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Intracellularly Expressed TLR2s and TLR4s Contribution to an Immunosilent Environment at the Ocular Mucosal Epithelium¹

Mayumi Ueta,^{*†‡} Tomonori Nochi,[‡] Myoung-Ho Jang,^{*} Eun Jeong Park,^{**‡} Osamu Igarashi,[‡] Ayako Hino,[‡] Satoshi Kawasaki,[†] Takashi Shikina,^{*} Takachika Hiroi,^{**‡} Shigeru Kinoshita,[†] and Hiroshi Kiyono^{2**§}

Epithelial cells are key players in the first line of defense offered by the mucosal immune system against invading pathogens. In the present study we sought to determine whether human corneal epithelial cells expressing Toll-like receptors (TLRs) function as pattern-recognition receptors in the innate immune system and, if so, whether these TLRs act as a first line of defense in ocular mucosal immunity. Incubation of human primary corneal epithelial cells and the human corneal epithelial cell line (HCE-T) with peptidoglycan or LPS did not lead to activation, at the level of DNA transcription, of NF- κ B or the secretion of inflammation-associated molecules such as IL-6, IL-8, and human β -defensin-2. However, when incubated with IL-1 α to activate NF- κ B, the production by these cells of such inflammatory mediators was enhanced. Human corneal epithelial cells were observed to express both TLR2- and TLR4-specific mRNA as well as their corresponding proteins intracellularly, but not at the cell surface. However, even when LPS was artificially introduced into the cytoplasm, it did not lead to the activation of epithelial cells. Taken together, our results demonstrate that the intracellular expression of TLR2 and TLR4 in human corneal epithelial cells fails to elicit innate immune responses and therefore, perhaps purposely, contributes to an immunosilent environment at the ocular mucosal epithelium. *The Journal of Immunology*, 2004, 173: 3337–3347.

The mucosal immune system coordinates the harmonious symbiosis that exists between the host and environmental microbes. Epithelial cells act as a first line of mucosal defense, in part through the use of innate immunity. For example, innate immune defenses make the intact corneal epithelium highly resistant to infection despite its continuous exposure to an array of microorganisms. Those bacteria must bind to the epithelial cell surface if they are to establish infection in vivo, but they are prevented from doing so by nonspecific ocular innate immune defense mechanisms, including blinking, tear flow, and mucin, which act to provide a physical barrier against infection under normal conditions. In addition to these mechanical defenses, the human tear film contains innate defense molecules with antibacterial properties, e.g., lysozyme, lactoferrin, and defensins (1). Thus, the ocular surface system creates 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 caused by *Pseudomonas aeruginosa* and

Staphylococcus aureus, the common causative pathogens (2, 3). Residing in the conjunctival sac or eyelid edge of the ocular surface are normal bacterial flora, including coagulase negative staphylococci, *Propionibacterium acnes*, and other Gram-positive and -negative bacteria (4, 5), but the corneal epithelium does not generally respond to such flora. In fact, in many cases, patients suffering from bacterial conjunctivitis show no signs of inflammation in their corneas.

Another important aspect of innate immune systems is the recent discovery of pattern recognition molecules for microbial pathogen-associated Ags. Toll was first identified as an essential molecule for embryonic patterning in *Drosophila* and was subsequently shown to be key to antifungal immunity as well (6). A homologous family of Toll receptors, the so-called TLRs, has been shown to exist in mammals (7). TLRs, a family of innate immune-recognition receptors, are involved in the pattern recognition of microbial pathogen-associated glycoproteins, proteins, and DNA, thereby providing an initial triggering signal for the induction of antimicrobial immune responses (8). Recent studies have revealed that a striking feature of TLRs is their ability to discriminate among different classes of pathogen-associated molecules. For example, TLR4 recognizes LPS (9), which is an integral component of the outer membranes of Gram-negative bacteria, whereas TLR2 recognizes peptidoglycan (PGN)³ and lipoproteins from Gram-positive bacteria (10, 11). Ten members of the TLR family have been identified in mammalian host immune-competent cells, such as dendritic cells and macrophages, which are the cells the most likely to come into direct contact with pathogens from the environment via the mucosal epithelia (12).

It has also been reported that several TLRs are expressed in mucosal epithelia, such as intestinal epithelial cells (13–17), tracheo-bronchial epithelial cells (18), renal epithelial cells (19), bladder epithelial cells (20, 21), and oral epithelial cells (22–24).

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³ Abbreviations used in this paper: PGN, peptidoglycan; hBD2, human β -defensin-2.

The respiratory epithelial cells and bladder epithelial cells were shown to be capable of responding to LPS (18, 20, 21). In the case of intestinal and oral epithelial cells, conflicting results were reported, with one group of studies finding that they were capable of responding to LPS (15–17, 24), and the other group of studies determining that they were not (13, 14, 22, 23). In contrast to dendritic cells and macrophages, which enjoy the relatively sterile environment of the peripheral lymphoid tissues where they are situated, mucosal epithelial cells are located in a harsh environment, where they are continuously exposed to large numbers of biologically active microbial products, such as LPS and PGN. Given this disparity in environments, the expression and responsive behaviors of TLRs in peripheral APCs and mucosal epithelial cells would be expected to be different.

The major aim of our study was to elucidate the expression and function of TLRs by corneal epithelial cells and to show the role these TLRs play in the first line of defense offered by the mucosal immune system at the ocular surface. Thus, we examined whether human corneal epithelial cells express TLRs and respond to bacterial components such as LPS and PGN, which are bacterial cell wall components associated with the ocular infectious diseases *P. aeruginosa* and *S. aureus*, respectively.

Materials and Methods

Human corneal epithelial cells

For RT-PCR, human corneal epithelial cells were obtained from corneal grafts after corneal transplantations for one bullous keratopathy and two keratoconus. For immunohistological analysis, human corneal tissue sections were prepared from the eyeball removed from a patient at Kyoto Prefectural University of Medicine (Kyoto, Japan). The eye was removed due to a malignant melanoma; however, the cornea was not affected. The purpose of the research and the experimental protocol were explained to all patients, and their informed consent was obtained. All experimental procedures have been conducted in accordance with the principles set forth in the Helsinki Declaration.

The human corneal epithelial cell line transformed with SV40 (HCE-T) (25) was maintained at Kyoto Prefectural University of Medicine and cultured in modified SHEM medium consisting of DMEM/F-12 medium (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Invitrogen Life Technologies), 10 ng/ml murine natural epidermal growth factor (Invitrogen Life Technologies), 5 µg/ml insulin from bovine pancreas (Sigma-Aldrich, St. Louis, MO), and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B; Invitrogen Life Technologies) at 37°C under 95% humidity and 5% CO₂ (26). Human primary corneal epithelial cells were obtained from KURABO (Osaka, Japan) and then cultured in a serum-free medium consisting of EpiLife (KURABO) supplemented with human corneal epithelial cell growth supplement containing 1 ng/ml murine epidermal growth factor, 5 µg/ml insulin from bovine pancreas, 0.18 µg/ml hydrocortisone, 0.4% bovine pituitary extract (all from KURABO), and 1% antibiotic-antimycotic solution consisting of 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B (Life Technologies) at 37°C under 95% humidity and 5% CO₂ (27).

