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

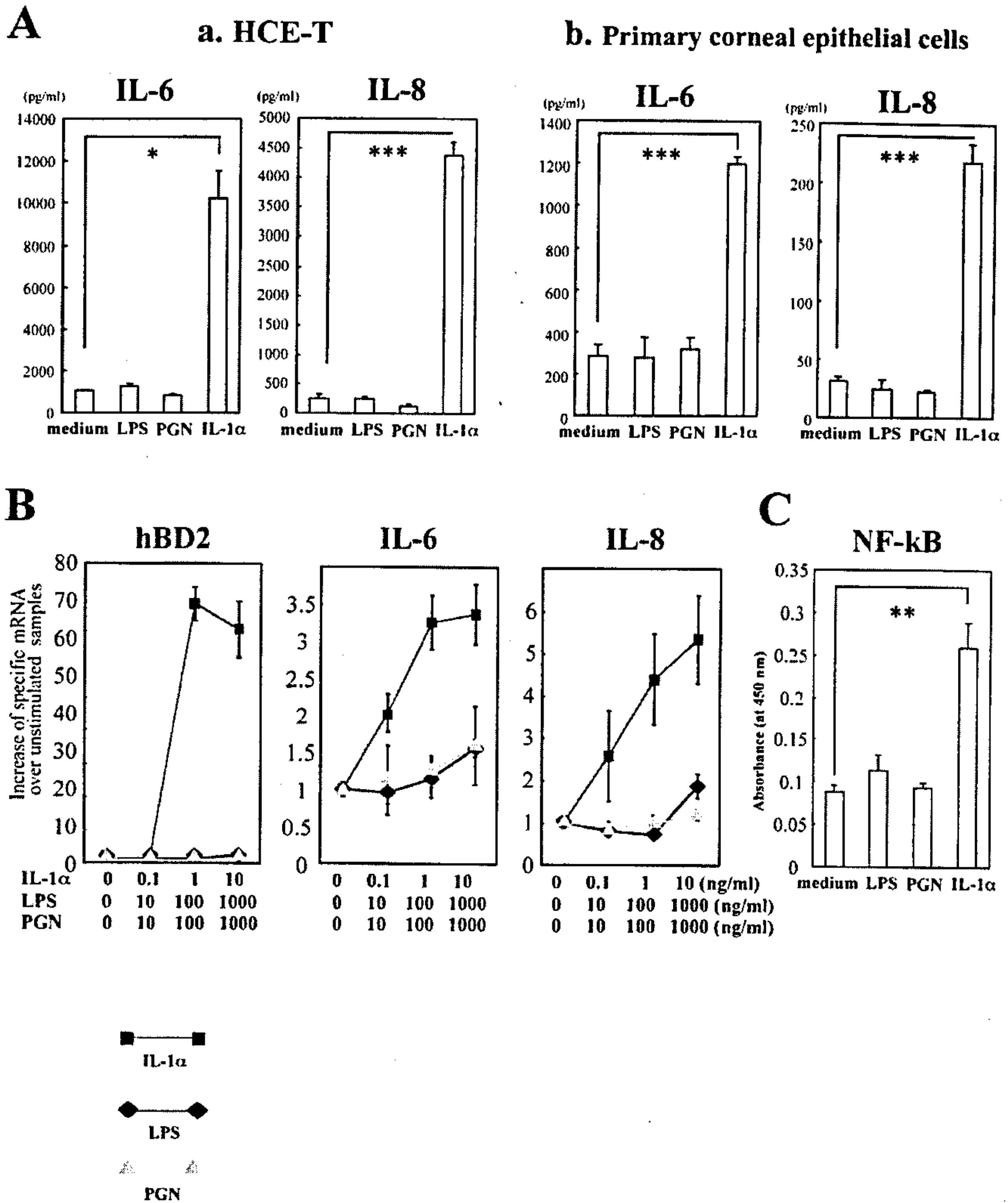


FIGURE 2. Human corneal epithelial cells fail to respond to LPS or PGN. To quantify inflammatory cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS, 1000 ng/ml PGN,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

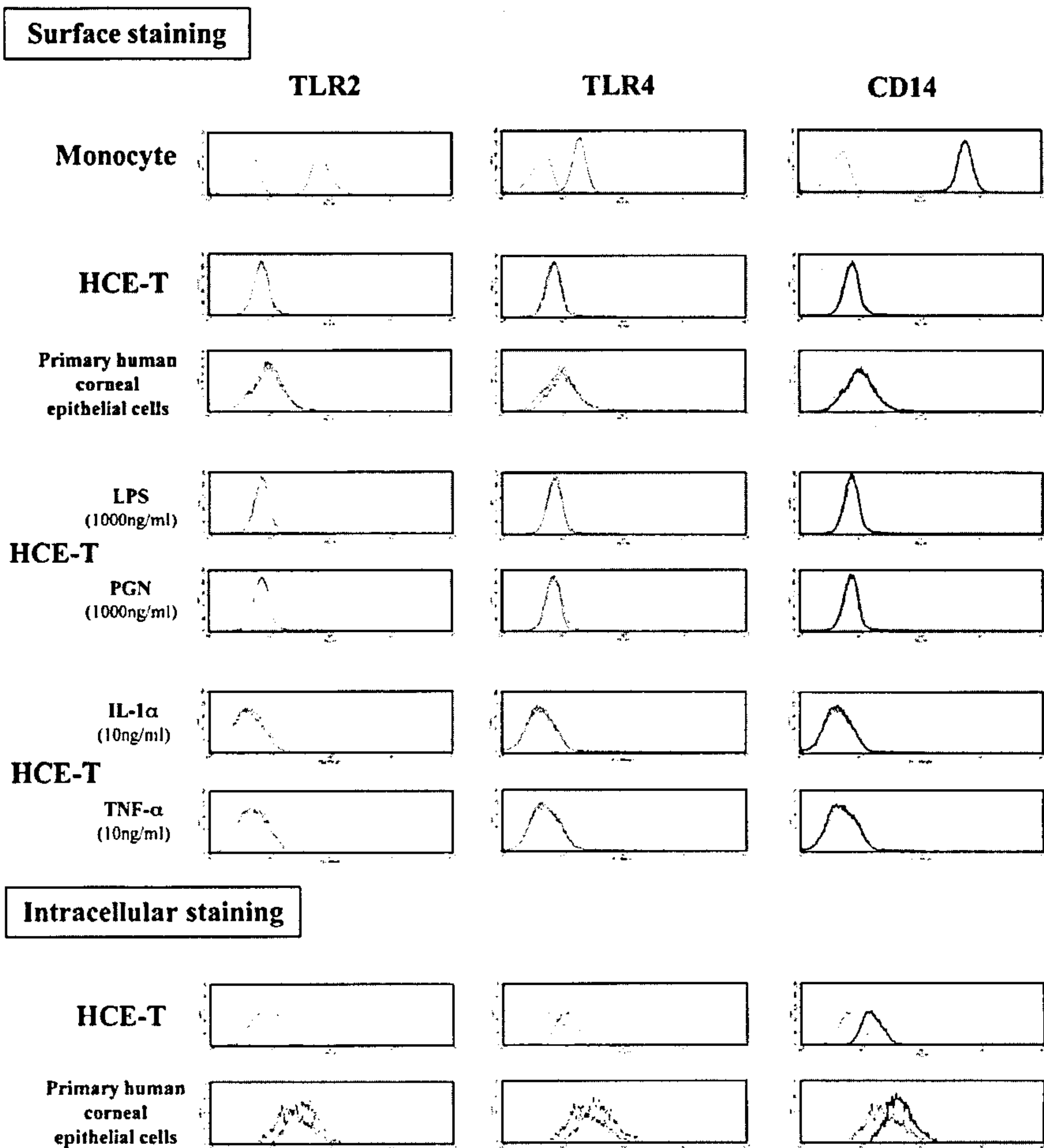


FIGURE 3. TLR2 and TLR4 are expressed intracellularly, but not on the cell surface of human corneal epithelial cells. Cell surface expressions of TLR2, TLR4, and CD14 in HCE-T and primary human corneal epithelial cells were examined by FACS. These cells were incubated with PE-conjugated mouse anti-human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

