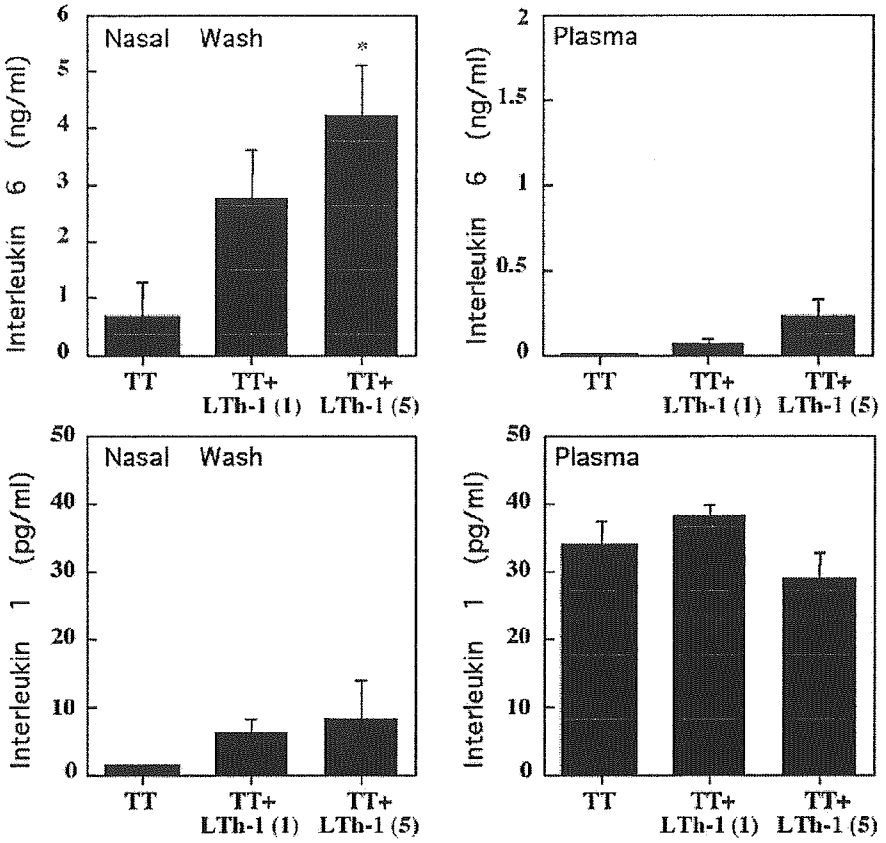

FIG. 8. Inflammatory cytokine expression in the nasal tract after nasal application of TT with or without LTh-1. Nasal washes and plasma were collected 12 h after nasal application of TT (10 μg) alone, TT and LTh-1 (1) (1.0 μg), or TT and LTh-1 (5) (5.0 μg). The levels of IL-6 and IL-1β in nasal washes and plasma were measured by ELISA. Indicated are the means plus standard errors of the mean for IL-6 and IL-1β of five mice per group. The asterisk indicates significantly elevated cytokine levels ($P < 0.05$) when LTh-1 given with TT was compared with TT given alone. The results are representative of three separate experiments.

epithelium in humans using nasal drops, while in mice this would be very reproducible (13). It could be argued that because of the above-outlined reasons nasal sprays would more consistently target the olfactory epithelium in humans than nasal drops.

The observation that nCT significantly reduces TT accumulation in NALT 3 h after nasal application compared to TT alone or TT plus nLTh-1 (Fig. 4) is interesting from the perspective that exposure to a low dose of soluble protein is associated with induction of a Th2-type T helper cell response (6, 20, 37, 47). The induction of potent Th2-type helper activity specific for antigens codelivered with nCT (33, 50) or mCT (30, 51, 53, 54) and the induction of a mixed Th1/Th2 response to antigen coadministered with nLTh-1 (2, 7, 42), mLTh-1 (2, 7), or LTIIb (34) coincide with decreased antigen accumulation in NALT with a strong Th2 response but not with the mixed Th1/Th2 response (Table 1). For example, antigen accumulation in NALT is approximately sixfold lower with nCT than with nLTh-1. It will be interesting to see in future studies whether this altered antigen level will translate into an altered cytokine environment in the NALT for induction of a TT-specific immune response.

In summary, the redirection of a vaccine protein into the olfactory tissues by enterotoxin-based mucosal adjuvants following nasal administration is associated with reactogenicity in the nasal mucosa. The differential accumulation of TT protein in NALT when administered with nCT or nLTh-1 may have consequences for the induced TT-specific T helper cell responses. The parameters controlling antigen redirection into the ON/E include ADP-ribosyltransferase activity of the A subunit and GM1 ganglioside binding by the B subunit. Thus, redirection of vaccine antigen into the ON/E by enterotoxin-based mucosal adjuvants, such as nCT and nLTh-1, clearly requires both ADP-ribosyltransferase activity and targeting of GM1 gangliosides.

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Prenatal Blockage of Lymphotoxin β Receptor and TNF Receptor p55 Signaling Cascade Resulted in the Acceleration of Tissue Genesis for Isolated Lymphoid Follicles in the Large Intestine[1]

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Signaling by lymphotoxin (LT) and TNF is essential for the organogenesis of secondary lymphoid tissues in systemic and mucosal compartments. In this study, we demonstrated that the progeny of mice treated with fusion protein of LTβR and IgGFc (LTβR-Ig) or LTβR-Ig plus TNFR55-Ig (double Ig) showed significantly increased numbers of isolated lymphoid follicles (ILF) in the large intestine. Interestingly, double Ig treatment accelerated the maturation of large intestinal ILF. Three-week-old progeny of double Ig-treated mice showed increased numbers of ILF in the large intestine, but not in the small intestine. Furthermore, alteration of intestinal microflora by feeding of antibiotic water did not affect the increased numbers of ILF in the large intestine of double Ig-treated mice. Most interestingly, mice that developed numerous ILF also had increased levels of activation-induced cytidine deaminase expression and numbers of IgA-expressing cells in the lamina propria of the large intestine. Taken together, these results suggest that ILF formation in the large intestine is accelerated by blockage of LTβR and TNFR55 signals in utero, and ILF, like colonic patches, might play a role in the induction of IgA response in the large intestine. *The Journal of Immunology,* **2005, 174: 4365–4372.**

The gut-associated lymphoid tissues are characterized as the initiation sites for the induction of IgA-mediated immunity and mucosally induced tolerance (1, 2). The mucosal immune system possesses a network of lymphoid organs that are composed of inductive sites (e.g., Peyer's patches (PP))[3] and effector sites (the intraepithelial and the lamina propria (LP) region) (1, 2). It had been believed that PP is the major inductive site for the initiation of Ag-specific IgA responses to a variety of exogenous Ag (3, 4); however, we and others have demonstrated that PP contribute to, but are not essential for, the induction of Ag-specific mucosal IgA responses (5–7). A recent study revealed the existence of isolated lymphoid follicles (ILF) in the small intestine that resemble PP in terms of architecture and cellular composition (8). The fact that ILF possess germinal centers and an overlying follicle-associated epithelium (FAE) containing M cells suggests their possible role as mucosal inductive sites (8).

