we proposed that continuous administration of FTY720 would prevent lymphocytes activated at inductive sites from homing into the CLP and thus interrupt the cycle of lymphocyte activation and recirculation. Actually, the numbers of $CD4^+$ T lymphocytes in CLP and the $CD4^+/CD8^+$ ratio in T lymphocytes from PB and CLP were significantly decreased (Fig. 6C). We also found decreased production of IFN-γ by CLP in conjunction with decreased severity of the chronic colitis.

Some authors have reported that FTY720 accelerates apoptosis.[18,19,50,51] The results of in vitro studies have suggested that selective apoptotic cell death of lymphocytes, particularly of certain T lymphocytes subsets, is the mechanism of action of this drug.[50–53] However, the concentrations (2–10 μM) needed for lymphocyte apoptosis in vitro were several orders of magnitude greater than the blood concentrations in rats administered FTY720 at the therapeutic dose of 1 mg/kg.[20,47] It remains unclear whether apoptosis by direct action of FTY 720 occurs in vivo. In the current study, we performed TUNEL assay and annexin labeling after FTY720 treatment. Quite a number of TUNEL positive cells could be detected in MLN and PP, but significant difference was not obtained also in the levels of annexin-positive/propidium iodide-negative lymphocytes between FTY720-treated and untreated control mice. Thus, we could find no evidence that FTY720 enhanced apoptosis of lymphocytes. However, there might be a possibility of apoptosis that could not been detected in vivo, because apoptotic lymphocytes had been promptly removed by activated macrophages. Actually it is well-known that intestinal macrophages are highly activated in IL-$10^{-/-}$ mice.[49] We have considered that apoptosis in this model would be gradually and continuously induced by activation-induced cell death, but not by programed cell death in case of irradiation or anticancer agents. It has been reported that by repeated activation, lamina propria T lymphocytes lead to the coexpression of two molecules, a death-inducing receptor (Fas) and its ligand (Fas-L), resulting in apoptotic cell death in situ.[54] Such activation-induced cell death might be caused also by FTY720 in the sequestrated secondary lymphoid tissues, such as MLN and PP. This possibility has not been addressed in the current study. Even if such a mechanism was operative in our animals, a new wave of the activated T lymphocytes was continuously supplied to the inflammatory site in untreated control mice. In FTY720-treated mice, however, it was considered that the activated T lymphocytes would not be supplied to the colon because of the inhibition of lymphocytes efflux from the secondary lymphoid tissues, by the direct mechanism of FTY720.

In conclusion, we have demonstrated that a novel lymphocyte homing reagent, FTY720, is effective in the treatment of established colitis in adult IL-$10^{-/-}$ mice. The most likely mechanism responsible for this effect is an accelerated sequestration of circulating lymphocytes into PP and MLN, resulting in a reduction in PB lymphocytes and lymphocytes migrating to the inflammatory site. The possibility that FTY720 or agents with a similar mode of action would be effective in the treatment of Crohn's disease deserves consideration.

ACKNOWLEDGMENTS

The authors thank Chika Ariga and Reiko Tsubouchi for technical assistance and Dr. William R. Brown and Dr. Yoshifumi Inoue for critical comments.

REFERENCES

1. Podolsky DK. Inflammatory bowel disease. *N Engl J Med.* 2002;347: 417–429.
2. Strober W, Neurath MF. Immunological disease of the gastrointestinal tract. In: Rich RR, ed. *Clinical Immunology.* St. Louis, MO: Mosby; 1995:1401–1428.
3. Shanahan F. Crohn's disease. *Lancet.* 2002;359:62–69.
4. Sands BE. Therapy of inflammatory bowel disease. *Gastroenterology.* 2000;118:S68–S82.
5. Scribano ML, Prantera C. Medical treatment of active Crohn's disease. *Aliment Pharmacol Ther.* 2002;16:S35–S39.
6. Rizzello F, Gionchetti P, Venturi A, et al. The management of refractory Crohn's disease. *Aliment Pharmacol Ther.* 2002;16:S40–S47.
7. Forbes A. Crohn's disease—the role of nutritional therapy. *Aliment Pharmacol Ther.* 2002;16:S48–S52.
8. Nakase H, Okazaki K, Tabata Y, et al. Development of an oral drug delivery system targeting immune-regulating cells in experimental inflammatory bowel disease: a new therapeutic strategy. *J Pharmacol Exp Ther.* 2000;292:15–21.
9. Sandborn WJ. A review of immune modifier therapy for inflammatory bowel disease: azathioprine, 6-mercaptopurine, cyclosporine, and methotrexate. *Am J Gastroenterol.* 1996;91:423–433.
10. Parronchi P, Romagnani P, Annunziato F, et al. Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease. *Am J Pathol.* 1997;150:823–832.
11. Shanahan F. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics, and ecotherapeutics. *Gastroenterology.* 2001;120:622–635.
12. Sandborn WJ, Hanauer SB. Infliximab in the treatment of Crohn's disease: a user's guide for clinicians. *Am J Gastroenterol.* 2002;97:2962–2972.
13. Sandborn WJ, Hanauer SB. Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. *Inflamm Bowel Dis.* 1999;5:119–133.
14. Ito H. Anti-interleukin-6 therapy for Crohn's disease. *Curr Pharm Des.* 2003;9:295–305.
15. Ding C, Jones G. Technology evaluation: MRA, Chugai. *Curr Opin Mol Ther.* 2003;5:64–69.
16. Kosaka T, Sawada K, Ohnishi K, et al. Effect of leukocytapheresis therapy using a leukocyte removal filter in Crohn's disease. *Intern Med.* 1999;38:102–111.
17. Matsui T, Nishimura T, Matake H, et al. Granulocytapheresis for Crohn's disease: a report on seven refractory patients. *Am J Gastroenterol.* 2003; 98:511–512.
18. Adachi K, Kohara T, Arita M, et al. Design, synthesis, and structure-activity relationships of 2-substituted-2-amino-1,3-propanodiols: discovery of a novel immunosuppressant, FTY720. *Bioorg Med Chem Lett.* 1995;5:853–856.
19. Fujita T, Yoneta M, Hirose R, et al. Simple compounds, 2-substituted-2-amino-1, 3-propanodiols have potent immunosuppressive activity. *Bioorg Med Chem Lett.* 1995;5:847–852.
20. Suzuki S, Enosawa S, Kakefuda T, et al. A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. *Transplantation.* 1996;6:200–205.
21. Chen S, Bacon KB, Garcia G, et al. FTY720, a novel transplantation drug, modulates lymphocyte migratory responses to chemokines. *Transplant Proc.* 2001;33:3057–3063.
22. Kuhn R, Lohler J, Rennick D, et al. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell.* 1993;75:263–274.
23. Sartor RB. Role of the intestinal microflora in pathogenesis and compli-

cations. In: Scholmerich JH, Goebell W, Kruis W, Hohenberger W, eds. *Inflammatory Bowel Diseases: Pathophysiology as Basis of Treatment.* London, England: Kluwer Academic, 1992;175–187.
24. Fujihashi K, McGhee JR, Kweon MN, et al. Gamma/delta T cell-deficient mice have impaired mucosal immunoglobulin A responses. *J Exp Med.* 1996;183:1929–1935.
25. Takahashi I, Kiyono H, Hamada S. CD4+ T-cell population mediates development of inflammatory bowel disease in T-cell receptor alpha chain-deficient mice. *Gastroenterology.* 1997;112:1876–1886.
26. Iijima H, Takahashi I, Kishi D, et al. Alteration of interleukin 4 production results in the inhibition of T helper type 2 cell-dominated inflammatory bowel disease in T cell receptor alpha chain-deficient mice. *J Exp Med.* 1999;190:607–615.
27. Corazza N, Eichenberger S, Eugster HP, et al. Nonlymphocyte-derived tumor necrosis factor is required for induction of colitis in recombination activating gene (RAG)2(−/−) mice upon transfer of CD4(+)CD45RB(hi) T cells. *J Exp Med.* 1999;190:1479–1492.
28. Fiocchi C. Inflammatory bowel disease: etiology and pathogenesis. *Gastroenterology.* 1998;115:182–205.
29. Davidson NJ, Leach MW, Fort MM, et al. T helper cell 1-type CD4+ T cells, but not B cells, mediate colitis in interleukin 10-deficient mice. *J Exp Med.* 1996;184:241–251.
30. Davidson NJ, Hudak SA, Lesley RE, et al. IL-12, but not IFN-gamma, plays a major role in sustaining the chronic phase of colitis in IL-10-deficient mice. *J Immunol.* 1998;161:3143–3149.
31. Berg DJ, Davidson N, Kuhn R, et al. Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest.* 1996;98:1010–1020.
32. Rennick DM, Fort MM, Davidson NJ. Studies with IL-$10^{-/-}$ mice: an overview. *J Leukoc Biol.* 1997;61:389–396.
33. Lindsay JO, Ciesielski CJ, Scheinin T, et al. The prevention and treatment of murine colitis using gene therapy with adenoviral vectors encoding IL-10. *J Immunol.* 2001;166:7625–7633.
34. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. *Science.* 1996;272:60–66.
35. Kiyono H, Kweon MN, Hiroi T, et al. The mucosal immune system: from specialized immune defense to inflammation and allergy. *Acta Odontol Scand.* 2001;59:145–153.
36. Iijima H, Takahashi I, Kiyono H. Mucosal immune network in the gut for the control of infectious diseases. *Rev Med Virol.* 2001;11:117–133.
37. Fiocchi C. The immune system in inflammatory bowel disease. *Acta Gastroenterol Belg.* 1997;60:156–162.
38. De Keyser F, Elewaut D, De Wever N, et al. The gut associated addressins: lymphocyte homing in the gut. *Baillieres Clin Rheumatol.* 1996; 10:25–39.
39. Ansel KM, Cyster JG. Chemokines in lymphopoiesis and lymphoid organ development. *Curr Opin Immunol.* 2001;13(2):172–179.
40. Picarella D, Hurlbut P, Rottman J, et al. Monoclonal antibodies specific for beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) reduce inflammation in the colon of scid mice reconstituted with CD45RBhigh CD4+ T cells. *Immunology.* 1997;158:2099–2106.
41. Ludviksson BR, Strober W, Nishikomori R, et al. Administration of mAb against alpha E beta 7 prevents and ameliorates immunization-induced colitis in IL-2−/− mice. *J Immunol.* 1999;62:4975–4982.
42. Ghosh S, Goldin E, Gordon FH, et al. Natalizumab for active Crohn's disease. *N Engl J Med.* 2003;348:24–32.
43. Chiba K, Yanagawa Y, Masubuchi Y, et al. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. I. FTY720 selectively decreases the number of circulating mature lymphocytes by acceleration of lymphocyte homing. *J Immunol.* 1998;160:5037–5044.
44. Yanagawa Y, Masubuchi Y, Chiba K. FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats, III. Increase in frequency of CD62L-positive T cells in Peyer's patches by FTY720-induced lymphocyte homing. *Immunology.* 1998;95:591–594.
45. Pinschewer DD, Ochsenbein AF, Odermatt B, et al. FTY720 immunosuppression impairs effector T cell peripheral homing without affecting induction, expansion, and memory. *J Immunol.* 2000;164:5761–5770.
46. Pyne S, Pyne NJ. Sphingosine 1-phosphate signalling in mammalian cells. *Biochem J.* 2000;349(Pt 2):385–402.
47. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J Biol Chem.* 2002; 277:21453–21457.
48. Mandala S, Hajdu R, Bergstrom J, et al. Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science.* 2002;296: 346–349.
49. Watanabe N, Ikuta K, Okazaki K, et al. Elimination of local macrophages in intestine prevents chronic colitis in interleukin-10-deficient mice. *Dig Dis Sci.* 2003;48:408–414.
50. Suzuki S, Li XK, Enosawa S, et al. A new immunosuppressant, FTY720, induces bcl-2-associated apoptotic cell death in human lymphocytes. *Immunology.* 1996;89:518–523.
51. Fujino M, Li XK, Guo L, et al. Activation of caspases and mitochondria in FTY720-mediated apoptosis in human T cell line Jurkat. *Int Immunopharmacol.* 2001;1:2011–2021.
52. Enosawa S, Suzuki S, Kakefuda T, et al. Induction of selective cell death targeting on mature T-lymphocytes in rats by a novel immunosuppressant, FTY720. *Immunopharmacology.* 1996;34:171–179.
53. Li XK, Tamura A, Fujino M, et al. Induction of lymphocyte apoptosis in rat liver allograft with ongoing rejection by FTY720. *Clin Exp Immunol.* 2001;123:331–339.
54. MacDonald TT. Effector and regulatory lymphoid cells and cytokines in mucosal sites. *Curr Top Microbiol Immunol.* 1999;236:113–135.

