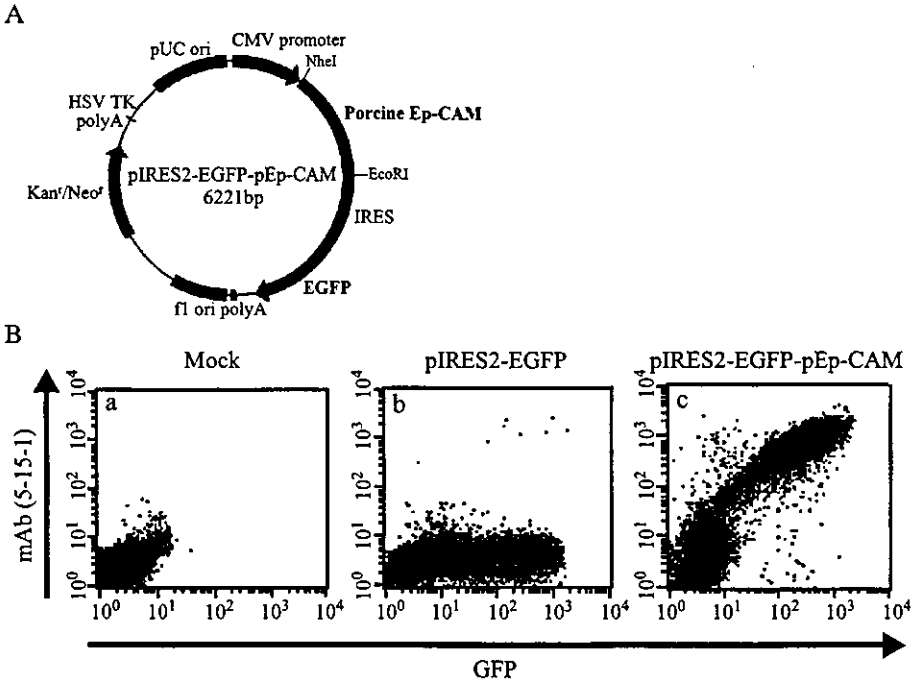

Fig. 6. Ep-CAM-specific 5-15-1 possessed specificity against COS-7 transfected with the cDNA of porcine Ep-CAM. Panel A shows the structure of the porcine Ep-CAM expression plasmid (pIRES2-EGFP-pEP-CAM). This plasmid (pIRES2-EGFP-pEp-CAM) is used for the expression of both porcine Ep-CAM and enhanced GFP (EGFP) because it contains the internal ribosome entry site (IRES). Panel B shows the flow cytometric analysis of nontransfected COS-7 (a), COS-7 transfected with empty vector (pIRES2-EGFP) (b), and pIRES2-EGFP-pEp-CAM (c) by using mAb (5-15-1). mAb (5-15-1) reacted only with COS-7 transfected with pIRES2-EGFP-pEp-CAM (c).

formally demonstrate that Ep-CAM is expressed by both IECs and CD45-positive IELs.

Discussion

The mucosal immune system has been shown to possess several biological characteristics that distinguish it from the systemic immune system [2]. For example, a sheet of intestinal epithelium provides a physical and immunological barrier against invading pathogens by forming an interdependent mucosal intranet between IECs and IELs. One IEL is usually surrounded by six to eight IECs [2]. When γδT cells representing approximately 50% of the murine IELs were removed, epithelial cell growth and MHC class II expression deteriorated [35]. This mutually beneficial relationship between IECs and IELs is reciprocally regulated by a group of mucosal cytokines including IL-7, IL-15, and SCF [17–19]. However, the physical and biological retention mechanism, which maintains IELs in the cellular pocket created by IECs in the intestinal epithelium, still remains unknown. To shed light on this issue, we sought to identify the novel molecule that helps retain IECs and IELs by generating a panel of mAbs specific for the intestinal epithelium. To identify the molecule on the cell surface that allows for physical cross-talk between IECs and IELs, we generated mAbs strongly reactive for the porcine epithelium by immunizing isolated and purified porcine IECs. After splenocytes isolated from immunized Balb/c mice were fused with myeloma (Sp2/0-Ag14), a total of 10 mAb-producing hybridomas were originally generated and screened for their specificity against isolated cells from the porcine intestine using flow cytometry (data not shown). Among these hybridomas, one, designated mAb 5-15-1, was found to strongly react with the basolateral surface of the intestinal epithelium (Fig. 1A). After immunoprecipitation, the antigen of 5-15-1 was detected at a molecular mass of 41–43 kDa by Western blot analysis under nonreducing conditions (Fig. 1B). Furthermore, the antigen was also characterized by using a G.P. Sensor, carbohydrate detection kit (Fig. 1B). Taken together, these data suggest that the surface antigen recognized by mAb 5-15-1 belongs to a family of membrane glycoproteins.

In order to identify the specific antigen of 5-15-1, we next attempted to purify the corresponding molecule from the lysate of the porcine small intestine using affinity chromatography with 5-15-1 (Fig. 4A). When the four major peaks identified by the MALDI-TOF-MS analysis of the purified trypsin-digested antigen (Fig. 4B) were further characterized by tandem MS, one peak was found to match with the peptide sequence of IADVAYYFEK, which corresponds to the human pan-carcinoma antigen epithelial glycoprotein (EGP). EGP was originally identified when various mAbs (e.g., MH99, AUA1, MOC31, 323/A3, KS1/4, GA733, HEA125) were developed and used for the

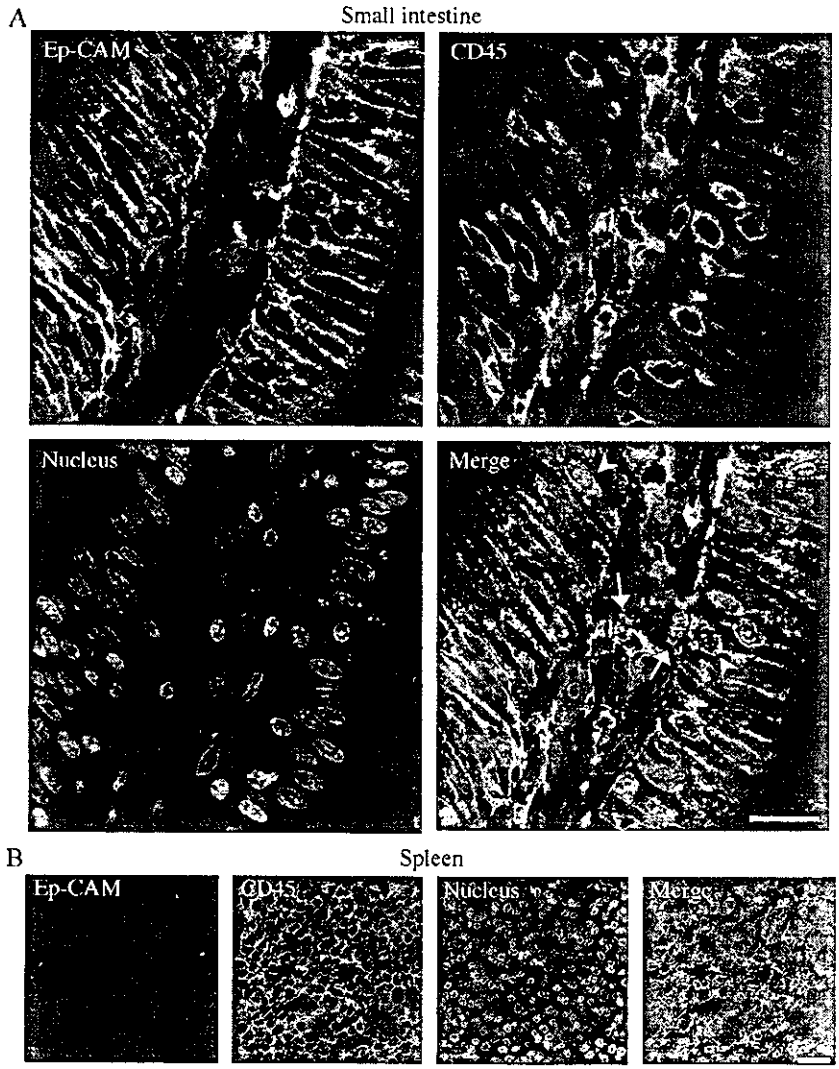

Fig. 7. Double immunohistochemical analysis of porcine small intestine and spleen with mAb 5-15-1 (anti-porcine Ep-CAM) and anti-porcine CD45 mAb. Tissue sections (5 μm) were incubated with anti-porcine Ep-CAM (5-15-1) and anti-porcine CD45 and then stained with FITC-conjugated anti-mouse IgG2b and rhodamine-conjugated anti-mouse IgG1, respectively. The slides were then counterstained by DAPI. In the small intestine, CD45-positive IELs (arrowhead) were costained with anti-porcine Ep-CAM (5-15-1), but such colocalization was not seen for lamina propria lymphocytes (LPLs, arrow) (A). Further, CD45-positive splenocytes did not react with anti-porcine Ep-CAM (5-15-1) (B). Scale bar = 20 μm.