Lymphotoxin (LT), a TNF family member, can be found in two forms: a membrane-bound heterotrimer and a soluble homotrimer (9, 10). The membrane-bound heterotrimer is comprised of two β-chains and one α-chain (LTα1β2) and is a ligand for LTβR, while the soluble homotrimer (LTα3) is ligand for both TNFR55 and TNFR75 (11, 12). Unlike the LTα trimer and TNF, which are secreted proteins, LT$\alpha\beta$ remains membrane bound and is expressed on the restricted hemopoietic lineage, particularly by T cells, B cells, and NK cells (13). The interaction of LT$\alpha\beta$ with LTβR is the critical molecular event triggering secondary lymphoid organ genesis and controlling spleen organization. For example, congenital lack of LTα, LTβ, or LTβR genes disrupted PP and lymph node (LN) organogenesis, and altered splenic architecture as characterized by the absence of distinct T and B cell areas and disruption of the marginal zone (14–17). Furthermore, administration of LTβR-Ig fusion protein to mice during the selected time window of embryogenesis disrupted LN and PP formation in the progeny (18, 19), suggesting that the molecular interaction of membrane-bound LT with LTβR during the gestational period is essential for the initiation of LN and PP development. In contrast to PP, a recent study demonstrated that ILF formation was not influenced by the blockage of LTβR signaling with LTβR-Ig fusion protein during gestation (8). However, no ILF was found in LT$\alpha^{-/-}$ or *aly/aly* mice, implying that ILF do require signals dependent on LT and NF-κB-inducing kinase, a critical downstream signaling molecule associated with LTβR, postgestation (8). It has recently been confirmed that, unlike PP formation, ILF formation requires LT-LTβR interaction in adulthood, as well as TNFR55-mediated signaling for their maturation (20).

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[3] Abbreviations used in this paper: PP, Peyer's patch; AID, activation-induced cytidine deaminase; CP, colonic patch; FAE, follicle-associated epithelium; ILF, isolated lymphoid follicle; LI-ILF, large intestinal ILF; LN, lymph node; LP, lamina propria; LT, lymphotoxin; PNA, peanut agglutinin.

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An additional component of the gut immune system is the colonic patch (CP). The cytoarchitectural components and immune functions of CP and PP were remarkably similar, despite differences in the surrounding environment of mucosa and luminal microbial exposure (21). The presence of organized lymphoid tissue with M cells and germinal centers in CP suggests that Ag uptake and recognition can take place in the rectum (22, 23). Similar to the PP, in utero treatment with LTβR-Ig fusion protein depleted CP formation in progeny (23). These results suggested that PP and CP were developmentally and functionally related components of the small intestine and large intestinal (colonic) immune systems, respectively. In addition to CP, it was shown that ~50 ILF were dispersed throughout the large intestine of BALB/c mice (8). Recently, it was reported that in utero treatment of mice with LTβR-Ig and TNFR55-Ig fusion proteins caused an increase in the number of submucosal lymphoid patches in the large intestine (24). This suggests that ILF in the small and large intestine are developmentally similar, although little else is known about these immunological structures and functions. In particular, the function of ILF in the large intestine and the precise contribution of LTβR and TNFR55 for their genesis, maturation, and the subsequent induction of IgA responses remain to be elucidated.

In this study, we provide several new findings regarding the unique contribution of the inflammatory cytokines LT and TNF in the genesis and function of ILF in the large intestine. In particular, the tissue genesis signals provided by the cytokine receptors of LTβR and TNFR55 are essential for the postnatal development of large intestinal ILF (LI-ILF). Our present findings suggest that the receptors behave as negative regulators for the genesis of LI-ILF because the blockage of prenatal LT/LTβR and TNF/TNFR55 signaling cascades accelerated the formation and maturation of ILF in the large intestine. Secondly, environmental factors, such as microflora-associated Ags, did not affect the formation and maturation of ILF in the large intestine. Finally, ILF in the large intestine play an important role for IgA^+ B cell development.

Materials and Methods

Mice

Timed pregnant BALB/c mice were purchased from Japan CLEA. These mice were maintained in the experimental facility under pathogen-free conditions in the Research Institute for Microbial Diseases at Osaka University and International Vaccine Institute and received sterilized food (certified diet MF; Oriental Yeast) and tap water ad libitum. TNF and LTα double-knockout ($TNF/LT\alpha^{-/-}$ mice; 129 × C57BL/6) mice were kindly provided by H. Bluethmann (Roche Center for Medical Genomics, Basel, Switzerland) (25). Germfree mice (BALB/c Yit) were kindly provided by H. Funabashi (Yakult Central Institute for Microbiological Research, Japan).

Fusion proteins and treatment protocol

Proteins comprised of the extracellular domain of either murine TNFR55 or LTβR fused to the hinge, C_H2, and C_H3 domains of human IgG1 (LTβR-Ig, TNFR55-Ig, and LFA-3-Ig, respectively) were used in our studies, as described elsewhere (19, 26, 27). Timed pregnant mice were injected i.v. with 200 μg of LTβR-Ig and/or 200 μg of TNFR55-Ig on gestational days 14 and 17, as described previously (5, 19). In some experiments, progeny of mice treated i.v. with LTβR-Ig and TNFR55-Ig on gestational period were further injected i.p. with 20 μg of LTβR-Ig, TNFR55-Ig, or human IgG1 (control) at weekly intervals from 7 days after birth and 50 μg of each Ig fusion protein from 4 wk old until age of 6 wk.