Purification of mononuclear cells from peripheral blood

Once the purpose of the research and the experimental protocol had been explained to and informed consent obtained from the volunteers, human venous blood samples were obtained from them. The blood sample was anticoagulated with heparin. Blood was then placed in sterile 50-ml polypropylene tubes. Blood was mixed with 1 vol of PBS⁻ (Ca²⁺ free), overlaid on Lymphoprep (Axis-Shield PoC, Oslo, Norway) and centrifuged for 20 min at 2000 rpm at 20°C. Mononuclear cells were gently aspirated from the interface and washed with PBS⁻.

RT-PCR analysis

A standard RT-PCR assay routinely performed in our laboratory was used in this study (28). Briefly, total RNA was isolated from HCE-T, human mononuclear cells, and human corneal epithelia using a TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's instructions. For RT reaction, the SuperScript preamplification system (Invitrogen Life Technologies) was applied. PCR amplification was

performed with DNA polymerase (AmpliAq; PerkinElmer Cetus, Norwalk, CT) for 38 cycles at 94°C for 1 min, at 52°C for 1 min, and at 72°C for 1 min using a commercial apparatus (GeneAmp; PerkinElmer Cetus). The primers used in this study are listed in the table shown in Fig. 1. The integrity of the RNA was assessed by electrophoresis in ethidium bromide-stained, 1.5% agarose gels.

ELISA

To quantify cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 12-well plates (1 × 10⁵ cells/well) and, after reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS from *P. aeruginosa* (Sigma-Aldrich), 1000 ng/ml PGN from *S. aureus* (Fluka, Buchs, Switzerland), or 10 ng/ml human IL-1α (R&D Systems, Minneapolis, MN) for 24 h. The concentrations of LPS, PGN, and IL-1α used in this study were optimal for the maximum induction of inflammatory cytokines (10, 29). The culture supernatants were harvested, and levels of IL-6 and IL-8 were measured by the respective human cytokine-specific ELISA (BioSource, Camarillo, CA).

Real-time quantitative PCR

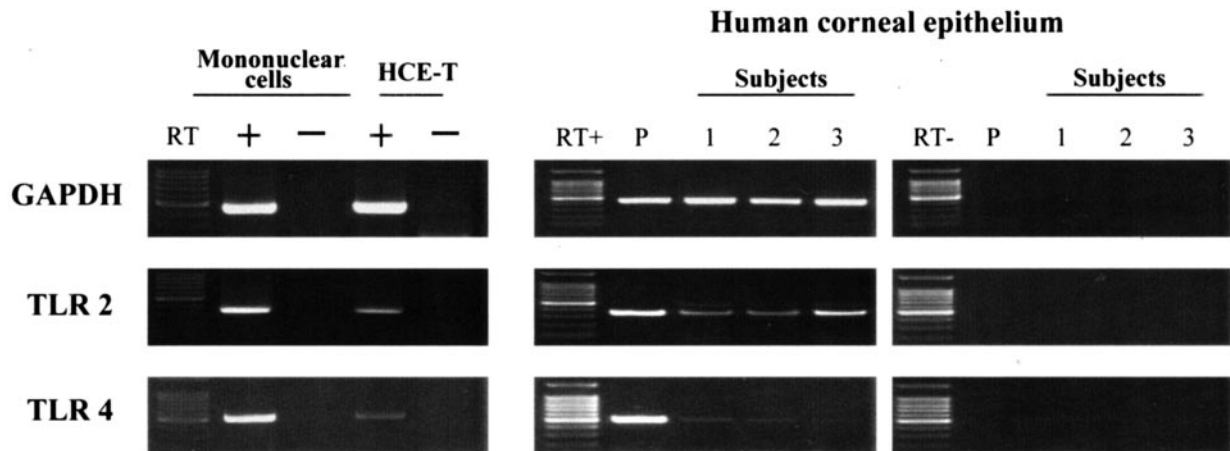
Real-time quantitative PCR was performed using a LightCycler (Roche, Mannheim, Germany) according to the previously described protocol (30) and manufacturer's instructions. For the amplification of IL-6, IL-8, and human β-defensin-2 (hBD2) cDNA, RT-PCR was performed in a 20-µl total volume in the presence of 2 µl of 10× reaction buffer (*Taq* polymerase, dNTPs, and MgCl₂; Roche), and 2 µl of cDNA (or water as a negative control, which was always included). MgCl₂ was added to a final concentration of 3 mM, and 5 pmol of each oligonucleotide primer was added. Real-time PCR was performed in glass capillaries. A calibration curve was automatically generated using the external standards, and samples were quantified accordingly by LightCycler analysis software (version 3; Roche). These quantification data were normalized to the expression of the housekeeping gene GAPDH. Listed below are the primers and probes used in this study because of their specificity for IL-6, IL-8, hBD2, and GAPDH (Table I).

NF-κB assay

To compare NF-κB production, HCE-T was plated in six-well plates (2 × 10⁵ cells/well) and, upon reaching subconfluence, were left untreated or were exposed to LPS (1000 ng/ml) from *P. aeruginosa*, PGN (1000 ng/ml) from *S. aureus*, or IL-1α (10 ng/ml) for 7 h. After incubation, the transcription NF-κB assay was performed using TransAM (Active Motif, Carlsbad, CA) according to the manufacturer's instructions (31). Briefly, cells were rinsed twice with cold PBS⁻ before being scraped and centrifuged for 10 min at 1,000 rpm. The pellet was then resuspended in 100 µl of the lysis buffer included in the kits. After 10 min on ice, the lysate was centrifuged for 20 min at 14,000 rpm. Twenty microliters of 10-fold diluted cell extracts were incubated with 30 µl of binding buffer in microwells coated with the probes containing the NF-κB consensus binding sequence. After 1-h incubation at room temperature with mild agitation, microwells were washed three times. Anti-NF-κB Abs were added to each well and incubated for 1 h at room temperature. Microwells were then washed three times before incubation with HRP-conjugated Abs for 1 h at room temperature. After incubation, microwells were washed four times and reacted with tetramethylbenzidine for 10 min at room temperature before the addition of stop solution. OD was then read at 450 nm with an iEMS microplate reader (Thermo LabSystem, Vantaa, Finland).

Flow cytometric analysis

HCE-T and human primary corneal epithelial cells were treated with 0.02% EDTA. Cell surface expression of TLR2, TLR4, and CD14 was examined by flow cytometry. Cells were incubated with the PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) mAb (eBioscience, San Diego, CA), PE-conjugated mouse anti-human CD14 mAb (BD Pharmingen, San Diego, CA), or isotype control mouse IgG2a (BD Pharmingen) for 1 h at room temperature. For intracellular FACS, the cell fixation/permeabilization kit (BD Pharmingen) was used. Cells were fixed with Cytofix/Cytoperm and then stained with the respective PE-conjugated mAbs, as described above, in Perm/Wash solution for 1 h at room temperature. Stained cells were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using CellQuest software (BD Biosciences).