analysis of human epithelial carcinoma [36–41]. Since EGP was initially shown to be expressed by human epithelial carcinoma, the molecule has become the target of EGP-specific immunotherapy and gene therapy strategies [42,43]. However, because EGP is an epithelial differentiation antigen but not a tumor-specific antigen, anti-EGP therapy can cause severe side effects [44]. A few years ago, EGP was shown to be capable of functioning as a Ca^{2+}-independent homophilic cell-to-cell adhesion molecule and received the new designation of epithelial cell adhesion molecule (Ep-CAM) [45,46]. Our present findings further demonstrate the physical homophilic cell-to-cell adhesion capability of Ep-CAM for the formation and maintenance of a sheet-like structure of intestinal epithelium. Thus, E-cadherin was shown to play a crucial role in the maintenance of the intestinal epithelium structure [47]. Ep-CAM may also play a physical role in sustaining the IELs population in the intestinal epithelium since IELs also possess specificity against 5-15-1 (Figs. 3 and 7). Thus, intestinal Ep-CAM is considered to be a key physical retention molecule for IECs and IELs and may provide a first line of defense against mucosal infection. It was recently reported that E-cadherin was recognized as the ligand molecule of internalin expressed by *Listeria monocytogenes* for their invasion to the host [48]. Although we still do not know exact molecular mechanism for the intercellular bacterial entry, Ep-CAM might be target molecule for several micro-organisms to enter the host via the destruction of mucosal epithelium created by IECs and IELs.

Only one of the peaks exhibited the peptide sequence of IADVAYYFEK, which corresponds to Ep-CAM (Fig. 4); the other three peaks identified by MALDI-TOF-MS

analysis were matched with the peptide sequence of actin by the subsequent tandem MS analysis (data not shown). Although the reactivity of 5-15-1 was only confirmed in mucosa-associated epithelium (Figs. 1 and 2), it is well known that anti-actin mAb generally reacts with most eukaryotic cells. Thus, actin is the major protein expressed by all of the mammalian tissues. When we performed the immunohistochemical staining for small intestinal epithelium using 5-15-1 and anti-actin mAb, epithelial cells reacted with both mAbs, as expected (data not shown). A previous study had reported that the cytoplasmic tail of Ep-CAM is capable of associating with the α-actinin [49]. This finding suggests the possibility of a biological association between Ep-CAM and the actin-based cytoskeleton in intestinal epithelial cells. This molecular interaction between the cytoplasmic tail of Ep-CAM and α-actinin has been considered to be a key component of the homophilic adhesion mechanism [49]. These findings lend further credibility to the possibility that the Ep-CAM expressed by both IECs and IELs may behave as a homophilic physical retention molecule for the heterologous cell-to-cell interaction in the intestinal epithelium.

To firmly confirm that 5-15-1 reacted specifically with porcine Ep-CAM, we cloned and sequenced porcine Ep-CAM and then examined the reactivity of 5-15-1 to COS-7 transfected with the isolated cDNA of porcine Ep-CAM (Fig. 6). The cloned cDNA contains an open reading frame (ORF) of 945 bp and encodes for 314 amino acids (Fig. 5). Compared to human, mouse, and rat sequences, the cloned porcine Ep-CAM displayed 71–83% and 77–83% homology at the nucleotide and amino acid levels, respectively (Fig. 5). Based on the characterization and comparison of the sequence with the known Ep-CAM in other species, the cloned porcine Ep-CAM was determined to be a type I transmembrane molecule with 12 conserved cystein residues and at least two N-glycosylation sites, like human, mouse, and rat Ep-CAM. Furthermore, the extracellular domain of porcine Ep-CAM also contained two epidermal growth factor (EGF)-like repeat motifs of $CX_1CX_8CX_7CX_1CX_{10}C$ (position 27-59, EGF-I) and $CX_{32}CX_{10}CX_5CX_1CX_{16}C$ (position 66-135, EGF-II), followed by a cysteine-poor domain (Fig. 5). The Ep-CAM polypeptide was originally shown to consist of 314 amino acids, including a 23 amino acid leader sequence, a 242 amino acid extracellular domain with two EGF-like repeats (EGF-I and EGF-II) within the cystein-rich N-terminal part, a 23 amino acid transmembrane domain, and a 26 amino acid cytoplasmic domain [50]. Among these different sections of the polypeptide, the EGF repeats of the extracellular domain of the Ep-CAM molecule are the most immunodominant epitopes expressed on the cell surface [50,51]. Thus, the focus has been on generating mAbs specific for the EGF-I and -II portion of the Ep-CAM in different species [50,51]. The majority of the currently existing mAbs possess specificity for the first EGF repeat. Based on the Western blot analysis (Fig. 1B), Ep-CAM was detected under nonreducing but not under reducing conditions. These findings suggest that 5-15-1 also recognized the cystein-rich domain of Ep-CAM (e.g., EGF-1, EGF-2). The 5-15-1 specifically reacted with COS-7 cells that had been transfected with the expression plasmid containing the cloned cDNA of porcine Ep-CAM (pIRES2-EGFP-pEp-CAM), but not with the cells transfected with the empty plasmid of pIRES2-EGFP (Fig. 6). Collectively, these results confirm that, by using the newly developed mAb 5-15-1, we have identified the porcine counterpart of Ep-CAM both at the level of the cloned gene and the protein.

It was interesting to note that the expression of 5-15-1 reactive Ep-CAM seemed always to be associated with the presence of IELs in the mucosal epithelium. When 5-15-1 was reacted with several other mucosal tissue-associated epithelial cells in addition to the small intestine (Fig. 2A), epithelial cells in the esophagus and stomach did not react with 5-15-1, while the other mucosa-associated epithelium did and at rates similar to those seen for the small intestinal epithelium. Further, the spleen and liver did not react with 5-15-1. Interestingly, because the esophageal and stomach epithelia are known not to contain IELs [52], they would have no need to express Ep-CAM as the retention molecule between IECs and IELs. Immunoprecipitation and Western blot analysis were used to confirm that all mucosa-associated tissues with the exception of the esophagus and stomach react with 5-15-1 (Fig. 2B). These findings suggest that Ep-CAM is strongly expressed by the mucosal epithelia that are covered by columnar epithelial cells. Since the stomach epithelium has been shown to possess physiological and immunological characteristics that distinguish it from the other mucosa-associated epithelia, its expression of Ep-CAM could be different as well. Taken together with the previous data [41], our current findings suggest that Ep-CAM plays a key role in the physical retention of IECs and IELs in the intestinal epithelium.

Moreover, we used flow cytometry with the appropriate fluorescence-conjugated 5-15-1 to show that Ep-CAM was expressed by IELs but not by splenocytes and PBMCs (Fig. 3). Further, the immunohistochemical analysis indicated that Ep-CAM and CD45 were colocalized on the cell surface of IELs but not of lamina propria lymphocytes (LPLs) (Fig. 7A). These findings further support our contention that a homophilic adhesion molecule of Ep-CAM plays a major biological role in the physical interaction between IECs and IELs. Previous studies reported that $\alpha_E\beta_7$ integrin mediates T cell adhesion to epithelial cells through its binding to E-cadherin, a member of the cadherin family of adhesion molecules that is expressed selectively on epithelial cells [53–55]. In fact, IELs decreased in number but did not disappear in α_E integrin-deficient mice [56]. These data suggest the interesting possibility that another adhesion mechanism mediated by Ep-CAM contributes to the formation and maintenance of an intact intestinal epithelium by physically retaining IELs in the cellular pocket created by IECs and may create an environment of mucosal intranet for

the maintenance of immunological homeostasis. In support of this possibility, a previous study reported that Ep-CAM was expressed in murine thymocyte and might contribute to adhesive interactions between thymocytes and thymus epithelial cells [57]. Currently, we are attempting to directly determine the physical adhesion mechanism via Ep-CAM between IECs and IELs by the creation of Ep-CAM-deficient mice.