Cell purification

The mononuclear cells from CP and ILF of the large intestine were obtained with modified method, as described previously (8). In brief, the large intestine was opened longitudinally along the mesenteric wall, and mucus and feces were vigorously washed in the RPMI 1640 medium and wiped with filter paper. Subsequently, a section of intestine ~30 mm long was pasted on a plastic culture dish. The structures of CP and ILF are both circular in appearance, but the CP are larger than triple diameter of the ILF. Morphologically, the center of CP forms protruding configuration, and thus CP appears as dome-shaped tissue under a transillumination seromicroscope (Olympus TH3). In contrast, the ILF are recognized as flat shape. Blind test was conducted to count the number of ILF by three independent investigators by a transillumination seromicroscope. Then CP were taken with two sharp forceps and isolated, and a tiny fragment of ILF was isolated using a sharp needle (23 gauge; inner diameter, μm). CP and ILF were separately digested with collagenase (type IV, 0.5 mg/ml in RPMI 1640 including 2% FBS; Sigma-Aldrich) for 20 min in a 37°C incubator. This step was repeated until the architecture of tissue was totally disrupted. The single cell suspensions were pooled, washed, and placed on a discontinuous 40 and 70% Percoll gradient (Pharmacia). After centrifugation for 20 min at 600 × *g*, the cells were collected from the interface (8). To isolate the LP lymphocytes from the large intestine, mononuclear cells were dissociated using the collagenase digestion method after removal of CP and ILF, as described previously (28).

Flow cytometric analysis

A single lymphoid cell suspension was incubated with anti-FcγRII/III mAb (BD Pharmingen) and stained with FITC- or PE-conjugated anti-B220, CD3, IgD, IgM, or IgA mAbs (BD Pharmingen). The other aliquots of cells were incubated with each isotype control mAb, including rat IgG2b or rat IgG2a (BD Pharmingen). The profiles were analyzed using FACScan with CellQuest software (BD Biosciences). Reactivity with peanut agglutinin (PNA) was demonstrated using biotinylated PNA (Vector Laboratories), followed by streptavidin-PE.

Histochemical analysis

For the H&E staining, the large intestine was fixed in 4% paraformaldehyde and embedded in paraffin. The tissues were cut into 5-μm sections and stained with H&E (28). The sections were mounted and viewed under ×20 optics using a digital light microscope. Each of the images was analyzed with Photoshop (Adobe Systems). For the immunohistochemical study, freshly obtained large intestine was rapidly frozen in OCT embedding medium (Tissue-Tek) and stored at −80°C until processing (28). Cryostat sections (5 μm) were fixed in ice-cold acetone for 10 min, dried, and preblocked with anti-FcγRII/III mAb (BD Pharmingen) in PBS. Cells were stained with FITC-conjugated anti-CD11c mAb (BD Pharmingen) and PE-conjugated anti-B220 or CD3 mAbs (BD Pharmingen). The other aliquots of cells were incubated with each isotype control mAb, including hamster IgG, rat IgG2b, or rat IgG2a (BD Pharmingen). IgA-containing cells were visualized by FITC-conjugated anti-IgA mAb (BD Pharmingen). The sections were mounted and viewed under a dual red/green filter by confocal microscopy (Bio-Rad). Each of the images was analyzed with Photoshop (Adobe Systems) in a consistent manner, followed by overlying of the green and red images in the screen mode.

Scanning electron microscope analysis

For scanning electron microscopy analysis, large intestinal fragments of the double Ig-treated mice were cleaned of mucus and fixed in 2% glutaraldehyde and 2% paraformaldehyde in PBS containing 100 mM HEPES for 1 h at room temperature. After being washed with PBS, specimens were treated with 1% osmium tetroxide for 1 h at room temperature and then dehydrated in graded ethanol solution. Dehydrated tissues were critical point dried with CO_2, and sputter coated and observed with a scanning electron microscope (Hitachi).

Treatment with antibiotic water

For the antibiotic treatment, each group of 6-wk-old progenies was given antibiotics in drinking water for a period of 4 wk. The antibiotic water contained 500 mg/L ampicillin, 1 g/L neomycin sulfate, and 2 g/L of streptomycin (29).

RT-PCR

A standard quantitative RT-PCR protocol was used for the study for GAPDH-based quantitative RT-PCR (30). Total RNA was extracted from mononuclear cells isolated from LP of the large intestine or CP of naive mice or ILF of double Ig-treated mice by using the RNeasy mini kit (Qiagen), according to the manufacturer's protocol. A total of 1 μg of total RNA was reverse transcribed into cDNA using *Taq* Man reverse transcription kit (Applied Biosystems). Activation-induced cytidine deaminase (AID) mRNA levels were measured by real-time quantitative PCR method performed on the ABI PRISM 7500 (Applied Biosystems). For each treatment, two distinct amplifications were conducted in parallel to amplify

AID cDNA and GAPDH cDNA. The amplification reactions were performed in 25 μl vol containing 100 ng of cDNA per treatment, 12.5 μl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems), and 1.25 μl of 20× Assays-on-Demand Gene Expression probe for AID (Applied Biosystems) or TaqMan GAPDH probe (Applied Biosystems). AID mRNA levels from each treatment were normalized to the corresponding amount of GAPDH mRNA levels. Water controls and samples without PCR mixtures were set up to eliminate the possibility of significant DNA contamination.

ELISPOT assay for total IgA Ab-forming cells

An ELISPOT assay was adopted to detect total numbers of IgA Ab-forming cells in the large intestine, as described previously (31).

Statistics

The data are expressed as the mean ± SE and compared using *t* test in Microsoft Excel Program.

Results

In utero blockade of LTβR- and TNFR-mediated signals induces the accelerated formation of ILF in the large intestine of mice

Using LTβR- and TNFR55-Ig fusion proteins as soluble antagonists, we tested whether LTαβ- and TNF-mediated signals influenced the formation of organized lymphoid tissues in the large intestine. For this purpose, mice were treated with TNFR55-Ig or LTβR-Ig or LTβR-Ig plus TNFR55-Ig (double Ig) fusion protein at gestational days 14 and 17. The exposure to LTβR-Ig or double Ig during the gestation period disrupted CP formation in the progeny, which confirmed our previous finding (23). Unexpectedly, however, careful light microscopy and H&E staining analysis revealed that numerous ILF existed throughout the mucosa of the large intestine of 6-wk-old mice treated in utero with LTβR-Ig or with double Ig fusion proteins (Fig. 1*A*). These changes in ILF development were not seen in the small intestine of these treated mice (6 wk old), but were unique to the large intestine. Interestingly, the progeny of mice treated with double Ig fusion protein in utero possessed more numerous ILF of larger size in the large intestine than those treated with LTβR-Ig alone. When the total number of ILF in the whole large intestine was counted under light microscopy, ~250 ILF were found in the progeny of mice treated with double Ig during gestation (Fig. 1*B*). In contrast, the numbers of ILF in control Ig-treated mice were 50 per large intestine (Fig. 1*B*). When mice were treated with TNFR55-Ig alone, no significant changes were seen in the total number of ILF in the large intestine. In all mice, ILF were preferentially located in the distal region of the large intestine (data not shown). An average of total recovered cell numbers of ILF isolated from the large intestine of the progeny of mice treated with double Ig fusion protein was 10-fold higher than control Ig-treated mice (3.0×10^6 vs 0.3×10^6) and 3-fold higher than LTβR-Ig-treated mice (3.0×10^6 vs 1.0×10^6). These observations suggest that ILF formation in the large intestine was accelerated by blockage of prenatal LTβR-mediated signals, and their maturation was further enhanced by the coblockage of TNFR-mediated signals during the selected gestational period.

