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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

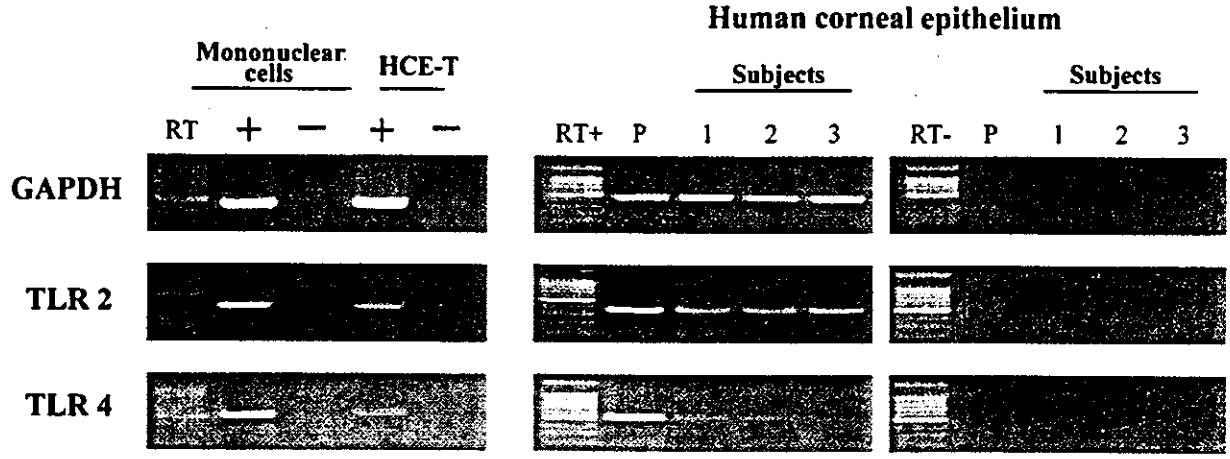
Real-time quantitative PCR was performed using a LightCycler (Roche, Mannheim, Germany) according to the previously described protocol (30) and manufacturer's instructions. For the amplification of IL-6, IL-8, and human β-defensin-2 (hBD2) cDNA, RT-PCR was performed in a 20-μl total volume in the presence 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

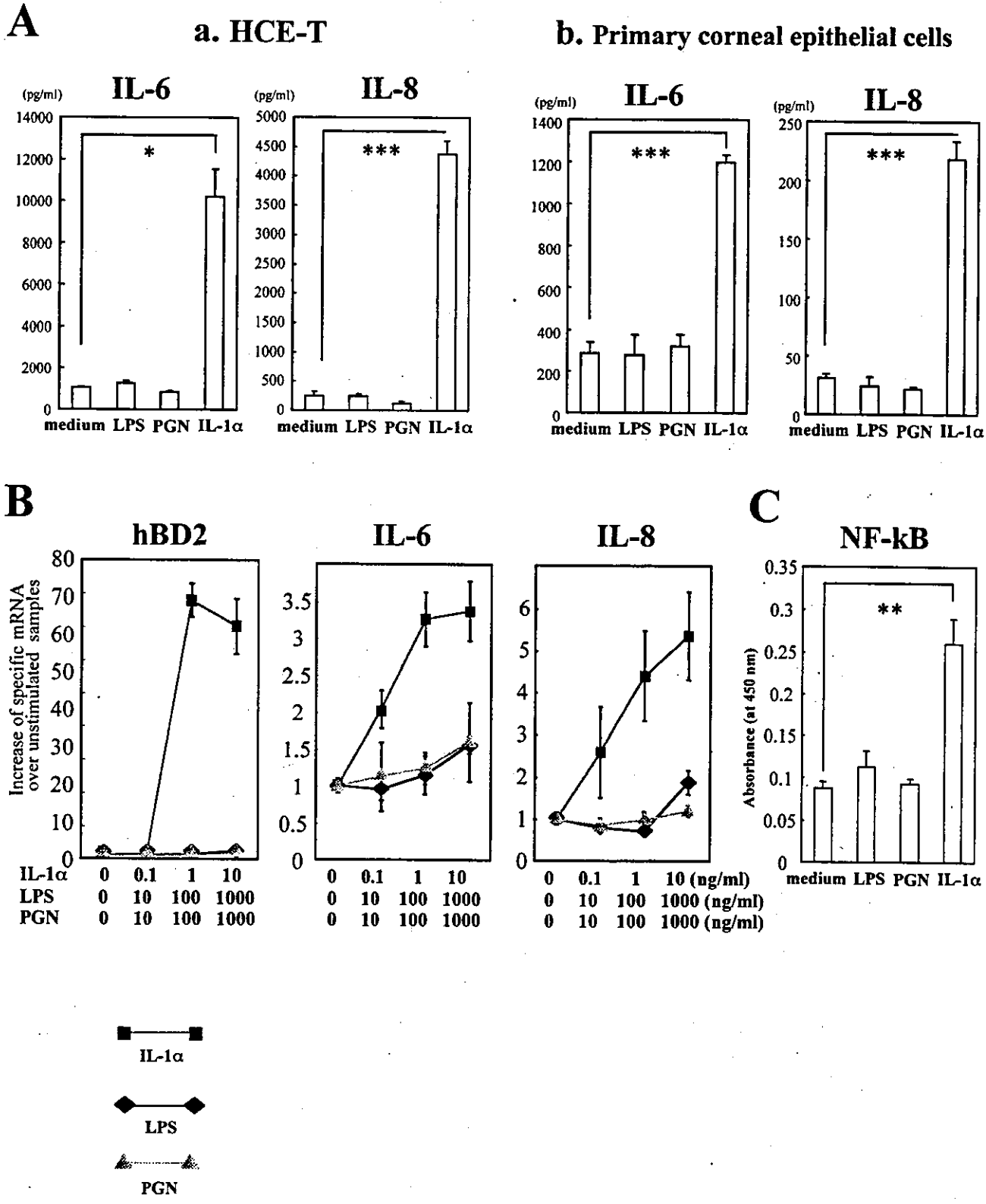


FIGURE 2. Human corneal epithelial cells fail to respond to LPS or PGN. To quantify inflammatory cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS, 1000 ng/ml PGN,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