In summary, we have used the newly generated mAb 5-15-1 to identify Ep-CAM expression by both intestinal intraepithelial lymphocytes and intestinal epithelial cells. The cell surface antigen recognized by 5-15-1 was a glycoprotein of Ep-CAM with a molecular mass of 41–43 kDa. The characterization of 5-15-1 affinity chromatography-purified glycoprotein by the use of MALDI-TOF-MS and tandem MS analyses demonstrated that the antigen was the porcine homologue of the human pan-carcinoma antigen epithelial glycoprotein, known as alias Ep-CAM. The cloning of the 5-15-1 reactive glycoprotein further confirmed the identification of Ep-CAM at the nucleotide and amino acid levels. The specificity of 5-15-1 was formally proved using COS-7 cells transfected with the cDNA of the cloned porcine Ep-CAM. Interestingly, not only IECs but also IELs reacted with 5-15-1. Since our data demonstrate that both IECs and IELs express homophilic adhesion molecules of Ep-CAM, it is plausible that Ep-CAM is an important element of the physical retention network responsible for retaining IECs and IELs in the intestinal epithelium for the generation of innate defense system.

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Intracellularly Expressed TLR2s and TLR4s Contribution to an Immunosilent Environment at the Ocular Mucosal Epithelium[1]

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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-κB or the secretion of inflammation-associated molecules such as IL-6, IL-8, and human β-defensin-2. However, when incubated with IL-1α to activate NF-κB, 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.

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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 Prefectual 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 Prefectual 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% CO_2 (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% CO_2 (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^- (Ca^{2+} 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 (AmpliTaq; 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 (1×10^5 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

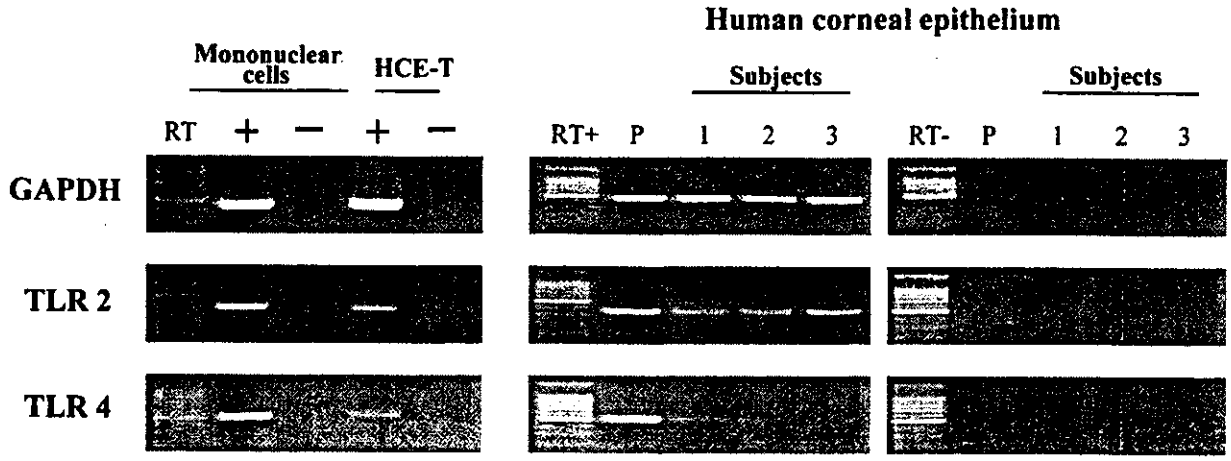
Real-time quantitative PCR was performed using a LightCycler (Roche, Mannheim, Germany) according to the previously described protocol (30) and manufacturer's instructions. For the amplification of IL-6, IL-8, and human β-defensin-2 (hBD2) cDNA, RT-PCR was performed in a 20-μl total volume in the presence of 2 μl of 10× reaction buffer (*Taq* polymerase, dNTPs, and $MgCl_2$; Roche), and 2 μl of cDNA (or water as a negative control, which was always included). $MgCl_2$ 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 (2×10^5 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 anti-sense	5'- CCATCACCATCTTCCAGGAG-3' 5'- CCTGCTTCACCACCTTCTTG-3'	(293-312) (849-868)	575bp
TLR2	XM003304	sense anti-sense	5'-GCCAAAGTCTTGATTGATTGG-3' 5'-TTGAAGTTCTCCAGCTCCTG-3'	(1783-1803) (2110-2129)	346bp
TLR4	XM005336	sense anti-sense	5'-TGGATACGTTTCCTTATAAG-3' 5'-GAAATGGAGGCACCCCTTC-3'	(1768-1787) (2256-2274)	506bp

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 μm) 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 μg/ml) was reacted with 5 μl/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-κB, 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 ± 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. 2*A*). 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-1α. 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-1α, 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. 2*B*). However, HCE-T responded to IL-1α in a dose-dependent manner for the enhancement of IL-6- and IL-8-specific mRNA (Fig. 2*B*). The expression of hBD2-specific mRNA was not induced by treatment with either LPS or PGN, but it was enhanced after exposure to IL-1α. 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-1α, whole-cell protein extracts were subjected to a DNA binding assay of NF-κB. As one might expect based on the results presented above, NF-κB-mediated signals were not enhanced by treatment of HCE-T with LPS or PGN, but were augmented by exposure to IL-1α (Fig. 2*C*).

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-1α 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 intracellulary, 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 perinucleic 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. 6*A*). 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 punctated fluorescein. Confocal scanning laser microscopy showed extensive loading of Alexa 488-LPS in the cytoplasm of human corneal epithelial cells (Fig. 6*A*).

After intracellularly exposing human corneal epithelial cells to LPS, we examined whether they secreted IL-6 and IL-8 (Fig. 6*B*). 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-1α. As a control, the medium pretreated epithelial cells were incubated with IL-1α. 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