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

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

Epithelial cells are key players in the first line of defense offered by the mucosal immune system against invading pathogens. In the present study we sought to determine whether human corneal epithelial cells expressing Toll-like receptors (TLRs) function as pattern-recognition receptors in the innate immune system and, if so, whether these TLRs act as a first line of defense in ocular mucosal immunity. Incubation of human primary corneal epithelial cells and the human corneal epithelial cell line (HCE-T) with peptidoglycan or LPS did not lead to activation, at the level of DNA transcription, of NF-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).

*Department of Mucosal Immunology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; †Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan; ‡Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; and §Core Research for Engineering, Science, and Technology of Japan Science and Technology, Tokyo, Japan

Received for publication September 9, 2003. Accepted for publication June 29, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the Uehara Science Foundation; the Core Research for Engineering, Science, and Technology of Japan Science and Technology; the Ministry of Education, Science, Sports, and Culture; the Ministry of Health and Welfare; and the Health Science Foundation, Japan.

[2] Address correspondence and reprint requests to Dr. Hiroshi Kiyono, Division of Mucosal Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Sirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail address: kiyono@ims.u-tokyo.ac.jp

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

0022-1767/04/$02.00

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

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

Materials and Methods

Human corneal epithelial cells

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

The human corneal epithelial cell line transformed with SV40 (HCE-T) (25) was maintained at Kyoto Prefectual University of Medicine and cultured in modified SHEM medium consisting of DMEM/F-12 medium (Invitrogen Life Technologies, Paisley, U.K.) supplemented with 10% FCS (Invitrogen Life Technologies), 10 ng/ml murine natural epidermal growth factor (Invitrogen Life Technologies), 5 μg/ml insulin from bovine pancreas (Sigma-Aldrich, St. Louis, MO), and 1% antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B; Invitrogen Life Technologies) at 37°C under 95% humidity and 5% CO_2 (26). Human primary corneal epithelial cells were obtained from KURABO (Osaka, Japan) and then cultured in a serum-free medium consisting of EpiLife (KURABO) supplemented with human corneal epithelial cell growth supplement containing 1 ng/ml murine epidermal growth factor, 5 μg/ml insulin from bovine pancreas, 0.18 μg/ml hydrocortisone, 0.4% bovine pituitary extract (all from KURABO), and 1% antibiotic-antimycotic solution consisting of 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Life Technologies) at 37°C under 95% humidity and 5% CO_2 (27).

Purification of mononuclear cells from peripheral blood

Once the purpose of the research and the experimental protocol had been explained to and informed consent obtained from the volunteers, human venous blood samples were obtained from them. The blood sample was anticoagulated with heparin. Blood was then placed in sterile 50-ml polypropylene tubes. Blood was mixed with 1 vol of PBS^- (Ca^{2+} free), overlaid on Lymphoprep (Axis-Shield PoC, Oslo, Norway) and centrifuged for 20 min at 2000 rpm at 20°C. Mononuclear cells were gently aspirated from the interface and washed with PBS^-.

RT-PCR analysis

A standard RT-PCR assay routinely performed in our laboratory was used in this study (28). Briefly, total RNA was isolated from HCE-T, human mononuclear cells, and human corneal epithelia using a TRIzol reagent (Invitrogen Life Technologies, Grand Island, NY) according to the manufacturer's instructions. For RT reaction, the SuperScript preamplification system (Invitrogen Life Technologies) was applied. PCR amplification was performed with DNA polymerase (AmpliTaq; PerkinElmer Cetus, Norwalk, CT) for 38 cycles at 94°C for 1 min, at 52°C for 1 min, and at 72°C for 1 min using a commercial apparatus (GeneAmp; PerkinElmer Cetus). The primers used in this study are listed in the table shown in Fig. 1. The integrity of the RNA was assessed by electrophoresis in ethidium bromide-stained, 1.5% agarose gels.

ELISA

To quantify cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 12-well plates (1×10^5 cells/well) and, after reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS from *P. aeruginosa* (Sigma-Aldrich), 1000 ng/ml PGN from *S. aureus* (Fluka, Buchs, Switzerland), or 10 ng/ml human IL-1α (R&D Systems, Minneapolis, MN) for 24 h. The concentrations of LPS, PGN, and IL-1α used in this study were optimal for the maximum induction of inflammatory cytokines (10, 29). The culture supernatants were harvested, and levels of IL-6 and IL-8 were measured by the respective human cytokine-specific ELISA (BioSource, Camarillo, CA).

Real-time quantitative PCR

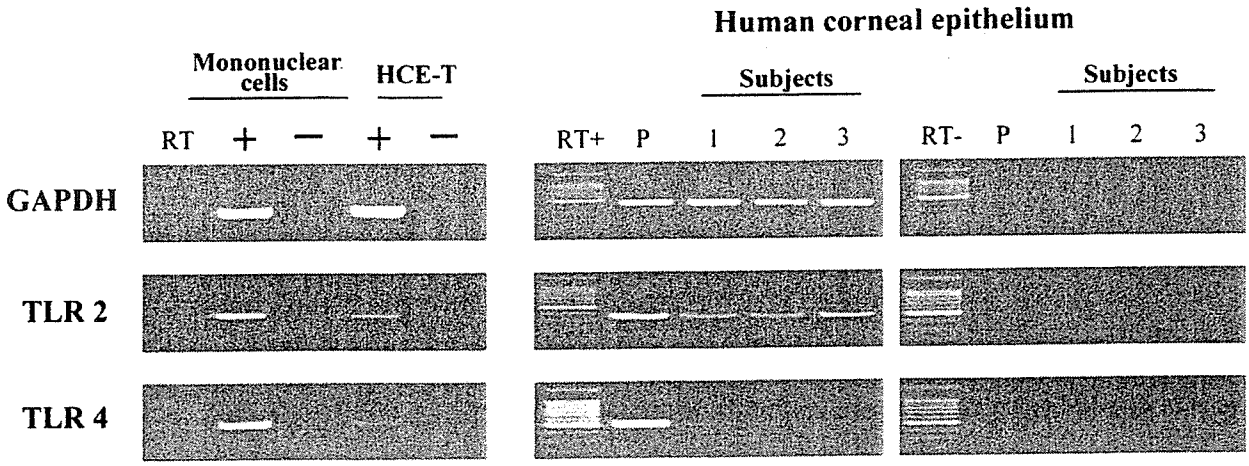
Real-time quantitative PCR was performed using a LightCycler (Roche, Mannheim, Germany) according to the previously described protocol (30) and manufacturer's instructions. For the amplification of IL-6, IL-8, and human β-defensin-2 (hBD2) cDNA, RT-PCR was performed in a 20-μl total volume in the presence of 2 μl of 10× reaction buffer (*Taq* polymerase, dNTPs, and $MgCl_2$; Roche), and 2 μl of cDNA (or water as a negative control, which was always included). $MgCl_2$ was added to a final concentration of 3 mM, and 5 pmol of each oligonucleotide primer was added. Real-time PCR was performed in glass capillaries. A calibration curve was automatically generated using the external standards, and samples were quantified accordingly by LightCycler analysis software (version 3; Roche). These quantification data were normalized to the expression of the housekeeping gene GAPDH. Listed below are the primers and probes used in this study because of their specificity for IL-6, IL-8, hBD2, and GAPDH (Table I).