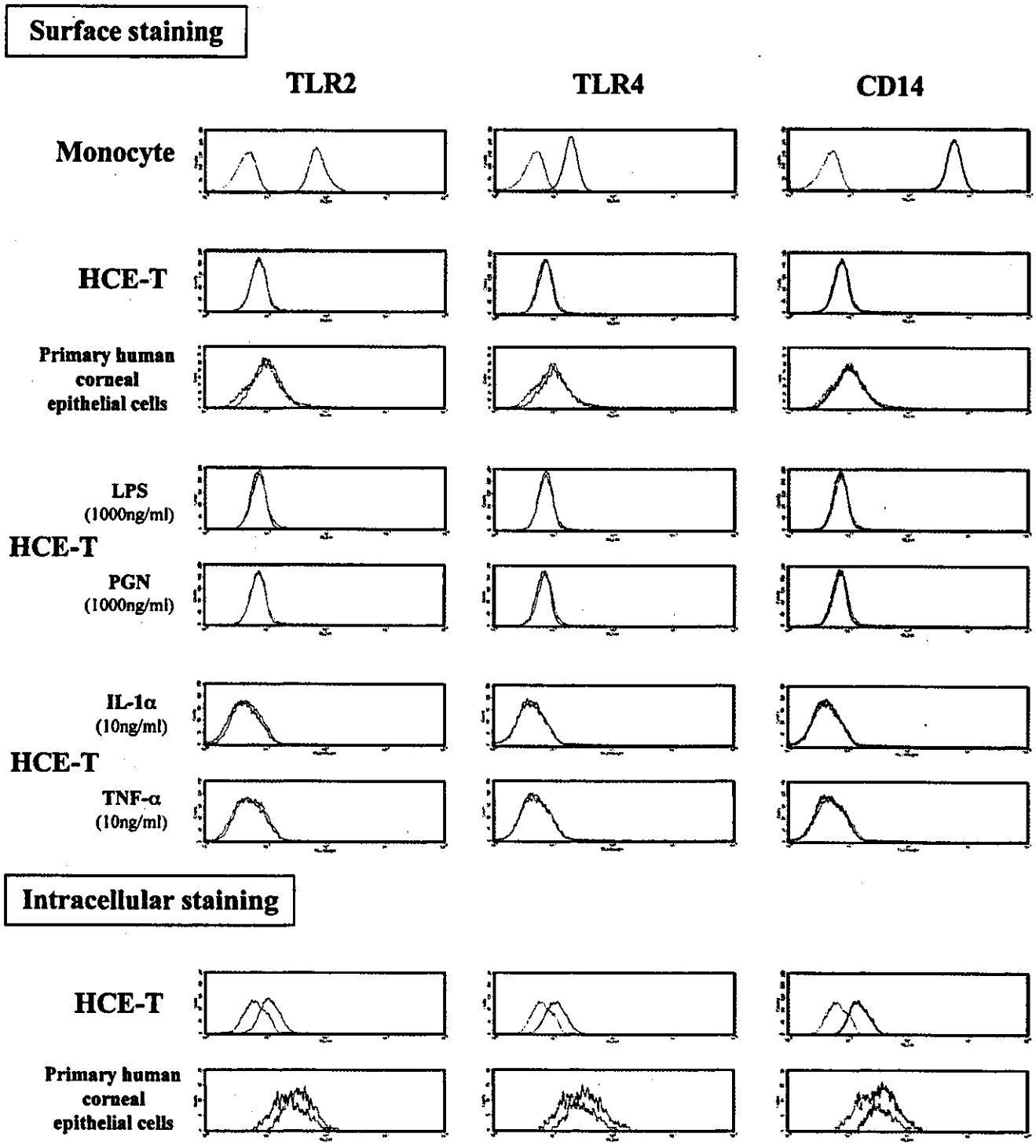


FIGURE 3. TLR2 and TLR4 are expressed intracellularly, but not on the cell surface of human corneal epithelial cells. Cell surface expressions of TLR2, TLR4, and CD14 in HCE-T and primary human corneal epithelial cells were examined by FACS. These cells were incubated with PE-conjugated mouse anti-human 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

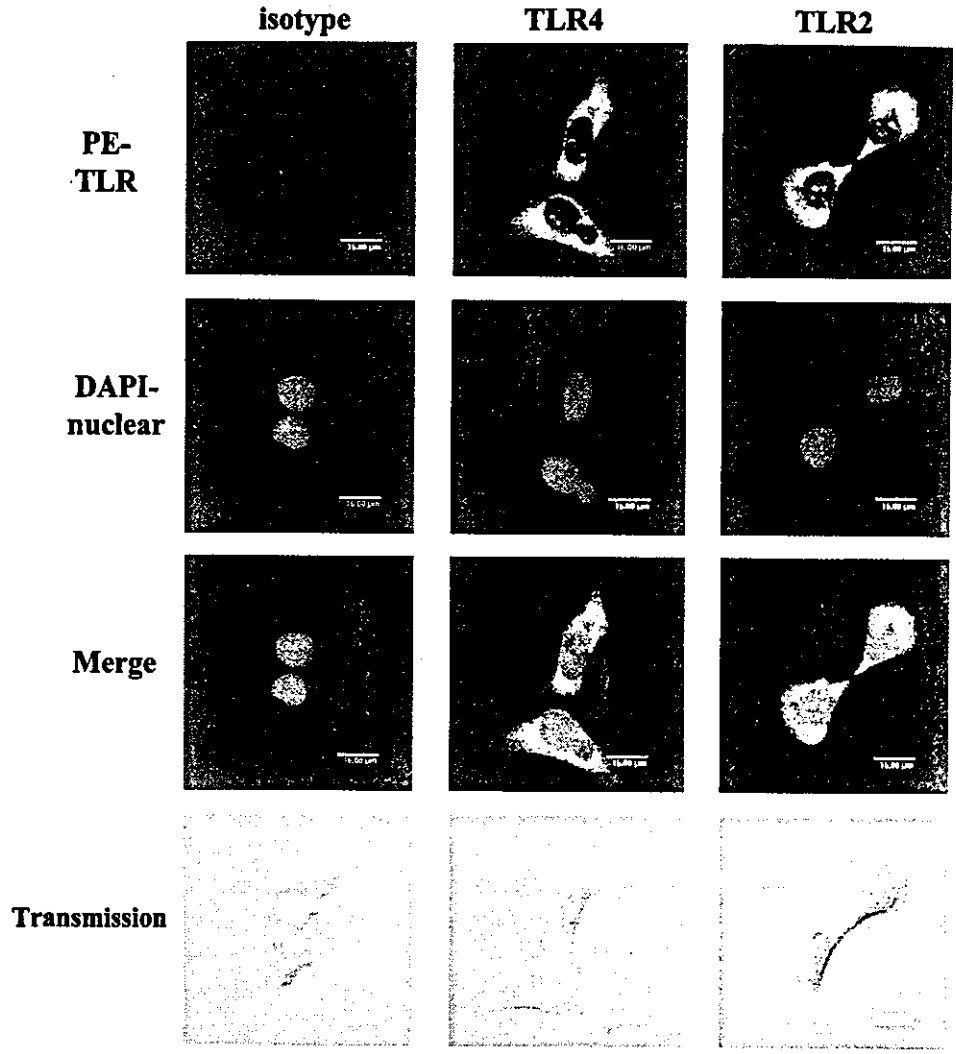


FIGURE 4. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in the human corneal epithelial cell line HCE-T. HCE-T was cultured on a slide chamber, washed with PBS^-, and air-dried. Slides were fixed with methanol for 30 min, then stained with PE-conjugated mouse anti-human 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