Gene	Accession No.		Primers	Bases	Product size
GAPDH	XM033263	sense	5'-CCATCACCATCTTCCAGGAG-3'	(293-312)	575bp
		anti-sense	5'-CCTGCTTCACCACCTTCTTG-3'	(849-868)	
TLR2	XM003304	sense	5'-GCCAAAGTCTTGATTGATTGG-3'	(1783-1803)	346bp
		anti-sense	5'-TTGAAGTTCTCCAGCTCCTG-3'	(2110-2129)	
TLR4	XM005336	sense	5'-TGGATACGTTTCCTTATAAG-3'	(1768-1787)	506bp
		anti-sense	5'-GAAATGGAGGCACCCCTTC-3'	(2256-2274)	

FIGURE 1. Normal human corneal epithelial cells express TLR-specific mRNA. Human corneal epithelial cells were obtained from corneal grafts after corneal transplantations for one bullous keratopathy and two keratoconus. Total RNA was isolated from human corneal cell lines (HCE-T), human mononuclear cells, and human corneal epithelial cells of three individuals. For RT reaction, the SuperScript preamplification system was applied. PCR amplification was performed with DNA polymerase. The primers used are indicated in the boxed column.

Immunocytoplasmic and histological staining

A standard immunocytoplasmic staining protocol was used in this study (32). Briefly, HCE-T was cultured in a chamber slide (Nalge Nunc International, Naperville, IL), washed with PBS⁻, and air-dried. Slides were fixed with methanol for 30 min and then stained with the PE-conjugated mouse mAbs anti-human TLR2 (TL2.1), TLR4 (HTA125), or isotype control mouse IgG2a (eBioscience) for 24 h at room temperature. Serial sections (6 μ m) of human cornea were prepared from normal human corneal tissue separated from an eyeball removed due to malignant melanoma; the cornea was not affected. After being air-dried and stored at -80°C , slides were fixed with methanol for 30 min and then stained with PE-conjugated mouse mAb anti-human TLR2 (TL2.1) or TLR4 (HTA125) or with isotype control mouse IgG2a (eBioscience) for 24 h at room temperature.

Internalization of LPS with DOTAP

For the internalization experiment, Alexa Fluor 488-conjugated LPS (Molecular Probes, Eugene, OR) and DOTAP Liposomal Transfection Reagent (Roche) were used (32). Alexa Fluor 488-conjugated LPS (1 $\mu\text{g}/\text{ml}$) was reacted with 5 $\mu\text{l}/\text{ml}$ DOTAP Liposomal Transfection Reagent according to the manufacturer's instructions. HCE-T and primary human corneal epithelial cells were then incubated with Alexa 488-LPS-DOTAP or Alexa 488-LPS alone. Five-, 7-, and 24-h incubations were conducted for immunostaining, NF- κ B, and ELISA, respectively. When the cell line of HCE-T was treated with DOTAP containing Alexa-LPS or DOTAP only, neither treatment influenced cell viability or morphology of the cells.

Data analysis

Data were expressed as the mean \pm SE and were evaluated by Student's *t* test using the Excel program.

Results

Normal human corneal epithelial cells and HCE-T express TLR2- and TLR4-specific mRNA

Among all the members of the TLR family, TLR2 and TLR4 have pattern recognition receptors that best suit them to target the most prominent microorganism-associated cell wall components of Gram-positive (e.g., PGN) and Gram-negative (e.g., LPS) bacteria, respectively (9–11). Thus, our initial experiment was aimed at elucidating whether HCE-T and normal human corneal epithelial cells harbor specific mRNA for TLR2 and TLR4. As one might expect, TLR2- and TLR4-specific mRNA was present in both HCE-T and normal human corneal epithelial cells. These PCR products were isolated, subcloned, and sequenced to ensure the expression of specific TLR. The sequences obtained for these PCR products were virtually identical (>95%) to those of human TLRs (Fig. 1). The specificity of the PCR product for TLR2 and TLR4 was also confirmed by the use of human mononuclear cells as a positive control.

Human corneal epithelial cells fail to respond to LPS or PGN

Inasmuch as human corneal epithelial cells and HCE-T were seen to express specific messages for TLR2 and TLR4, the next logical step was to elucidate whether human corneal epithelial cells could respond to LPS or PGN. At first, we examined the production of inflammatory cytokines by HCE-T and primary human corneal epithelial cells after exposure to LPS and PGN (Fig. 2A). Stimulation with LPS or PGN did not induce the secretion of IL-6 and IL-8; therefore, levels of IL-6 and IL-8 production in the treated

Table I. *Primers and probes used in this study*

mRNA	Accession No.	Forward Primer	Reverse Primer	Probe (3'-Fluorescein)	Probe (LCRed640-5')	Product Length
GAPDH	XM033263	601–620	1033–1052	884–904	906–928	451 bp
hBD2	XM031794	24–44	258–278	143–167	115–141	254 bp
hIL-6	NM000600	379–398	620–639	480–504	506–530	260 bp
hIL-8	XM031289	143–162	346–365	222–251	194–220	222 bp

supernatants remained essentially the same as those in unstimulated HCE-T or primary human corneal epithelial cells. However, both IL-6 and IL-8 secretions were up-regulated by the stimulation of HCE-T and primary human corneal epithelial cells with IL-1 α . These findings demonstrate that HCE-T and primary human corneal epithelial cells proved incapable of responding to exogenous microbial stimuli (e.g., LPS and PGN.)

This finding was further confirmed at the level of mRNA. After *in vitro* incubation of HCE-T with various concentrations of LPS, PGN, and IL-1 α , quantitative RT-PCR was performed for the respective cytokines. The levels of IL-6- and IL-8-specific mRNA were not elevated in HCE-T stimulated with LPS or PGN (Fig. 2B). However, HCE-T responded to IL-1 α in a dose-dependent manner for the enhancement of IL-6- and IL-8-specific mRNA (Fig. 2B). The expression of hBD2-specific mRNA was not induced by treatment with either LPS or PGN, but it was enhanced after exposure to IL-1 α . These results confirm our original finding that human corneal epithelial cells express TLR2- and TLR4-specific mRNA, but fail to respond to PGN and LPS, respectively.

The unresponsiveness of human corneal epithelial cells to LPS and PGN was further demonstrated at the level of nucleus transcription. After the incubation of HCE-T with optimal concentrations of LPS, PGN, or IL-1 α , whole-cell protein extracts were subjected to a DNA binding assay of NF- κ B. As one might expect based on the results presented above, NF- κ B-mediated signals were not enhanced by treatment of HCE-T with LPS or PGN, but were augmented by exposure to IL-1 α (Fig. 2C).

Taken together, these results show that human corneal epithelial cells were unable to respond to LPS from *P. aeruginosa* or to PGN from *S. aureus* despite the evidence that these epithelial cells harbor specific messages for TLR4 and TLR2, respectively.