Postnatal blockade of LTβR-mediated signals inhibits the accelerated formation of ILF in the large intestine of mice

To establish an exact role of TNFR and LTβR signaling after birth on the accelerated formation of ILF in the large intestine, the

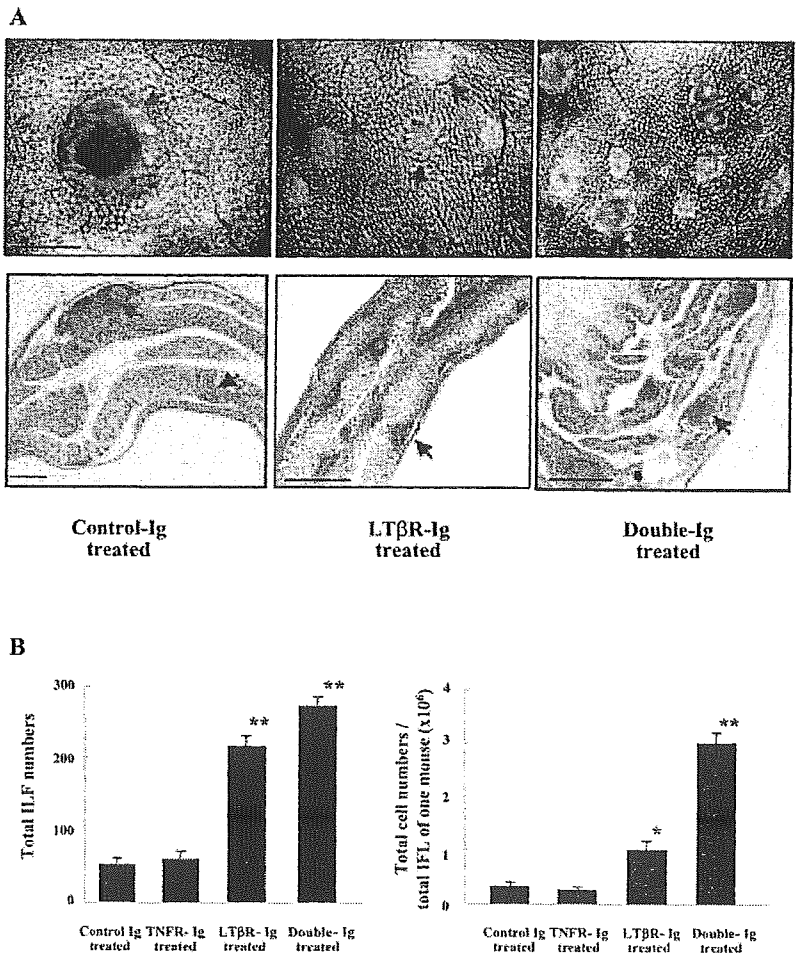


FIGURE 1. Effects of prenatal blockage of LT/LTβR and TNF/TNFR55 signal on the formation of ILF in the large intestine. Timed pregnant BALB/c mice were injected i.v. with TNFR55-Ig or LTβR-Ig and/or TNFR55-Ig (double IgG1) on gestational days 14 and 17. Purified human Ig was used as control Ig. *A*, Morphology of colonic patch (red arrow) and ILF (blue arrow) in the large intestine of each 6-wk-old progeny. The large intestine was opened longitudinally along the mesenteric wall, and a ~30-mm-long length of intestine was pasted onto the plastic culture dish. The picture was taken under the stereomicroscopy (*upper*). For the H&E staining, the large intestine was fixed in 4% paraformaldehyde and embedded in paraffin. The sections were viewed under ×20 optics with a digital light microscope (*below*). The bar indicates 50 μm. *B*, Total number of ILF in a whole large intestine was counted under a light microscope (*left*). Each ILF was then taken off with a sharp needle, and mononuclear cells were isolated and counted (*right*). The results are expressed as the mean ± SE from three mice per group and from a total of three experiments. *, $p < 0.05$, and **, $p < 0.01$ when compared with the number of ILF in the large intestine of control Ig-treated mice.

progenies of double Ig-treated mice on gestational period were further injected i.p. with LTβR-Ig or TNFR55-Ig or control Ig at weekly intervals from 1 wk after birth to age of 6 wk. Although the numbers of ILF were slightly lower, no significant difference was detected on the numbers of ILF in the large intestine of TNFR55-Ig-treated progenies compared with the control Ig-treated one. Interestingly, however, the large intestine of progenies postnatal treated with LTβR-Ig did not possess any ILF (Fig. 2). Furthermore, we have assessed the presence of ILF in the large intestine of TNF/LTα double-knockout mice that lack signaling pathways through both TNFR55 and LTβR. Interestingly, there are no ILF in the large intestine of those mice (Fig. 2). These findings indicate that the tissue genesis signaling pathway through LTβR, but not TNFR55, is required for the formation of ILF in the large intestine.

ILF in the large intestine contain B220$^+$, CD11c$^+$, and CD3$^+$ cells

To clarify the cell population in ILF of the large intestine, flow cytometric and immunohistochemical analyses were conducted using 6-wk-old progeny of mice treated in utero with control Ig or double Ig. Similar to the ILF in the small intestine (8, 20), immunohistochemical study revealed that LI-ILF were enriched with B cells (B220$^+$), with a limited frequency of dendritic stromal cells (CD11c$^+$) and T cells (CD3$^+$) (Fig. 3). Their cell population is phenotypically similar to the CP, but LI-ILF possesses more B220$^+$ cells and less CD3$^+$ cells than CP (Fig. 3). A major population of B220$^+$ cells in LI-ILF belongs to the IgD$^+$ and IgM$^+$ cells with some B220$^+$IgA$^-$ cells (Fig. 3). Similar to the phenotype of CP, LI-ILF possess detectable levels of B220$^+$IgA$^+$ cells, implying a role of LI-ILF as an inductive site for mucosal IgA responses. Interestingly, LI-ILF of mice treated with double Ig fusion protein in utero showed a higher density of CD3$^+$ cells when compared with those of control Ig-treated progeny (Fig. 3). Although the frequency of CD3$^+$ cells is increased in the treated LI-ILF, the follicle is still considered a B cell-enriched tissue because the frequency of B220$^+$ cells outnumbers CD3$^+$ cells (Fig. 3). In addition, flow cytometry analysis showed that LI-ILF of control Ig-treated progeny contained low numbers of germinal center-forming PNA$^+$ B cells; however, blockage of LTβR and TNFR55 signals during gestation enhanced the formation of germinal center-forming PNA$^+$ B cells (Fig. 3). These findings suggest that LI-ILF, similar to ILF in the small intestine, is one of key