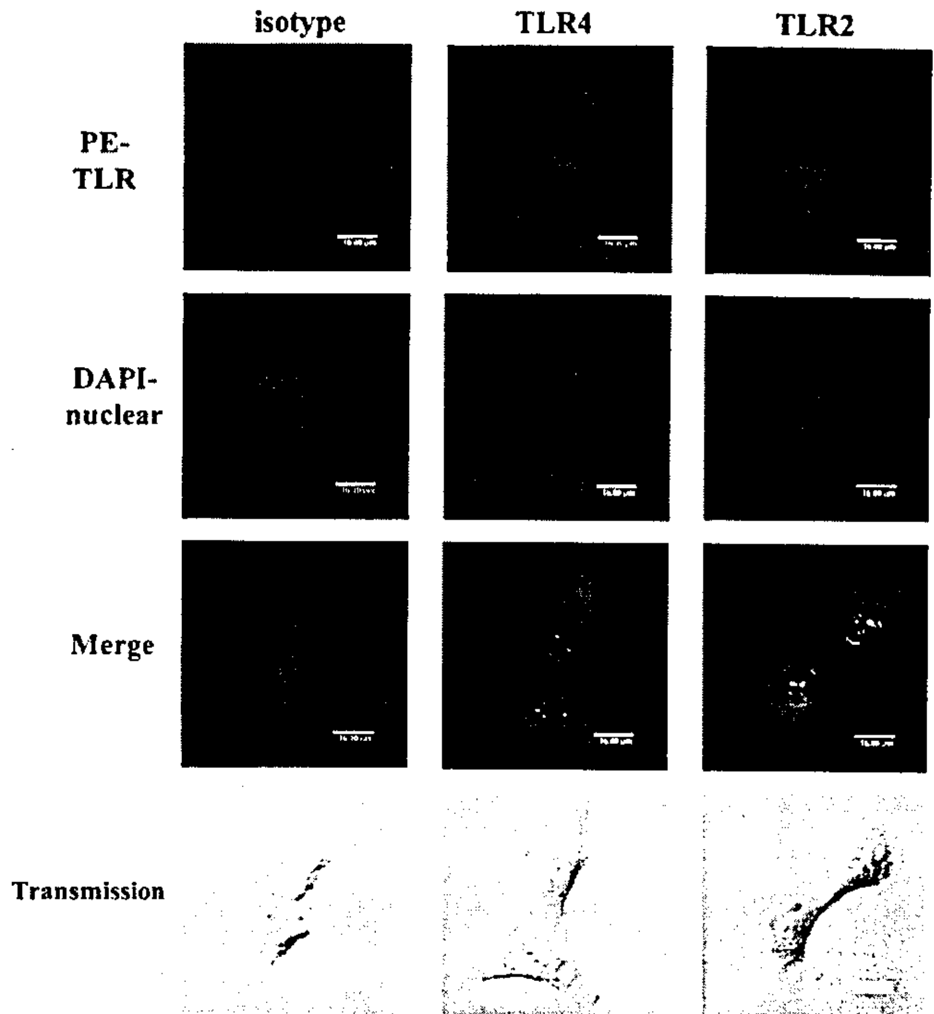


FIGURE 4. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in the human corneal epithelial cell line HCE-T. HCE-T was cultured on a slide chamber, washed with PBS⁻, and air-dried. Slides were fixed with methanol for 30 min, then stained with PE-conjugated mouse anti-human TLR2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

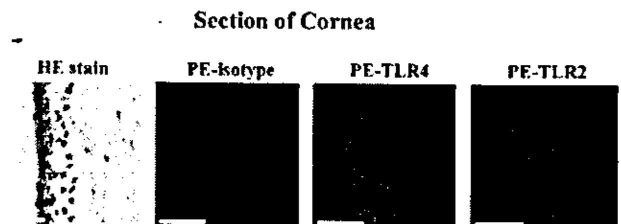
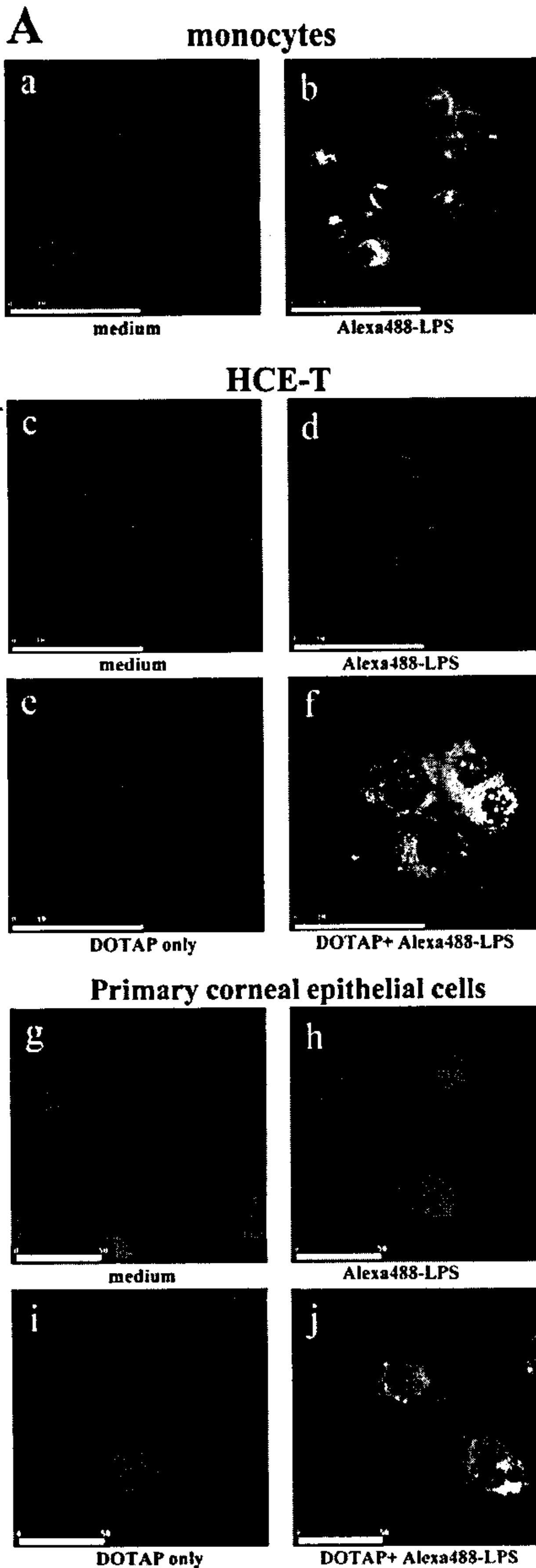


FIGURE 5. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelium. Slides of tissue sections were fixed with methanol for 30 min and then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs or isotype control mouse IgG2a for 24 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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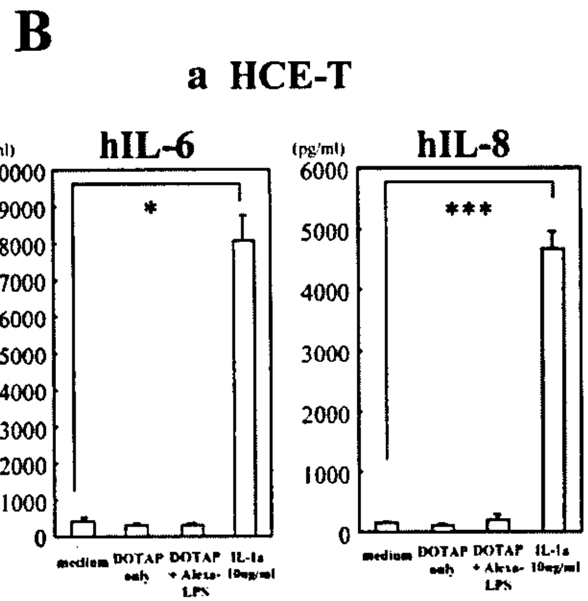
Primary corneal epithelial cells
g
h
medium
Alexa488-LPS
i
j
DOTAP only
DOTAP+ Alexa488-LPS


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
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3000
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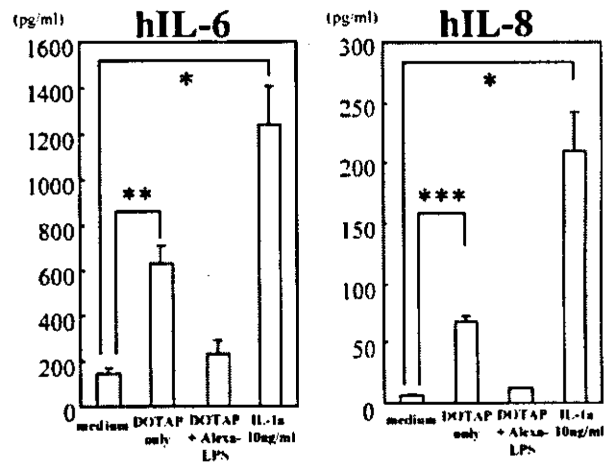
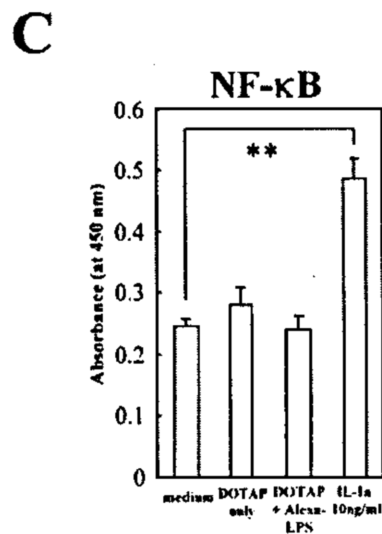
***
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml


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.