HCE-T and primary human corneal epithelial cells express TLR2 and TLR4 intracellularly, but not at the cell surface

The next logical step was to investigate whether human corneal epithelial cells express TLR2 and TLR4 at their cell surface. To make this determination, we examined the cell surface expression of TLR2, TLR4, and CD14 on HCE-T and primary human corneal epithelial cells (Fig. 3). No surface expression of TLR2, TLR4, or CD14 was detected for the cell line or for primary human corneal epithelial cells. Because monocytes were used as a positive control in this study, the expressions of TLR2, TLR4, and CD14 were confirmed by the analysis of human peripheral blood monocytes. Stimulation of HCE-T with LPS and PGN failed to induce the expression of TLR2 and TLR4, respectively. Moreover, even stimulation of HCE-T with an optimal concentration of 10 ng/ml IL-1 α or 10 ng/ml TNF- α did not induce the expression of TLR2, TLR4, and CD14. However, FACS analysis showed that TLR2, TLR4, and CD14 were intracellularly expressed by HCE-T and primary human corneal epithelial cells (Fig. 3). Taken together, these findings demonstrate that human corneal epithelial cells express TLR2, TLR4, and CD14 intracellularly, but not at the cell surface.

Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelial cells

To directly demonstrate the intracellular expression of TLR2 and TLR4 by human corneal epithelial cells, immunohistological examination was performed using confocal image analysis. After the intracellular staining of HCE-T with mAbs specific for TLR2 and TLR4, the confocal image analysis of HCE showed cytoplasmic staining of TLR2 and TLR4 in the perinuclear region (Fig. 4). Furthermore, immunoprecipitation of cell lysates prepared from HCE-T with polyclonal anti-human TLR4 (Imgenex, San Diego, CA), followed by Western blotting with biotinylated mAb anti-human TLR4 (HTA125), resulted in the detection of a 120-kDa protein corresponding to TLR4 (data not shown). These findings were further supported by immunohistochemical analysis of a tissue section of human cornea, which showed that specific staining of TLR2 and TLR4 was localized in the cytoplasm (Fig. 5). These results directly demonstrate that TLR2 and TLR4 are present intracellularly in human corneal epithelial cells.

Intracellular TLR4 in human corneal epithelial cells fails to respond to LPS

Once human corneal epithelial cells were known to express cytoplasmic TLRs, it became important to examine whether intracellular TLRs are biologically capable of responding to internalized corresponding bacterial cell wall components. To address this issue, our next experiment was aimed at elucidation of the intracellular TLR4/LPS interaction (Fig. 6). At first, the cell line HCE-T, primary human corneal epithelial cells, and monocytes were cocultured with Alexa 488-coupled LPS (Alexa 488-LPS) and then examined by confocal image analysis. HCE-T and primary human corneal epithelial cells cocultured with Alexa 488-LPS did not internalize Alexa 488-LPS, but monocytes did (Fig. 6A). For the next experiment, Alexa 488-LPS was artificially translocated into the HCE-T and primary human corneal epithelial cells using the DOTAP liposomal transfection reagent. Although the free form of Alexa 488-LPS was not taken up by human corneal epithelial cells, the epithelial cells coincubated with the DOTAP preparation of Alexa 488-LPS showed punctuated fluorescein. Confocal scanning laser microscopy showed extensive loading of Alexa 488-LPS in the cytoplasm of human corneal epithelial cells (Fig. 6A).

After intracellularly exposing human corneal epithelial cells to LPS, we examined whether they secreted IL-6 and IL-8 (Fig. 6B). We found that the production of IL-6 and IL-8 was not up-regulated even when LPS was intracellularly delivered to TLR4 expressed in the cytoplasm of HCE-T. To negate the possibility that the artificial introduction of LPS by the DOTAP system might influence the functional capacity of cytokine synthesis by the epithelial cells, HCE-T cells pretreated with DOTAP-Alexa-LPS or DOTAP alone were further incubated with IL-1 α . As a control, the medium pretreated epithelial cells were incubated with IL-1 α . These DOTAP-pretreated epithelial cells responded to the cytokine and thus resulted in the similar levels of IL-6 (25,000–30,000 pg/ml) and IL-8 (7,500–9,000 pg/ml) synthesis compared with the