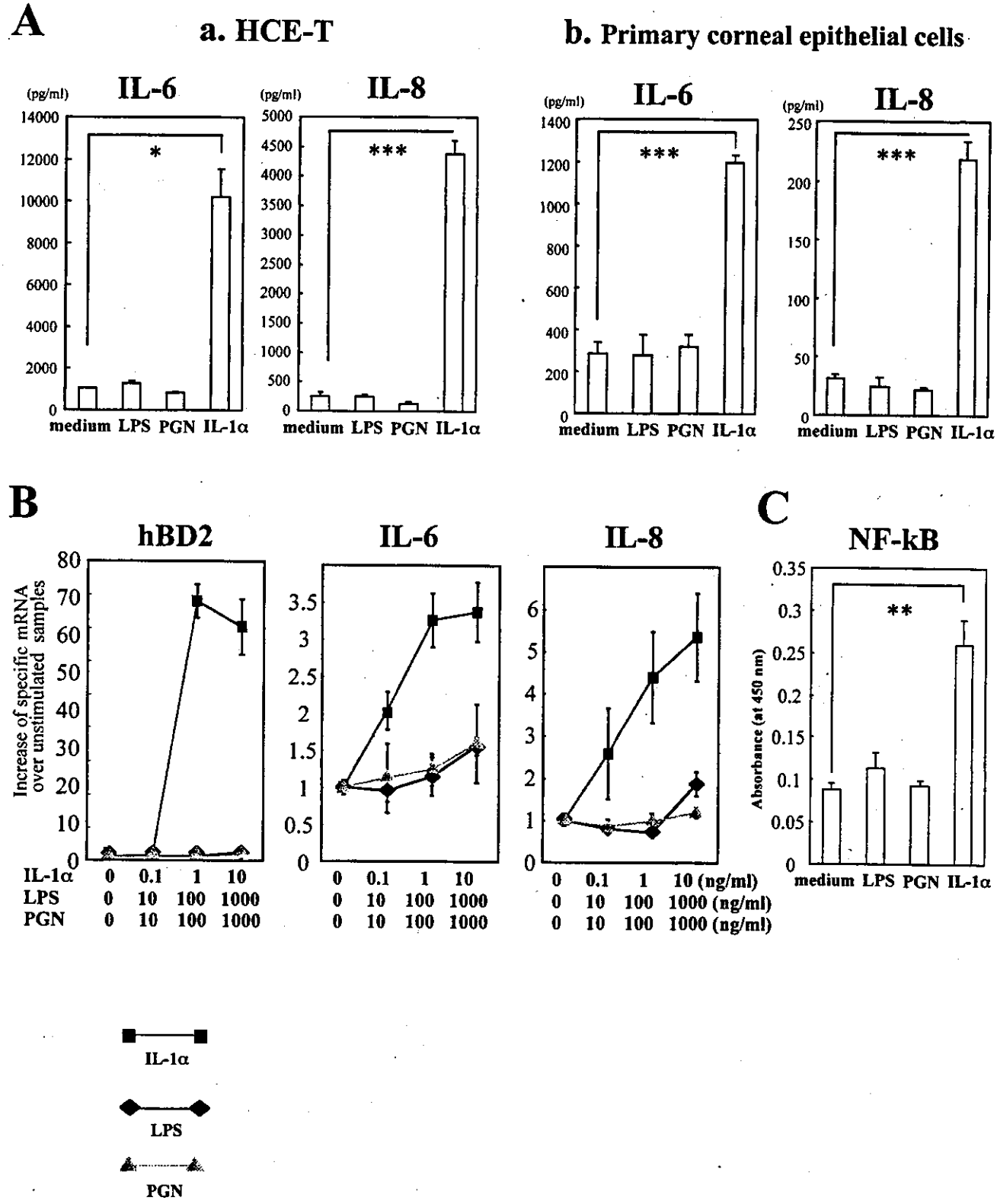


FIGURE 2. Human corneal epithelial cells fail to respond to LPS or PGN. To quantify inflammatory cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS, 1000 ng/ml PGN, 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. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

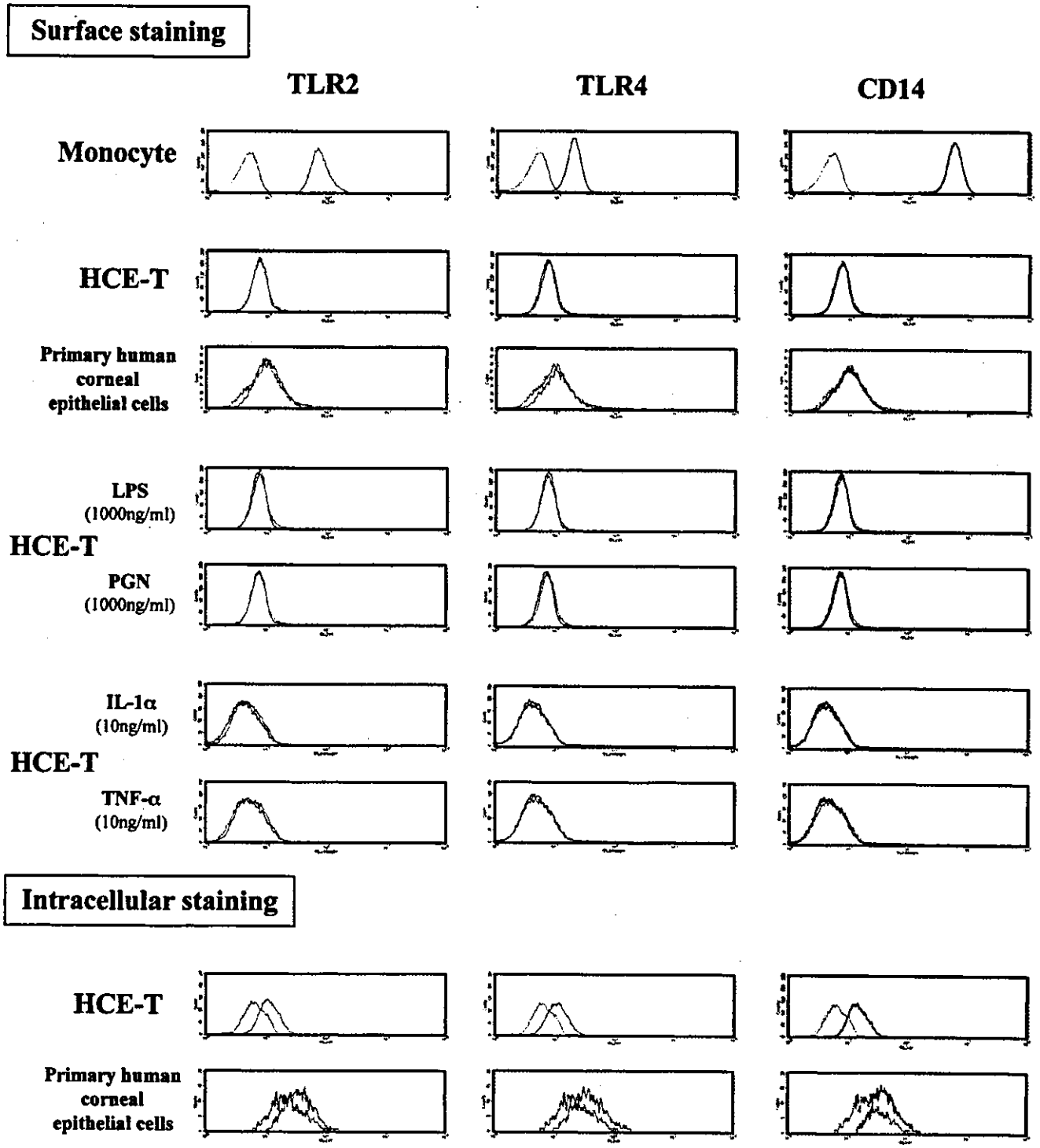


FIGURE 3. TLR2 and TLR4 are expressed intracellularly, but not on the cell surface of human corneal epithelial cells. Cell surface expressions of TLR2, TLR4, and CD14 in HCE-T and primary human corneal epithelial cells were examined by FACS. These cells were incubated with PE-conjugated mouse anti-human 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-κB signaling was up-regulated by the intracellular delivery of LPS into HCE-T. We found that internalization of

