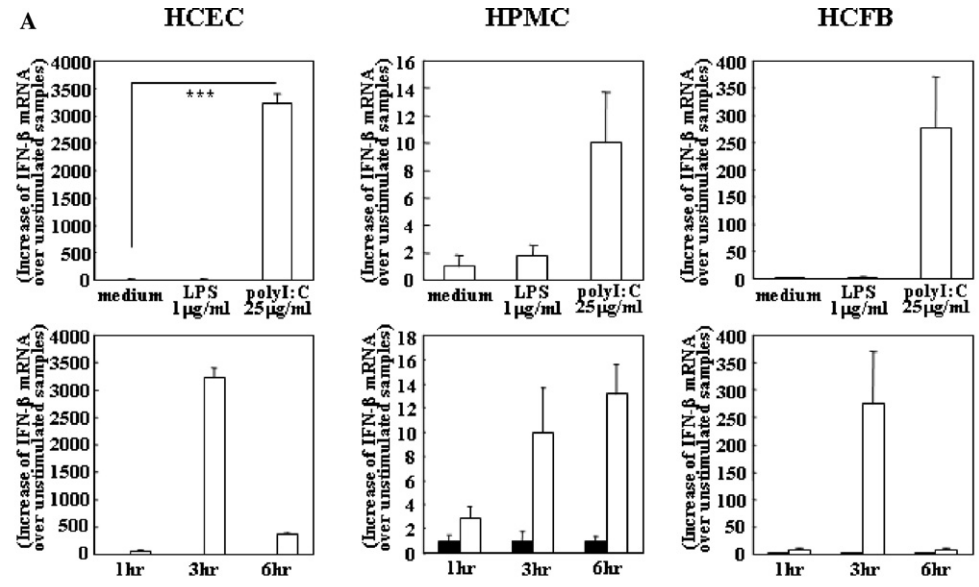


Fig. 3. The production and mRNA expression of IL-6 and IL-8 by primary HCEC. Primary HCEC and HCFB were cultured to sub-confluence and exposed to 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. HPMC were cultured at a density of about 1×10^6 cells/ml and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. (A) The culture supernatants were harvested and assayed by cytokine-specific ELISA for IL-6 and IL-8. (B) Total RNA was isolated from these cells with the Trizol reagent (Life Technologies). The RT reaction was performed with the SuperScript preamplification system (Invitrogen). Real-time semi-quantitative PCR was on an ABI-prism 7700. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show means ± SEM from an experiment carried out in triplicate wells (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$; and ****$p < 0.001$).

novel role for TLRs in ocular surface physiology. The new findings presented here contribute to a better understanding of innate ocular surface immunity.

A novel IκB protein, IκBζ/MAIL, induced by IL-1 and PAMPs regulates NF-κB in the cell nucleus. The induction of IκBζ is controlled by NF-κB, which, in turn, is negatively regulated by IκBζ, thereby forming an autonomous negative-feedback loop [34]. We postulate that IκBζ in ocular surface epithelium negatively regulates the pathological progression of ocular surface inflammation [35].

IκBζ was originally reported as a regulator of NF-κB induced by IL-1 and LPS [36]. LPS stimulation induced IκBζ in macrophages [31,34], but not primary HCEC (Fig. 4C). Furthermore, IκBζ is reportedly indispensable for IL-6 production in response to TLR ligands and is a positive regulator of NF-κB in the two-step process of IL-6 gene activation [37]. The implications of the



B The ratio of IFN-β/GAPDH mRNA at 3hr

	HCEC	ratio to HCEC	HPMC	ratio to HCEC	HCFB	ratio to HCEC	MRC-5	ratio to HCEC	HeLa	ratio to HCEC
unstimulated samples	0.00074 ±0.00014	1	0.01821 ±0.01295	24.6	0.00031 ±0.00002	4/10	0.00003 ±0.00002	4/100	0.0004 ±0.00013	5/10
stimulated with polyI:C	2.39136 ±0.13371	1	0.14682 ±0.06039	6/100	0.11038 ±0.01085	5/100	0.0462 ±0.00201	2/100	0.0468 ±0.00338	2/100
increasing ratio	3224	1	8	2/1000	360	1/10	1540	5/10	117	4/100