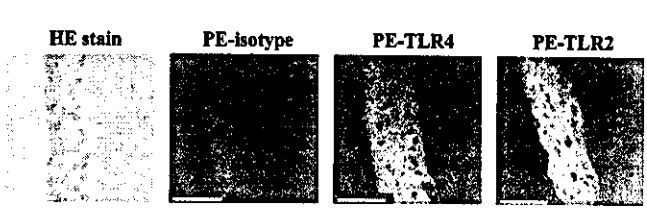
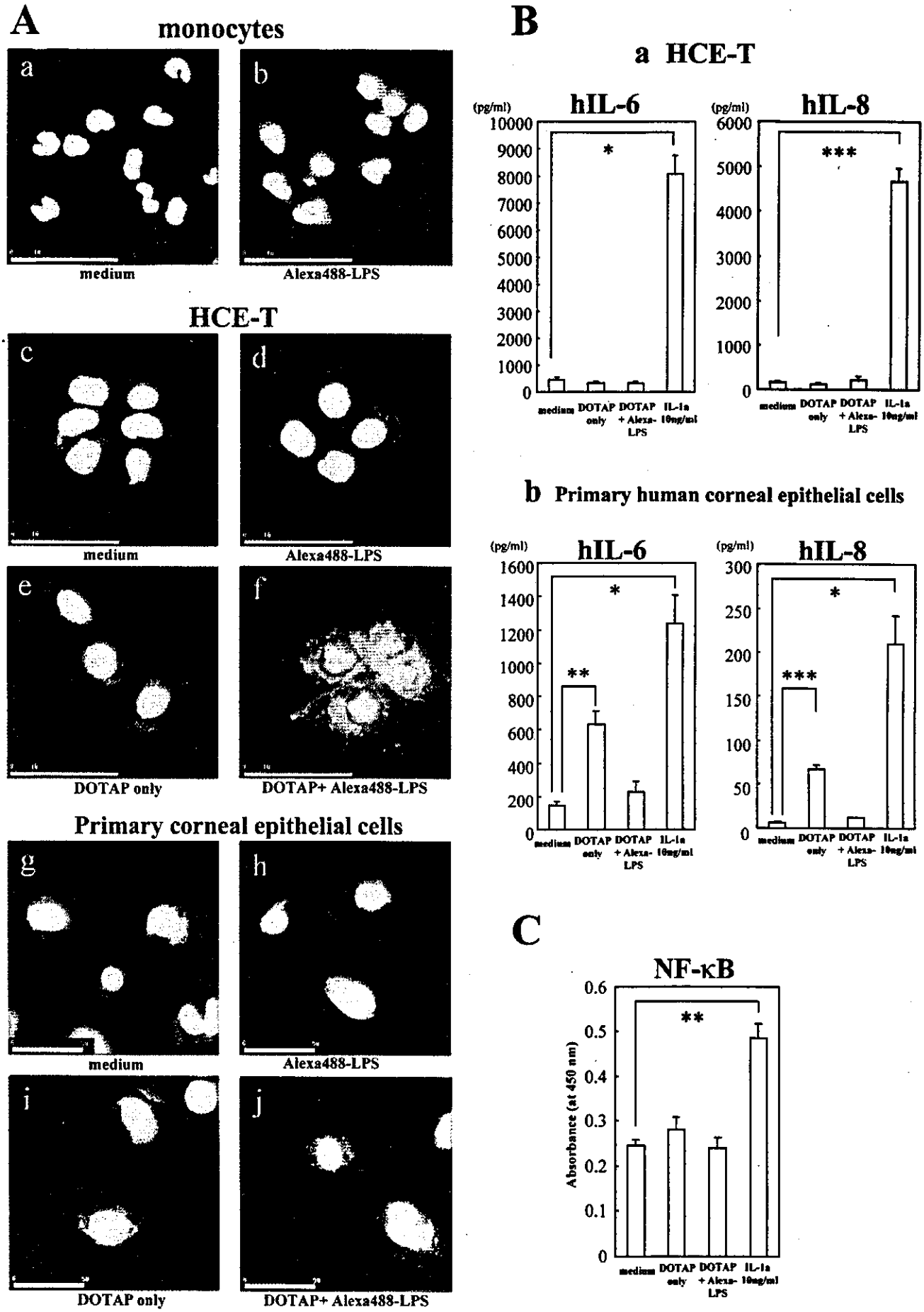


FIGURE 5. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelium. Slides of tissue sections were fixed with methanol for 30 min and then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs or isotype control mouse IgG2a for 24 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.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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 ± 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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Roles of a conserved family of adaptor proteins, Lnk, SH2-B, and APS, for mast cell development, growth, and functions: *APS*-deficiency causes augmented degranulation and reduced actin assembly

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Abstract

Lnk, SH2-B, and APS form a conserved adaptor protein family. All of those proteins are expressed in mast cells and their possible functions in signaling through c-Kit or FcεRI have been speculated. To investigate roles of Lnk, SH2-B or APS in mast cells, we established IL-3-dependent mast cells from *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, and *APS*$^{-/-}$ mice. IL-3-dependent growth of those cells was comparable. Proliferation or adhesion mediated by c-Kit as well as degranulation induced by cross-linking FcεRI were normal in the absence of Lnk or SH2-B. In contrast, *APS*-deficient mast cells showed augmented degranulation after cross-linking FcεRI compared to wild-type cells, while c-Kit-mediated proliferation and adhesion were kept unaffected. *APS*-deficient mast cells showed reduced actin assembly at steady state, although their various intracellular responses induced by cross-linking FcεRI were indistinguishable compared to wild-type cells. Our results suggest potential roles of APS in controlling actin cytoskeleton and magnitude of degranulation in mast cells.

Keywords: Actin cytoskeleton; Adaptor protein; BMMC; c-Kit; Cytokine; Cytokine receptor; Degranulation; FcεRI; IgE; Signal transduction; Tyrosine kinase

Mast cells play critical roles in allergic and inflammatory responses. Mast cells express the high affinity IgE receptor FcεRI and cross-linking of IgE bound to FcεRI by antigens initiates a series of molecular events in mast cells, which lead to degranulation and release of a wide variety of chemical mediators such as histamine, arachidonic acid metabolites, and soluble proteins including neutral proteases and cytokines [1–3]. Even in the absence of antigen, binding of monomeric IgE to FcεRI induces cytokine production and cell survival [4]. Mast cells differentiate from hematopoietic progenitor cells. Stem cell factor (SCF), which is also known as mast cell growth factor, and IL-3 provide signals for their differentiation, proliferation, and survival mediated through c-Kit receptor tyrosine kinase and IL-3 receptor, respectively. SCF also regulates chemotaxis and adhesion of mature mast cells [1,5].

Lnk, SH2-B, and APS form a conserved family of adaptor proteins, whose members share a homologous N-terminal region with proline rich stretches, PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site [6–9]. Lnk plays a critical role in regulating production of B cell precursors and hematopoietic progenitor cells, and functions as a negative regulator of c-Kit-mediated signaling. We have shown that *lnk*$^{-/-}$ mice show enhanced B cell production because of the hypersensitivity of B cell precursors to SCF [8]. In addition, *lnk*$^{-/-}$ mice exhibit increased numbers of hematopoietic progenitors in the bone marrow, and the ability of hematopoietic progenitors to repopulate

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irradiated host animals was greatly enhanced by the absence of Lnk [10]. Independently, Velazquez et al. [11] have reported *lnk*-deficiency results in abnormal modulation of SCF and IL-3-mediated signaling pathways and augmented growth of bone marrow cells or splenocytes. SH2-B is originally identified as a protein associated with immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRI γ-chain by a modified two-hybrid (tribrid system) screening [6]. We have shown that SH2-B is a critical molecule for the maturation of reproduction organs that is at least in part mediated by insulin-like growth factor I (IGF-I) receptor signaling [12]. APS is identified as a potential substrate of c-Kit by two-hybrid system [7]. We also independently isolated the murine counterpart of APS as a protein homologous to Lnk and SH2-B [9]. APS is phosphorylated upon stimulation with various growth factors, including EPO-R, PDGF-R, insulin, nerve growth factor (NGF), and cross-linking B cell receptor (BCR) [9,13–16]. Recently, we generated *APS*$^{-/-}$ mice and found that B-1 cells in peritoneal cavity were increased, and humoral immune responses to type-2 antigen significantly enhanced in *APS*$^{-/-}$ mice [17].