NF-κB assay

To compare NF-κB production, HCE-T was plated in six-well plates (2×10^5 cells/well) and, upon reaching subconfluence, were left untreated or were exposed to LPS (1000 ng/ml) from *P. aeruginosa*, PGN (1000 ng/ml) from *S. aureus*, or IL-1α (10 ng/ml) for 7 h. After incubation, the transcription NF-κB assay was performed using TransAM (Active Motif, Carlsbad, CA) according to the manufacturer's instructions (31). Briefly, cells were rinsed twice with cold PBS^- before being scraped and centrifuged for 10 min at 1,000 rpm. The pellet was then resuspended in 100 μl of the lysis buffer included in the kits. After 10 min on ice, the lysate was centrifuged for 20 min at 14,000 rpm. Twenty microliters of 10-fold diluted cell extracts were incubated with 30 μl of binding buffer in microwells coated with the probes containing the NF-κB consensus binding sequence. After 1-h incubation at room temperature with mild agitation, microwells were washed three times. Anti-NF-κB Abs were added to each well and incubated for 1 h at room temperature. Microwells were then washed three times before incubation with HRP-conjugated Abs for 1 h at room temperature. After incubation, microwells were washed four times and reacted with tetramethylbenzidine for 10 min at room temperature before the addition of stop solution. OD was then read at 450 nm with an iEMS microplate reader (Thermo Labsystem, Vantaa, Finland)

Flow cytometric analysis

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



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

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

Immunocytoplasmic and histological staining

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

Internalization of LPS with DOTAP

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

Data analysis

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

Results

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

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

Human corneal epithelial cells fail to respond to LPS or PGN

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

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

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

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

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

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

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

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

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

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

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

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

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

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

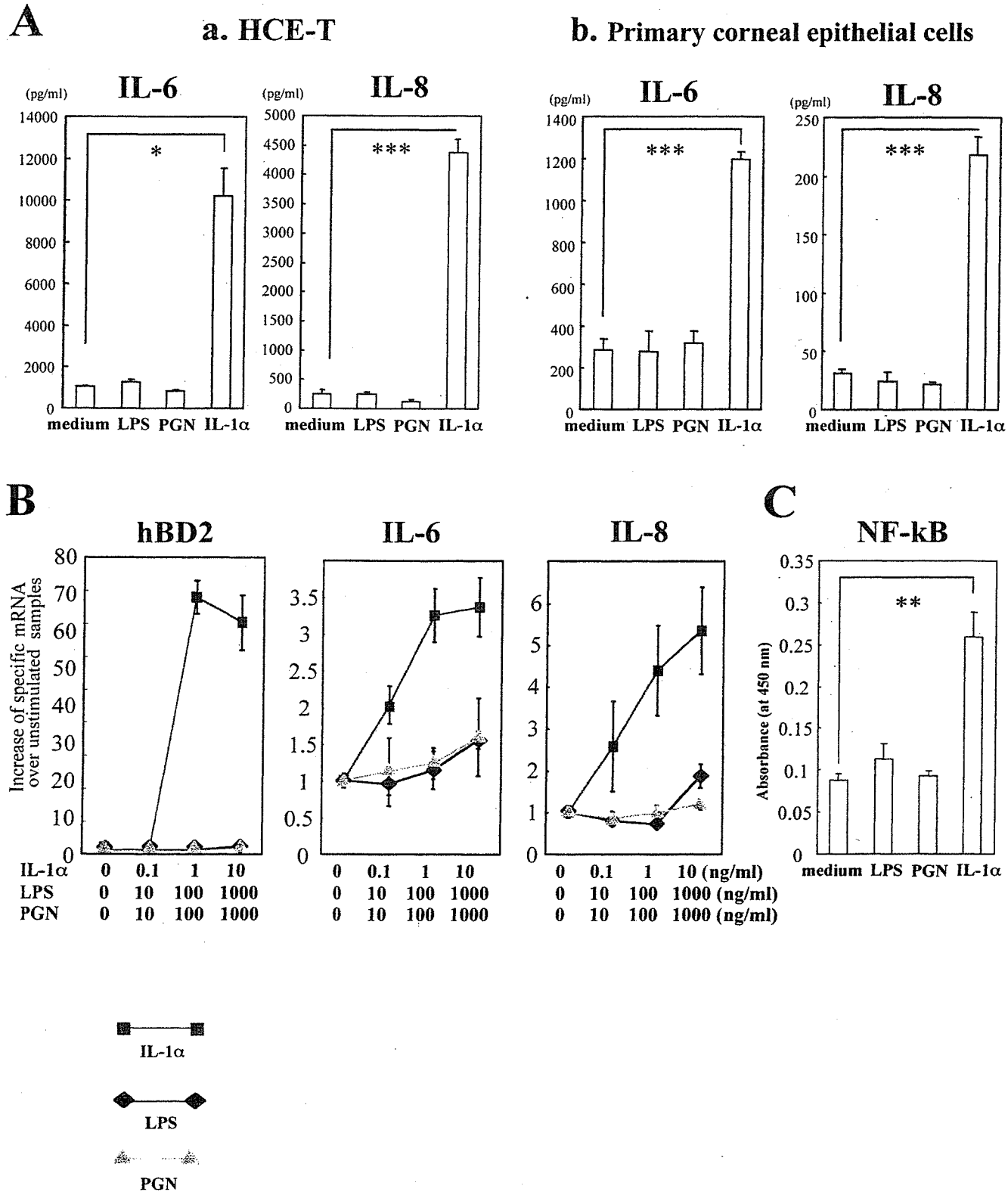


FIGURE 2. Human corneal epithelial cells fail to respond to LPS or PGN. To quantify inflammatory cytokine secretion, HCE-T and primary human corneal epithelial cells were plated in 24-well plates and, upon reaching subconfluence, were left untreated or were exposed to 1000 ng/ml LPS, 1000 ng/ml PGN,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

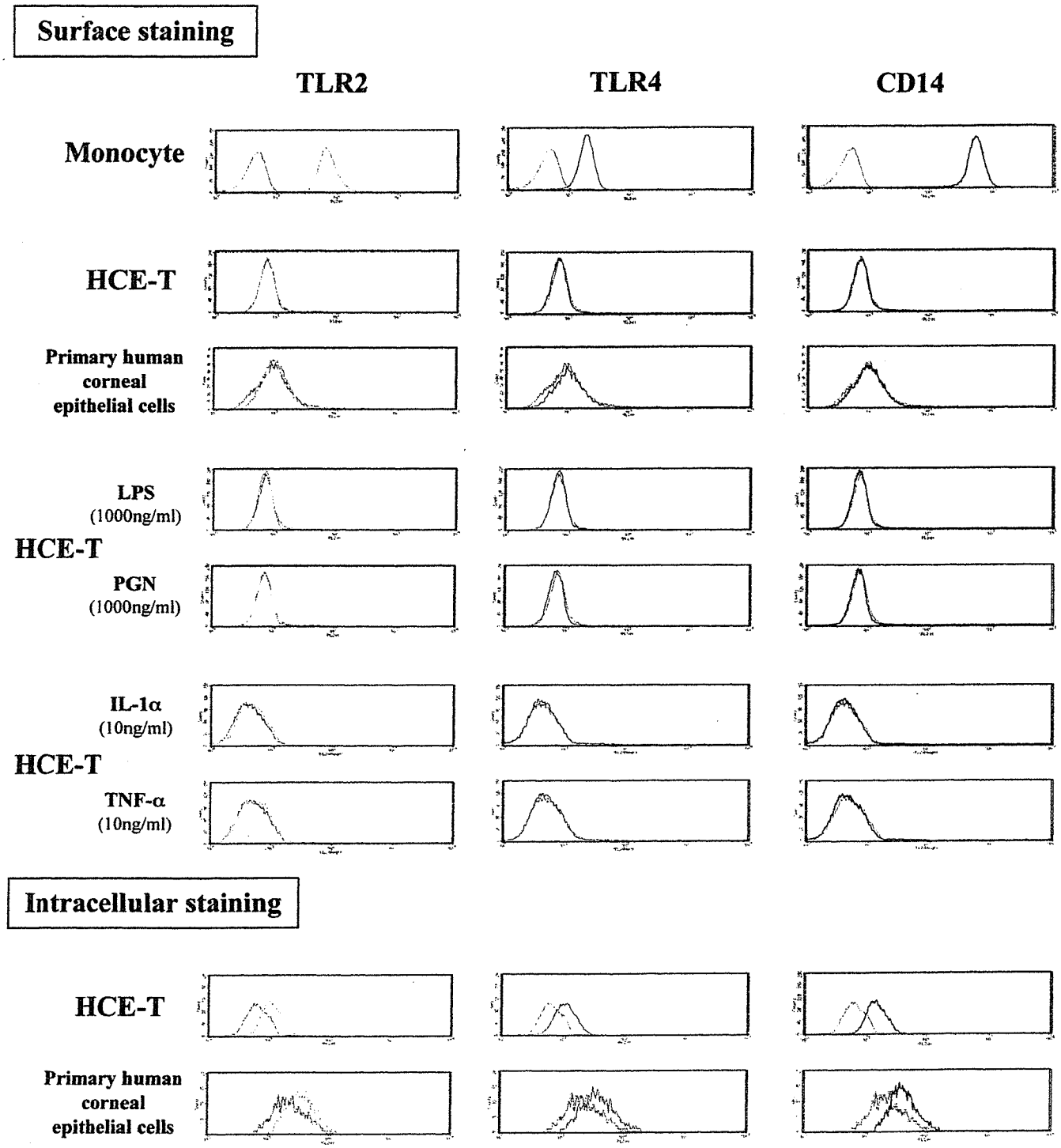


FIGURE 3. TLR2 and TLR4 are expressed intracellularly, but not on the cell surface of human corneal epithelial cells. Cell surface expressions of TLR2, TLR4, and CD14 in HCE-T and primary human corneal epithelial cells were examined by FACS. These cells were incubated with PE-conjugated mouse anti-human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