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IL-4-Induced GATA-3 Expression Is a Time-Restricted Instruction Switch for Th2 Cell Differentiation[1]

Noriyasu Seki,*† Mayumi Miyazaki,* Wataru Suzuki,* Katsuhiko Hayashi,‡ Kazuhiko Arima,§ Elmarie Myburgh,‖ Kenji Izuhara,§¶ Frank Brombacher,‖ and Masato Kubo[2]*#

An initial activation signal via the TCR in a restricted cytokine environment is critical for the onset of Th cell development. Cytokines regulate the expression of key transcriptional factors, T-bet and GATA-3, which instruct the direction of Th1 and Th2 differentiation, through changes in chromatin conformation. In this study, we investigated the kinetics of IL-4-mediated signaling in a transgenic mouse, expressing human IL-4R on a mouse IL-4αR-deficient background. These experiments, allowing induction with human IL-4 at defined times, demonstrated that an IL-4 signal was required at the early stage of TCR-mediated T cell activation for lineage commitment to Th2, along with structural changes in chromatin, which take place in the conserved noncoding sequence-1 and -2 within the IL-4 locus. At later times, however, IL-4 failed to promote efficient Th2 differentiation and decondensation of chromatin, even though GATA-3 was clearly induced in the nuclei by IL-4 stimulation. Moreover, IL-4-mediated Th2 instruction was independent from cell division mediated by initial TCR stimulation. The role of IL-4 signaling may have a time restriction during Th2 differentiation. In late stages of initial T cell activation, the chromatin structure of the IL-4 locus retains condensation state. These results demonstrate that IL-4-induced GATA-3 expression is time-restriction switch for Th2 differentiation. ***The Journal of Immunology,*** **2004, 172: 6158–6166.**

Helper T cells exhibit cytokine expression patterns that divide them into at least two functionally distinct subsets. Th1 cells secrete IL-2, IFN-γ, and TNF-α, which promote cellular immune responses against intracellular pathogens and viruses, mediate delayed-type hypersensitivity responses, and may lead to organ-specific autoimmune diseases. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13, which promote humoral immune responses mainly against extracellular pathogens. IL-4 and IL-5 regulate the immune response via mast cells and eosinophils, especially in atopic and allergic conditions. The development of Th cells is determined by cytokines present at the early stages of T cell activation, upon encounter with Ag on APCs. The most critical role for the instruction of Th1 and Th2 development is played by cytokines such as IL-12 and IL-4, which act through the STAT4 pathway, or through the STAT6 signaling pathway, respectively (1–4).

Differential cytokine production within Th1 and Th2 cells is controlled at the level of gene transcription. During differentiation, IL-4 transcription is amplified by TCR stimulation in the presence of IL-4. TCR stimulation regulates the expression of a *trans* activator of the IL-4 promoter, c-*maf*, and also regulates the activation of NF-AT and AP-1 family members (5–7). IL-4R signaling controls expression of GATA-3, a Th2-specific transcription factor that regulates lineage commitment to Th2 (8, 9). TCR-mediated signaling, together with CD28 stimulation, augments GATA-3 expression through activating NF-κB (10). GATA-3 protein induces further GATA-3 expression in an autocrine manner, leading to massive up-regulation of GATA-3 transcription (4, 11, 12). In the absence of IL-4-mediated STAT6 activation, ectopic overexpression of GATA-3 inhibits IL-12Rβ2 expression (8) and chromatin remodeling at the IL-4 locus, as well as Th2 cytokine gene expression (12–14). Therefore, in Th2 differentiation, the role of the IL-4 signal is thought to be the initial induction of GATA-3.

Decondensation of chromatin is characterized by hyperacetylation of histones H3 and H4, as well as by increased accessibility to restriction enzymes, DNase I, and transcription factors (15–17). During Th2 differentiation, the chromatin structure in the IL-13/IL-4 locus changes, allowing transcription of the Th2 cytokine genes. In Th cells, TCR stimulation in the presence of IL-4 elicits a cluster of DNase I-hypersensitive sites (18, 19) and histone acetylation (20). Agarwal et al. (21, 22) have found six Th2-specific hypersensitive sites, named HS-I to -V and HS-Va, in the region spanning the IL-4 promoter to the KIF3 locus. Takemoto et al. (23) found additional sites, named HSS1 to HSS3, in the noncoding sequences between the IL-13 and IL-4 genes. The sequences of HSS1–3 and HS-Va are highly conserved between humans and mice, and are designated as conserved noncoding sequence-1 and -2, respectively (24). The deletion of these sequences either in a human YAC transgene or in the endogenous mouse locus reduces secretion of Th2 cytokines during restimulation (24–26). In both Th1 and Th2 lineages, core histone acetylation seems to occur within the first 48 h in the promoter, HS-Va, or conserved

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noncoding sequence-1 and -2. The IL-4 signal then further regulates Th2-specific acetylation, subsequently leading to chromatin remodeling (20, 27). HSS1–3, HS-II, intronic enhancer (IE),[3] and HS-Va have all the consensus sequence for GATA-3 binding. Indeed, in Th2 cells, the HS-Va region is precipitated by Ab against GATA-3 (20, 22). This suggests that GATA-3 binding to conserved noncoding sequence-1 and -2 could regulate Th2-specific histone acetylation and chromatin remodeling. However, the mechanisms of how GATA-3 regulates these structural changes remain unclear.

IL-2 and IFN-γ mRNA are induced within 6 h in G_1 to S phase, while IL-4 mRNA is induced after 48 h at a time point when T cells have undergone more than three cell cycles. This indicates that cell division might be needed to induce Th2 cytokine transcription (28). However, other reports using a cell cycle blocker and IL-4 withdrawal to control early IL-4R signaling showed that IL-4 instructed IL-4 production in the first S phase (29, 30). The coordination of IL-4R and TCR signaling regulates IL-4 gene transcription (31, 32). Nevertheless, the individual roles of IL-4R signaling and TCR signaling remain unclear, because both occur around the same time, and their effects are experimentally difficult to separate.

To overcome this problem, we established a transgenic (Tg) mouse model, expressing human IL-4Rα (hIL-4Rα), under the control of an IE from the Ig H chain Eμ locus, to allow specific expression in lymphocytes only. The chimeric IL-4R molecule, composed of the hIL-4Rα chain and the mouse common γ-chain is responsive to hIL-4, therefore allowing us to control IL-4R signaling independent of endogenous IL-4 in lymphocytes only. In this study, we found that IL-4 signaling regulates the competence of effector cytokine production during a restricted phase of initial T cell activation, irrespective of progressive cell divisions. We discuss the importance of the timing of IL-4-mediated GATA-3 expression on Th2-specific chromatin remodeling and on the Th2 lineage commitment.

Materials and Methods

Mice

Tg constructs for the hIL-4R were expressed under the control of an IE of Ig H chain locus (Eμ) promoter. A Tg line that expressed hIL-4R on T and B cells at similar level to endogenous mouse (m)IL-4R (hIL-4RαTg) was selected for this study. The hIL-4R Tg mice were backcrossed to BALB/c genetic background for more than 10 generations. OVA-specific TCR (DO11.10) Tg mice on BALB/c background were kindly provided by K. Murphy (Washington University, St. Louis, MO). IL-4Rα-deficient (mIL-4Rα knockout (KO)) mice were generated on a BALB/c genetic background (33) and crossed with hIL-4RαTg mice. BALB/c mice were purchased from Sankyo (Tokyo, Japan).