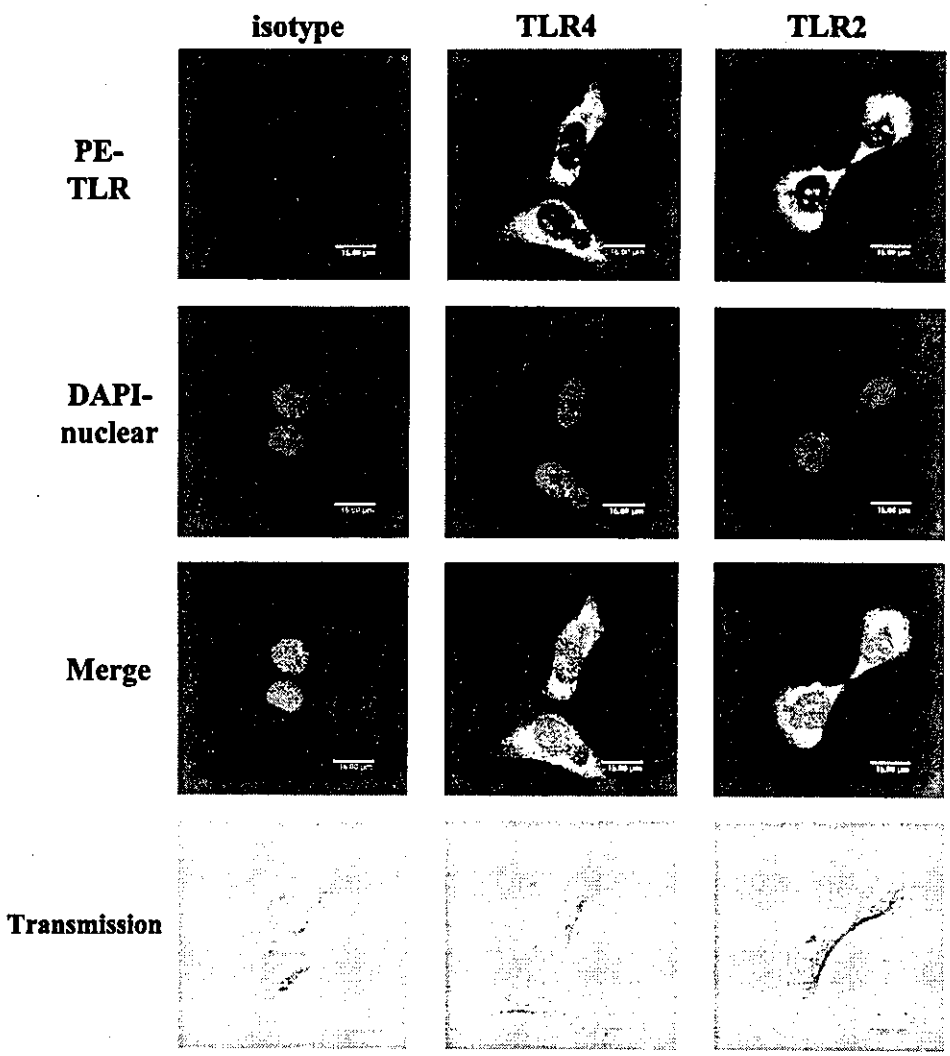


FIGURE 4. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in the human corneal epithelial cell line HCE-T. HCE-T was cultured on a slide chamber, washed with PBS^-, and air-dried. Slides were fixed with methanol for 30 min, then stained with PE-conjugated mouse anti-human 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 perinucleic region or cytoplasm. DAPI were used for counterstaining. Each bar represents a length of 50 μm.

Alexa 488-LPS into HCE-T did not lead to the enhancement of NF-κB-mediated signals (Fig. 6*C*). 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-κB 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-κB-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 bacterial 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

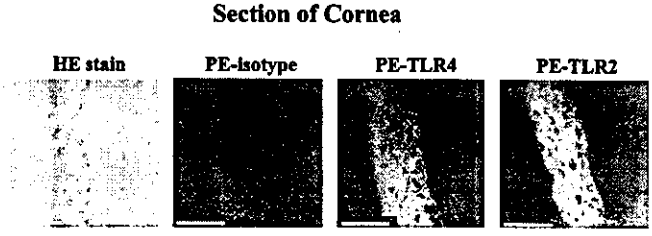
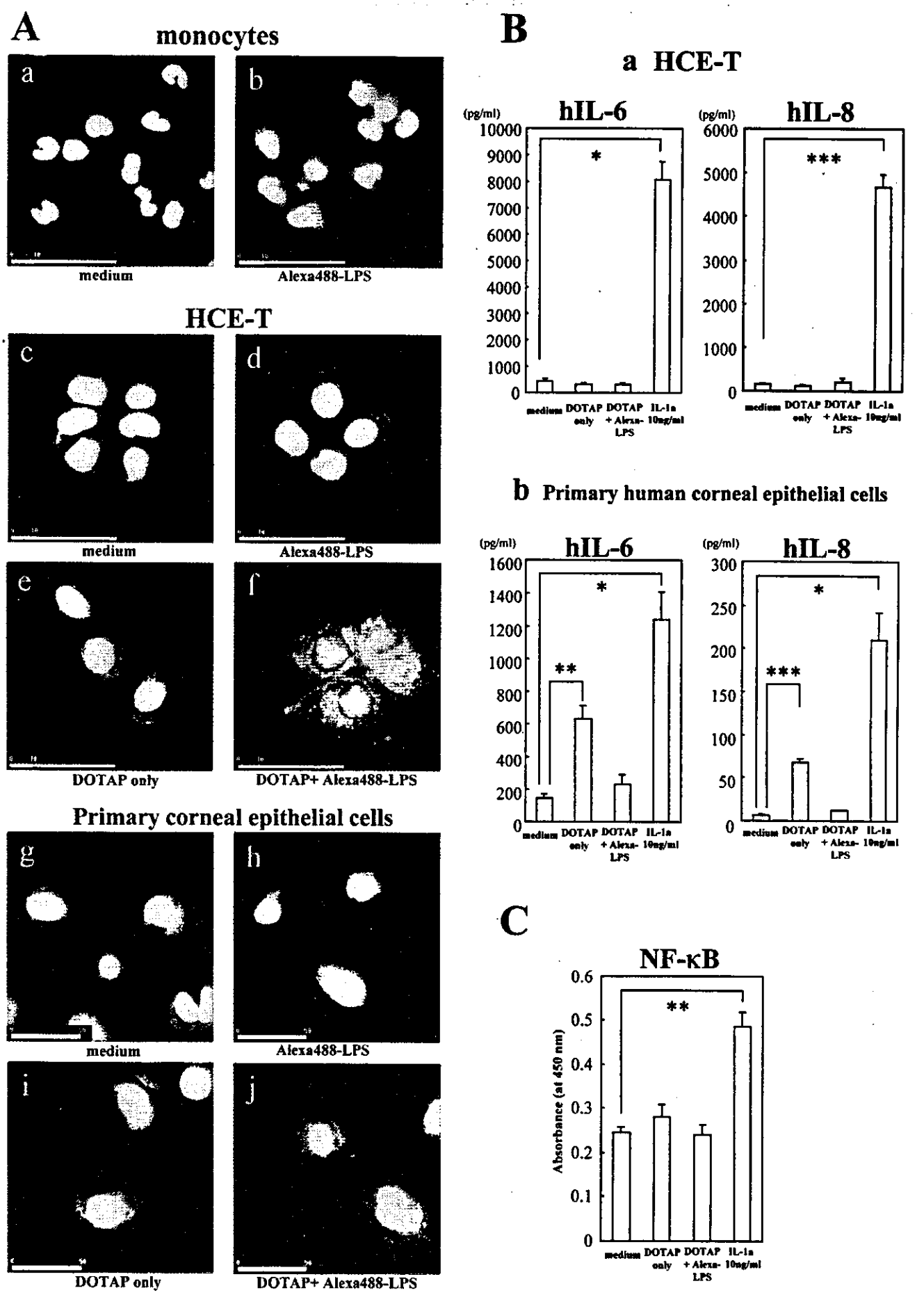


FIGURE 5. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelium. Slides of tissue sections were fixed with methanol for 30 min and then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs or isotype control mouse IgG2a for 24 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 μm.


A
monocytes
a
b
medium
Alexa488-LPS
HCE-T
c
d
medium
Alexa488-LPS
e
f
DOTAP only
DOTAP+ Alexa488-LPS
Primary corneal epithelial cells
g
h
medium
Alexa488-LPS
i
j
DOTAP only
DOTAP+ Alexa488-LPS
B
a HCE-T
hIL-6
(pg/ml)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
hIL-8
(pg/ml)
6000
5000
4000
3000
2000
1000
0
***
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
b Primary human corneal epithelial cells
hIL-6
(pg/ml)
1600
1400
1200
1000
800
600
400
200
0
*
**
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
hIL-8
(pg/ml)
300
250
200
150
100
50
0
*
***
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
C
NF-κB
Absorbance (at 450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
**
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml

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 trachebronchia (18). After exposure to LPS, human trachebronchial 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-1α) (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-κB 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-1α 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 μg/ml Alexa-LPS and 5 μl/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 μl/ml) alone, DOTAP with Alexa-LPS (1000 ng/ml), or human IL-1α (10 ng/ml) for 24 h. The culture supernatants were then harvested for measurement of IL-6 and IL-8 (*B*). To examine NF-κB activation, HCE-T were plated in six-well plates and, upon reaching subconfluence, were left untreated or were exposed to DOTAP (5 μl/ml) alone, DOTAP with Alexa-LPS (1000 ng/ml), or human IL-1α (10 ng/ml) for 7 h. After the stimulation, the NF-κB assay was performed using TransAM (*C*). ELISA and NF-κB 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 μm.

NF-κB 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.

Acknowledgments

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NALT- VERSUS PEYER'S-PATCH-MEDIATED MUCOSAL IMMUNITY

Hiroshi Kiyono and Satoshi Fukuyama

Abstract | Recent studies indicate that the mechanism of nasopharynx-associated lymphoid tissue (NALT) organogenesis is different from that of other lymphoid tissues. NALT has an important role in the induction of mucosal immune responses, including the generation of T helper 1 and T helper 2 cells, and IgA-committed B cells. Moreover, intranasal immunization can lead to the induction of antigen-specific protective immunity in both the mucosal and systemic immune compartments. Therefore, a greater understanding of the differences between NALT and other organized lymphoid tissues, such as Peyer's patches, should facilitate the development of nasal vaccines.