Lnk-family adaptor proteins, Lnk, SH2-B, and APS, are all expressed in bone marrow-derived mast cells (BMMCs) [12]. In addition, various experiments using cell lines overexpressing those Lnk-family adaptor proteins suggested their possible functions in signaling mediated through c-Kit or FcεRI. We investigated and compared for the first time consequences of the deficiency either of Lnk, SH2-B or APS in mast cell functions using primary cultured cells. We established BMMCs from bone marrow progenitors of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$ mice, and their respective control wild-type mice. IL-3-dependent BMMCs were equally established even in the absence of Lnk, SH2-B or APS. SCF-dependent proliferation or adhesion was also not compromised and was comparable among *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, and *APS*$^{-/-}$ BMMCs. Although FcεRI-mediated degranulation was not affected by the absence of Lnk or SH2-B, *APS*$^{-/-}$ BMMCs showed enhanced degranulation after cross-linking FcεRI. *APS*$^{-/-}$ BMMCs showed reduced filamentous actin (F-actin) assembly at steady state and was resistant to inhibitors disrupting F-actin microfilaments in FcεRI-mediated degranulation responses. These results suggest that APS plays a role in negative regulation of mast cell degranulation by controlling actin dynamics.

Materials and methods

Cells and culture. Bone marrow cells were obtained from 8- to 10-week-old *lnk*$^{-/-}$ [8], *SH2-B*$^{-/-}$ [12], *APS*$^{-/-}$ mice [17], and their respective wild-type littermates, and cultured in RPMI1640 supplemented with 5 ng/ml murine IL-3 (PeproTech), 8% fetal calf serum (FCS), nonessential amino acids (Gibco-BRL), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 μM of 2-mercaptoethanol. Cells were split and supplied with fresh medium every 4 or 5 days. After 4 weeks of cultivation, greater than 95% of cells were c-Kit and FcεRI positive as assessed by flow cytometry.

Flow cytometry and cytochemistry. For the detection of FcεRI, BMMCs were incubated in a supernatant of IGEL a2 (15.3) hybridoma containing mouse anti-DNP IgE monoclonal antibody (mAb) and then stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgE mAb (LO-ME-2, Oxford Biomarketing, UK). For the detection of c-Kit, cells were stained with phycoerythrin (PE)-conjugated anti-CD117 mAb (2B8, Pharmingen). For measurements of F-actin content, cells were fixed in 3.7% formaldehyde for 6 h at 4 °C permeabilized with 0.2% Triton X-100 in PBS for 30 min and then stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 1 h. Stained cells were then analyzed by flow cytometry using a FACSCalibur (Becton–Dickinson).

Unstimulated or stimulated BMMCs were resuspended in PBS and deposited onto microscope slides using a Cytospin 3 (Shandon Scientific, Cheshire, England). After staining with May–Gruenwald's and Giemsa's solutions (MERCK), cellular morphology was assessed by a light microscope.

Proliferation and survival assays. BMMCs (5×10^4) were cultured in 0.2 ml of flesh medium containing various concentrations of SCF (PeproTech) in a 96-well multi-well plate for 72 h. Cells were pulsed with [^{3}H]thymidine (0.2 μCi/well) in the last 12 h of culture and harvested and incorporated [^{3}H]thymidine was measured in triplicate determination using a MATRIX 96 Direct Beta Counter (Packard, Meriden, CT). Cells were cultured in media alone or in the presence of various concentrations of anti-DNP IgE mAb (SPE-7, Sigma). Percentage of viable cells was determined by trypan blue exclusion.

Adhesion assay. Adhesion assays to fibronectin were performed as previously described [18]. In brief, 5×10^4 BMMCs labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy fluorescein (BCECF: Molecular Probes, Eugene, OR) were incubated in triplicate in a 96-well polystyrene plate (Lynbro-Titertek, Aurora, OH) coated with fibronectin (Sigma) in the presence of various concentrations of SCF or 10 ng/ml PMA at 37 °C for 30 min. Unbound cells were removed by washing the plates with binding medium RPMI 1640 containing 10 mM Hepes (pH 7.4), and 0.03% BSA four times. Adhered cells were quantified by measuring fluorescence of input and bound cells using a Fluorescence Concentration Analyzer (IDEXX Laboratories, Westbrook, ME).

Degranulation assay. BMMCs were sensitized with anti-DNP IgE at 37 °C for 18 h, washed, and resuspended in Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, and 0.1% BSA). Cells (5×10^5 in 0.2 ml) were then stimulated with various concentrations of DNP-BSA or 10 ng/ml PMA plus 400 ng/ml ionomycin at 37 °C for 1 h. Enzymatic activities of β-hexosaminidase in supernatants and cells solubilized in 0.5% Triton X-100 Tyrode's buffer were measured using *p*-nitrophenyl *N*-acetyl-β-D-glucosaminidase (Sigma) as substrates. Degranulation was calculated as the percentage of β-hexosaminidase released from cells in the total amount of the enzyme in the supernatants and cell pellets as described before [18]. For the experiment using latrunculin, sensitized BMMCs were pretreated with various concentrations of latrunculin for 15 min at 37 °C before assays. Histamine released into culture supernatants after degranulation was measured using ELISA kit (Immunotech, Marseille, France) according to manufacturer's recommendation.

Calcium measurements. Sensitized BMMCs were incubated with 6 μM Fura PE3/AM (TEFLABS, Austin, TX) in PBS containing 20 mM Hepes (pH 7.4), 5 mM glucose, 0.025% BSA, and 1 mM $CaCl_2$ (HBS) at 37 °C for 60 min. Cells were washed and resuspended in HBS (1×10^5 cells/0.1 ml) in a stirring cuvette. Fluorescence was monitored continuously with a fluorescence spectrophotometer (CAF-110: JASCO, Osaka, Japan) at an emission wavelength of 500 nm and two different excitation wavelengths (340 and 380 nm).

Immunoblotting. Cell lysates from stimulated BMMCs were subjected to immunoprecipitation and Western blot analysis as previously

described [9]. The proteins were resolved by SDS–8% PAGE and transferred to PVDF membranes (Immobilon, Millipore). After blocking with 5% BSA, membranes were probed with anti-phosphotyrosine mAb (4G10, Upstate Biotechnology) and incubated with HRP-conjugated secondary antibodies. Blots were washed in 0.05% Tween 20/Tris-buffered saline, pH 7.6, and proteins were detected by chemiluminescence (Perkin–Elmer Life Sciences).