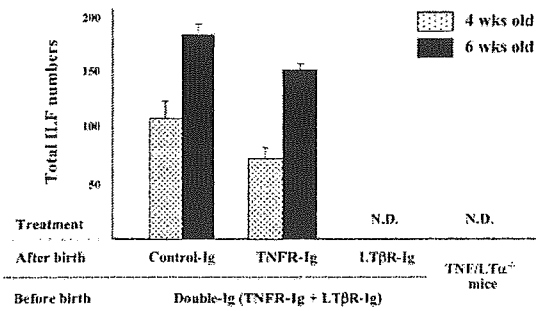
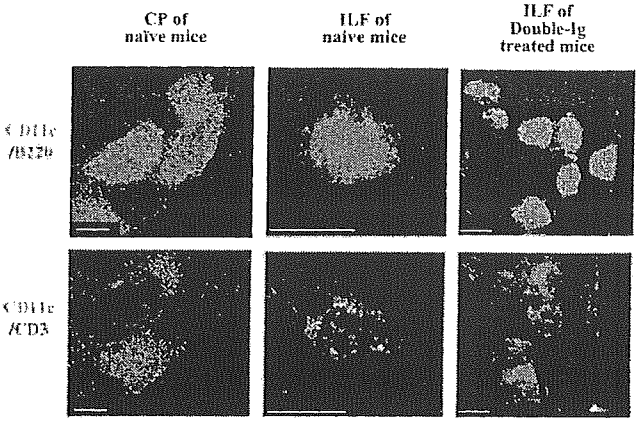


FIGURE 2. Effects of postnatal blockage of LT/LTβR or TNF/TNFR55 signal on the formation of ILF in the large intestine of the progenies treated in utero with the LTβR-Ig and TNFR55-Ig (double Ig). Progeny of mice treated i.v. with double Ig on gestational days 14 and 17 were further injected i.p. with 20 μg of LTβR-Ig or TNFR55-Ig or control Ig at weekly intervals from 7 days after birth and 50 μg of each Ig fusion protein from 4 wk old. Total numbers of ILF in a whole large intestine were counted under the stereomicroscope. The results are expressed as the mean ± SE from three mice per group and from a total of two experiments. N.D., not detectable.



Mononuclear Cells	CP	ILF	
	Control-Ig	Control-Ig	Double-Ig
B220$^+$	77.4± 2.5	86.9±3.4	86.2±3.9
CD3$^+$	13.3±1.2	5.7± 0.5	10.0± 2.2
IgD$^+$/IgM$^+$	57.5±3.8	32.0± 1.8	44.8± 2.2
PNA$^+$/B220$^+$	5.4±1.1	1.0± 0.2	6.4±1.0
B220$^+$/IgA$^+$	10.5±1.2	5.6± 1.0	6.3± 0.2

FIGURE 3. Characterization of mononuclear cells in the LI-ILF and CP of mice in utero treated with LTβR-Ig and TNFR55-Ig fusion proteins, as described in Fig. 1 legend. Immunofluorescence staining (*upper picture*) and FACS analysis of mononuclear cells isolated from CP and ILF of the large intestine (table). For the immunohistochemical study, the large intestine of 6-wk-old progenies was rapidly frozen, and cryostat sections were stained with FITC-conjugated anti-CD11c mAb and PE-conjugated anti-B220 mAb or PE-conjugated anti-CD3 mAb. The FACS results are expressed as the mean ± SE from three mice per group and from a total of two experiments. The bar indicates 50 μm.

mucosal inductive tissues for initiation of IgA responses. Furthermore, LTβR- and TNFR55-mediated signal play a critical role in the control of LI-ILF development.

Chronological analysis of escalated LI-ILF formation in double Ig-treated mice

Because we found large numbers of ILF in the large intestine of 6-wk-old progeny following the gestational blockage of LTβR- and TNFR55-mediated signals, we further examined the effect of double Ig treatment on kinetics of their development. For this purpose, we compared ILF number in the small and large intestine of progeny of mice treated with control Ig or double Ig at age of 3, 10, and 24 wk. When the control Ig-treated mice were examined, the numbers of ILF in both small and large intestine were gradually increased (Fig. 4). Interestingly, maximum numbers of LI-ILF were already reached as early as 3 wk old among progeny of mice treated with double Ig (Fig. 4). The total numbers of ILF were then maintained up to 24 wk old. In contrast, ILF in the small intestine of progeny treated with control Ig or double Ig fusion protein gradually developed and reached the maximum numbers at the age of 24 wk old. It was also noted that total numbers of small intestinal ILF were higher in mice treated with the double Ig in utero when compared with the control Ig-treated mice. The fact that the accelerated ILF formation in the large intestine by gestational blockage of LTβR and TNFR55 signals occurred very early after birth implied that their formation and development might not be influenced by exogenous environmental factors, such as gut microflora.