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

Results for primary human corneal epithelial cells were similar where the cells also did not respond to intracellularly introduced LPS, except that, in contrast to HCE-T, they secreted some IL-6 and IL-8 when cocultured with DOTAP alone. It is possible that DOTAP may provide activation signals for primary human corneal epithelial cells, but as of yet the specific signaling mechanism remains unknown. We also examined whether NF-κB signaling was up-regulated by the intracellular delivery of LPS into HCE-T. We found that internalization of

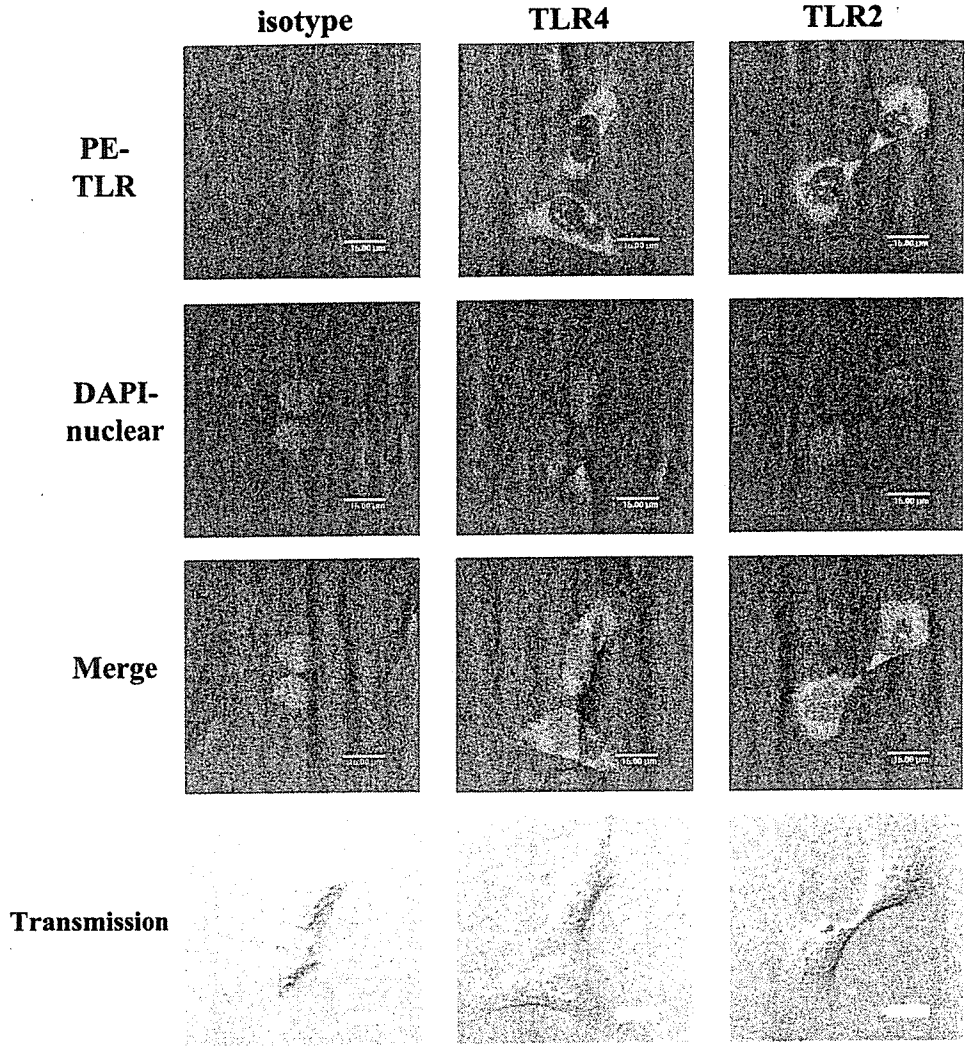


FIGURE 4. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in the human corneal epithelial cell line HCE-T. HCE-T was cultured on a slide chamber, washed with PBS^-, and air-dried. Slides were fixed with methanol for 30 min, then stained with PE-conjugated mouse anti-human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

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

Discussion

Interestingly, our results indicate that ocular surface epithelial cells, which are an important component of the mucosal immune system, express TLR-specific mRNA for two well-characterized pattern recognition receptors, TLR2 and TLR4. However, incubation with PGN and LPS failed to induce the secretion by HCE-T and primary human corneal epithelial cells of inflammation-associated cytokines such as IL-6 and IL-8. Further, NF-κB activation was not up-regulated by the stimulation of HCE-T with LPS or PGN. These results show that human corneal epithelial cells are incapable of responding to LPS from *P. aeruginosa* and to PGN from *S. aureus*. To support the finding, we subsequently used FACS and immunohistochemical analyses to show that human corneal epithelial cells express TLR2 and TLR4 intracellularly, but not at the cell surface. Even when LPS was artificially delivered to intracellularly expressed TLR4 in the cytoplasm, it did not lead to the subsequent activation of NF-κB-mediated signaling for the induction of IL-6 and IL-8. These findings suggest the interesting possibility that the ocular surface epithelial cell-associated mucosal immune system may create an immunosilent condition for TLR-mediated innate immunity to prevent unnecessary inflammatory responses to normal bacterial flora. However, it has been shown that Langerhans cells and macrophages are located at the basal layer of the corneal epithelium and corneal stroma (33). Thus, these APCs may immediately respond to microbial products via TLRs.

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

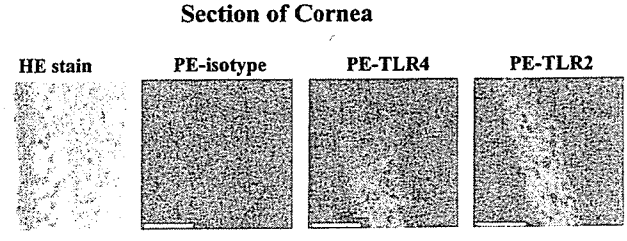
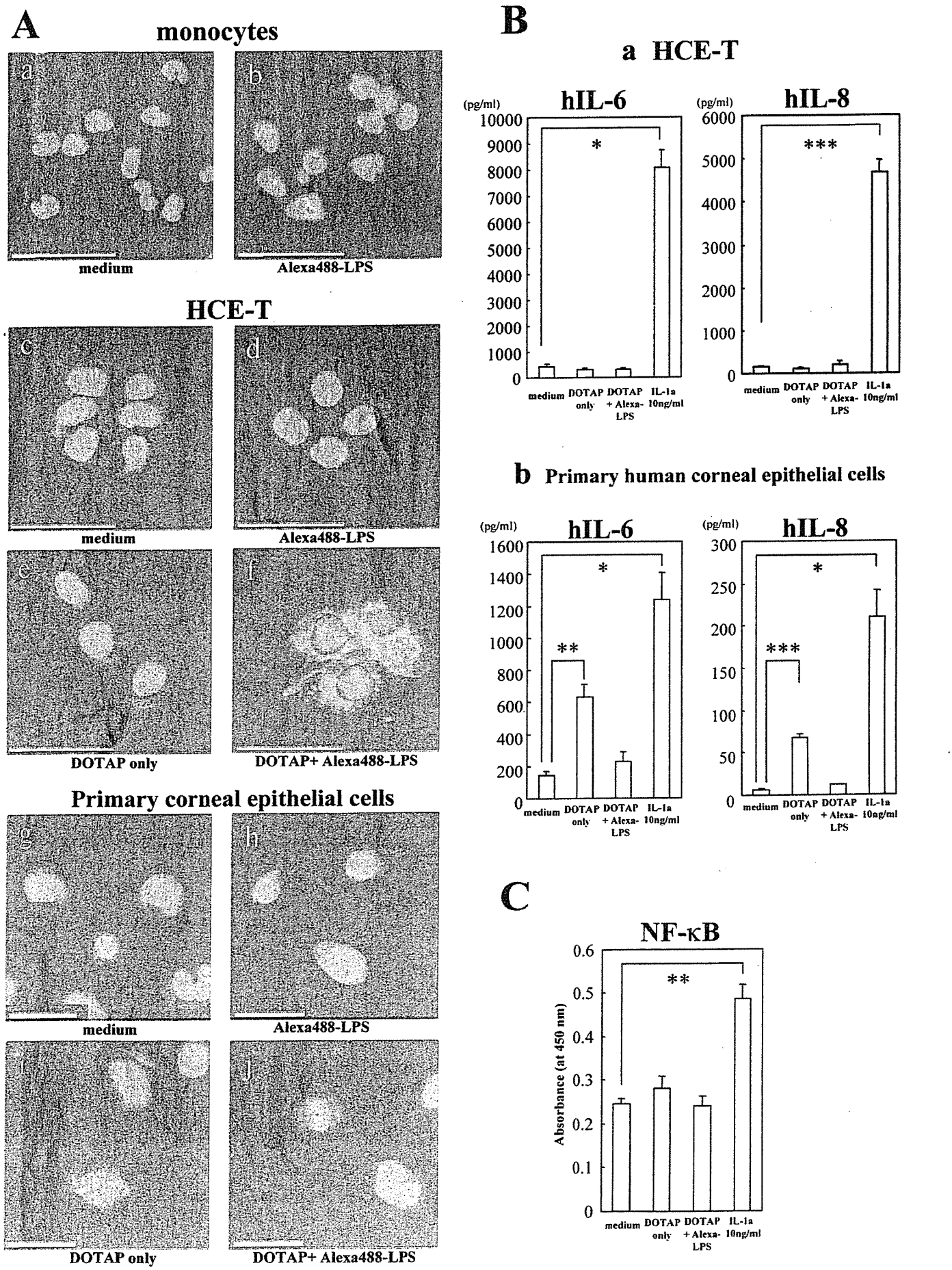


FIGURE 5. Immunohistochemical analysis for the detection of cytoplasmic TLR2 and TLR4 in human corneal epithelium. Slides of tissue sections were fixed with methanol for 30 min and then stained with PE-conjugated mouse anti-human TLR2 (TL2.1) or TLR4 (HTA125) mAbs or isotype control mouse IgG2a for 24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


A
monocytes
a
b
medium
Alexa488-LPS
HCE-T
c
d
medium
Alexa488-LPS
e
f
DOTAP only
DOTAP+ Alexa488-LPS
Primary corneal epithelial cells
g
h
medium
Alexa488-LPS
i
j
DOTAP only
DOTAP+ Alexa488-LPS
B
a HCE-T
(pg/ml)
hIL-6
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
(pg/ml)
hIL-8
6000
5000
4000
3000
2000
1000
0
***
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
b Primary human corneal epithelial cells
(pg/ml)
hIL-6
1600
1400
1200
1000
800
600
400
200
0
*
**
medium
DOTAP only
DOTAP + Alexa-LPS
IL-1a 10ng/ml
(pg/ml)
hIL-8
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 ± 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

We thank S. Terawaki and the members of Department of Mucosal Immunology, Osaka University, and Division of Mucosal Immunology, University of Tokyo, for their helpful discussions and suggestions. We also thank Drs. S. Akashi and K. Miyake (University of Tokyo) for their helpful discussions and suggestions.