MICROFOLD (M) CELLS
Specialized antigen-sampling cells that are located in the follicle-associated epithelium of the organized mucosa-associated lymphoid tissues. M cells deliver antigens by transepithelial vesicular transport from the aero-digestive lumen directly to the subepithelial lymphoid tissues of nasopharynx-associated lymphoid tissue and Peyer's patches.

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The mucosal immune system is responsible both for mediating the symbiotic relationship between the host and endogenous microorganisms (commensal bacteria), and for functioning as a first line of physical and immunological defence against invading pathogens[1]. Through innate and acquired immunity, the mucosal immune system maintains immunological homeostasis along the vast expanse of the epithelial surface area, ranging from the oral and nasal cavities to the respiratory, intestinal and genito-urinary tracts.

The initiation of antigen-specific immune responses occurs at special 'gateways', which comprise MICROFOLD (M) CELLS located in the epithelium overlying follicles of the mucosa-associated lymphoid tissues (MALT). These contain all of the immunocompetent cells that are required for the generation of an immune response (that is, T cells, B cells and antigen-presenting cells). Peyer's patches, in the gut, and nasopharynx-associated lymphoid tissue (NALT) — two of the main components of MALT — are important inductive tissues for the generation of mucosal immunity through the ingestion and inhalation of antigen in the intestinal and respiratory tracts respectively[1] (FIG. 1). The COMMON MUCOSAL IMMUNE SYSTEM (CMIS) connects these inductive sites (that is, the Peyer's patches and NALT) with effector sites (such as the lamina propria of the intestinal and respiratory tracts, and glandular tissues) for the generation of antigen-specific T helper 2 (T_H2)-cell-dependent IgA responses, and T_H1-cell- and cytotoxic T lymphocyte (CTL)-dependent immune responses, which function as the first line of defence at mucosal surfaces[1,2].

In this review, we discuss three issues concerning the biology of the NALT immune system: first, we focus on the unique characteristics of its tissue genesis compared with that of Peyer's patches; second, we examine the immunological function of NALT; and third, we discuss manipulation of the NALT immune system to develop mucosal vaccines.

Distinct features of NALT organogenesis

Despite the functional similarity of NALT and Peyer's patches in terms of their role as mucosal inductive sites, their programmes of lymphoid organogenesis are distinct. On the basis of recent studies, the unique characteristics of NALT development compared with those of Peyer's patches have become clear in terms of both kinetics and cytokine requirements.

Chronological development. In normal mice, NALT is a bell-shaped tissue that is characterized by an accumulation of lymphoid cells. In contrast to the HIGH ENDOTHELIAL VENULES (HEVs) of Peyer's patches, which express mucosal vascular addressin cell-adhesion molecule 1 (MADCAM1) (REF. 3), NALT-associated HEVs express peripheral-node addressin (PNAD). Vascular cell-adhesion molecule 1 (VCAM1) has been

shown to be associated with the tissue genesis of Peyer's patches, because a cluster of VCAM1$^+$ stromal cells occurs at the site of Peyer's-patch development on day 15.5 after coitus[4]. To determine when NALT develops, we used immunohistochemistry to analyse PNAD expression in wild-type mice of various ages. NALT formation was not observed during embryogenesis or in newborn mice[5] (FIG. 2), whereas Peyer's patches were already present in the embryo as dome-shaped lymphoid tissues[6]. Instead, PNAD$^+$ HEVs with associated lymphocytes were first detected bilaterally in nasal tissue at 1 week after birth, and the complete formation of bell-shaped NALT (including lymphoid cells) was not observed until 5–8 weeks after birth[5] (FIG. 2). In rats, the development of NALT is also observed postnatally as a small accumulation of lymphoid cells[7]. These findings indicate a prenatal initiation of lymphoid organogenesis for Peyer's patches and a postnatal initiation for NALT. An intriguing possibility is that the NALT-genesis programme is triggered after birth through stimulatory signals that are provided by environmental antigens and mitogens. This view is supported by the finding that nasal administration of cholera toxin, a well-known mucosal immunogen with adjuvant activity, resulted in the acceleration of NALT organogenesis and the development of the bell-shaped lymphoid tissue[5]. Therefore, environmental stimulation might be essential for NALT organogenesis, although

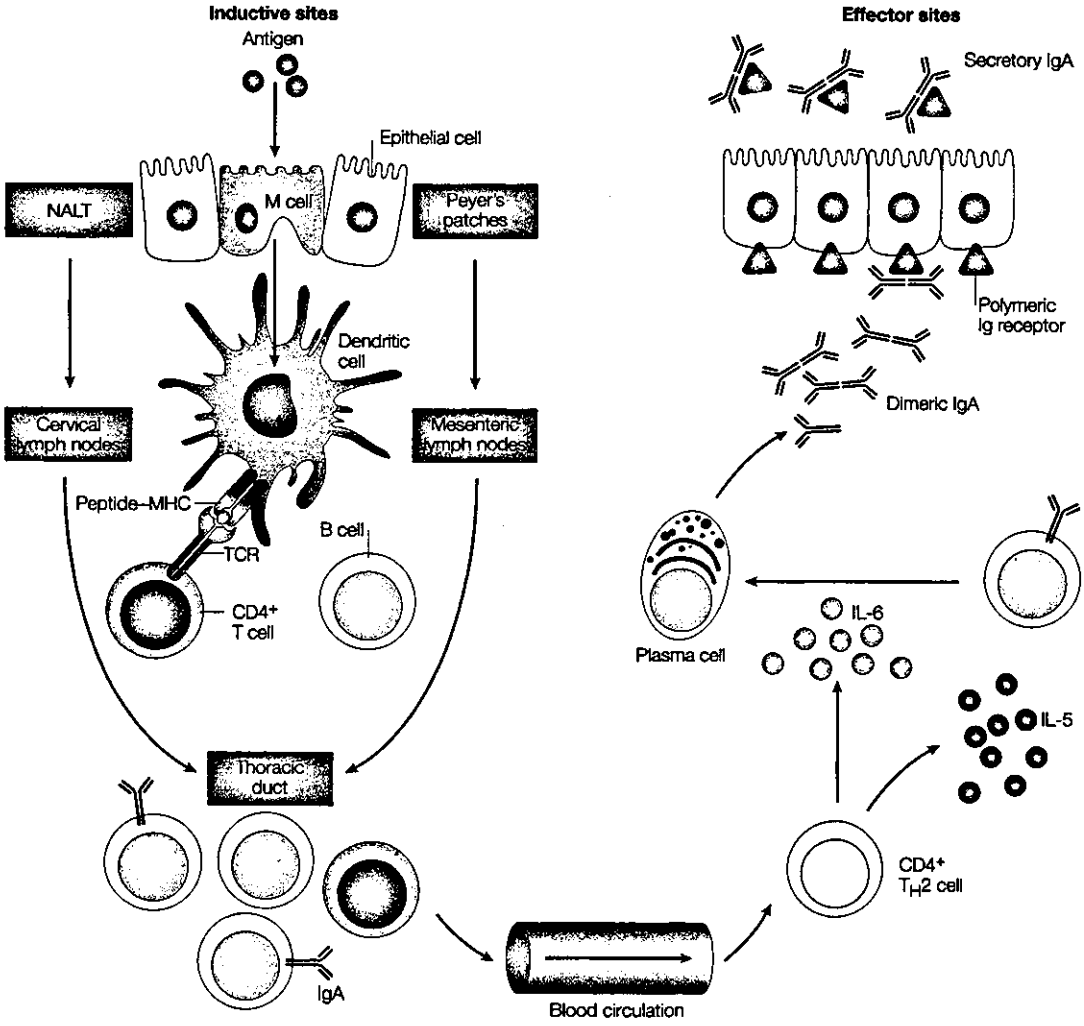

Figure 1 | **The common mucosal immune system.** Luminal antigens are transported to the nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches through microfold (M) cells that are present in the epithelium overlying NALT and Peyer's-patch follicles. Dendritic cells process and present antigens to T cells in these lymphoid tissues. CD4$^+$ T cells that are stimulated by dendritic cells then preferentially induce IgA-committed B-cell development in the germinal centre of the lymphoid follicle. After IgA class switching and affinity maturation, B cells rapidly migrate from NALT and Peyer's patches to the regional cervical lymph nodes and mesenteric lymph nodes respectively, through the efferent lymphatics. Finally, antigen-specific CD4$^+$ T cells and IgA$^+$ B cells migrate to effector sites (such as the nasal passage and intestinal lamina propria) through the thoracic duct and blood circulation. IgA$^+$ B cells and plasmablasts then differentiate into IgA-producing plasma cells in the presence of cytokines (such as interleukin-5 (IL-5) and IL-6) that are produced by T helper 2 (T_H2) cells, and they subsequently produce dimeric (or polymeric) forms of IgA. These dimeric forms of IgA then become secretory IgA by binding to polymeric Ig receptors (which become the secretory component in the process of secretory IgA formation) that are displayed on the monolayer of epithelial cells lining the mucosa. Secretory IgA is then released into the nasal passage and intestinal tract. TCR, T-cell receptor.