Results

Establishment of BMMCs lacking either Lnk, SH2-B or APS

Lnk, SH2-B, and APS were all expressed in normal BMMCs [12]. To investigate possible functions of those adaptor proteins in mast cells, we established IL-3-dependent BMMCs from bone marrow progenitors of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, and *APS*$^{-/-}$ mice and their responses were compared with those of BMMCs established from respective control wild-type littermates. IL-3-dependent growth of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ bone marrow progenitor cells was almost comparable to that of respective control progenitor cells (Fig. 1A). Established *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ BMMCs were not distinguishable from the wild-type BMMCs in terms of surface expression of FcεRI and c-Kit (Fig. 1B). Mast cell differentiation and proliferation induced by IL-3 was not affected at all even in the absence of Lnk, SH2-B or APS.

Functions of lnk$^{-/-}$, *SH2-B*$^{-/-}$ *or APS*$^{-/-}$ *BMMCs*

First, we examined proliferative responses of established BMMCs to SCF and found no difference among *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$, and respective control BMMCs (Fig. 2A). Adhesion to fibronectin induced by SCF or PMA was also not affected in the absence of Lnk, SH2-B or APS (Fig. 2B). We then examined degranulation of those BMMCs induced by cross-linking FcεRI by measuring β-hexosaminidase and histamine released after stimulation. Degranulation from *lnk*$^{-/-}$ or *SH2-B*$^{-/-}$ BMMCs was almost comparable to that from control wild-type BMMCs (Fig. 2C). In contrast, *APS*$^{-/-}$ BMMCs showed enhanced degranulation responses upon cross-linking FcεRI (Fig. 2C). Degranulation from *APS*$^{-/-}$ BMMCs, determined by β-hexosaminidase releasability, was 130–140% of that from control cells at each stimulation condition, and the enhancement was statistically significant at the concentrations of DNP-BSA over 0.5 μg/ml (Table 1). Histamine released after cross-linking FcεRI was also augmented in *APS*$^{-/-}$ BMMCs (data not shown).

FcεRI-mediated cellular responses in APS$^{-/-}$ *BMMCs*

To clarify the possible molecular mechanisms leading to the enhanced degranulation in the absence of APS,

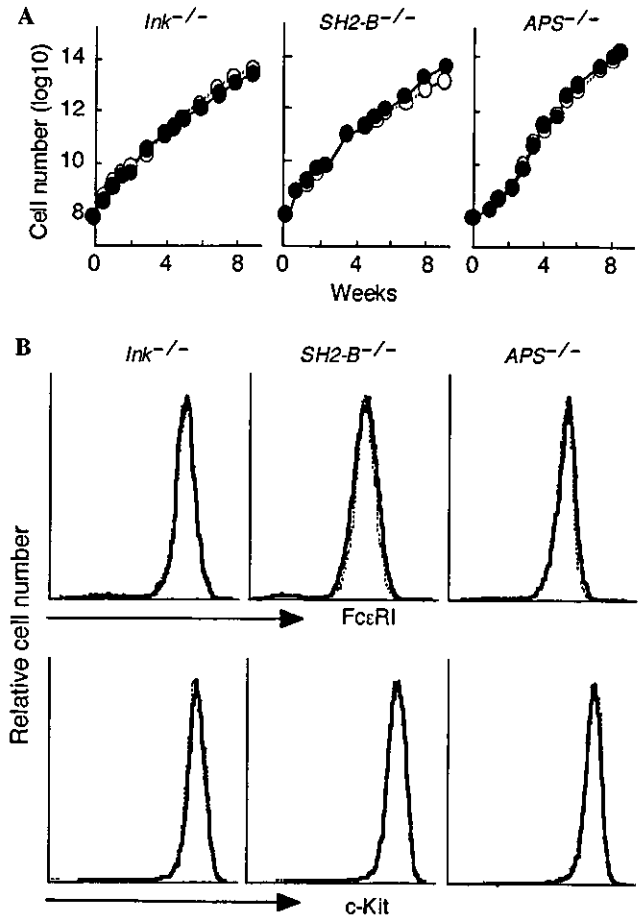

Fig. 1. (A) Cumulative cell numbers of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$ (closed circles), and respective wild-type control (open circles) BMMCs. Differentiation of BMMCs from progenitors and their cell growth induced by IL-3 was comparable in the absence of either Lnk, SH2-B or APS. Representative results obtained from multiple independent pairs of BMMCs are shown. (B) Surface expressions of FcεRI (upper panels) or c-Kit (lower panels) on *lnk*$^{-/-}$, *SH2-B*$^{-/-}$, *APS*$^{-/-}$ (bold lines), and respective wild-type control (dotted lines) BMMCs. After IgE sensitization, BMMCs were stained with anti-c-Kit or anti-IgE antibodies and analyzed by flow cytometry. Representative results of multiple independent experiments are shown.

we tried to evaluate various cellular events induced by cross-linking FcεRI. We first cytochemically evaluated the proportion of degranulated BMMCs after stimulation. Percentage of degranulated cells increased in a dose-dependent manner as the concentration of antigens increased. Importantly, the ratio of degranulated BMMCs in each stimulation condition was comparable between *APS*$^{-/-}$ and wild-type BMMCs (Fig. 3A). The enhanced degranulation from *APS*$^{-/-}$ BMMCs was thus due to augmented degranulation from each mast cell but not to increased proportion of cells that underwent degranulation. We then analyzed calcium influx induced by cross-linking FcεRI, however, we did not observe significant difference in initial peak and following sustained increase of intracellular free calcium between *APS*$^{-/-}$ and control BMMCs (Fig. 3B). Cell survival

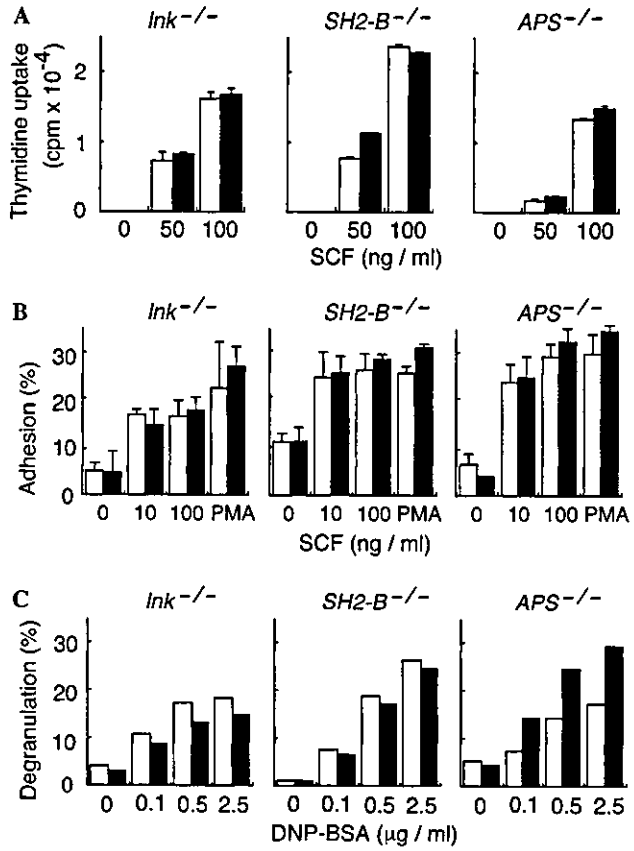

Fig. 2. Responses of *lnk*$^{-/-}$, *SH2-B*$^{-/-}$ or *APS*$^{-/-}$ BMMCs (filled bars) and of respective control BMMCs (open bars) induced by activation of c-Kit or FcεRI. (A) Proliferation upon stimulation with various concentrations of SCF. Values shown are the mean cpm ± SD of triplicate determinations. (B) Adhesion to fibronectin induced by various concentrations of SCF or 10 ng/ml PMA. Shown are average ± SD of triplicate measurements. (C) Degranulation after cross-linking FcεRI. Cells sensitized with anti-DNP IgE mAb were stimulated with the various concentrations of DNP-BSA. Shown is the percentage of β-hexosaminidase activity released into culture supernatants out of the total β-hexosaminidase initially stored in cells. *APS*$^{-/-}$ BMMCs showed augmented degranulation responses (see also Table 1). Representative results of three independent experiments are shown from (A) through (C).