References

1. Haynes, R. J., P. J. Tighe, and H. S. Dua. 1999. Antimicrobial defensin peptides of the human ocular surface. *Br. J. Ophthalmol. 83:737.*
2. Aswad, M. I., T. John, M. Barza, K. Kenyon, and J. Baum. 1990. Bacterial adherence to extended wear soft contact lenses. *Ophthalmology 97:296.*
3. Gudmundsson, O. G., L. D. Ormerod, K. R. Kenyon, R. J. Glynn, A. S. Baker, J. Haaf, S. Lubars, M. B. Abelson, S. A. Boruchoff, C. S. Foster, et al. 1989. Factors influencing predilection and outcome in bacterial keratitis. *Cornea 8:115.*
4. Doyle, A., B. Beigi, A. Early, A. Blake, P. Eustace, and R. Hone. 1995. Adherence of bacteria to intraocular lenses: a prospective study. *Br. J. Ophthalmol. 79:347.*
5. Hara, J., F. Yasuda, and M. Higashitsutsumi. 1997. Preoperative disinfection of the conjunctival sac in cataract surgery. *Ophthalmologica 211(Suppl. 1):62.*
6. Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann. 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. *Cell 86:973.*
7. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394.*
8. Medzhitov, R., and C. Janeway, Jr. 2000. Innate immune recognition: mechanisms and pathways. *Immunol. Rev. 173:89.*
9. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085.*
10. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443.*
11. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem. 274:17406.*
12. Hornung, V., S. Rothenfusser, S. Britsch, A. Krug, B. Jahrsdorfer, T. Giese, S. Endres, and G. Hartmann. 2002. Quantitative expression of Toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol. 168:4531.*
13. Abreu, M. T., P. Vora, E. Faure, L. S. Thomas, E. T. Arnold, and M. Arditi. 2001. Decreased expression of Toll-like receptor-4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. *J. Immunol. 167:1609.*
14. Abreu, M. T., E. T. Arnold, L. S. Thomas, R. Gonsky, Y. Zhou, B. Hu, and M. Arditi. 2002. TLR4 and MD-2 expression is regulated by immune-mediated signals in human intestinal epithelial cells. *J. Biol. Chem. 277:20431.*
15. Cario, E., D. Brown, M. McKee, K. Lynch-Devaney, G. Gerken, and D. K. Podolsky. 2002. Commensal-associated molecular patterns induce selective Toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. *Am. J. Pathol. 160:165.*
16. Cario, E., I. M. Rosenberg, S. L. Brandwein, P. L. Beck, H. C. Reinecker, and D. K. Podolsky. 2000. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing Toll-like receptors. *J. Immunol. 164:966.*
17. Hornef, M. W., T. Frisan, A. Vandewalle, S. Normark, and A. Richter-Dahlfors. 2002. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J. Exp. Med. 195:559.*
18. Becker, M. N., G. Diamond, M. W. Verghese, and S. H. Randell. 2000. CD14-dependent lipopolysaccharide-induced β-defensin-2 expression in human tracheobronchial epithelium. *J. Biol. Chem. 275:29731.*
19. Wolfs, T. G., W. A. Buurman, A. van Schadewijk, B. de Vries, M. A. Daemen, P. S. Hiemstra, and C. van't Veer. 2002. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-γ and TNF-α mediated up-regulation during inflammation. *J. Immunol. 168:1286.*
20. Schilling, J. D., M. A. Mulvey, C. D. Vincent, R. G. Lorenz, and S. J. Hultgren. 2001. Bacterial invasion augments epithelial cytokine responses to *Escherichia coli* through a lipopolysaccharide-dependent mechanism. *J. Immunol. 166:1148.*
21. Backhed, F., M. Soderhall, P. Ekman, S. Normark, and A. Richter-Dahlfors. 2001. Induction of innate immune responses by *Escherichia coli* and purified lipopolysaccharide correlate with organ- and cell-specific expression of Toll-like receptors within the human urinary tract. *Cell. Microbiol. 3:153.*
22. Uehara, A., S. Sugawara, and H. Takada. 2002. Priming of human oral epithelial cells by interferon-γ to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptidoglycans. *J. Med. Microbiol. 51:626.*
23. Uehara, A., S. Sugawara, R. Tamai, and H. Takada. 2001. Contrasting responses of human gingival and colonic epithelial cells to lipopolysaccharides, lipoteichoic acids and peptidoglycans in the presence of soluble CD14. *Med. Microbiol. Immunol. 189:185.*
24. Krisanaprakornkit, S., J. R. Kimball, A. Weinberg, R. P. Darveau, B. W. Bainbridge, and B. A. Dale. 2000. Inducible expression of human β-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect. Immun. 68:2907.*
25. Araki-Sasaki, K., Y. Ohashi, T. Sasabe, K. Hayashi, H. Watanabe, Y. Tano, and H. Handa. 1995. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Vis. Sci. 36:614.*
26. Jumblatt, M. M., and A. H. Neufeld. 1983. β-Adrenergic and serotonergic responsiveness of rabbit corneal epithelial cells in culture. *Invest. Ophthalmol. Vis. Sci. 24:1139.*
27. Hayashida-Hibino, S., H. Watanabe, K. Nishida, M. Tsujikawa, T. Tanaka, Y. Hori, Y. Saishin, and Y. Tano. 2001. The effect of TGF-β1 on differential gene expression profiles in human corneal epithelium studied by cDNA expression array. *Invest. Ophthalmol. Vis. Sci. 42:1691.*
28. Ohta, N., T. Hiroi, M. N. Kweon, N. Kinoshita, Jang, M. H., T. Mashimo, J. Miyazaki, and H. Kiyono. 2002. IL-15-dependent activation-induced cell death-resistant Th1 type CD8 $\alpha\beta^{+}NK1.1^{+}$ T cells for the development of small intestinal inflammation. *J. Immunol. 169:460.*
29. Takeuchi, O., K. Takeda, K. Hoshino, O. Adachi, T. Ogawa, and S. Akira. 2000. Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. *Int. Immunol. 12:113.*
30. Yura, M., I. Takahashi, M. Serada, T. Koshio, K. Nakagami, Y. Yuki, and H. Kiyono. 2001. Role of MOG-stimulated Th1 type "light up" (GFP^{+}) $CD4^{+}$ T cells for the development of experimental autoimmune encephalomyelitis (EAE). *J. Autoimmun. 17:17.*
31. Renard, P., I. Ernest, A. Houbion, M. Art, H. Le Calvez, M. Raes, and J. Remacle. 2001. Development of a sensitive multi-well colorimetric assay for active NFκB. *Nucleic Acids Res. 29:E21.*
32. Eldstrom, J. R., K. La, and D. A. Mathers. 2000. Polycationic lipids translocate lipopolysaccharide into HeLa cells. *BioTechniques 28:510.*
33. Dana, M. R. 2004. Corneal antigen-presenting cells: diversity, plasticity, and disguise: the Cogan lecture. *Invest. Ophthalmol. Vis. Sci. 45:722.*
34. Kim, J. M., L. Eckmann, T. C. Savidge, D. C. Lowe, T. Witthoft, and M. F. Kagnoff. 1998. Apoptosis of human intestinal epithelial cells after bacterial invasion. *J. Clin. Invest. 102:1815.*
35. Mowat, A. M. 2003. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat. Rev. Immunol. 3:331.*
36. Hentschel, U., U. Dobrindt, and M. Steinert. 2003. Commensal bacteria make a difference. *Trends Microbiol. 11:148.*
37. Song, P. I., T. A. Abraham, Y. Park, A. S. Zivony, B. Harten, H. F. Edelhauser,

S. L. Ward, C. A. Armstrong, and J. C. Ansel. 2001. The expression of functional LPS receptor proteins CD14 and Toll-like receptor 4 in human corneal cells. *Invest. Ophthalmol. Vis. Sci. 42:2867.*