Preparation of Th cell

Single spleen cell suspensions were incubated with anti-CD8 mAb for 30 min on ice, and $CD8^+$ cells and B cells were eliminated with rabbit anti-mouse Ig-coated dishes. The enriched $CD4^+$ T cells were suspended in RPMI 1640 medium containing 10% (v/v) FCS, 10 mM HEPES-KOH, pH 7.4, 2 mM L-glutamine, and 50 μM 2-ME, and were stimulated with a combination of plate-coated anti-TCR mAb (H57-597) and soluble anti-CD28 mAb (PV-1), as previously described (34). For DO11.10 Tg T cells, cells were stimulated with 1 μM $OVA_{323-339}$ and irradiated APCs. After 48 h, 30 U/ml IL-2 was added, and cells were cultured another 5 days. Cells from BALB/c and mIL-4Rα KO mice were stimulated in the presence of anti-IL-4 mAb (11B11). Th2 development in hIL-4Rα Tg was conducted with rhIL-4 (10 U/ml) (PeproTech, London, U.K.). Th1 and Th2 cells were prepared with the induction culture by the addition of either 10 U/ml rIL-12 and anti-IL-4 mAb (11B11) or 100 U/ml rIL-4 and anti-IL-12 mAb (C15.6 and C17.8; The Wister Institute, Philadelphia, PA), respectively.

Intracellular cytokine staining

The activated $CD4^+$ T cells were restimulated with anti-TCR mAb for 6 h in the presence of 2 μM monensin (Sigma-Aldrich, St Louis, MO). The cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. After blocking with 3% BSA-PBS, cells were stained with anti-IFN-γ (XMG1.2) FITC and anti-IL-4 (11B11) PE Abs (34). Flow cytometric analysis was conducted on a FACSort and analyzed by CellQuest software (BD Biosciences, San Diego, CA).

Proliferation assay

$CD4^+$ T cells were stimulated with either mouse or hIL-4 in the presence of PMA for 48 h. The cells were pulsed with [^{3}H]thymidine (0.5 μCi/well) for the last 12 h, and the incorporation of [^{3}H]thymidine was measured by a beta counter.

Analysis of cell division and cytokine production. The enriched $CD4^+$ T cells were labeled with CFSE (Molecular Probes, Eugene, OR) by incubating 1×10^7 cells/ml in PBS with 10 μM CFSE for 8 min at room temperature. The labeling process was stopped with the addition of one aliquot FCS and subsequent three washing steps. Cell divisions were analyzed by FACSort with intracellular cytokine staining after restimulation with anti-TCR mAb, as described above.

Northern blot analysis

Total cytoplasmic RNA was isolated from cells using a TRIzol reagent (Life Technologies, Rockville, MD). Two micrograms of RNA was separated on a 1% (w/v) agarose gel containing 2.2 M formaldehyde. Transfer of RNA onto a Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, NJ), hybridization, and washing were performed according to the procedure supplied by the manufacturer (Roche). The probes used were digoxigenin-labeled antisense riboprobes transcribed from the cDNA template of *IL-12Rβ2*, T-bet, *GATA-3*, and *G3PDH*. Signals were visualized using an alkaline phosphatase-conjugated, anti-digoxigenin Ab (Roche, Mannheim, Germany).

Separation of nuclear and cytoplasmic fraction and Western blot analysis

The nuclear and cytoplasmic fractions were separated by nuclear and cytoplasmic extraction reagent (Pierce, Rockford, IL). The nuclear and cytoplasmic protein (25 μg) were loaded on SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blotted with anti-GATA-3 mAb (HG3-35) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NF-AT1 mAb (4G6-G5) (Santa Cruz Biotechnology), anti-NF-AT2 mAb (7A6) (Alexis, San Diego, CA), and anti-c-*maf* rabbit serum (M-153) (Santa Cruz Biotechnology). The blots were visualized with HRP-conjugated goat anti-mouse or anti-rabbit Ig (DAKO, Glostrup, Denmark).

Restriction enzyme accessibility assay

Enriched $CD4^+$ T cells (5×10^6 cells) were used for each condition. Nuclei were prepared, as described previously (20), and resuspended in buffer F (100 mM NaCl, 50 mM Tris (pH 8.0), 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM 2-ME). *Xho*I and *Hga*II (100 U; Toyobo, Osaka, Japan) were added and incubated at room temperature for 1 h. Purified DNA was digested with either *Eco*RI or *Hin*dIII, ethanol precipitated, and resuspended in TE buffer, and the concentration was measured at absorbance of 260 nm. The digested DNA (5 μg) was transferred onto a Hybond N membrane (Amersham Pharmacia Biotech), and blotted with digoxigenin-labeled appropriated probes.

Retrovirus infection

The protocol for retroviral infection has been previously described in detail (12). Briefly, the murine *GATA-3* cDNA was inserted into pMX-GFP (green fluorescent protein) vector (pMX-GFP-GATA-3). To ensure that GATA-3 and GFP were translated bicistronically, an internal ribosomal entry site was ligated upstream of the GFP. The pMX-GFP-GATA-3 and pMX-GFP control plasmid were transfected into a packaging cell line, PLAT-E, using FuGENE6 (Roche), and, after incubation for 24~48 h, the culture supernatant was harvested and condensed as a viral stock. $CD4^+$-enriched T cells were stimulated with anti-TCR and anti-CD28 mAbs and infected with a viral stock at the indicated time point after primary stimulation. The viral infected $CD4^+$ T cells were restimulated with anti-TCR mAb for 6 h in the presence of 2 μM monensin, and subsequent intracellular staining was conducted.

[3] Abbreviations used in this paper: IE, intronic enhancer; GFI, growth-factor independent; GFP, green fluorescent protein; hIL, human IL; KO, knockout; mIL, mouse IL; Tg, transgenic.

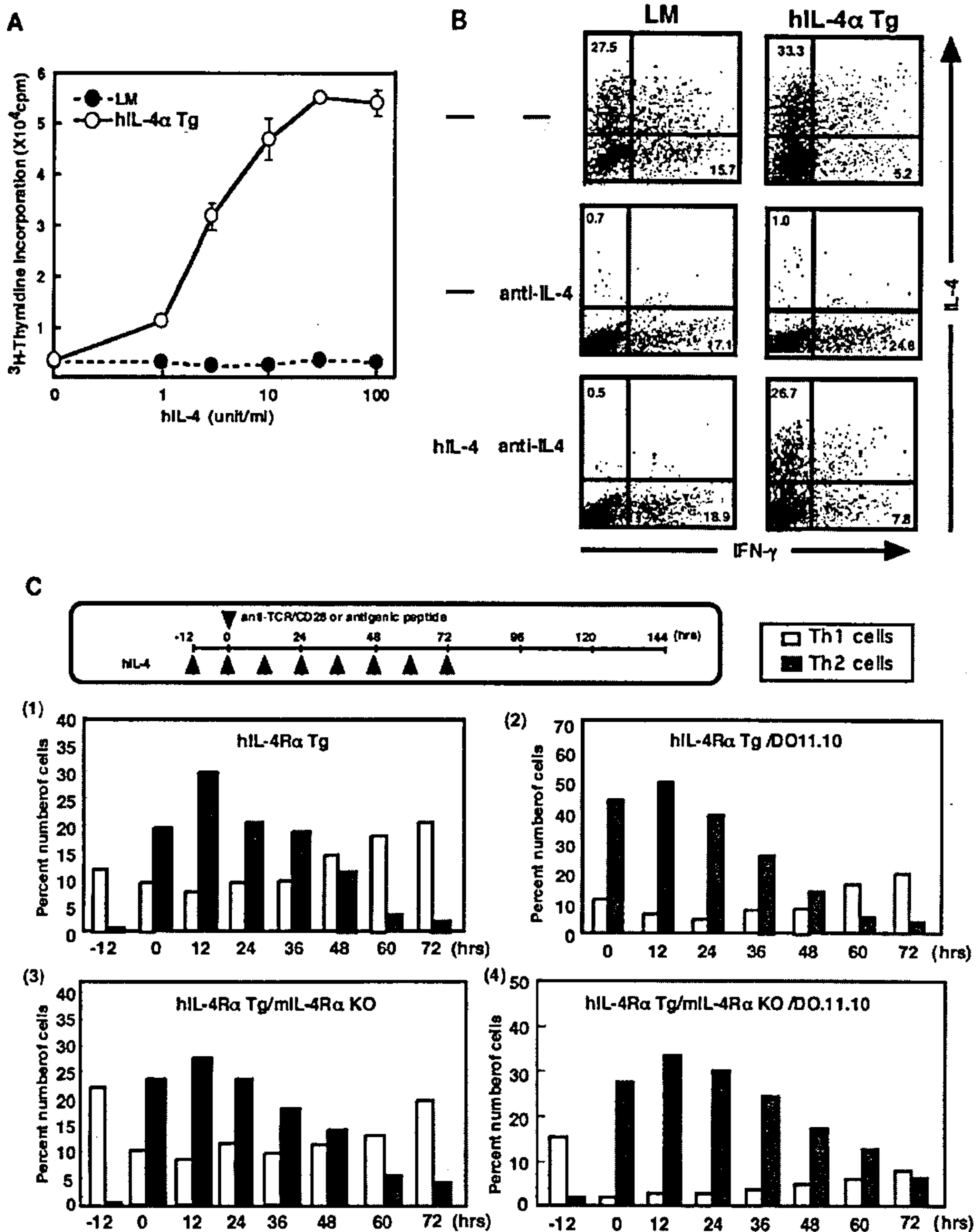
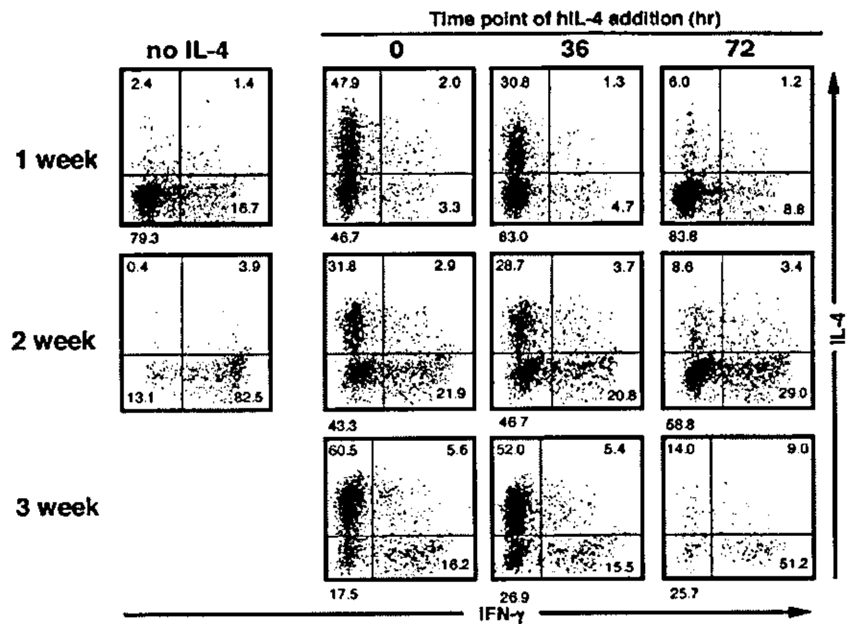