mediated by binding of monomeric IgE to FcεRI was also comparable (Fig. 3C). Tyrosine phosphorylation of various cellular proteins was rapidly induced after cross-linking FcεRI in mast cells and was comparable between *APS*$^{-/-}$ and wild-type BMMCs. Phosphorylation of neither Akt nor PKCδ molecules was affected in the absence of APS (data not shown).

Decreased actin assembly in APS$^{-/-}$ *BMMCs*

It has been shown that Lnk associates with an actin binding protein ABP-280 [19] and that SH2-B plays a role in actin reorganization and cell motility mediated by growth hormone receptor [20,21]. We recently found that Lnk facilitates actin reorganization in transfected fibroblast cells (S.M.K. and S.T., unpublished data). In addition, a negative correlation between actin polymerization and FcεRI-mediated degranulation from RBL-2H3 mast cell line has been presented [22,23].

We speculated APS may regulate actin cytoskeleton, which potentially has regulatory process for degranulation in mast cells. Therefore, we investigated consequences of inhibition of actin polymerization induced by cross-linking FcεRI in BMMCs and its effect on degranulation by treatment with latrunculin. Treatment of sensitized BMMCs with latrunculin resulted in the reduction of F-actin contents as demonstrated by rhodamine–phalloidine binding (Fig. 4A, left panel). Cross-linking FcεRI induced reduction of F-actin contents in stimulated BMMCs. Consistent with observations using RBL-2H3 cells, inhibition of actin assembly by treatment with latrunculin enhanced degranulation from normal BMMCs in a dose-dependent manner (Fig. 4A, right). Interestingly, sensitized *APS*$^{-/-}$ BMMCs showed reduced F-actin content (about 70% of control) compared to wild-type cells (Fig. 4B, left). The reduction in F-actin contents became less evident in cells treated with latrunculin. Finally, the effect of latrunculin on degranulation was compared between *APS*$^{-/-}$ and control BMMCs. As shown in Fig. 4B, augmented degranulation by *APS*$^{-/-}$ BMMCs became less evident by treatment with latrunculin, which was well correlated with difference in F-actin contents between latrunculin treated *APS*$^{-/-}$ and control cells. These results suggested that *APS*-deficiency in mast cells made actin assembly at relatively low levels and that resulted in facilitated degranulation process after cross-linking FcεRI.

Table 1
Enhancement of FcεRI-induced degranulation in *APS*$^{-/-}$ BMMC

DNP-BSA(μg/ml)	Degranulation (% maximal response induced by PMA plus ionomycin)			
	0	0.1	0.5	2.5
+/+ ($n = 11$)	5.6 ± 0.9	19.4 ± 2.4	28.9 ± 2.5	29.4 ± 2.1
−/− ($n = 11$)	5.0 ± 0.7	25.8 ± 3.9	39.5 ± 3.9*	40.6 ± 3.4**
(% +/+ response)	(89%)	(133%)	(136%)	(138%)

Sensitized BMMCs were stimulated with the various concentrations of DNP-BSA or 10 ng/ml PMA plus 400 ng/ml ionomycin. Values represent the mean ± SE of % β-hexosaminidase activity normalized by the value induced with PMA plus ionomycin as 100%. *$p < 0.05$, **$p < 0.01$ compared to +/+ BMMCs by Student's t test.