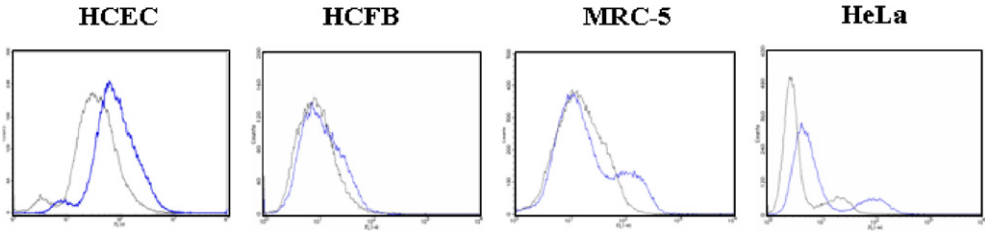


Fig. 4. Comparison of IFN-β mRNA expression and TLR3 cell-surface expression. (A) HCEC, HCFB, and HPMC were cultured as in Fig. 3 and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 3 h. Subsequent procedures were as in the assay of mRNA expression. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of two separate experiments and show means ± SEM from an experiment carried out in triplicate wells (***$p < 0.005$). (B) The methods were as in (A). The actual ratio of IFN-β/GAPDH mRNA and the relative ratio in HCEC are summarized in the table. (C) Cell-surface expression of TLR3 was examined by flow cytometry. Cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. The histogram data are representative of three separate experiments.

induction by polyI:C of IκBζ/MAIL in primary HCEC remain to be determined.

The epithelial expression of TLRs may be of importance in inflammation and immunity in response to pathogens [38–41]. Unique patterns of TLR expression appear to exist at different host-environment tissue interfaces. Under physiological conditions, the corneal epithelium appears to be hyporesponsive to commensal bacteria to which it is consistently exposed. We previously reported that HCEC failed to respond functionally to LPS or PGN because they lack TLR2 and TLR4 on their cell surface [20]. Despite the presence of TLR2 and TLR4 in their cytoplasm, HCEC did not respond to experimentally translocated LPS [20]. This is indicative of a characteristic difference between HCEC and immune-competent cells such as macrophages. The selective expression of TLR3 in human corneal epithelium (Fig. 1) contrasts with the ubiquitous expression of TLR family members in HPMC and indicates that the regulation and localization of TLR3 are different

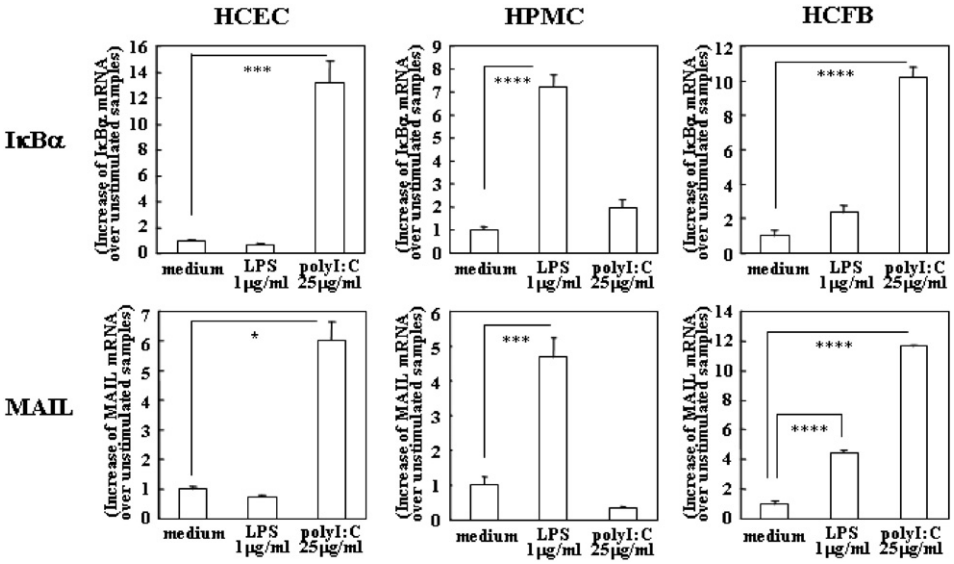


Fig. 5. Augmentation of MAIL and IκB-α gene expression in polyI:C-stimulated primary HCEC. HCEC, HCFB, and HPMC were cultured as in Fig. 3 and exposed to either 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 3 h, subsequent procedures were as in the assay of mRNA expression. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of two separate experiments and show means ± SEM from an experiment carried out in triplicate wells (*$p < 0.05$; ***$p < 0.005$; and ****$p < 0.001$).