FIGURE 1. *A*, Enriched CD4$^+$ T cells, obtained either from control BALB/c littermates (●) or Tg animals containing hIL-4R (hIL-4R Tg) (○), were stimulated with hIL-4 in the presence of PMA (50 ng/ml) and anti-mIL-4 mAb for 48 h. The proliferative response was measured by the incorporation of [^{3}H]thymidine. *B*, Th cell differentiation was induced with a combination of anti-TCR and anti-CD28 mAbs in the presence or absence of anti-mIL-4 mAb and/or hIL-4. After 7 days, cells were restimulated with anti-TCR mAb and stained for intracellular cytokine. *C*, CD4$^+$ T cells from hIL-4Rα Tg (1) and hIL-4Rα Tg/mIL-4R KO (3) were stimulated with the combination of anti-TCR and anti-CD28 mAbs at time point 0. For CD4$^+$ T cells from hIL-4Rα Tg/DO11.10Tg mice (2) and hIL-4Rα Tg/mIL-4Rα KO/DO11.10 mice (4), cells were stimulated with OVA peptide in context of APCs at time point 0. For hIL-4Rα Tg (1) and DO11.10/hIL-4Rα Tg (2), endogenous IL-4 was neutralized with anti-mIL-4 mAb. hIL-4 (10 U/ml) was added at different time points before or after initial T cell activation (−12 and 0–72 h). For time point −12 h, after 7 days from time point 0, cells were restimulated with anti-TCR mAb, and intracellular cytokine staining was conducted. The numbers represent the percentage of either IL-4- or IFN-γ-producing cells. The experiment was independently performed three times with similar results.

FIGURE 2. $CD4^+$ T cells from hIL-4R Tg were stimulated by anti-TCR and anti-CD28 mAbs in the presence of anti-mIL-4 mAb. hIL-4 was added 0, 36, and 72 h after initial activation. These precultured cells were repeatedly stimulated with anti-TCR and anti-CD28 mAbs in the presence of hIL-4 at weekly intervals. Cells were restimulated with anti-TCR mAb, and the cytokine production profile was assessed by intracellular cytokine staining. The experiment was independently performed three times with similar results.



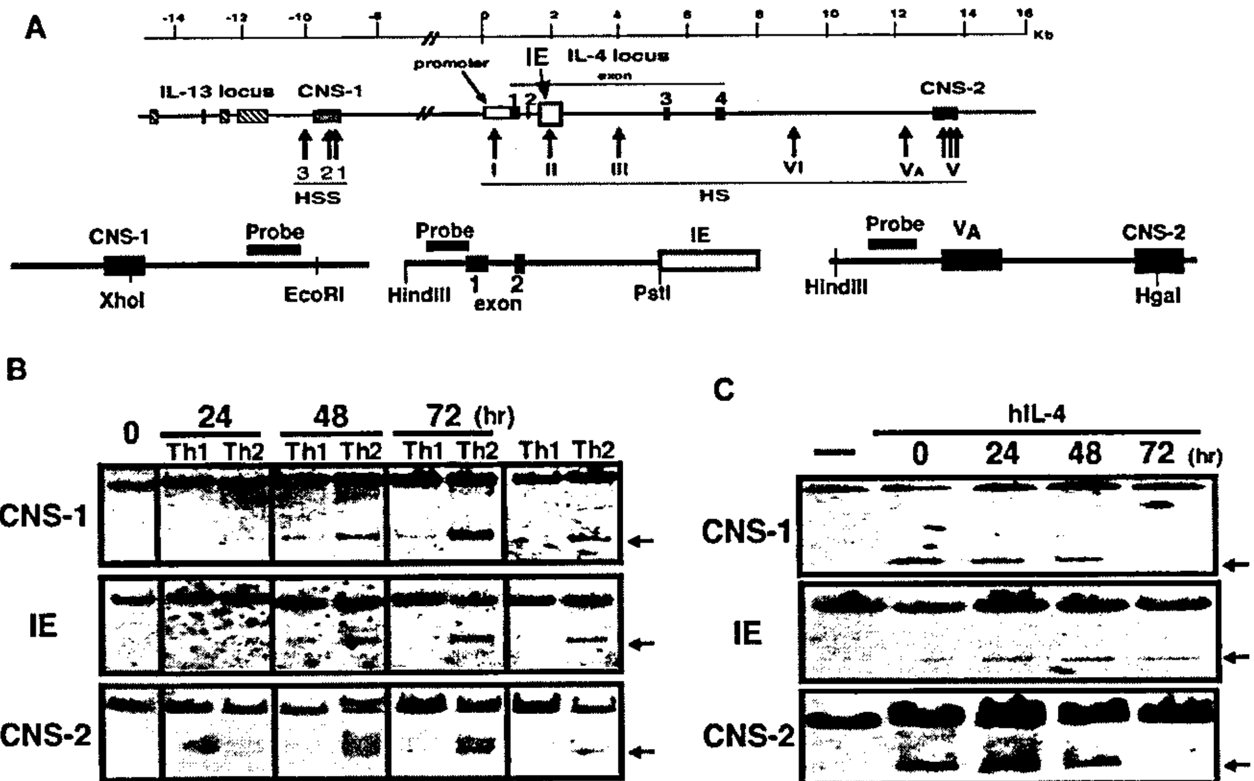


FIGURE 3. *A*, Position of conserved noncoding sequence-1, IE, and conserved noncoding sequence-2 region on the IL-4 locus (*upper panel*). Restriction enzyme site and probe for the detection of conserved noncoding sequence-1, IE, and conserved noncoding sequence-2 (*lower left*). HSS1~3 and HS I~V indicate DNase I-hypersensitive site. *B*, Nuclei were prepared from naive $CD4^+$ T, Th1, and Th2 cells (5×10^6 cells) and digested with *XhoI* for conserved noncoding sequence-1, *PstI* for IE, or *HgaI* for conserved noncoding sequence-2. DNA was purified and digested with either *EcoRI* for conserved noncoding sequence-1 or *HindIII* for IE and conserved noncoding sequence-2 and analyzed by Southern blotting, using probes for conserved noncoding sequence-1 or conserved noncoding sequence-2. The arrow indicates *XhoI*, *PstI*, and *HgaI* sites on conserved noncoding sequence-1, IE, and conserved noncoding sequence-2. $CD4^+$ T cells from DO11.10 Tg mice were stimulated with OVA peptide and APCs in Th1- and Th2-skewing condition. Nuclei were prepared at different time points after initial T cell activation and the restriction enzyme assay was conducted, as described in *A*. *C*, $CD4^+$ T cells from hIL-4R/mIL-4R KO/DO11.10 mice were stimulated with OVA peptide in the context of APCs. hIL-4 was added at different time points, and cells were further cultured until 7 days after the initial activation. Nuclei were prepared from the precultured cells, and the restriction enzyme assay was conducted. The experiment was independently performed three times with similar results.