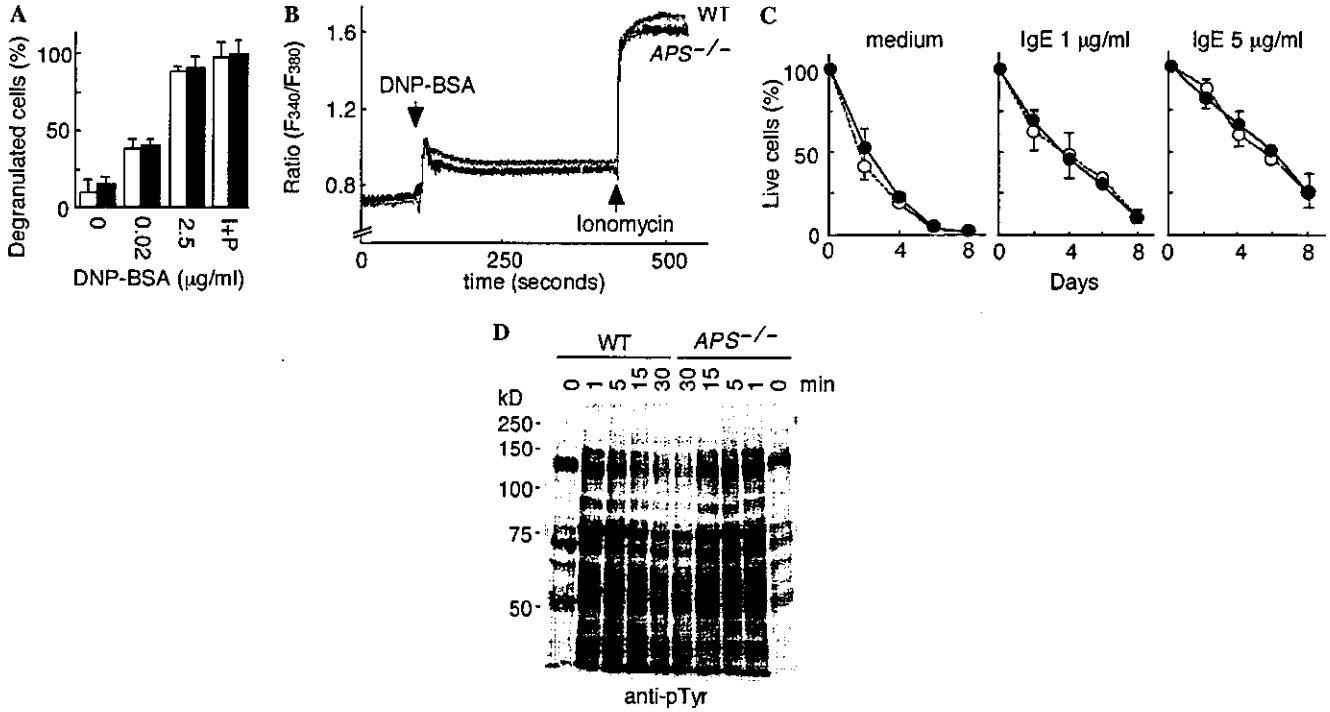

Fig. 3. Cellular responses of *APS*$^{-/-}$ BMMCs mediated through cross-linking FcεRI. (A) Proportion of degranulated cells after cross-linking FcεRI with various concentrations of antigens was determined by cytochemistry. Percentages of degranulated cells were comparable between *APS*$^{-/-}$ (closed bars) and wild-type control mice (open bars). The average ± SD of three independent experiments are shown. (B) Calcium influx induced upon cross-linking FcεRI in *APS*$^{-/-}$ (lower line) and wild-type (upper line) BMMCs. After IgE sensitization, BMMCs were loaded with Fura PE3 and stimulated with 5 μg/ml DNP-BSA and 10 μg/ml ionomycin at the indicated time points (arrows), and fluorescence intensity ratio at 340–380 nm was measured. Representative results of two independent experiments are shown. (C) Survival of *APS*$^{-/-}$ (closed circles) and wild-type (open circles) BMMCs by binding of monomeric IgE to FcεRI. Cells were cultivated in the absence or in the presence of various concentrations of monomeric IgE and percentages of live cells were measured. The average ± SD of three independent experiments are shown. (D) Tyrosine phosphorylation of total cellular proteins after cross-linking FcεRI. Sensitized BMMCs were stimulated with 2.5 μg/ml DNP-BSA for the indicated times. Total cell lysates were separated through SDS–PAGE and subjected to immunoblot using anti-phosphotyrosine mAb (4G10). Representative results of three experiments are shown.

Discussion

We investigated functions of Lnk, SH2-B or APS in mast cells, since possible regulatory roles of Lnk-family adaptor proteins in signaling through c-Kit or FcεRI had been suggested. We established BMMCs lacking either Lnk, SH2-B or APS and examined their cellular responses. None of those mutant BMMCs showed altered responses against IL-3 or SCF, the c-Kit ligand. *APS*-deficiency resulted in enhanced FcεRI-mediated degranulation, while both *lnk*$^{-/-}$ and *SH2-B*$^{-/-}$ BMMCs did not show any abnormal responses induced by cross-linking FcεRI.

We have shown that Lnk negatively regulates c-Kit signaling in B cell precursors and hematopoietic progenitor cells [8,10]. We did not observe significant enhancement in SCF-dependent growth of *lnk*$^{-/-}$ BMMCs in contrast to a previous report by Velazquez et al. [11]. SCF-dependent adherence was also comparable to normal cells. Expression levels of *lnk* transcripts are rather low in BMMCs compared to B-lineage cells or hematopoietic progenitor cells (unpublished data). It is likely that *lnk*-deficiency alone hardly affects mast cell function because of low expression of Lnk in mast cells.

APS had been cloned as a possible candidate substrate for the c-Kit [7]. However, *APS*$^{-/-}$ BMMCs did not show any altered responses upon stimulation with SCF. Instead, they showed enhanced FcεRI-mediated degranulation. *APS*$^{-/-}$ BMMCs showed reduced actin assembly at steady state compared to normal BMMCs. Inhibition of actin assembly in normal BMMCs by latrunculin resulted in enhanced degranulation similar to *APS*$^{-/-}$ BMMCs. In *APS*$^{-/-}$ mice, B-1 cells in peritoneal cavity increased and showed reduced F-actin contents. Conversely, in transgenic mice overexpressing APS in lymphocytes, B cells were reduced and showed enhanced actin assembly [17]. These results suggest that APS may negatively regulate degranulation process by controlling actin dynamics in mast cells. In RBL-2H3 mast cells, F-actin assembly induced by cross-linking FcεRI negatively controls degranulation as well as calcium signaling [22,23]. Oka et al. [24] recently reported that monomeric IgE binding induced actin assembly and that inhibition

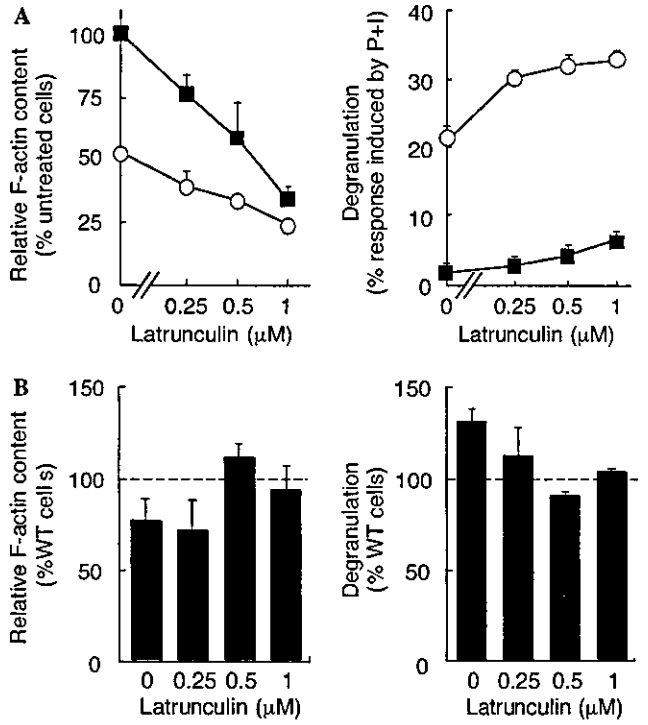

Fig. 4. Enhanced degranulation correlated with reduced F-actin contents in BMMCs treated with inhibitor of actin assembly, latrunculin or by APS-deficiency. (A) Treatment with latrunculin inhibited actin assembly and resulted in reduced F-actin content in BMMCs. Sensitized wild-type BMMCs were incubated with the various concentrations of latrunculin, kept unstimulated (squares) or stimulated with 2.5 μg/ml DNP-BSA (circles). F-actin contents of cells were then analyzed by rhodamine–phalloidin staining and flow cytometry, and the results are shown as relative F-actin contents compared with that of unstimulated cells in the absence of latrunculin (left). Degranulation was determined by measuring β-hexosaminidase activity released into culture supernatants, and results were shown as percent maximal responses induced by PMA and ionomycin treatment (right). (B) F-actin content of *APS*$^{-/-}$ BMMCs in the absence or the presence of various concentrations of latrunculin was measured and relative F-actin contents compared with those of control cells treated with the same concentrations of latrunculin were shown (left). Degranulation from *APS*$^{-/-}$ BMMCs treated with latrunculin was measured, and shown as percent reaction compared with those from wild-type control cells in the same conditions (right). Results shown are means ± SE of values obtained from three independent experiments.

of IgE-induced actin assembly by cytochalasin D initiates calcium influx and degranulation. Although enhancement of calcium influx in *APS*$^{-/-}$ BMMCs was not observed, reduction of actin assembly in *APS*$^{-/-}$ BMMCs may lead to augmented degranulation in analogy with those observed in RBL-2H3 mast cells. The molecular mechanisms for APS-mediated actin assembly as well as APS function downstream of cross-linking FcεRI remain to be elucidated.

APS function in insulin-R signaling has been also indicated in various experiments using cell lines [15,16,25–27]. *APS*$^{-/-}$ mice exhibited increased sensitivity to insulin and enhanced glucose tolerance [28]. It is intriguing to examine whether effect of *APS*-deficiency on insulin sensitivity is also mediated by actin dynamics. Regulation of actin cytoskeleton seems one of the common functions of Lnk-family adaptor proteins. Lnk associates with an actin binding protein ABP-280 [19] and facilitates actin assembly in overexpressed fibroblasts by activating Vav and Rac (S.M.K. and S.T., unpublished data). SH2-B is required for actin reorganization and regulates cell motility induced by GH-R activation [20,21].