COMMON MUCOSAL IMMUNE SYSTEM (CMIS). An integrated pathway that allows communication between the organized mucosa-associated lymphoid tissues (inductive sites) and the diffuse mucosal tissues (effector sites), enabling the induction and regulation of host-protective immunity against pathogenic microorganisms.

HIGH ENDOTHELIAL VENULES (HEVs). Venules (small veins that join capillaries to larger veins) that have a high-walled endothelium and are present in the paracortex of lymph nodes and tonsils, as well as in the interfollicular areas of Peyer's patches. HEVs are essential for lymphocyte homing to secondary lymphoid organs.

NASOPHARYNX-ASSOCIATED LYMPHOID-TISSUE (NALT) ANLAGEN
The site for the initiation of NALT development. At this site, the accumulation of CD3⁻CD4⁺CD45⁺ cells and the expression of peripheral-node addressin (PNAD) by venules are observed in infant nasal tissues.

we have observed the formation of NALT in adult mice that were born and raised under germ-free conditions (H.K. and S.F., unpublished observations). Nonetheless, it is a strong possibility that initiation of NALT genesis is programmed to be activated after birth, and the subsequent maturation process is controlled by environmental antigens.

Contribution of cytokines to Peyer's-patch and lymph-node organogenesis. To show that cytokine-mediated NALT organogenesis is unique, it is important to summarize the mechanisms of Peyer's-patch and lymph-node organogenesis for comparative purposes. A family of pro-inflammatory cytokines that consists of lymphotoxin (LT) and tumour-necrosis factor (TNF), and their corresponding receptors (LT-β receptor (LT-βR), TNF receptor p55 (TNFRp55) and TNFRp75), creates a condition of 'programmed inflammation', which controls secondary lymphoid-tissue genesis[8,9] (TABLE 1). LTs are essential for secondary lymphoid-tissue organogenesis that is associated with the mucosal immune system, because deletion of either the genes that encode LT or the LT receptors, or artificial blockade of the interaction between the cytokine and its receptor during the embryonic period, results in the inhibition of both Peyer's-patch and peripheral lymph-node development[8,10,11]. For example, deletion of the *Lt-α* gene prevented Peyer's-patch formation and greatly limited the number of lymph nodes that developed[8]. LT-α forms LT-$\alpha_1\beta_2$ heterotrimers that can transduce an activation signal through the LT-βR, contributing to the organization of secondary lymphoid tissues[10]. When an LT-βR–Ig fusion protein was infused to antagonize the biological function of the LT-$\alpha_1\beta_2$ heterotrimer, lymphoid tissue formed at different anatomical locations depending on which embryonic stage was perturbed by introduction of the fusion protein[10]. This finding shows that the timing of secondary lymphoid-tissue development is regulated during embryogenesis[10,12]. We also found that the infusion of LT-βR–Ig between embryonic day (E) 15 and E17 suppressed Peyer's-patch development but had no effect on the formation of lymph nodes[13]. These studies clearly indicate the importance of the programmed inflammation that is mediated by LT-$\alpha_1\beta_2$ and the LT-βR for the genesis of Peyer's patches (TABLE 1; FIG. 3), but it is also known that another membrane-bound member of the TNF family, LIGHT, can bind to the LT-βR[14]. However, lymph nodes and Peyer's patches develop in the absence of LIGHT[15]. These findings indicate that the LT-$\alpha_1\beta_2$–LT-βR interaction is the essential component of programmed inflammation that initiates Peyer's-patch genesis at a particular time during the gestational period.

An additional cytokine that is associated with the mucosal immune system, namely interleukin-7 (IL-7), also has a crucial role in the initiation of Peyer's-patch genesis. IL-7 is produced by both mouse and human intestinal epithelial cells[16,17], and it provides stimulation and growth signals for neighbouring intestinal intraepithelial γδ T CELLS[16,18]. In mice that are deficient in the IL-7 receptor α-chain (*Il-7rα*$^{-/-}$), only the formation of Peyer's patches, and not lymph nodes, was impaired[19]. Similarly, when IL-7Rα function was blocked by administration of a single injection of an antagonistic monoclonal antibody to pregnant mothers on E15.5, the resulting offspring were deficient in Peyer's patches but showed normal lymph-node development[12]. These findings further emphasize that the LT-βR- and IL-7R-mediated tissue-genesis programme is crucial for the initiation of Peyer's-patch formation at the appropriate stage of embryogenesis (E14–E17) (FIGS 2,3).

Recently, a model that describes the development of Peyer's patches was proposed on the basis of this evidence. It was shown that lymphoid-lineage

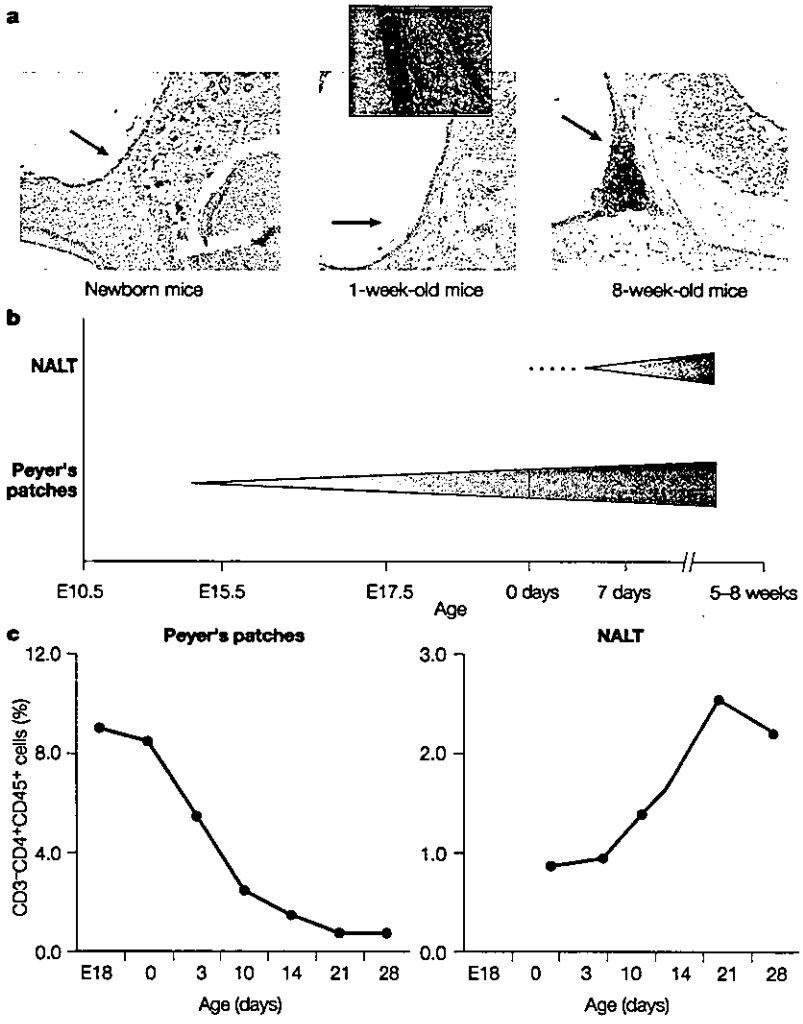

Figure 2 | **Chronological differences between NALT- and Peyer's-patch tissue genesis.** **a** | Nasal tissue from newborn mice (day 0) is characterized by an absence of peripheral-node addressin (PNAD)-expressing high endothelial venules (HEVs). The NASOPHARYNX-ASSOCIATED LYMPHOID-TISSUE (NALT) ANLAGEN from one-week-old mice shows a small accumulation of lymphoid cells around a single PNAD-expressing HEV in the nasal tissue. In eight-week-old mice, NALT contains numerous PNAD-expressing HEVs. This figure is reproduced with permission from REF. 5 © Elsevier (2002). **b** | The formation of NALT therefore starts after birth, whereas the development of Peyer's patches is initiated during embryogenesis. **c** | These kinetic differences in the initiation of tissue genesis of NALT and Peyer's patches are also supported by the appearance and frequency of CD3⁻CD4⁺CD45⁺ inducer cells in nasal and intestinal tissues. The inducer cells accumulate postnatally at the site of NALT formation, whereas high numbers of these cells are observed in Peyer's patches during the gestational period. E, embryonic day.