in these cells. This may reflect the participation of cell type-specific multiple pathways in antiviral IFN induction via TLR3 [42]. However, the previous work does not exclude the role of inflammation-dependent TLRs on HCEC or on antigen-presenting cells infiltrating corneal tissue. IFN-α up-regulated TLR3 mRNA expression in epithelial cells and IFN-γ enhanced TLR3 expression in epithelial- and endothelial cells [43]. In macrophages, TLR3 expression is inducible by both TLR3 and TLR4 ligands, although these stimuli fail to induce TLR4 expression. Furthermore, TLR3 and TLR4 require the IFN-β autocrine/paracrine feedback mechanism to induce TLR3 expression and to activate and/or enhance genes required for antiviral activity [32]. IFN-α/β is critical for the measles virus-mediated up-regulation of TLR3 induction [44]. Given that cell-surface TLR3 expression was up-regulated by an agonist of TLR3, polyI:C (Fig. 6), and that polyI:C was able to induce the gene expression of IFN-β in HCEC (Fig. 4B), it is conceivable that IFN-β is crucial for the innate immune response of the ocular surface to pathogenic and nonpathogenic viruses and bacteria.

LPS up-regulates TLR3 expression in murine phagocytic cells through autocrine IFN-β induction. In humans, however, the IFN-β-induced up-regulation of TLR3 was blocked by pretreatment with LPS [45]. This observation coincides with our present results that TLR3 expression was not up-regulated by an agonist of TLR4, LPS (Fig. 6), and that LPS was incapable of inducing the gene expression of IFN-β in HCEC (Fig. 4A). The species-specific differences between humans and mice in their responses to LPS coincide with the presence of different, evolutionary nonconserved promoter sequences in both species. The intriguing functionality of TLR3 in HCEC and the far more potent induction of IFN-β by a TLR3 agonist in HCEC than fibroblasts require elucidation of the molecular mechanisms that regulate TLR3 expression on HCEC.

Although all TLRs activate NF-κB, not all TLRs activate IRF3 or induce IFN-β expression. TLR3 and TLR4 are the best-characterized TLRs known to activate IRF3 [30]. The TIR domain-containing adapter-inducing IFN-β (TRIF) is also an adapter for TLR3 and TLR4 [46,47]. Unlike the other TLRs including TLR4 that use the common MyD88-dependent pathway, TLR3 seems to employ only MyD88-independent TRIF-dependent pathways. These biochemical findings may account for the distinctly different responses elicited by polyI:C and LPS in the present study.

Type I IFN is induced not only by viral but also by bacterial infection [22,48]. An understanding of the role of the TLR3-IFN-β-link is crucial for understanding the involvement of type I IFN in TLR3-induced biological effects on the ocular surface. O'Conell et al. [49] reported that type I IFNs play a different role in bacterial and viral infections. The sophisticated interplay between bacteria and viruses may culminate in the exacerbation of pathological inflammation on the ocular surface.

In summary, the innate immune responses in mucosal epithelial cells such as HCEC differ from those in immune-competent cells such as macrophages. The elucidation of the unique innate immune response in mucosal epithelium is critical for a better understanding of the symbiotic relationship between mucosal epithelial cells and commensal bacteria inhabiting the mucosal surface.