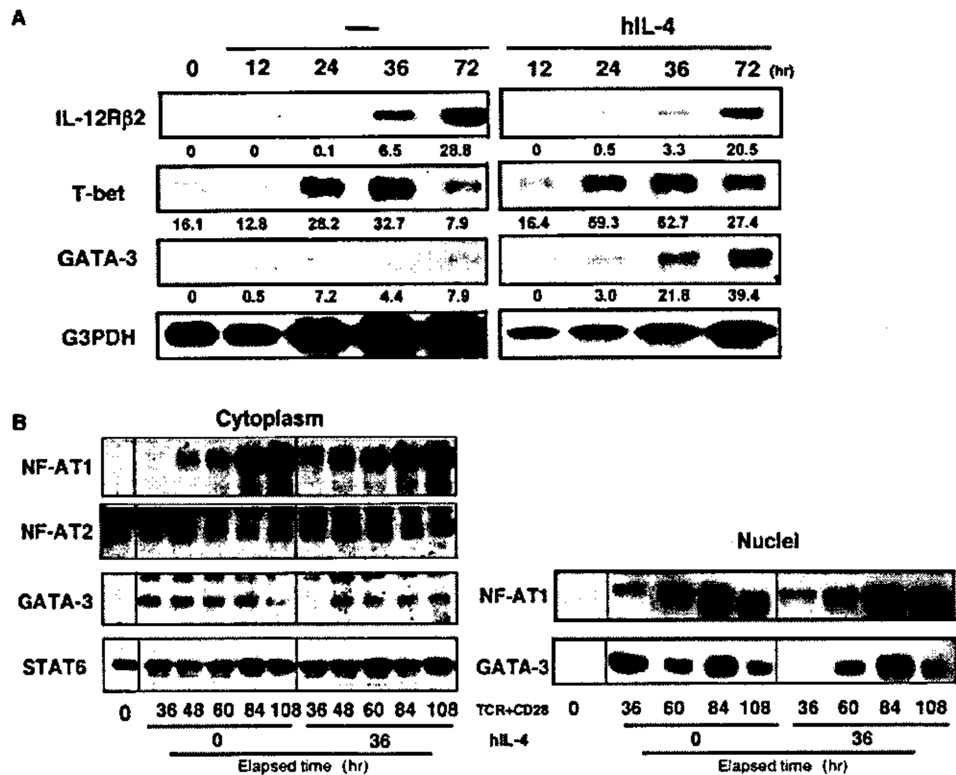


FIGURE 4. *A*, Enriched hIL-4R Tg/IL-4KO CD4$^+$ T cells were stimulated with anti-TCR and anti-CD28 mAbs in the presence or absence of hIL-4. Cells were harvested at the indicated time points after the initial activation, and the expression of IL-12β2, T-bet, GATA-3, and G3PDH mRNAs was measured by Northern blotting analysis. The number indicates the analyzed time points. *B*, Enriched hIL-4R Tg cells were stimulated with anti-TCR and anti-CD28 mAbs in the presence of anti-mIL-4 mAb. hIL-4 was added at time 0 or 36 h after initial activation, and protein expressions of NF-AT1, NF-AT2, GATA-3, and STAT6 in cytoplasmic (*left*) or nuclear (*right*) fraction were examined by Western blotting. The levels of the transcript were quantitated with densitometry and normalized with G3PDH.

Results

hIL-4R substitutes for the function of the mIL-4R

We established a series of Tg mouse lines expressing hIL-4Rα chain under the control of the Ig H chain promoter, and selected lines in which T cells apparently expressed hIL-4Rα chain for backcrossing to a BALB/c genetic background. As expected, T (Fig. 1*A*) and B (data not shown) cells from Tg mice, but not from control littermates, responded to hIL-4 in a proliferation assay. Furthermore, splenic CD4$^+$ T cells from the Tg animals, stimulated with exogenous hIL-4 in the presence of anti-mIL-4 mAb, showed Th2 differentiation at levels comparable to those seen with endogenous IL-4 (Fig. 1*B*). These results suggest that hIL-4 is able to fully substitute for endogenous mIL-4, resulting in Th2 polarization.

To study the kinetics of IL-4-mediated Th2 instruction, T cells from hIL-4Rα Tg and DO11.10/hIL-4α Tg were stimulated with anti-TCR and anti-CD28 mAbs and OVA antigenic peptide in the presence of BALB/c APCs. To eliminate the effect of endogenous IL-4, cells were cultured in anti-IL-4 mAb. To exclude signaling via endogenous IL-4 completely, hIL-4Rα Tg mice were also crossed with mIL-4Rα KO mice on a BALB/c genetic background (hIL-4Rα Tg/IL-4Rα KO). Time 0 was the initial stimulation with either anti-TCR and anti-CD28 mAb, or OVA peptide, and 10 U/ml hIL-4 was added at different times (−12 to 72 h; Fig. 1*C*, *top panels*).

First, we defined the kinetic relationship between initial TCR signaling and IL-4 signaling. Before T cell activation though TCR, CD4$^+$ T cells of hIL-4Rα Tg were cultured in the presence of hIL-4 for 12 h, and then after hIL-4 withdrawal, cells were stimulated with either anti-TCR and anti-CD28 mAbs, or Ag. The IL-4-pretreated T cells failed to induce IL-4-producing cells upon restimulation with anti-TCR mAb (Fig. 1*C*). When T cells from hIL-4Rα Tg, hIL-4Rα Tg/DO11.10 double Tg, or hIL-4Rα/mIL-4Rα KO/DO11.10 mice were initially stimulated with TCR cross-linking or OVA in the absence of hIL-4, T cells differentiated no or few Th2 cells. Th2 cells first appeared when hIL-4 was added between 0 and 36 h after initial TCR stimulation (Fig. 1*C*). However, their frequency rapidly declined thereafter. At 60∼72 h after initial TCR stimulation, the frequency of IL-4-producing Th2 cells was drastically reduced in both initial TCR cross-linking and antigenic stimulation (Fig. 1*C*). These results demonstrate that TCR activation is needed before IL-4 signaling to induce Th2 differentiation, and that the IL-4 signal is effective 0∼48 h after initial TCR activation.

However, we cannot exclude the possibility that an unskewed population, which has remained at day 7, could subsequently differentiate into Th2 cells. To investigate this possibility, hIL-4Rα Tg CD4$^+$ T cells that had been treated with hIL-4 at 0, 36, or 72 h were constitutively restimulated with Ag and hIL-4 at weekly intervals, and their differentiation profile was determined after 1, 2, or 3 wk. In cells that had been treated with hIL-4 at 0 or 36 h, the frequency of Th2 cells increased during the 3-wk restimulation (Fig. 2). In contrast, in cells that had been treated with hIL-4 at 72 h, Th2 differentiation was impaired, and consecutive Ag and hIL-4 restimulation did not restore it (Fig. 2), although these T cells retained hIL-4 responsiveness in a proliferation assay (data not shown). These results suggest that CD4$^+$ T cells lost their capability to differentiate into Th2 cells during initial TCR activation.