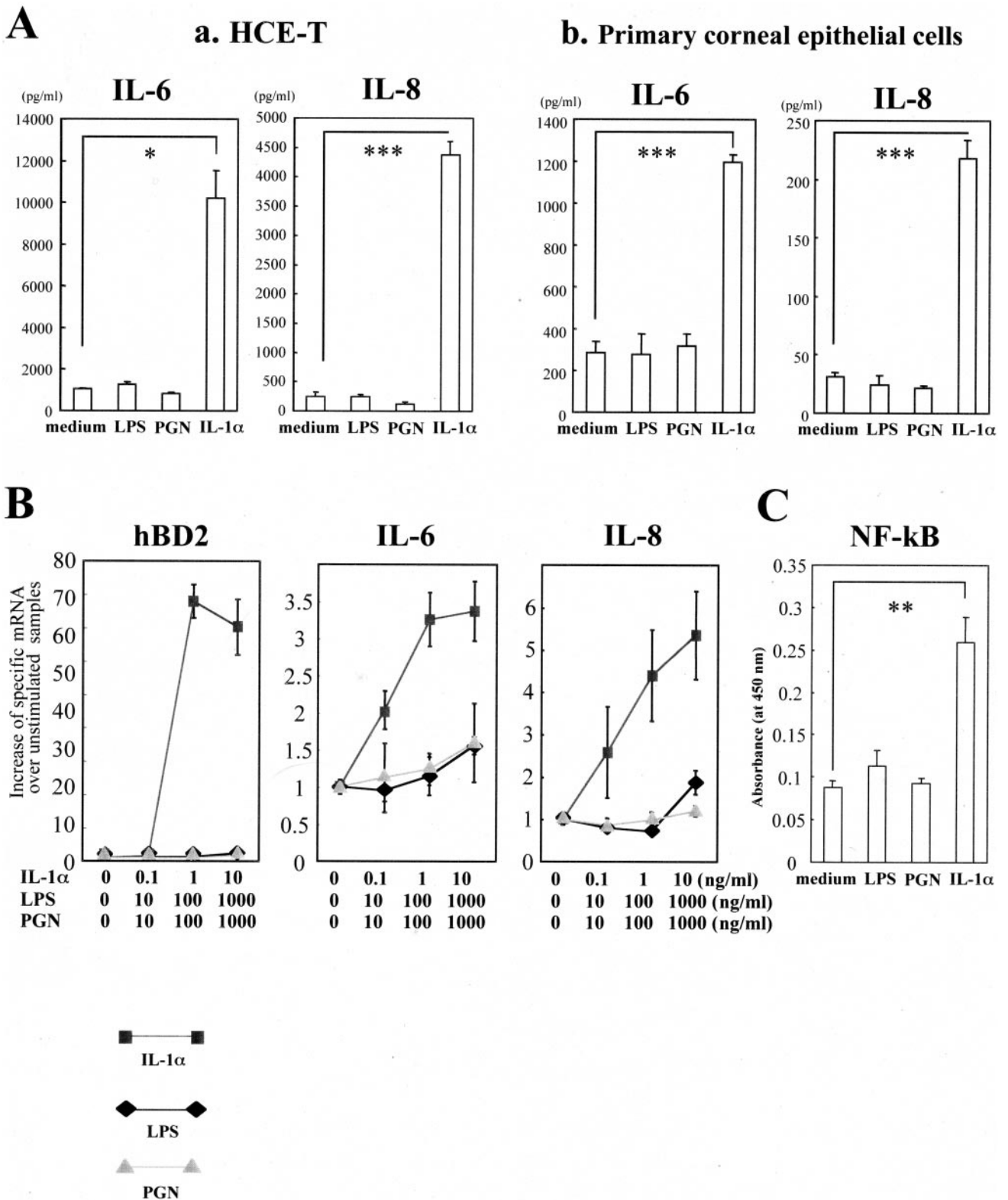


FIGURE 2. Human corneal epithelial cells fail to respond to LPS or PGN. To quantify inflammatory cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS, 1000 ng/ml PGN, or 10 ng/ml human IL-1α for 24 h. The culture supernatants were harvested for measurement of IL-6 and IL-8 (A). Quantitative RT-PCR was used to measure the expression of IL-6, IL-8, and hBD2 mRNA in HCE after treatment with LPS, PGN, or IL-1α. Real-time quantitative PCR was performed using a LightCycler. The quantification data were normalized to the expression of the housekeeping gene GAPDH. The y-axis shows an increase in specific mRNA over unstimulated samples (B). Primers and probes of IL-6, IL-8, hBD2, and GAPDH are listed in Table I. To characterize NF-κB activation, HCE were plated in six-well plates and, upon reaching subconfluence, were left untreated or were exposed to LPS (1000 ng/ml), PGN (1000 ng/ml), or IL-1α (10 ng/ml) for 7 h. After the stimulation, the NF-κB assay was performed using TransAM (C). Data represent the mean ± SEM from an experiment with triplicate wells. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

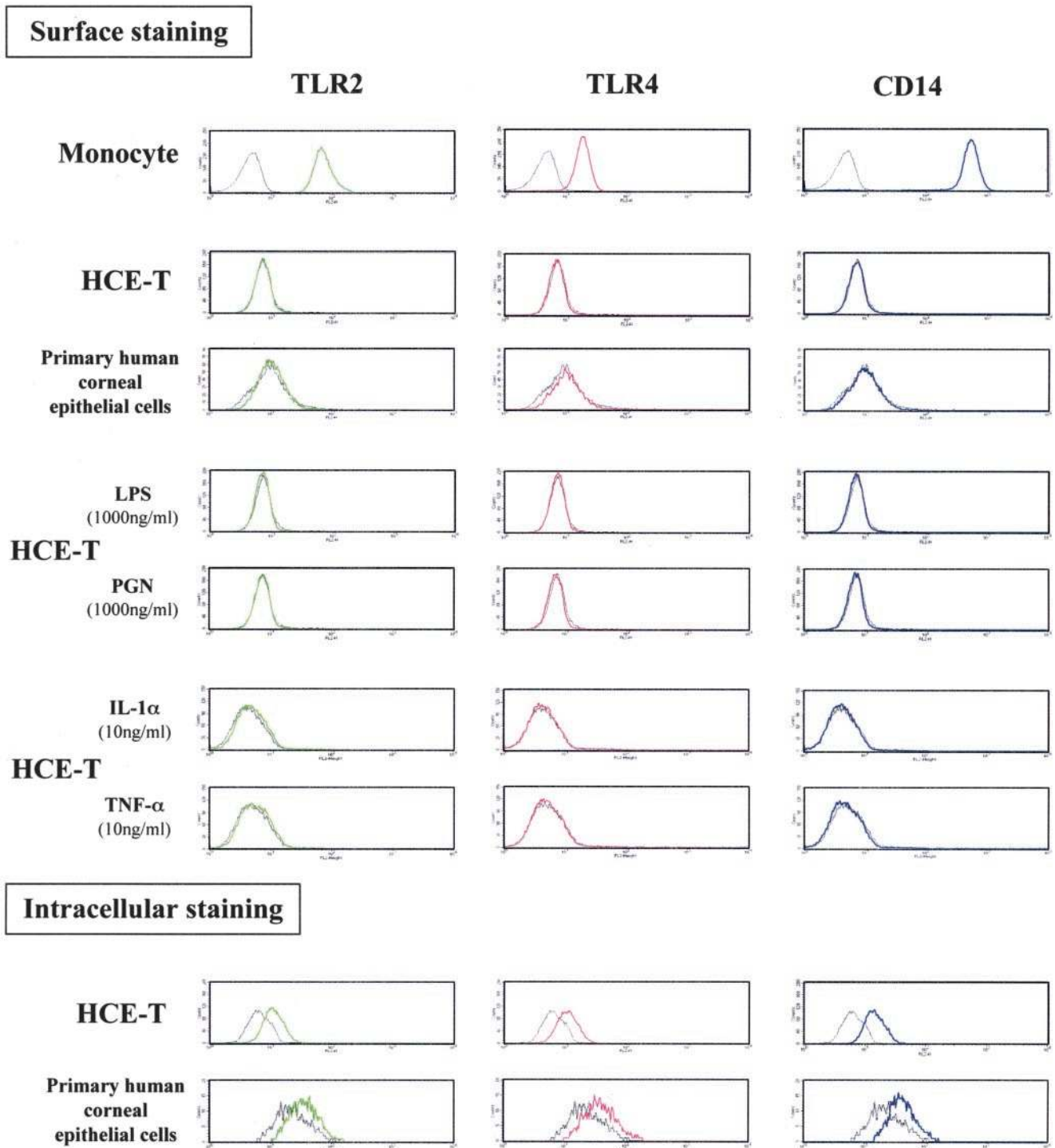


FIGURE 3. TLR2 and TLR4 are expressed intracellularly, but not on the cell surface of human corneal epithelial cells. Cell surface expressions of TLR2, TLR4, and CD14 in HCE-T and primary human corneal epithelial cells were examined by FACS. These cells were incubated with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs, PE-conjugated mouse anti-human CD14 mAbs, or isotype control mouse IgG2a for 1 h at room temperature. In these studies monocytes served as a positive control. In some experiments the epithelial cells were stimulated with LPS or PGN, then examined for the expression of TLR2 and TLR4. For intracellular FACS analysis of TLR2 and TLR4, Cell Fixation/Permeabilization kits were used. Human corneal epithelial cells were fixed with Cytofix/Cytoperm and then stained with their respective mAbs in Perm/Wash solution for 1 h at room temperature as described above. Histogram data are representative of three separate experiments.

medium-pretreated HCE-T (IL-6, 24,000–28,000 pg/ml; IL-8, 7,000–8,000 pg/ml).