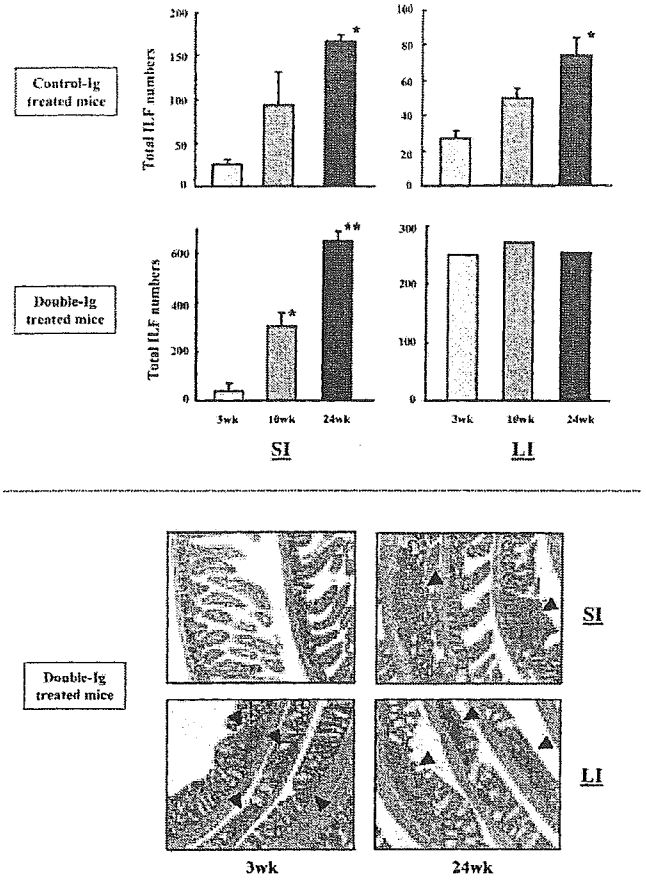


FIGURE 4. Chronological influences on the increased LI-ILF development in mice in utero treated with LTβR-Ig and TNFR55-Ig (double Ig). Numbers of ILF in the small and large intestines (SI and LI, respectively) of mice treated in utero with control Ig or double Ig fusion protein under the stereomicroscopy, as described in Fig. 1 legend. For the H&E staining, the sections were viewed under ×20 optics with a digital light microscope. The progenies were sacrificed, and the total number of ILF in a whole small and large intestine at 3, 10, and 24 wk old was counted. The results are expressed the mean ± SE from three mice per group and from a total of two experiments. *, $p < 0.05$, and **, $p < 0.01$ when compared with the number of ILF of the 3-wk-old progeny.

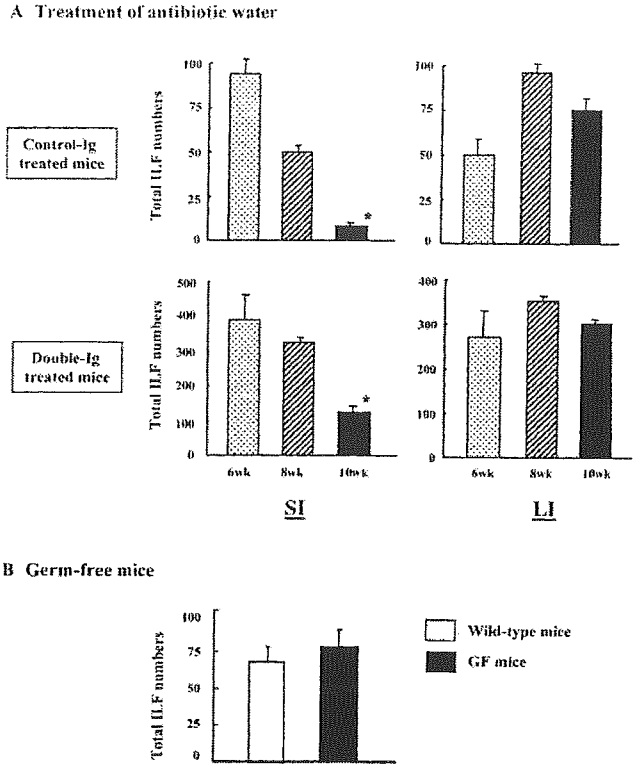


FIGURE 5. Effects of gut microflora on the formation and development of ILF in the small and large intestine. *A*, Both groups of 6-wk-old mice treated with control or LTβR-Ig and TNFR55-Ig (double Ig) fusion protein in utero were fed for 4 wk with antibiotic water containing ampicillin, neomycin sulfate, and streptomycin. At 2 wk (8 wk old) or 4 wk (10 wk old) after antibiotic treatment, the total number of ILF in a whole small and large intestine was counted under light microscope, as described in the Fig. 1 legend. *B*, The total number of ILF in the large intestine of germfree mice was counted. The results are expressed as the mean ± SE from three mice per group and from a total of three experiments. *, $p < 0.05$ when compared with non-antibiotic-treated mice.

The development of LI-ILF is independent from influences of gut microflora

Several previous studies have demonstrated a critical role of gut microflora on the ILF formation in the small intestine (8, 20, 29). To determine the potential involvement of intestinal microflora on the ILF hyperplasia in the large intestine following the prenatal blockage of LTβR and TNFR55 signals, we altered the microflora by oral administration of antibiotic water, which reduced both aerobic and anaerobic bacteria (29). Both groups of 6-wk-old progenies were fed antibiotic water for 4 wk and sacrificed at 2 and 4 wk after feeding. To see the effectiveness of the antibiotic treatment, the bacteria load was determined in the feces of control Ig- and double Ig-treated mice before and after antibiotic treatment. Essentially, no bacteria were remaining after the feeding of antibiotic water (data not shown). As shown in Fig. 5*A*, the antibiotic water treatment drastically decreased ILF formation in the small intestine in both groups of mice treated in utero with control Ig or double Ig. However, strikingly, LI-ILF formation in the double Ig-treated progeny was not influenced by the antibiotic treatment, and maximum numbers of LI-ILF were not changed even after prolonged antibiotic water treatment (Fig. 5*A*). Furthermore, the numbers of LI-ILF were comparable in the germfree mice to those of wild-type mice (Fig. 5*B*). These results indicate that development of ILF in the large intestine is not influenced by the gut bacterial flora.

LI-ILF possess M cells on the FAE region and express AID mRNA

To determine whether LI-ILF possess the ability of Ag uptake from the lumen of intestine, we examined the presence of M cells on the FAE of LI-ILF from the double Ig-treated mice. As indicated in Fig. 6*A*, LI-ILF revealed a hallmark feature of M cells, i.e., a depressed surface with short and irregular microvilli. These results suggest that LI-ILF might play as Ag sampling site as like as the other organized mucosa-associated lymphoid tissues, e.g., colonic patches.

To assess the ability of IgA class switching in the LI-ILF, the expression levels of AID mRNA, which plays an essential role in class switching recombination and somatic hypermutation of Ig genes, were determined (32). The mononuclear cells isolated from LI-ILF of mice treated with double Ig in utero expressed AID mRNA (Fig. 6*B*). In contrast to ILF, AID mRNA was not detectable in the diffused LP region of the large intestine (Fig. 6*B*). These findings suggest that the levels of μ to α class switching are increased in the large intestine of mice treated with double Ig in utero due to the maximum increase of ILF numbers. Thus, one can