Kinetics of Th2-specific chromatin remodeling at conserved noncoding sequence-1 and -2 on IL-4 locus was related to the requirement of the IL-4 signal

A conformational change in chromatin structure to allow access to the transcriptional factors is critical for cell lineage commitment (21, 23). To understand the behavior of chromatin at the IL-4 locus during Th2 differentiation, nuclear fractions were collected at different times after primary stimulation, and treated with restriction

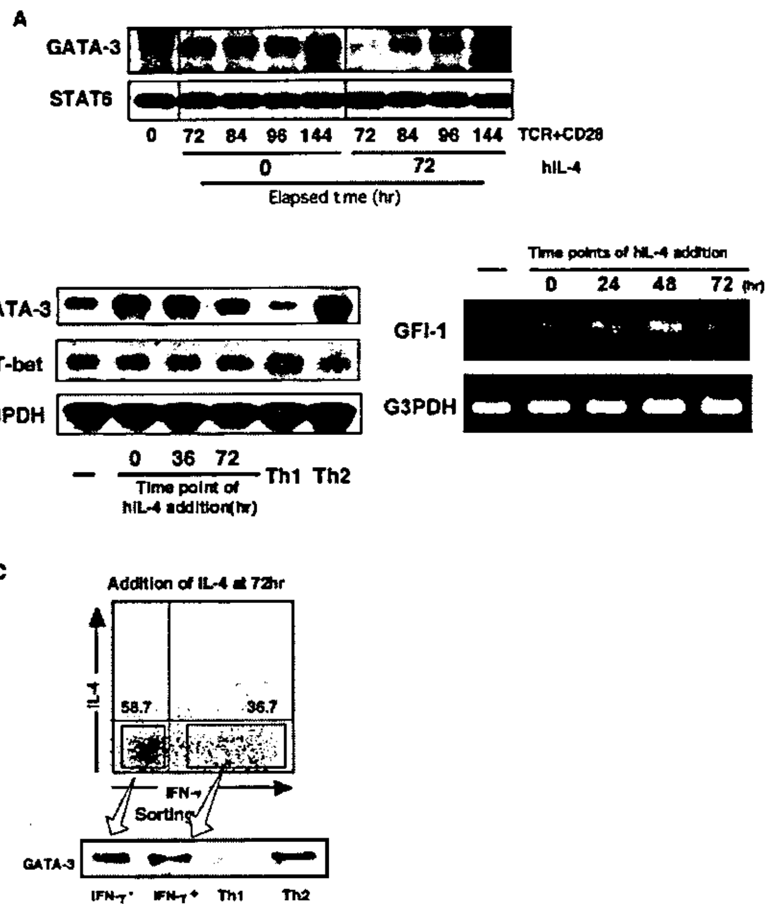


FIGURE 5. *A*, Enriched hIL-4R Tg cells were stimulated with anti-TCR/CD28 mAbs (time point 0) in the presence of anti-mIL-4 mAb. hIL-4 was added at 0 or 72 h, and cytoplasmic GATA-3 and STAT6 were assessed by Western blotting. The experiment was performed three times independently with similar results. *B*, Enriched hIL-4R Tg/mIL-4R KO $CD4^+$ T cells were stimulated with anti-TCR and anti-CD28 mAbs. hIL-4 was added at 0, 36, and 72 h, and expression of T-bet, GATA-3, and G3PDH mRNA was analyzed by Northern blotting. Expression of GF-1 was analyzed by RT-PCR. *C*, TCR-activated $CD4^+$ T cells from hIL-4R Tg/mIL-4R KO mice were stimulated with hIL-4 at 72 h. After 5 days, cells were restimulated with anti-TCR mAb, and intracellular cytokine staining was conducted. IFN-γ-producing and nonproducing cells were sorted, and expression of GATA-3 was examined by Western blotting.

enzymes that cut in each regulatory region, the conserved noncoding sequence-1, conserved noncoding sequence-2, and IE (Fig. 3*A*). $CD4^+$ T cells from DO11.10 Tg mice were stimulated with OVA in the presence of APCs, and 48 h later, Th2-specific alterations in the chromatin structure were observed in the conserved noncoding sequence-1 and -2 regions and IE (Fig. 3*B*).

We next examined whether the requirement for an IL-4 signal correlated with the kinetics of Th2-specific chromatin remodeling at conserved noncoding sequence-1 and -2. $CD4^+$ T cells from hIL-4Rα/mIL-4Rα KO/DO11.10 mice were stimulated with Ag, and hIL-4 was added at different time points. Seven days after the initial stimulation, chromatin structure was examined as above. When hIL-4 was given 0–48 h after initial stimulation, Th2-specific remodeling was seen in the conserved noncoding sequence-1 and -2 regions; but when hIL-4 was given 72 h after initial stimulation, remodeling was absent (Fig. 3*C*). However, the IE that mainly acts on mast cells showed distinct profile in the remodeling. Decondensation was observed even when hIL-4 was given 72 h after initial stimulation (Fig. 3*C*). These results indicated that the requirement for IL-4 signaling for the competence to secrete IL-4 in restimulation strictly corresponds with the alteration of the chromatin structure at conserved noncoding sequence-1 and -2 regions.

Influence of the IL-4 signal on the expression of transcriptional factors regulating IL-4 gene expression

GATA-3 is a master regulator controlling the lineage commitment of Th2 cells (12, 14), and its expression is tightly regulated by the IL-4-mediated STAT6 activation pathway (11). Therefore, selective expression of GATA-3 occurs in committed Th2 cells only. We examined the kinetics of *GATA-3*, *T-bet*, and *IL-12Rβ2* mRNA expression. $CD4^+$ T cells from hIL-4Rα Tg/mIL-4Rα KO mice were stimulated with anti-TCR/CD28 mAbs. In the absence of hIL-4, T cells predominantly differentiated into Th1 cells and no GATA-3 expression was found. When hIL-4 was added at time 0, GATA-3 expression clearly appeared from 24 h after initial TCR/CD28 stimulation (Fig. 4*A*). These results demonstrate that initial GATA-3 expression was regulated by the IL-4 signal. In contrast,

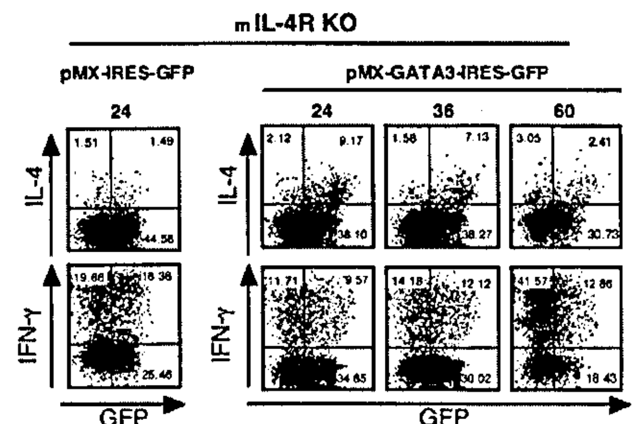


FIGURE 6. $CD4^+$ T cells from mIL-4R KO were stimulated with anti-TCR and anti-CD28 mAbs, and infected with the retrovirus vector, pMX-GATA3-IRES-GFP, at different time points. At day 7 after the initial activation, cytokine production was examined by intracellular staining. The experiment was performed three times independently with similar results.

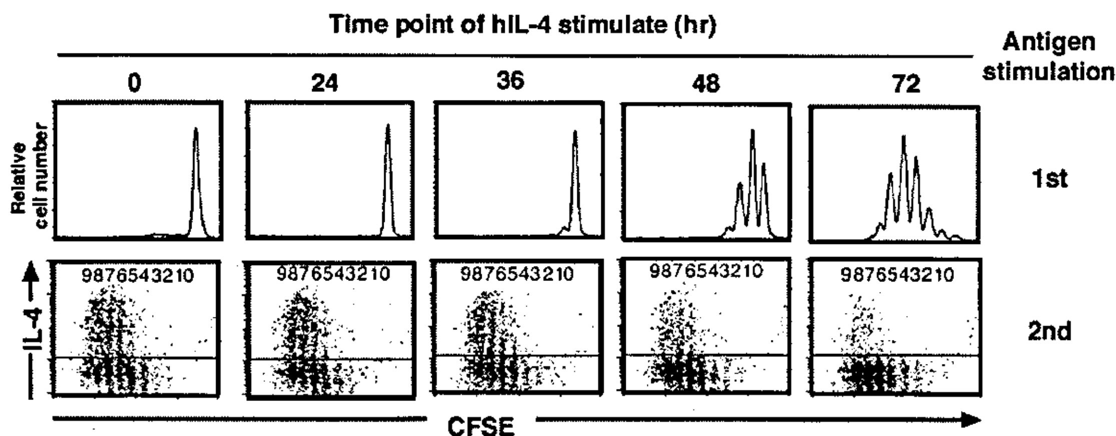


FIGURE 7. CFSE-labeled DO11.10 CD4$^+$ T cells were stimulated with OVA peptide in the presence of BALB/c APCs, and cell division was assessed by the intensity of green fluorescence at indicated time point after initial T cell stimulation (1st). The *x*-axis represents the intensity of green fluorescence, with progression of cell division moving *right* to *left* owing to dilution of intracellular dye. To examine IL-4 production ability in recall response, cells were restimulated with anti-TCR mAb (2nd) after 7 days, and IL-4-producing cells were detected, as described for Fig. 1. 0–9, Indicates the number of cell divisions. The experiment was performed three times independently with similar results.

T-bet and IL-12Rβ2 expression kinetics were not affected by the IL-4 signal (Fig. 4*A*).