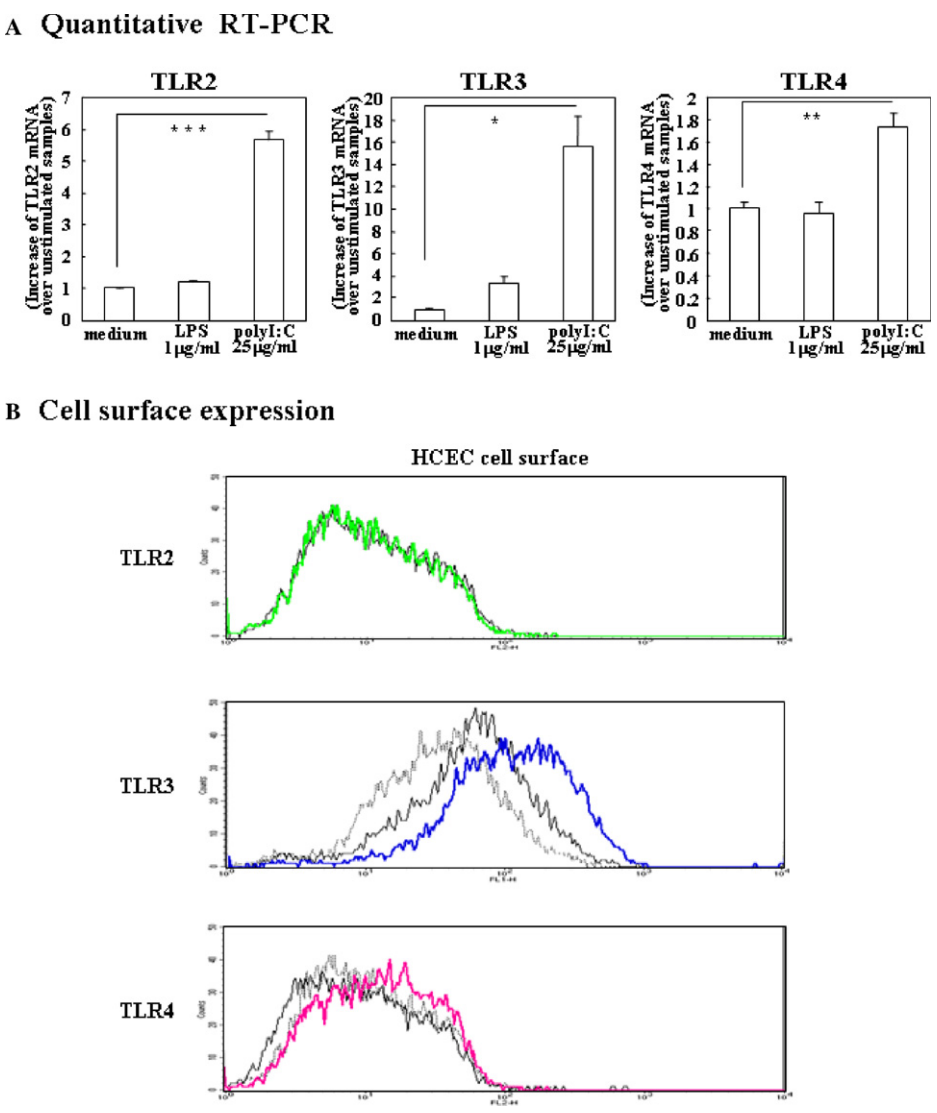


Fig. 6. Augmentation of TLR3 expression on HECE by polyI:C stimulation. (A) Augmentation of TLR3 gene expression in polyI:C-stimulated primary HCEC. Primary HCEC were cultured to sub-confluence in 25 cm^2 flasks (2×10^6 cells/flask) and exposed to 1 μg/ml LPS from *P. aeruginosa* or 25 μg/ml polyI:C for 6 h. RNA extraction, RT reaction, and real-time semi-quantitative PCR were as in Fig. 3. The *Y* axis shows the increase of specific mRNA over unstimulated samples. Data are representative of three separate experiments and show means ± SEM from an experiment carried out in triplicate wells. (B) Augmentation of TLR3 cell-surface expression on polyI:C-stimulated primary HCEC. Primary HCEC were cultured to sub-confluence in 75 cm^2 flasks (6×10^6 cells/flask) and untreated or exposed to 25 μg/ml polyI:C for 6 h. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated (30 min, 4 °C) with mouse anti-human TLR3 monoclonal antibody or isotype control mouse IgG1. Alexa Fluor 488 goat anti-mouse IgG (H + L) was the secondary antibody. For TLR2 and TLR4 expression, cells were incubated (30 min, 4 °C) with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody, or isotype control mouse IgG2a. The histogram data are representative of two separate experiments (dotted line, isotype control; thin line, untreated; and bold line, stimulated with 25 μg/ml polyI:C for 6 h).

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