Results for primary human corneal epithelial cells were similar where the cells also did not respond to intracellularly introduced LPS, except that, in contrast to HCE-T, they secreted some IL-6 and IL-8

when cocultured with DOTAP alone. It is possible that DOTAP may provide activation signals for primary human corneal epithelial cells, but as of yet the specific signaling mechanism remains unknown. We also examined whether NF- κ B signaling was up-regulated by the intracellular delivery of LPS into HCE-T. We found that internalization of

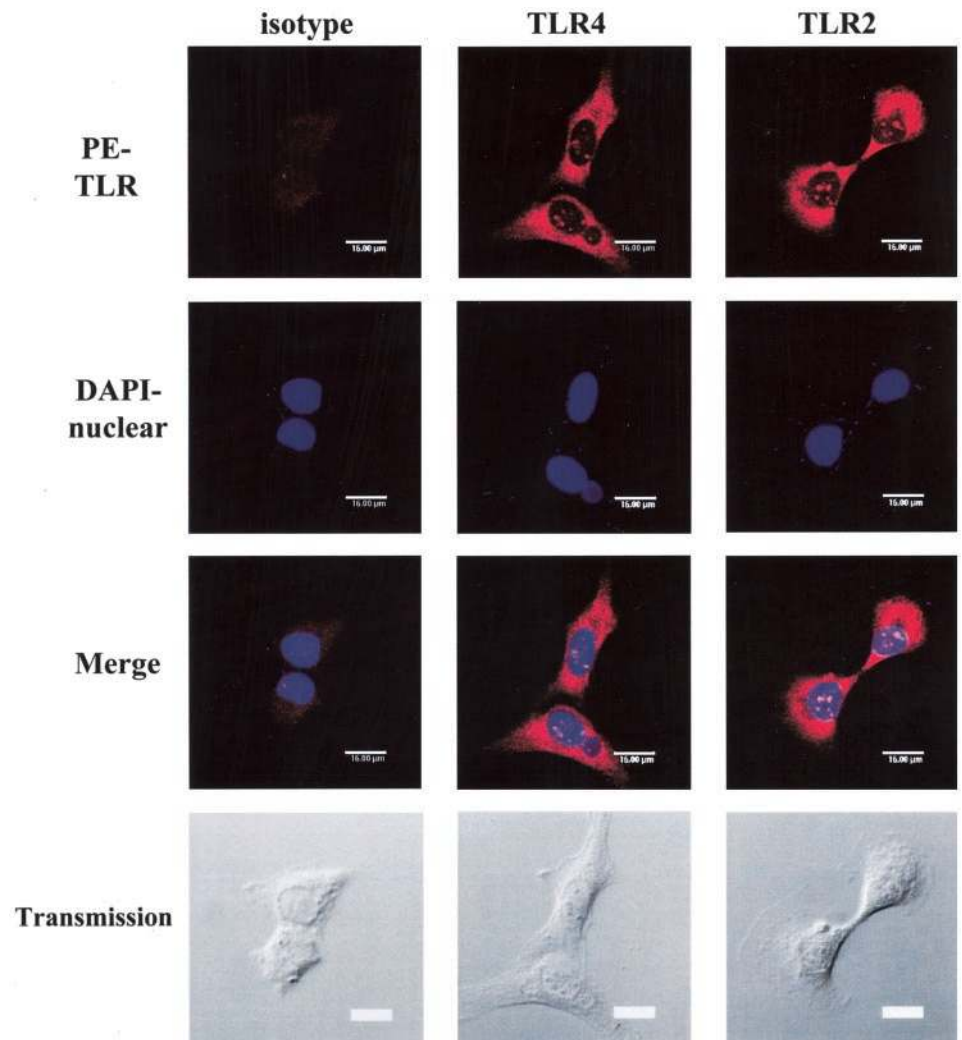


FIGURE 4. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in the human corneal epithelial cell line HCE-T. HCE-T was cultured on a slide chamber, washed with PBS⁻, and air-dried. Slides were fixed with methanol for 30 min, then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAb or isotype control mouse IgG2a for 24 h at room temperature. Confocal images of HCE-T showed specific staining with anti-TLR2 and -TLR4 mAb in the perinuclear region or cytoplasm. DAPI were used for counterstaining. Each bar represents a length of 50 μ m.

Alexa 488-LPS into HCE-T did not lead to the enhancement of NF- κ B-mediated signals (Fig. 6C). These findings suggest that cytoplasmically expressed TLR4 is not capable of responding to LPS even when the endotoxin is intracellularly introduced.

Discussion

Interestingly, our results indicate that ocular surface epithelial cells, which are an important component of the mucosal immune system, express TLR-specific mRNA for two well-characterized pattern recognition receptors, TLR2 and TLR4. However, incubation with PGN and LPS failed to induce the secretion by HCE-T and primary human corneal epithelial cells of inflammation-associated cytokines such as IL-6 and IL-8. Further, NF- κ B activation was not up-regulated by the stimulation of HCE-T with LPS or PGN. These results show that human corneal epithelial cells are incapable of responding to LPS from *P. aeruginosa* and to PGN from *S. aureus*. To support the finding, we subsequently used FACS and immunohistochemical analyses to show that human corneal epithelial cells express TLR2 and TLR4 intracellularly, but not at the cell surface. Even when LPS was artificially delivered to intracellularly expressed TLR4 in the cytoplasm, it did not lead to the subsequent activation of NF- κ B-mediated signaling for the induction of IL-6 and IL-8. These findings suggest the interesting possibility that the ocular surface epithelial cell-associated mucosal immune system may create an immunosilent condition for TLR-mediated innate immunity to prevent unnecessary inflammatory responses to normal bac-

terial flora. However, it has been shown that Langerhans cells and macrophages are located at the basal layer of the corneal epithelium and corneal stroma (33). Thus, these APCs may immediately respond to microbial products via TLRs.

Epithelial cells have long been thought to protect the integrity of mucosal surfaces mainly by acting as a physical barrier to invading pathogens. In fact, the mucosal epithelium serves as a critical immunological barrier against invasion by bacteria and viruses. As well as constituting a physical barrier, mucosal epithelial cells are