Other transcriptional factors or coactivators, NF-AT and c-*maf*, are also involved in commitment to the Th2 lineage (35–38). Thus, we examined IL-4 responsiveness on the expression of these transcriptional factors. The hIL-4 Tg CD4$^+$ T cells expressed a small amount of NF-AT2, and no NF-AT1, c-*maf*, or GATA-3. Following TCR stimulation, expression of NF-AT2 increased rapidly for 48 h and then declined independently of IL-4. Similarly, NF-AT1 expression increased following TCR stimulation, however, in later time points (Fig. 4*B*). IL-4 delayed the induction of NF-AT1 expression in the cytoplasm, and c-*maf* expression was not detected in the nuclear fraction (data not shown). Furthermore, IL-4 made no difference to TCR-induced dephosphorylation of NF-AT1 or its translocation to the nucleus (Fig. 4*B*). Together, these results suggest that the IL-4 signal did not influence the kinetics of NF-AT1 and -2 expression, or of TCR-induced nuclear localization.

Administration of hIL-4 at 0 or 36 h after initial TCR stimulation rapidly induced GATA-3 protein in cytoplasm and nuclei (Fig. 4*B*), again confirming IL-4-dependent GATA-3 expression at early stages of initial activation. We next studied whether the hIL-4R-mediated signal was able to induce GATA-3 expression even later, as IL-4R failed to transduce the signal in Th1 cells. Thus, we analyzed expression of GATA-3 at 72 h, in which most T cells were differentiating into Th1 cells. We also examined the expression of growth-factor independent 1 (GFI-1), which was induced by IL-4-STAT6 signaling. Both GATA-3 mRNA and protein as well as GFI-1 mRNA clearly appeared at this late time point (Fig. 5, *A* and *B*).

Next, we studied whether GATA-3 protein is only expressed in the residual uncommitted population of T cells. Subsequent GATA-3 expression in Th1-committed and uncommitted T cells was examined when hIL-4 was added at 72 h. At day 7, cells were restimulated with anti-TCR mAb, IFN-γ-producing or nonproducing subpopulations were sorted, and their GATA-3 protein was analyzed by Western blotting. Both Th1-committed and uncommitted T cells expressed GATA-3 to a similar extent (Fig. 5*C*). These results indicate that IL-4 signaling through the hIL-4R induced GATA-3 expression, even in a Th1-skewing condition.

Timing of GATA-3 expression regulates the efficiency of Th2 development

Ectopic expression of GATA-3 can mimic the function of IL-4 signal, by inducing Th2 cytokines and Th2-specific chromatin remodeling, but our results address that timing may be critical. To further investigate this, we examined whether ectopic expression of GATA-3 late in the differentiation of CD4$^+$ T cells could result in efficient development of Th2 cells. GATA-3 and GFP were coexpressed in T cells by a bicistronic retrovirus construct (pMX-GATA3-GFP), and the proportion of IL-4-producing GFP-positive population was assessed. The mIL-4R KO T cells were stimulated with anti-TCR/CD28 mAbs, and then infected with pMX-GATA3-GFP after 24, 36, or 60 h. Infection at 24 h resulted in 9% GATA-3- and IL-4-coproducing T cells, which corresponds to ~20% of the total GFP$^+$ cells (Fig. 6). Infection at 60 h reduced the proportion of T cells producing IL-4, to ~7% of total GFP$^+$ cells (2.4% GFP$^+$IL-4$^+$ from 33.1% GFP$^+$ cells). These results confirm that the timing of GATA-3 expression is critical for determining lineage commitment. Nevertheless, the presence of some IL-4$^+$/GFP^{high} cells after infection at 60 h indicates that even at this late stage, high levels of GATA-3 expression can induce commitment to the Th2 lineage.

Relationship between cell division and lineage-specific IL-4 gene expression

Previous reports concluded that lineage-specific IL-4 gene expression is regulated by the number of cell divisions. Thus, we studied the relationship between cell division and IL-4 signaling required for Th2 lineage commitment. CFSE-labeled hIL-4Rα Tg/mIL-4Rα KO/DO11.10 CD4$^+$ T cells were stimulated with OVA peptide. Cell division was profiled in a time kinetic (Fig. 7, *upper panel*), and the proportion of IL-4-producing cells following restimulation with anti-TCR mAb was examined at day 7 (Fig. 7, *lower panel*). Consistent with previous observations (26), IL-4-producing cells appeared after five generations (Fig. 7, *lower panel*). At 36 h poststimulation, in which hIL-4 efficiently induced Th2 differentiation (Fig. 1*C*), most T cells remained at the stage before undergoing into first cell division, while at 72 h poststimulation, all cells entered into successive cell division (Fig. 7, *upper panel*). These results suggest that IL-4 signaling may be able to

instruct Th2 lineage commitment before progression to cell division.

Discussion

This study investigated the relevance of IL-4 signaling for the regulation of Th2 differentiation. We established a Tg mouse model allowing us to separate IL-4 signaling from TCR-mediated stimulation. IL-4-mediated signaling regulates chromatin structure at the IL-4 locus and thereby influences the competence to secrete effector cytokines during Th2 differentiation. In this study, we show evidence that the initial 48 h is a critical period for Th2 commitment. This commitment depended on IL-4-induced GATA-3 expression, as we demonstrated that Th2 cells differentiated from naive T cells only, when GATA-3 was expressed at appropriate times. Furthermore, we found that IL-4-mediated lineage commitment was independent of cell division.

During lineage commitment, TCR and IL-4 signaling act synergistically on Th2-specific alterations of chromatin structure, by hyperacetylation of core histones H3 and H4 (20). TCR stimulation causes the formation of BAG or Brm associated factor (BAF) complexes with the nuclear matrix, leading to decondensation of heterochromatin (39). However, the regulatory regions of the IL-4 locus are acetylated equally in the first 24 h in both Th1- and Th2-skewing conditions (20), suggesting that initial TCR signaling promotes the decondensation of heterochromatin and the early increase of histone acetylation. Th2-specific decondensation in the regulatory regions of the IL-4 locus became visible only in a time window between 36 and 48 h (Fig. 3*C*). These observations suggest that if the chromatin remains condensed for long times, it eventually becomes incapable of Th2-type condensation. The major role of IL-4 signaling is to induce GATA-3 expression, and the induced GATA-3 then initiates the Th2-specific chromatin remodeling, subsequently leading to competence to secrete IL-4 upon restimulation (12). Therefore, the timing of GATA-3 expression may be critical for permissive chromatin alteration. This notion is supported by evidence that the timing of Th2-specific chromatin remodeling in the conserved noncoding sequence-1 and -2 regions matches the kinetics of the IL-4-mediated GATA-3 expression.

Previous work has examined whether cell division was necessary for the instruction of IL-4 production upon restimulation (28). It has been reported that T cells have to complete a certain number of cell cycles to become competent to secrete relatively high amounts of IL-4 upon restimulation. We confirmed this as five cell divisions were needed until substantial IL-4 production upon restimulation (Fig. 7, *lower panel*). However, the previous report did not clearly indicate the timing of IL-4 signaling during Th2 differentiation (28), because it ignored the possible involvement of IL-4 secreted from naive T cells. Richter et al. (29) argued that cell division is not critical for instruction into IL-4-producing cells upon restimulation, because use of L-mimosine, which blocks the cell cycle before S phase, did not affect IL-4-induced Th2 differentiation. Furthermore, they showed that coordination of TCR and IL-4R signaling is necessary for the recall production of IL-4, and that this coordination is able to control Th2 commitment for at least 1 day. Their results resemble our data using hIL-4R Tg mice, as IL-4 was able to induce a substantial number of Th2 cells, even before the cells undergo cell division. Taken together, our data suggest that the timing of GATA-3 expression, rather than cell division, is the important factor in the acquisition of competence to secrete IL-4.

The recent discovery of GFI-1 provides an alternative explanation for the role of IL-4 signaling, namely that IL-4 simply selects a subpopulation that responds to the IL-4-STAT6 signaling, rather than instructing the entire population into Th2 differentiation (4, 40). Previous reports have demonstrated that IL-4R signaling is selectively impaired in Th1-committed cells (41–43). This selective defect causes the selective expansion of IL-4-responding Th2 cells. However, in this study, we showed that the IL-4 signal introduced through hIL-4R is able to induce GATA-3 and GFI-1 expression, even in Th1-committed cells. In these cells, the expressed GATA-3 and GFI-1 expression dose not promote Th2 differentiation, suggesting that IL-4 signaling may act by instruction, rather than selection. However, further investigation will be required to clarify the role of IL-4 signaling on Th2 lineage commitment.

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