38. Solomon, A., M. Rosenblatt, D. Li, Z. Liu, D. Monroy, Z. Ji, B. Lokeshwar, and S. Pflugfelder. 2000. Doxycycline inhibition of interleukin-1 in the corneal epithelium. *Invest. Ophthalmol. Vis. Sci. 41:2544.*
39. Del Vecchio, P. J., and J. B. Shaffer. 1991. Regulation of antioxidant enzyme expression in LPS-treated bovine retinal pigment epithelial and corneal endothelial cells. *Curr. Eye Res. 10:919.*
40. Dighiero, P., F. Behar-Cohen, Y. Courtois, and O. Goureau. 1997. Expression of inducible nitric oxide synthase in bovine corneal endothelial cells and keratocytes in vitro after lipopolysaccharide and cytokines stimulation. *Invest. Ophthalmol. Vis. Sci. 38:2045.*
41. Sekine-Okano, M., R. Lucas, D. Rungger, T. De Kesel, G. E. Grau, P. M. Leuenberger, and E. Rungger-Brandle. 1996. Expression and release of tumor necrosis factor-α by explants of mouse cornea. *Invest. Ophthalmol. Vis. Sci. 37:1302.*
42. Shams, N. B. K., M. M. Sigel, and R. M. Davis. 1989. Interferon-γ, *Staphylococcus aureus*, and lipopolysaccharide/silica enhance interleukin-1β production by human corneal cells. *Reg. Immunol. 2:136.*
43. Schultz, C. L., D. W. Morck, S. G. McKay, M. E. Olson, and A. Buret. 1997. Lipopolysaccharide induced acute red eye and corneal ulcers. *Exp Eye Res. 64:3.*
44. Khatri, S., J. H. Lass, F. P. Heinzel, W. M. Petroll, J. Gomez, E. Diaconu, C. M. Kalsow, and E. Pearlman. 2002. Regulation of endotoxin-induced keratitis by PECAM-1, MIP-2, and Toll-like receptor 4. *Invest. Ophthalmol. Vis. Sci. 43:2278.*
45. Schultz, C. L., A. G. Buret, M. E. Olson, H. Ceri, R. R. Read, and D. W. Morck. 2000. Lipopolysaccharide entry in the damaged cornea and specific uptake by polymorphonuclear neutrophils. *Infect. Immun. 68:1731.*
46. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol. 3:667.*
47. Randow, F., and B. Seed. 2001. Endoplasmic reticulum chaperone gp96 is required for innate immunity but not cell viability. *Nat. Cell. Biol. 3:891.*

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

Hiroshi Kiyono and Satoshi Fukuyama

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

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

Division of Mucosal Immunology, Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. Correspondence to H.K. e-mail: kiyono@ims.u-tokyo.ac.jp
doi:10.1038/nri1439

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

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

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

Distinct features of NALT organogenesis

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

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

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

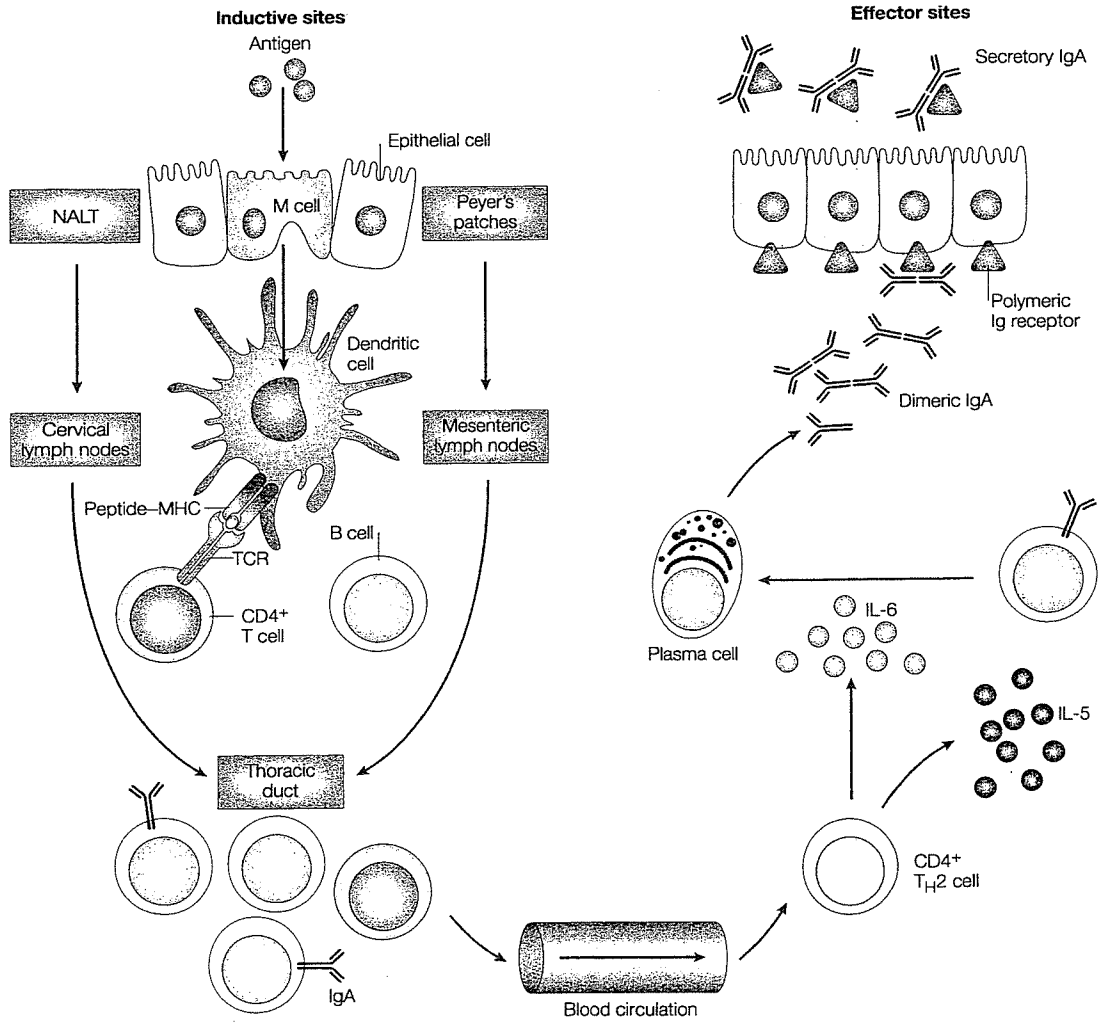


Figure 1 | **The common mucosal immune system.** Luminal antigens are transported to the nasopharynx-associated lymphoid tissue (NALT) and Peyer's patches through microfold (M) cells that are present in the epithelium overlying NALT and Peyer's-patch follicles. Dendritic cells process and present antigens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

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

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

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

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

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

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

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

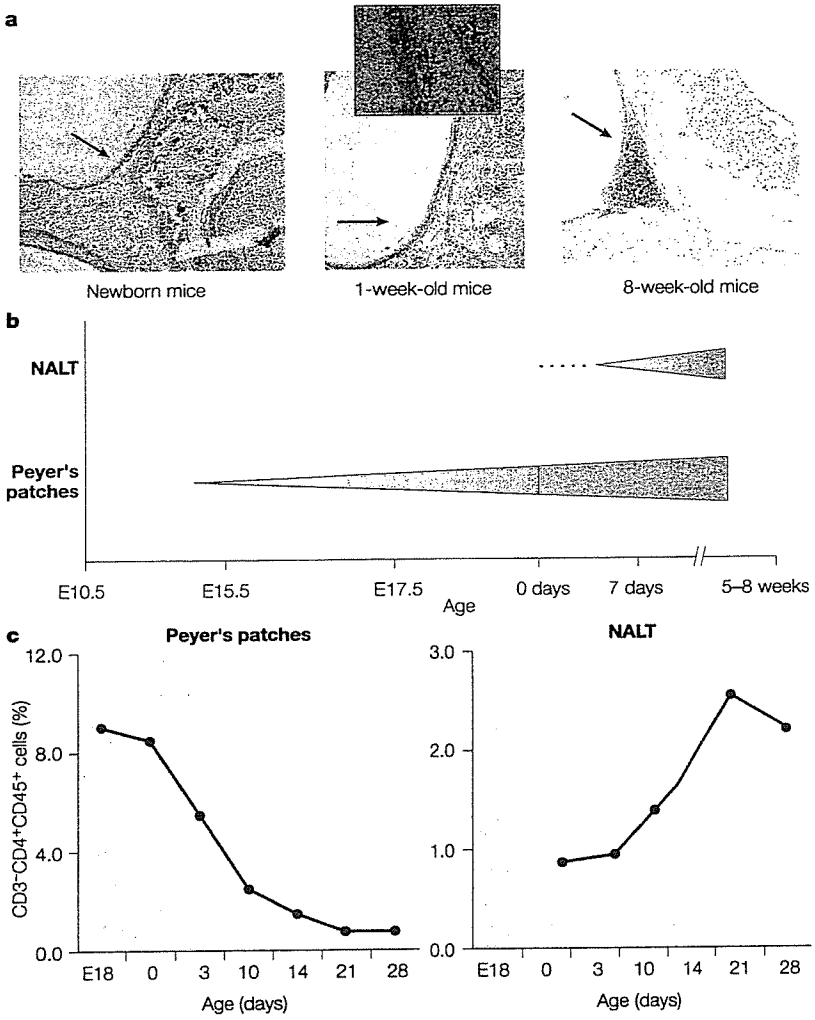


Figure 2 | **Chronological differences between NALT- and Peyer's-patch tissue genesis.** **a** | Nasal tissue from newborn mice (day 0) is characterized by an absence of peripheral-node addressin (PNAD)-expressing high endothelial venules (HEVs). The NASOPHARYNX-ASSOCIATED LYMPHOID-TISSUE (NALT) ANLAGEN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

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

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

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

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

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

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

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

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

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

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

the genes that is responsible for the induction of these $CD3^-CD4^+CD45^+$ inducer cells[30]. Not surprisingly, deletion of the *Id2* gene completely impaired the genesis of all secondary lymphoid tissues, including both NALT and Peyer's patches[5,30]. $CD3^-CD4^+CD45^+$ inducer cells were shown to accumulate at the site of NALT formation after birth[5] (FIG. 2), thereby clarifying the role of these cells in the induction of NALT development. To directly show that $CD3^-CD4^+CD45^+$ cells are responsible for the genesis of NALT, fetal liver cells were adoptively transferred from wild-type $Id2^{+/+}$ mice to newborn $Id2^{-/-}$ mice. Seven days after this transfer, $CD3^-CD4^+$ cells were observed to have migrated to the site of NALT formation, and 7 weeks after transfer, a NALT-like structure was detected[5]. These findings are the first to show directly *in vivo* that $CD3^-CD4^+CD45^+$ inducer cells are essential for the initiation of organogenesis of secondary lymphoid tissues (such as NALT).