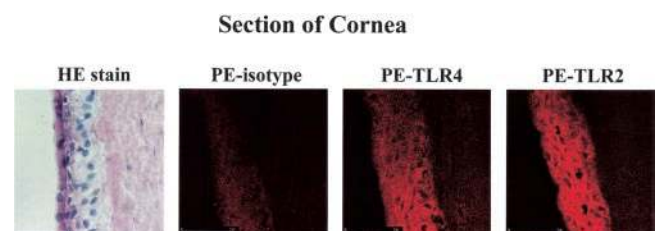
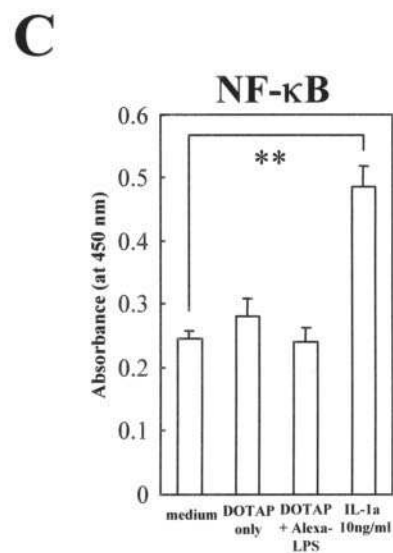
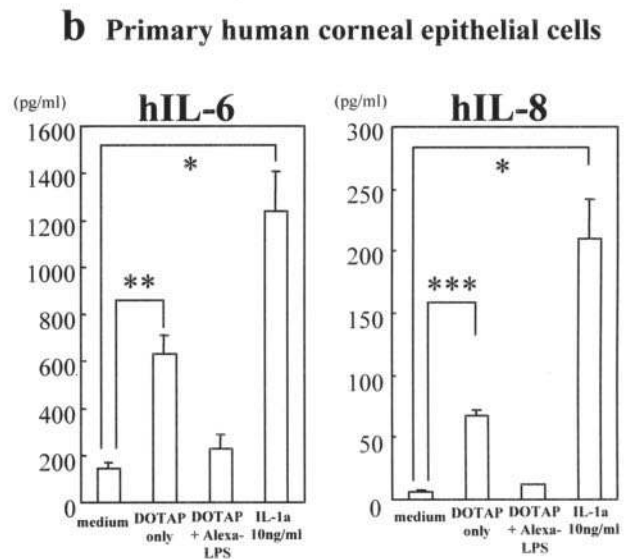
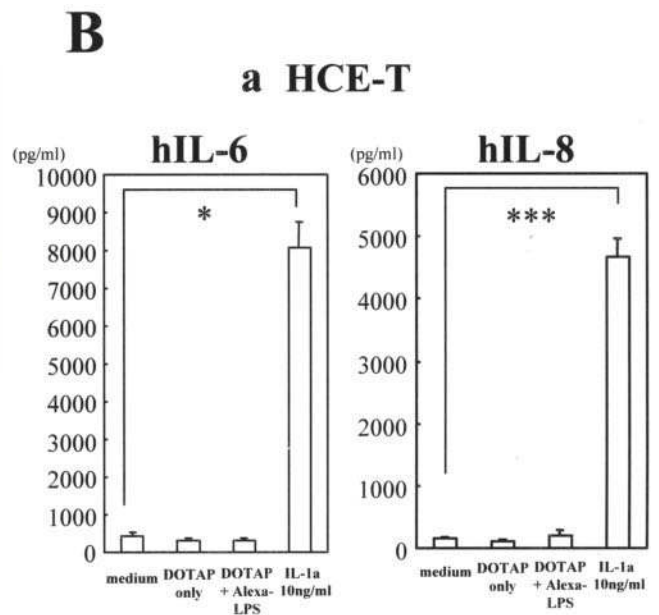
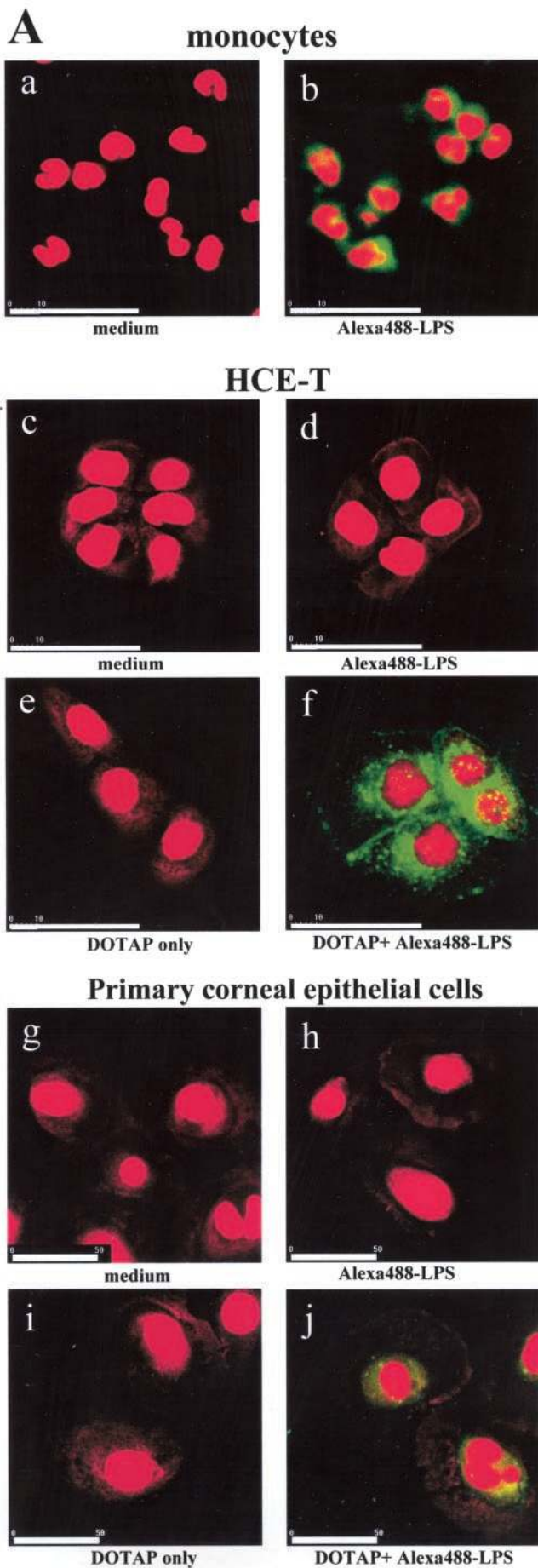


FIGURE 5. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelium. Slides of tissue sections were fixed with methanol for 30 min and then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs or isotype control mouse IgG2a for 24 h at room temperature. Tissue sections of human cornea showed specific staining with anti-TLR2 and -TLR4 mAb in the cytoplasm. Each bar represents a length of 50 μ m.



active participants in innate and acquired mucosal immune responses. When invaded by respiratory or intestinal pathogens, mucosal epithelial cells elicit proinflammatory gene expression, secretion of cytokines and chemokines, and recruitment of inflammatory cells to the site of infection (34). These findings suggest that epithelial cells play a major role in innate immune responses, which probably evolved to limit the infection by pathogenic bacteria at the invasion site. Alternatively, epithelial cells may initiate a sequence of innate and acquired immunity phases for the induction of Ag-specific immunity in both mucosal and systemic compartments. It is thus logical to assume that epithelial cells residing at the mucosal surface continuously express an array of TLR family members as sensors to detect and recognize invading pathogens. To this end, it has been shown that several TLRs, including TLR2 and TLR4, are expressed in the mucosal epithelium of the human tracheobronchia (18). After exposure to LPS, human tracheobronchial epithelial cells were activated for the expression of increased hBD2 mRNA. Bladder epithelial cells have also been reported to express TLR4 as well as increased levels of proinflammatory cytokines after incubation with LPS (20). In total contrast to these previous results, our findings suggest that the corneal epithelia do not express TLR2 and TLR4 at their cell surface.

To understand these seemingly conflicting findings, one must revisit the immunological and microbiological conditions prevailing in the mucosal epithelium. Even in the absence of pathogens, the mucosal epithelium is continuously exposed to great numbers of commensal bacteria, both Gram-positive and -negative (35, 36). Despite the high density of these commensal bacteria and their biologically active products observed under these physiological circumstances, the mucosal epithelium generally does not activate proinflammatory signaling cascades against them. These commensal bacteria are generally regarded as beneficial microflora for the host because they can suppress pathogens by displacing them from a microbial niche or by secreting antimicrobial substances (36). Normal bacterial flora residing in the conjunctival sac or along the eyelid edge making contact with the corneal surface include coagulase negative staphylococci, *P. acnes*, and others (4, 5). Commensal flora are also key to creating a symbiotic host-parasite interaction for the intestinal mucosa, especially in the large intestine. It is our contention that corneal epithelial cells purposely do not express TLRs (e.g., TLR2 or TLR4) so as to prevent inappropriate immune responses against such commensal bacteria, which, it must be admitted, are seen in lesser quantities at the ocular surface than in the large intestine.