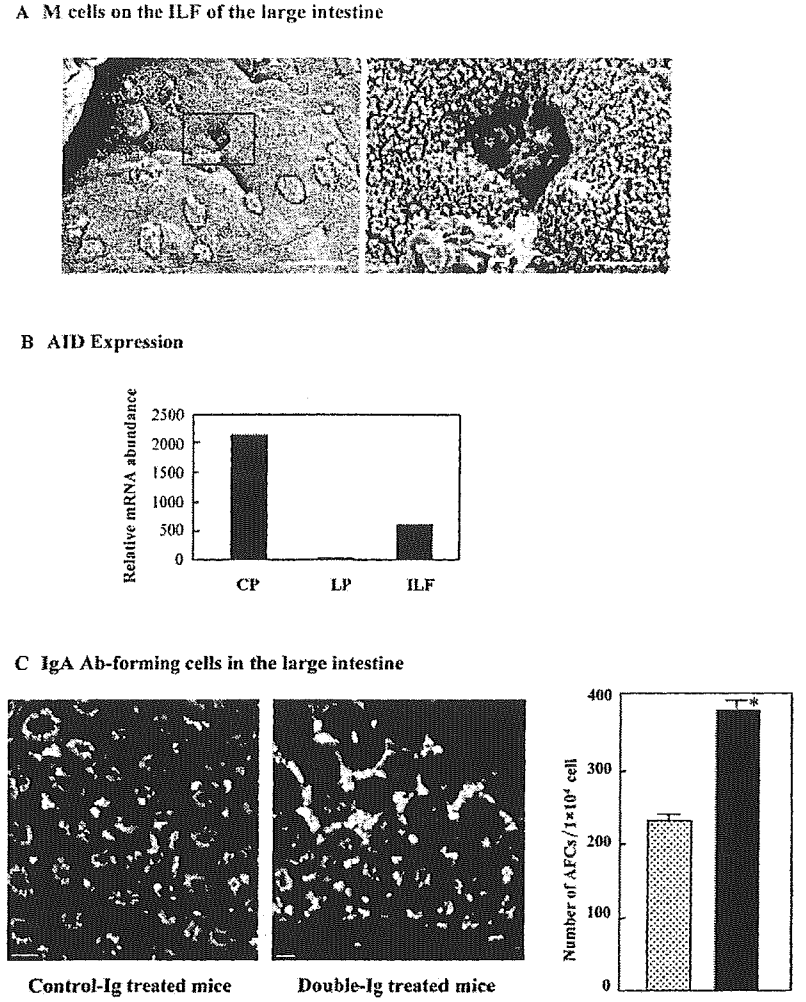


FIGURE 6. *A*, The presence of M cells on the FAE of LI-ILF of mice prenatally treated with LTβR-Ig and TNFR55-Ig (double Ig) fusion protein. *B* and *C*, The mRNA expression of AID and the number of IgA Ab-secreting cells in the large intestine of mice treated with control Ig or double Ig fusion protein in utero. A quantitative RT-PCR was performed using mononuclear cells isolated from LP in the large intestine of control Ig-treated mice and from ILF in the large intestine of double Ig-treated mice. As positive control for a quantitative RT-PCR, CP of control Ig-treated mice were adopted. For the immunohistochemical study (*C*, *left*), the large intestine of 6-wk-old progenies of both groups were rapidly frozen, and cryostat sections were stained with FITC-conjugated anti-IgA. ELISPOT assay was adopted to further confirm the number of IgA Ab-forming cells in the LP of the large intestine of mice treated with control Ig (▨) or double Ig (■) fusion protein in utero (*C*, *right*). The results are expressed as the mean ± SE from five mice per group and from a total of two experiments. The bar indicates 10 μm (*A*, *left*) and 2.5 μm (*A*, *right*).

predict the subsequent elevation of IgA-producing cells in the large intestine of these mice. The numbers of IgA-producing cells were therefore assessed, using the large intestine of the 6-wk-old mice treated in utero with control Ig or double Ig. Interestingly, increased numbers of IgA-expressing cells were detected in the LP region of the large intestine of in utero double Ig-treated mice when compared with the control mice (Fig. 6*C*, *left*). The results were also confirmed by the analysis of single cells using ELISPOT assay. The numbers of IgA-producing cells were increased in mononuclear cells isolated from the large intestine of mice treated with double Ig in utero when compared with the control Ig-treated mice (Fig. 6*C*, *right*). These results further support a notion that ILF may play a role for induction of IgA^+ B cells in the large intestine.

Discussion

In general, a family of inflammatory cytokine-mediated signals provided via LTβR and TNFR55 is considered to be critical for the organogenesis of secondary lymphoid tissue (18, 33). One can consider the organogenic steps that take place during development as a form of programmed inflammation, given what we now know about the steps underlying the genesis of tertiary lymphoid structures in chronic disease settings (34). In this study, we demonstrated that prenatal blockage of LT/LTβR signaling cascade resulted in the acceleration of ILF formation in the large intestine, and further that prenatal blockage of TNF/TNFR55 signal enhanced their maturation. The hyperplasia of LI-ILF was not due to stimulation of exogenous environmental stimuli, such as microflora Ags. Most interestingly, LI-ILF expressed AID mRNA, and therefore might be critically involved in the generation of IgA-committed B cells. Thus, our present study is the first one to show the unique characteristics of LI-ILF as IgA-inductive sites, whose development is accelerated by the blockage of LTβR and TNFR55 in utero.

Normal numbers of ILF were present in the small intestine of mice treated with LTβR-Ig fusion protein in utero, but were absent in the $LT\alpha^{-/-}$ mice and *aly/aly*$^{-/-}$ mice, suggesting that ILF formation in the small intestine did not require gestational LT/LTβR-dependent event, but needed the postnatal signals (8). In addition, a recent study demonstrated that, unlike PP, postnatal LT/LTβR signals are required for ILF formation in the small intestine, and additional TNF/TNFR55 signal and exogenous stimuli were needed for their maturation (20). In contrast, limited information is currently available on the immunological function and tissue genesis of ILF (or solitary lymphoid aggregates) in the large intestine. For example, in utero treatment with LTβR-Ig ablated the formation of CP, but scattered B cell aggregates in the mucosal layer of large intestine were retained (23). Furthermore, the progeny of mice treated with LTβR-Ig plus TNFR55-Ig fusion protein were reported to have some submucosal lymphoid patches in the large intestine (24). In the present study, we demonstrate that prenatal blockage of LT/LTβR signal enhanced the formation of LI-ILF,

and prenatal blockage of LT/LTβR plus TNF/TNFR signals further accelerated this phenomenon and resulted in extreme hyperplasia of LI-ILF. It is clear that LI-ILF used LT/LTβR and TNF/TNFR signals in a different manner than other gut-associated lymphoid tissues such as PP and CP. An interesting possibility is that LTβR- and TNFR-mediated signaling might behave as a negative regulation for the LI-ILF genesis in the gestational period.