The transcriptional regulator ROR-γ has also been shown to be required for the development of $CD3^-CD4^+CD45^+$ inducer cells[29,32]. Deletion of the gene that encodes ROR-γ suppressed Peyer's-patch and lymph-node organogenesis[29,33]. However, NALT development has been reported in ROR-γ-deficient mice[31]. This might indicate that although NALT and Peyer's patches have inducer cells of the same phenotype — that is, $CD3^-CD4^+CD45^+$ — those inducer cells can be classified into two distinct groups on the basis of their dependence on ROR-γ and ID2 (FIG. 4). We think that a population of IL-7R-expressing $CD3^-CD4^+CD45^+$ inducer cells that are essential for Peyer's-patch tissue genesis are regulated by both ROR-γ and ID2, whereas a subset of inducer cells that lack IL-7R expression and are required for NALT genesis are regulated by ID2 but not ROR-γ (FIG. 4) — although this has not been proven experimentally. So, the two $CD3^-CD4^+CD45^+$ inducer-cell populations for NALT and Peyer's-patch organogenesis might be determined or programmed at the level of the transcriptional regulator ROR-γ (FIG. 4). In addition, it is also possible that a population of $CD3^-CD4^+CD45^+$ inducer cells is absent in both $Id2^{-/-}$ mice and $Ror\text{-}\gamma^{-/-}$ mice, and instead, another as-yet-undefined cell population — which can substitute for the classical inducer cells during the formation of NALT — is present in $Ror\text{-}\gamma^{-/-}$ mice but not $Id2^{-/-}$ mice. Further studies are required to investigate these possibilities and others.

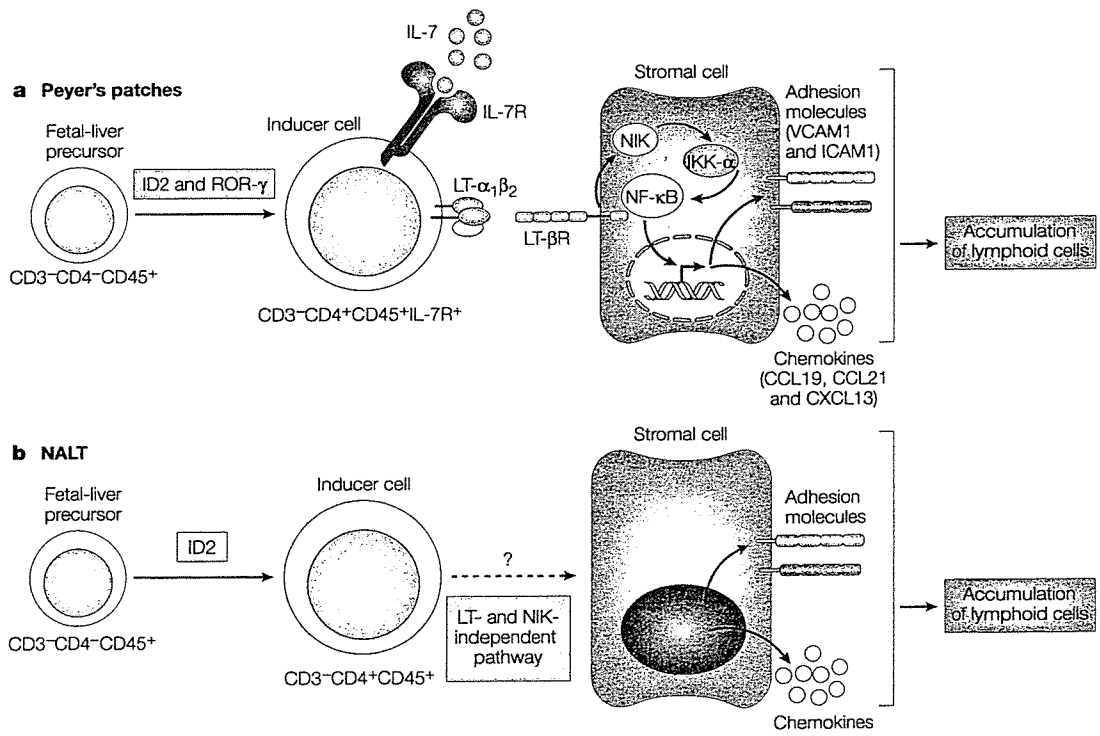


Figure 3 | **Comparison of the organogenesis programme of NALT and Peyer's patches.** $CD3^-CD4^+CD45^+$ cells are considered to be the common inducers of secondary lymphoid tissue. ID2 (inhibitor of DNA binding 2) is indispensable for the induction and differentiation of these inducer cells from their fetal-liver precursors (which have the phenotype $CD3^-CD4^-CD45^+$). **a** | For Peyer's patches, after activation through the interleukin-7 receptor (IL-7R) or TRANCE (tumour-necrosis-factor-related activation-induced cytokine), these $CD3^-CD4^+CD45^+$ cells express the lymphotoxin-$\alpha_1\beta_2$ ($LT\text{-}\alpha_1\beta_2$) heterotrimer, which then binds to the LT-β receptor (LT-βR) displayed on stromal cells and induces signal transduction through NIK (nuclear factor-κB (NF-κB)-inducing kinase). In turn, NIK promotes the expression of adhesion molecules and/or chemokines. These homing molecules trigger the accumulation of lymphoid cells at the site of Peyer's patches. So, the IL-7R- and LT-βR-mediated signals are essential for the tissue genesis of Peyer's patches. **b** | The development of $CD3^-CD4^+CD45^+$ cells in nasopharynx-associated lymphoid tissue (NALT) also requires ID2; however, the initiation of NALT organogenesis is independent of signalling that involves the IL-7R, $LT\text{-}\alpha_1\beta_2$–LT-βR interactions and NIK. CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; ICAM1, intercellular adhesion molecule 1; IKK-α, inhibitor of NF-κB (IκB) kinase-α; ROR-γ, retinoic-acid-receptor-related orphan receptor-γ; VCAM1, vascular cell-adhesion molecule 1.

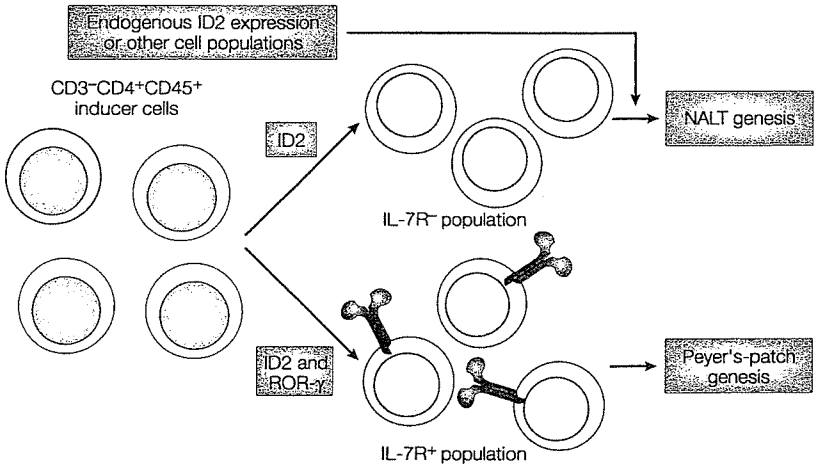


Figure 4 | **Model for the induction of organogenesis of NALT and Peyer's patches by two subsets of CD3⁻CD4⁺CD45⁺ inducer cells.** CD3⁻CD4⁺CD45⁺ cells differentiate from fetal-liver progenitors. We propose that both ROR-γ (retinoic-acid-receptor-related orphan receptor-γ) and ID2 (inhibitor of DNA binding 2) are essential for the generation of interleukin-7 receptor (IL-7R)-expressing CD3⁻CD4⁺CD45⁺ cells for the induction of Peyer's-patch organogenesis. By contrast, the generation of the IL-7R⁻CD3⁻CD4⁺CD45⁺ inducer cells that are involved in nasopharynx-associated lymphoid tissue (NALT) organogenesis is regulated by ID2 but not ROR-γ. However, this model remains to be tested experimentally, and other possibilities exist (see main text). NALT genesis in ID2-deficient mice can be initiated by the adoptive transfer of CD3⁻CD4⁺CD45⁺ cells from wild-type mice, but the maturation of NALT formation is incomplete. This indicates that other cell populations, or the endogenous expression of ID2 at the site of NALT, might be required for the full maturation of NALT.

WALDEYER'S RING
Human nasopharynx-associated lymphoid tissues, including the palatine tonsils and adenoids, which are considered to have an important role in the induction and modulation of mucosal immunity in the upper respiratory tract.

MIDDLE CONCHA
Bony plate that extends from the central section of the lateral wall of the nasal cavity.

T_H0 CELLS
Precursors of T helper 1 (T_H1) cells and T_H2 cells, which produce both interferon-γ and interleukin-4. This T-cell population has the capacity to become T_H1- and/or T_H2 cells.

CLASS-SWITCH RECOMBINATION
Molecular alteration of the constant-region gene of the immunoglobulin heavy chain (C_H) that leads to a switch in expression from the Cμ (or Cδ) region to one of the other C_H genes. This leads to a switch in the class of the immunoglobulin that is displayed on the cell-surface of the B cell (and that subsequently differentiating plasma cells produce) — from IgM (or IgD) to IgG, IgA or IgE — without altering the specificity of the immunoglobulin.

Immunological features of NALT

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered analogous to WALDEYER'S RING in humans[34,35]. Also, in a recent study, a NALT-like structure of lymphocyte aggregates that form follicles was identified in human nasal mucosa, particularly in the MIDDLE CONCHA of children less than two years of age[36], indicating that an equivalent to mouse NALT can develop in humans. NALT consists of follicle-associated epithelium (FAE), HEVs, and T-cell- and B-cell-enriched areas. Antigen-sampling M cells are present in the epithelium of NALT, which is specialized for antigen uptake similar to the FAE of Peyer's patches[7,37]. Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are also found in NALT[38]. So, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens that are delivered to the nasal cavity. For example, the intranasal administration of reovirus resulted in the formation of germinal centres in NALT, leading to the clonal expansion of antigen-induced IgA⁺ B cells and the subsequent generation of reovirus-specific IgA in the respiratory and intestinal tracts[39]. Moreover, reovirus-specific CTLs were also induced in NALT with a high frequency. These findings show that NALT can be a potent inductive site for the mucosal immune system. In addition to the induction of positive immune responses, the nasal deposition of antigen has been shown to be effective for the induction of systemic unresponsiveness — a form of mucosally induced tolerance[40]. So, NALT has been shown to be involved in the generation of positive- and negative-regulatory signals for the induction of antigen-specific immunity and tolerance respectively. The cellular and molecular contributions of the immunocompetent cells present in NALT to the generation of tolerance to mucosally exposed antigens are unknown. Because little is known about the induction of nasally induced tolerance, we focus here on the role of NALT in the induction of protective immunity.