Table 1 | **Unique organogenesis of NALT characterized by study of gene-manipulated mice**

Mice	NALT	Lymph nodes	Peyer's patches	References
Il-7rα$^{-/-}$	Disorganized	+	–	5,19,31
Lt-α$^{-/-}$	Disorganized	–	–	5,8,31
Lt-β$^{-/-}$	Disorganized	CLN and MLN	–	5,11
Lt-βr$^{-/-}$	ND	–	–	9
LT-βR–Ig	+	+/–	–	5,10
aly/aly (*Nik*$^{-/-}$)	Disorganized	–	–	5,27,28
Id2$^{-/-}$	–	–	–	5,30
Ror-γ$^{-/-}$	+	–	–	29,31,33
Trance$^{-/-}$	Disorganized	–	+	31
Cxcr5$^{-/-}$*Cxcl13*$^{-/-}$	ND	CLN and MLN	Reduced number	21,24

aly/aly, alymphoplasia mouse; CLN, cervical lymph node; *Cxcl13*, CXC-chemokine ligand 13; *Cxcr5*, CXC-chemokine receptor 5 (receptor for Cxcl13); *Id2*, inhibitor of DNA binding 2; *Il-7r*, interleukin-7 receptor; *Lt*, lymphotoxin; *Lt-βr*, Lt-β receptor; LT-βR–Ig, lymphotoxin-β-receptor–Ig fusion protein; MLN, mesenteric lymph node; NALT, nasopharynx-associated lymphoid tissue; ND, not determined; *Nik*, nuclear-factor-κB-inducing kinase; *Ror-γ*, retinoic-acid-receptor-related orphan receptor-γ; *Trance*, tumour-necrosis-factor-related activation-induced cytokine.

IL-7R$^+$CD3$^-$CD4$^+$CD45$^+$ cells that are considered to be PEYER'S-PATCH INDUCERS express CXC-chemokine receptor 5 (CXCR5) and can produce membrane-associated LT-$\alpha_1\beta_2$ heterotrimer, whereas mesenchymal-lineage VCAM1$^+$ and intercellular adhesion molecule 1 (ICAM1)$^+$ PEYER'S-PATCH ORGANIZERS express the LT-βR[20,21] (FIG. 3). Following stimulatory signals that are provided through the IL-7R, Peyer's-patch inducers express LT-$\alpha_1\beta_2$, which activates Peyer's-patch organizers through the LT-βR; and in turn, Peyer's-patch organizers produce chemokines, such as CXC-chemokine ligand 13 (CXCL13) and CC-chemokine ligand 19 (CCL19), which stimulate Peyer's-patch inducers through CXCR5 and CC-chemokine receptor 7 (CCR7) (REF. 22). The reciprocal interaction between inducer and organizer cells through chemokine and cytokine receptors is essential for the formation of Peyer's patches (FIG. 3), and the loss of any component of either of the signalling programmes is sufficient to disrupt secondary lymphoid-tissue development, as indicated by the loss of Peyer's patches in LT-βR-deficient and IL-7Rα-deficient mice[9,23]. Furthermore, deletion of the gene that encodes CXCR5 partially reduces the formation and number of Peyer's patches[24] (TABLE 1). The lack of Peyer's patches and lymph nodes in alymphoplasia (*aly/aly*) mice, which have defective NIK (nuclear factor-κB (NF-κB)-inducing kinase) function, also fits this model, because recent analyses have established that NIK is essential for the transduction of signals through the TNFR family, including those through the LT-βR[25,26]. So, *aly/aly* mice lack Peyer's patches because the NIK mutation inhibits the reciprocal interaction between Peyer's-patch inducers and organizers that is mediated through LT-$\alpha_1\beta_2$ and the LT-βR[27,28]. Further evidence in support of this model comes from studies showing that mice that lack the CD3$^-$CD4$^+$CD45$^+$ inducer cells, owing to genetic deletion of the transcriptional regulators ID2 (inhibitor of DNA binding 2) or ROR-γ (retinoic-acid-receptor-related orphan receptor-γ), also lack Peyer's patches and lymph nodes[29,30].

LT-βR- and IL-7R-independent NALT organogenesis. Because Peyer's-patch formation requires a cytokine-signalling cascade that involves the IL-7R and the LT-βR (TABLE 1; FIG. 3), we examined whether an identical receptor-signalling cascade would trigger NALT development. The formation of NALT was studied in mice lacking Peyer's patches and/or lymph nodes, including *Lt-α*$^{-/-}$, *Lt-β*$^{-/-}$ and *aly/aly* mice, and mice that were treated *in utero* with the LT-βR–Ig fusion protein[5] (TABLE 1). Nasal lymphoid tissue was detected in all mouse strains lacking Peyer's patches or both Peyer's patches and lymph nodes because of a deficiency in the LT-βR-mediated pro-inflammatory cytokine cascade[5]. A separate study by Harmsen and colleagues[31] confirmed the formation of NALT in the absence of LT-βR-mediated signalling. The authors also showed that NALT formation was reconstituted in mice that were deficient in both TNF and LT-α by the adoptive transfer of wild-type bone marrow, even though Peyer's patches did not develop in these mice[31]. These findings further support the idea that NALT development does not conform with the model of programmed inflammation that is required for the genesis of Peyer's patches (FIG. 3).

Because Peyer's-patch formation has also been shown to require the IL-7R-mediated signalling pathway, in addition to the LT-βR cascade, NALT development was examined in IL-7R-deficient mice. NALT, but not Peyer's patches, was found to develop in IL-7R-deficient mice[5,31]. Taken together, these findings directly show that NALT formation is independent of IL-7R- and LT-βR-mediated tissue genesis (FIG. 3).

CD3$^-$CD4$^+$CD45$^+$ cells in NALT organogenesis. A unique subset of mononuclear cells that are characterized as being CD3$^-$CD4$^+$CD45$^+$ have been shown to function as inducer cells for the organogenesis of secondary lymphoid tissues, including Peyer's patches[21]. So, a high frequency of CD3$^-$CD4$^+$CD45$^+$ cells is observed in the intestinal tract at embryonic stages of development (FIG. 2). Furthermore, *Id2* has been identified as one of

γδ T CELLS
T cells that express heterodimers consisting of the γ- and δ-chains of the T-cell receptor. They are present mainly in the intestinal epithelium as intraepithelial lymphocytes (IELs). Although the exact function of γδ T cells (or IELs) is still unknown, it has been suggested that mucosal γδ T cells are involved in the innate immune responses of the mucosal immune system.

PEYER'S-PATCH INDUCERS
CD3$^-$CD4$^+$CD45$^+$ cells that express the interleukin-7 receptor and lymphotoxin-$\alpha_1\beta_2$. They differentiate from fetal liver cells and can induce Peyer's-patch formation during the embryonic stage.

PEYER'S-PATCH ORGANIZERS
Lymphotoxin-β-receptor-positive stromal cells that are present in the anlagen of Peyer's patches and also express both VCAM1 (vascular cell-adhesion molecule 1) and ICAM1 (intercellular adhesion molecule 1). Peyer's-patch development is initiated with the cooperation of Peyer's-patch inducers.