SH2-B has been identified as a possible adaptor binding to ITAMs of FcεRI γ chain [6]. However, all examined responses induced by FcεRI ligation were normal with *SH2-B*$^{-/-}$ *BMMCs*. It seems *SH2-B*-deficiency do not affect mast cell function. However, it should be notified that interaction of SH2 domains of Lnk-family proteins with c-Kit or ITAM of FcεRI γ chain had been demonstrated in overexpression systems with different combinations, for example, SH2-B with FcεRI γ chain, APS with c-Kit. *SH2-B*$^{-/-}$ mice showed mild growth retardation and infertility due to impaired maturation of gonad organs [12]. Thus, SH2-B seemed to have a true target except FcεRI, worked as a positive regulator of signal transduction in contrast to Lnk and APS that function as negative regulators as shown in previous studies and in this study. Despite the significant structural similarities between APS, Lnk, and SH2-B, their functions appear to be quite different from each other. However, possible common functions of those adaptor proteins in vivo should be examined by generating mutant mice lacking APS, Lnk or SH2-B in various combinations.

In conclusion, our studies describe roles of Lnk family adaptor proteins on BMMCs. Both Lnk and SH2-B were dispensable for various mast cell responses mediated through c-Kit, FcεRI as well as IL-3-R. APS plays a role in controlling FcεRI-induced degranulation response but not in c-Kit-mediated proliferation or adhesion. APS may regulate degranulation by controlling actin dynamics in mast cells.

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The Role of IL-5 for Mature B-1 Cells in Homeostatic Proliferation, Cell Survival, and Ig Production[1]

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B-1 cells, distinguishable from conventional B-2 cells by their cell surface marker, anatomical location, and self-replenishing activity, play an important role in innate immune responses. B-1 cells constitutively express the IL-5R α-chain (IL-5Rα) and give rise to Ab-producing cells in response to various stimuli, including IL-5 and LPS. Here we report that the IL-5/IL-5R system plays an important role in maintaining the number and the cell size as well as the functions of mature B-1 cells. The administration of anti-IL-5 mAb into wild-type mice, T cell-depleted mice, or mast cell-depleted mice resulted in reduction in the total number and cell size of B-1 cells to an extent similar to that of IL-5Rα-deficient (IL-5Rα$^{-/-}$) mice. Cell transfer experiments have demonstrated that B-1 cell survival in wild-type mice and homeostatic proliferation in recombination-activating gene 2-deficient mice are impaired in the absence of IL-5Rα. IL-5 stimulation of wild-type B-1 cells, but not IL-5Rα$^{-/-}$ B-1 cells, enhances CD40 expression and augments IgM and IgG production after stimulation with anti-CD40 mAb. Enhanced IgA production in feces induced by the oral administration of LPS was not observed in IL-5Rα$^{-/-}$ mice. Our results illuminate the role of IL-5 in the homeostatic proliferation and survival of mature B-1 cells and in IgA production in the mucosal tissues. *The Journal of Immunology,* **2004, 172: 6020–6029.**

B-1 cells differ from conventional B-2 cells in their surface phenotype, anatomical localization, self-replenishing activity, and V_H usage of IgM (1, 2). B-1 cells constitutively express three different markers, namely Mac-1 (CD11b/CD18), FcεR (CD23), and the IL-5R α-chain (IL-5Rα).[3] Mac-1 is present in peritoneal and pleural cavity B-1 cells but is not expressed on B-2 cells, whereas FcεR is preferentially expressed on B-2 cells in the peritoneal cavity and in the spleen (3, 4). IL-5Rα is constitutively expressed on all B-1 cells, but is expressed on a small proportion (2–4%) of resting B-2 cells in the spleen (5).

The progenitors of B-1 cells are abundant in the fetal omentum and liver but are missing in the bone morrow of adult animals (6, 7). In contrast with B-2 cells, which are supplied from progenitors in the bone marrow throughout life, B-1 cells maintain their number in adult animals by their self-replenishing capacity (3, 7). In the adult, these self-replenishing B-1 cells are clearly enriched in the peritoneal and pleural cavities, and a low frequency is seen in the spleen, but B-1 cells are virtually absent from the lymph nodes, Peyer's patches (PP), and peripheral blood, where most conventional B-2 cells are localized (3). B-1 cells are categorized into B-1a cells that express CD5 and B-1b cells that express cell surface markers similar to those of B-1a cells, except for the low expression, if any, of CD5 (2, 3).

B-1 cells are believed to be the primary source of natural IgM Ab, although they can become Ig-producing cells for all isotypes. Consistent with a major role of B-1 cells in natural IgM production, a number of specificities of natural IgM Ab have been identified in the B-1 repertoire. These include specificities for LPS, phosphorylcholine, undefined determinants on *Escherichia coli* and *Salmonella* spp., phosphatidylcholine, and complement-binding Abs (3). Furthermore, B-1 cells in the peritoneal cavity serve as an important source of IgA-producing plasma cells at mucosal sites. These findings are largely supported by transfer experiments of peritoneal B-1 cells in irradiated mice or otherwise B cell-depleted mice and by analysis of genetically altered immune-deficient mice (8). The role of B-1 cells in IgA production in the gut is further supported by evidence that mice with a selective B-1 cell reduction in number showed decreased frequencies of IgA-producing cells in the lamina propria (LP) (9). The helper T cell dependency of B-1 cells on gut-associated IgA production is still controversial (9, 10).

Studies of gene-targeted and transgenic mice have revealed that B cell receptor (BCR) signaling is critical for B-1 cell development or maintenance. Mutant mice that lack Bruton's tyrosine kinase protein kinase C β, CD19, the p85α subunit of phosphatidylinositol-3 kinase, $p95^{vav}$, CD21/CD35, and CD81, which are strongly associated with BCR signaling, have substantial depletion of B-1 cells but largely spare B-2 cells (11–18). Conversely, mutation or overexpression of Src homology protein-1, CD22, or CD72 that induces enhanced BCR signaling results in an expanded B-1 cell compartment (19–21).

IL-5, mainly produced by activated Th2 cells and mast cells, acts on B-1 and B-2 cells to induce proliferation and differentiation into Ig-producing cells (22–25). IL-5 also controls the production and functions of eosinophils and basophils. The IL-5R consists of two distinct membrane proteins, IL-5Rα and βc, each of which is a member of the cytokine receptor superfamily (5). The binding of IL-5 occurs through the IL-5Rα, and the βc forms a high-affinity IL-5R in combination with the IL-5Rα, transducing signals into nuclei. Although the molecular mechanisms for IL-5 signal

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[3] Abbreviations used in this paper: IL-5Rα, IL-5R α-chain; PP, Peyer's patch; LP, lamina propria; BCR, B cell receptor; RAG, recombination-activating gene; PEC, peritoneal exudate cell; MLN, mesenteric lymph node; s, surface; TLR, Toll-like receptor 4; HPRT, hypoxanthine phosphoribosyltransferase; m, murine; CD40L, CD40 ligand.

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