In support of our view are recent studies providing new evidence that intestinal epithelial cells, perhaps in a bid to create a quiescent condition, express extremely low levels of TLR4 and no MD-2, a critical coreceptor of TLR4, and therefore do not respond to LPS (13, 14). These findings contradict earlier reports, which demonstrated that intestinal epithelial cells expressed TLR4 and thus were activated by LPS (16, 17). It has also been shown that nondifferentiated T84 cells obtained from colon cancers did not

respond to LPS, because TLR4 was expressed in the cytoplasmic compartment and not at the apical surface (15). In contrast, differentiated T84 cells expressing TLR4 at the apical surface were found to be capable of responding to LPS (15). Together with our results, these findings suggest that mucosal epithelial cells, which continuously interact with commensal bacteria, are capable of down-regulating the expression of TLR2 and TLR4. It is only natural that peripheral dendritic cells and macrophages, situated as they are in immunologically sanitary conditions, respond immediately to pathogen-associated molecules such as LPS via TLR4 to initiate immune responses. In contrast, epithelial cells, directly exposed as they are to external environmental Ags along with resident commensals, must behave in a totally different manner with regard to TLR-mediated immune responses. Moreover, on the ocular surface of humans, differentiated corneal and conjunctival epithelial cells are exposed to commensal bacteria and therefore would be expected to possess a down-regulatory mechanism for the TLR-mediated stimulation cascades. However, a previous report found just the opposite; human corneal epithelial cells were capable of responding to LPS via TLR4 expressed on their cell surface (37). One possible explanation could be that the previous study based its conclusion on the basis of a single line of corneal epithelial cells (10.014 pRSV-T) (37). In addition, another previous study demonstrated that human corneal epithelium were capable of responding to LPS, which resulted in the production of inflammatory cytokines (e.g., IL-1 α) (38). Because this study used human corneal limbal epithelium cultured from explants prepared from limbal rings of donor cornea, one cannot neglect the possibility that other alien cells in the explant responded to LPS. To this end, corneal endothelial cells, keratocytes, and fibroblasts associated with oculus from human and animals have been shown to respond to LPS (39–42). Further a previous report showed that explants of corneal rims yielded in the outgrowth of epithelial cells together with some single or clustered spindle-shaped cells resembling fibroblasts (42). It has been also shown that endotoxin-induced keratitis occurred in mice after administration of LPS to cornea (43–45). However, it should be noted that LPS-induced keratitis only occurred when corneal epithelium was abraded. Although we cannot pinpoint the reason for this discrepancy with the previous studies, we believe that our results convincingly demonstrate that although the corneal epithelial cell line and primary corneal epithelial cells express TLR2 and TLR4 in the cytoplasm, they remain unresponsive to PGN and LPS, respectively, as evidenced by the lack of inflammatory cytokine production, mRNA expression, and NF- κ B activity.

Our study also presents the novel finding that human corneal epithelial cells express TLR2 and TLR4 intracellularly, but not at the cell surface. Our experiments further show that even when stimulated with IL-1 α or TNF- α , HCE express neither TLR2 nor TLR4 on their cell surface. However, such cytokine treatment did activate corneal epithelial cells by means of the activation of

FIGURE 6. HCE-T and primary human corneal epithelial cells fail to respond to LPS even when LPS is translocated into the cytoplasm. When cocultured with Alexa 488-LPS, human corneal epithelial cells did not internalize it (*d* and *h* of *A*), but monocytes did (*b* of *A*). To examine whether intracellular TLR4 of human corneal epithelial cells can respond to LPS, Alexa 488-LPS was translocated into HCE-T and primary human corneal epithelial cells using DOTAP liposomal transfection reagent. Although human corneal epithelial cells did not spontaneously take up Alexa 488-LPS from the culture medium, the cells coincubated with 1 μ g/ml Alexa-LPS and 5 μ l/ml DOTAP showed punctated fluorescein (*f* and *j* of *A*). Confocal scanning laser microscopy showed extensive Alexa 488-LPS loading in the cytoplasm of human corneal epithelial cells. SYTOX Orange nucleic acid stain was used for counterstaining. In some experiments HCE-T and primary human corneal epithelial cells were cultured in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to DOTAP (5 μ l/ml) alone, DOTAP with Alexa-LPS (1000 ng/ml), or human IL-1 α (10 ng/ml) for 24 h. The culture supernatants were then harvested for measurement of IL-6 and IL-8 (*B*). To examine NF- κ B activation, HCE-T were plated in six-well plates and, upon reaching subconfluence, were left untreated or were exposed to DOTAP (5 μ l/ml) alone, DOTAP with Alexa-LPS (1000 ng/ml), or human IL-1 α (10 ng/ml) for 7 h. After the stimulation, the NF- κ B assay was performed using TransAM (*C*). ELISA and NF- κ B assay data represent the mean \pm SEM from an experiment with triplicate wells. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. Each bar represents a length of 50 μ m.

NF- κ B and the production of inflammatory cytokines, including IL-6 and IL-8. Thus, even when activated, human corneal epithelial cells did not recruit cytoplasmically expressed TLR4 to the cell surface. Further, our experiments showed that human corneal epithelial cells failed to respond to LPS even when LPS was artificially translocated into them. At the moment, we do not have any specific explanation for this unique finding. However, it was recently shown that a deficiency of MD-2, an associated molecule of the extracellular domain of TLR4, resulted in the lack of cell surface TLR4 expression (46). When embryonic fibroblasts from LPS-nonresponsive MD-2^{-/-} mice were examined, it was discovered that TLR4 could not reach the plasma membrane, but instead accumulated predominantly in the Golgi apparatus. In contrast, TLR4 was distributed at the leading edge surface of cells in wild-type embryonic fibroblasts (46). Moreover, TLRs were shown to be retained intracellularly in the absence of endoplasmic reticulum chaperone gp96, and thus the mutant cells of gp96 deficiency did not respond to microbial stimuli (47). Based on these results, it would seem plausible that cell surface TLR expression could be regulated at the level of TLR4-associated molecules (e.g., MD-2) and chaperon. These interesting possibilities will, of course, be the subject of our future investigations.

In summary, the data presented in this study demonstrate that human corneal epithelial cells fail to respond to PGN and LPS due to their inability to express TLR2 and TLR4, respectively, on their cell surfaces. Although both TLR2 and TLR4 were observed in the cytoplasm of human corneal epithelial cells, translocation of LPS to the cytoplasm did not elicit a response by those cells. These findings suggest that human corneal epithelial cells possess a unique regulatory mechanism for the inhibition of TLR2- and TLR4-mediated innate immunity.

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