T_H0 environment. Characterization of the mRNA that encodes T_H1 and T_H2 cytokines in CD4⁺ T cells isolated from mouse NALT revealed a dominant cytokine profile of T_H0 CELLS, indicating that these T cells are capable of becoming T_H1 or T_H2 cells immediately after antigen exposure of the nasal tract[41–43]. CD4⁺ T cells isolated from NALT of naive wild-type mice are T_H0 cells[42], so they can become either T_H1 or T_H2 cells depending on the identity of the nasally administered antigen. Nasal delivery of protein antigens (such as bacterial cell-wall components or virus-associated antigens) together with cholera toxin as a mucosal adjuvant induces antigen-specific T_H2-type responses that promote the generation of antigen-specific IgA-producing B cells, both in the nasal passages and at distant mucosal effector sites, including the genito-urinary, respiratory and intestinal tracts[41,44,45]. By contrast, intranasal vaccination with antigen-expressing recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG) results in T_H1-cell-mediated immunity[43].

IgA class switching. Peyer's patches have long been thought to be the sites for the initiation of CLASS-SWITCH RECOMBINATION (CSR) of μ- to α-gene expression in the gastrointestinal tract, because they contain all of the cellular and microarchitectural elements that are required for the generation of IgA-committed B cells, including germinal-centre-containing B-cell follicles, a FOLLICULAR DC network and an interfollicular T-cell area[1,46,47]. The germinal-centre region contains a high frequency of IgM⁺B220⁺ B cells that express activation-induced cytidine deaminase (AID), which is essential for μ- to α-gene conversion[48]. In an early study, it was shown that incubation of IgM⁺IgA⁻ B cells isolated from Peyer's patches in the presence of the cytokine transforming growth factor-β (TGF-β) resulted in the generation of IgM⁻IgA⁺ B cells[49–51]. These post-switch IgA-committed B cells then migrated to mucosal effector tissues (such as the intestinal lamina propria), a process mediated by a group of homing and chemokine receptors and their ligands (such as MADCAM1–$\alpha_4\beta_7$-integrin and CCL25–CCR9 interactions)[52,53] (discussed later). In the intestinal lamina propria, these cells became IgA⁺ plasma cells in the presence of the IgA-enhancing cytokines IL-5, IL-6 and IL-10 (REFS 50,54–58). So, it was generally accepted that organized lymphoid structures of MALT, such as Peyer's patches, function as the inductive sites for generating IgA-committed B cells through μ- to α-gene CSR, whereas the diffuse tissues of the intestinal lamina propria function as effector sites for the production of IgA[1,2] (FIG. 1). However, the finding that IgA class switching can occur in the intestinal lamina propria without involvement of

the Peyer's patches[59] cast these assumptions into doubt. Stromal-cell-derived TGF-β present in the intestinal lamina propria was shown to trigger IgM$^+$B220$^+$ cells to switch to IgA$^+$ B cells[59]. Mice with a deficiency in the programmed-inflammation-associated cytokine LT-α do not form Peyer's patches, so the levels of IgA responses are reduced compared with those of wild-type mice[60]. However, reconstitution of *Lt-α*$^{-/-}$ mice with LT-expressing bone-marrow cells, or transplantation of an intestinal segment from recombination-activating gene (*Rag*)$^{-/-}$ mice to *Lt-α*$^{-/-}$ mice, resulted in the recovery of IgA responses[60]. These findings imply that at least some IgA-committed B cells can develop, even in the absence of Peyer's patches. *Lt-α*$^{-/-}$ mice have also been shown to be capable of inducing antigen-specific IgA responses to orally administered *Salmonella typhimurium*, despite the absence of organized lymphoid tissues associated with the mucosal compartment[61]. Although these findings provide supporting evidence that the environment of the diffuse lamina propria region is self-sufficient for the μ- to α-gene CSR that leads to the generation of IgA-committed B cells, it is also possible that unidentified, programmed-inflammation-independent miniature lymphoid aggregates, and recently characterized isolated lymphoid follicles[62], have a role in mounting IgA responses in cases of Peyer's-patch deficiency.

The finding that IgA-specific CSR can occur in diffuse mucosal effector tissues indicates that organized mucosal tissue is not essential for the generation of IgA-committed B cells in the digestive tract[59], although this new view is still controversial[63,64]. Because it has been shown that intestinal IgA is produced by two groups of cells, B1 cells and B2 CELLS[63,65,66], an interesting possibility is that IgA-specific CSR of B1 cells does not require organized lymphoid structures, whereas these structures are essential for IgA-isotype switching in B2 cells. In support of this view, we have shown that most B cells in gut-associated lymphoid tissues are B2 cells, whereas B1 cells are located preferentially in the intestinal lamina-propria region[66]. So, we think that the initial antigenic stimulation for the triggering of IgA-isotype switching in B2 cells might be provided by antigen sampling through M cells that are located in the dome epithelium (or FAE) of Peyer's patches. By contrast, the IgA-specific CSR process that occurs for B1 cells might be triggered by antigens sampled through newly identified villous M cells that are located adjacent to lamina-propria regions that do not contain observable lymphoid-like structures[67].

Because NALT is of similar importance for the initiation of IgA$^+$ B-cell responses as Peyer's patches, we have investigated whether IgA-isotype switching can also occur in the diffuse tissue of the nasal passage. IgM$^+$B220$^+$ B cells, which are a prerequisite for CSR, were found in the organized inductive sites (NALT) but were mostly absent from the diffuse effector tissues (nasal passage) of the respiratory mucosal immune system[68]. Similarly, IgM$^+$B220$^+$ B cells were observed in the organized Peyer's patches of the intestinal tract but not in the intestinal lamina propria. So, in this study, IgM$^+$B220$^+$ B cells that are preconditioned to undergo IgA class switching are selectively located in the organized mucosa-associated inductive tissues of NALT and Peyer's patches[68]. This finding was confirmed by the molecular analysis of IgA CSR-associated mRNA specific for AID, Iα-Cμ CIRCULAR TRANSCRIPTS and Iμ-Cα TRANSCRIPTS. Because the expression of AID and the Iα-Cμ circular transcript are upregulated preferentially during μ- to α-gene conversion and then quickly down-regulated, these molecular events are considered to be a hallmark of B cells that are undergoing IgA class switching[69]. The expression of Iμ-Cα transcripts indicates the completion of IgA-specific CSR[59]. This analysis showed that the expression of AID-, Iα-Cμ circular transcript- and Iμ-Cα transcript-specific mRNA was restricted to the organized mucosal inductive tissues of NALT and Peyer's patches but was not found in the diffuse effector tissues of the nasal passage and intestinal lamina propria[68]. Furthermore, these organized mucosal lymphoid tissues are known to be associated with B2 cells[66]. So, these findings indicate that the IgA class switching, at least for B2 cells, requires the organized lymphoid structures of NALT and Peyer's patches in the aero-digestive tract. NALT was also recently shown to be an important site for the generation of memory B cells, which produce high-affinity IgA[70]. Taken together, these findings show that NALT contains all of the immunocompetent cells that are required for the induction and regulation of antigen-specific T_H1- or T_H2-cell-mediated responses and B-cell immune responses.

Differences between NALT- and Peyer's-patch-initiated immune responses. NALT and Peyer's patches are thought to have similar immunological characteristics and biological functions, as well as to contain the same types of resident immunocompetent cell. So, similar to oral immunization, nasal immunization can stimulate antigen-specific T_H1- or T_H2-cell-mediated responses and IgA responses in distant mucosal effector tissues[1,2,41,43–45]. However, in general, NALT-targeted immunization effectively induces antigen-specific immunity in the respiratory and reproductive tissues, whereas Peyer's-patch-targeted immunization promotes the generation of protective immunity in the gastrointestinal-tract tissues[1,2]. Further support for a compartmentalized CMIS was provided recently when it was shown that nasal immunization induces the expression of high levels of CCR10 and $\alpha_4\beta_1$-integrin by IgA-committed B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts, which express the corresponding ligands, CCL28 and VCAM1 (REFS 71,72). By contrast, orally induced IgA-committed B cells express CCR9 and CCR10, as well as $\alpha_4\beta_7$- and $\alpha_4\beta_1$-integrins, so they migrate to sites such as the small intestine, which express CCL25 and/or CCL28 together with MADCAM1 and/or VCAM1 (REF. 73).

So, despite NALT and Peyer's patches both belonging to the mucosal immune system, the subtle differences that we have discussed indicate that the tissue genesis and biological functions of NALT and Peyer's patches might differ because of their anatomically and

FOLLICULAR DC (FDC). Cell with a dendritic morphology that is present in lymph nodes. These cells display on their surface intact antigens that are held in immune complexes, and B cells present in the lymph node can interact with these antigens. FDCs are of non-haematopoietic origin and are not related to dendritic cells.

B2 CELLS IgM^{low}IgD^{hi}MAC1$^-$B220^{hi}CD23$^+$ cells that originate from bone marrow and are distributed to mucosal and systemic immune compartments for the continuous secretion of antibodies with high affinity and fine specificity.

Iα-Cμ CIRCULAR TRANSCRIPTS Circular DNA molecules that are present in activated B cells and consist of Iα (intervening region-α) and Cμ genes. They are a hallmark of B cells that are in the process of IgA class switching.

Iμ-Cα TRANSCRIPTS Germline transcripts that can be detected in IgA-committed B cells after class switching.