Several recent studies demonstrated a critical involvement of gut flora on the development of ILF in the small intestine. It has been reported that normal numbers of ILF were detected in the small intestine of germfree mice (8). However, another study showed that the development of ILF in the small intestine did not occur in germfree mice, but, if germfree mice were conventionalized, modest number of mature ILF developed (20). Further augmentation of ILF formation in the small intestine was induced by in utero treatment with LTβR-Ig fusion protein (20). Fagarasan et al. (29) recently revealed that alteration of bacteria flora by antibiotic treatment abolished ILF hyperplasia and germinal center enlargement in the small intestine that was provoked by the genetic deficiency of AID. Furthermore, the number of anaerobic bacteria was 100-fold increased in the small intestines of $AID^{-/-}$ mice than $AID^{+/-}$; however, no significant changes were detected in the large intestine of $AID^{-/-}$ mice (29). Those results are consistent with our present results showing that the total number of ILF in the small intestine of naive mice and mice treated with double Ig in utero was significantly reduced by antibiotic treatment. However, the formation of LI-ILF of both naive and the double Ig-treated mice was not influenced by the antibiotic treatment. The fact that extreme hyperplasia of ILF in the large intestine of double Ig-treated mice was not due to stimulation of gut microflora suggests that bacterial Ag may not be involved in the development and maturation of LI-ILF. Hence, although the immunological nature of ILF in the small and large intestine seems identical in terms of cell populations and morphologic features, regulatory factors associated with programmed inflammation for their tissue genesis and maturation might be significantly different due to the different exogenous environments. We are investigating the exact regulatory factors that are specifically involved in the development of LI-ILF. In particular, an understanding of the identification and ontogeny of the cells required to induce local LI-ILF formation will be critical, as it appears these cells require LTβR and TNFR signals during embryogenesis that regulate their activity. Furthermore, the identity and regulation of the specific molecules critical for induction of LI-ILF need further study, as it is likely that these are further involved in the regulation of colon-associated diseases.

We have also demonstrated that ILF are abundantly developed in the large intestine in the absence of CP. These plentiful ILF that developed in CP-null large intestine could be a key site for the continuous generation of IgA-committed B cells. Interestingly, our present study showed mononuclear cells isolated from CP and ILF of normal mice, but not from intestinal LP, expressed high levels of AID mRNA that play an essential role for isotype switching recombination and somatic hypermutation of Ig. Furthermore, enhanced numbers of IgA-producing cells were noted in the LP region of large intestine of progenies with numerous numbers of ILF, but no CP by the gestational blockage of LT/LTβR and TNF/TNFR55 signaling pathways. Based upon their cell phenotype and micro- and macromorphology, it is reasonable to classify LI-ILF as an inductive site for intestinal IgA production in addition to CP. Therefore, LI-ILF and CP may form a reciprocal inductive network in mucosal immunity. Thus, the increased IgA response seen in the CP-null mice could be explained as a compensatory response in the absence of CP. Taken together, both ILF and CP in the large intestine are integrated inductive tissues that can compensate each other for the induction of mucosal IgA responses.

A possible contribution of LI-ILF to the development of colon-restricted inflammatory disease is just beginning to be understood. A previous study described that elimination of PP and CP, but not scattered aggregates of B cells by in utero treatment with LTβR-Ig fusion protein, resulted in the prevention of trinitrobenzenesulfonic acid-induced Th2 cell type colitis development (23). In contrast, another group found a more severe type of dextran sodium sulfate-induced colitis in PP- and mesenteric lymph node-null mice generated by the in utero treatment with LTβR-Ig and TNFR55-Ig fusion proteins (24). Although these mice did not possess peripheral lymphoid tissue, they found that there were submucosally located lymphoid follicles in the large intestine consisting of B and T cell areas. Furthermore, dextran sodium sulfate-induced colitis accelerated additional formation of these lymphoid follicles (24). In all cases of ulcerative colitis patients, the colonic LP contain numerous basal lymphoid aggregates composed of T and B lymphocytes and dendritic cells (35). Furthermore, these lymphoid aggregates increase in number and size with severity of disease (35). Overall, it seems likely that LI-ILF could be site for the generation of regulatory and/or pathogenic lymphocytes. Thus, both in human patients and in mouse disease models, LI-ILF hyperplasia is associated with colonic disease. Furthermore, whether ILF induces regulatory type responses or pathogenic type responses may depend on surrounding environmental and immunological conditions. To further address these questions, our murine model of LI-ILF hyperplasia will be useful to clarify their precise role in the control of large intestine-restricted diseases.

Disclosures

The authors have no financial conflict of interest.

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Intestinal γδ T Cells Develop in Mice Lacking Thymus, All Lymph Nodes, Peyer's Patches, and Isolated Lymphoid Follicles[1]

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Through analysis of athymic (*nu/nu*) mice carrying a transgenic gene encoding GFP instead of RAG-2 product, it has recently been reported that, in the absence of thymopoiesis, mesenteric lymph nodes and Peyer's patches (PP) but not gut cryptopatches are pivotal birthplace of mature T cells such as the thymus-independent intestinal intraepithelial T cells (IEL). To explore and evaluate this important issue, we generated *nu/nu* mice lacking all lymph nodes (LN) and PP by administration of lymphotoxin-β receptor-Ig and TNF receptor 55-Ig fusion proteins into the timed pregnant *nu/+* mice that had been mated with male *nu/nu* mice (*nu/nu* LNP$^-$ mice). We also generated *nu/nu aly/aly* (*aly*, *alymphoplasia*) double-mutant mice that inherently lacked all LN, PP, and isolated lymphoid follicles. Although γδ-IEL were slightly smaller in number than those in *nu/nu* mice, substantial colonization of γδ-IEL was found to take place in the intestinal epithelia of *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice. Notably, the population size of a major CD8αα$^+$ γδ-IEL subset was maintained, the use of TCR-γ-chain variable gene segments by these γδ-IEL was unaltered, and the development of cryptopatches remained intact in these *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice. These findings indicate that all LN, including mesenteric LN, PP, and isolated lymphoid follicles, are not an absolute requirement for the development of γδ-IEL in athymic *nu/nu* mice. *The Journal of Immunology,* **2005, 174: 1906–1912.**

Over the past 2 decades, it has been revealed that numerous intestinal intraepithelial T cells (IEL)[3] have cellular and behavioral characteristics distinct from those of thymus-derived peripheral T cells (1–8). In mice, IEL are enriched with TCR-γδ T cells (γδ-IEL) (9, 10), and virtually all γδ-IEL and many αβ-IEL, unlike thymus-derived CD8αβ T cells that use the ζ-chain as part of their CD3 complex, express the unique CD8αα homodimer (11–14) and can use the FcRγ-chain in place of the ζ-chain (15–17). Along these findings, growing evidence has indicated thymus-independent (TI) development of such CD8αα-expressing IEL (TI-IEL) (5, 7, 11, 12, 18). Detection of RAG-1 and RAG-2 transcripts (12, 19–22) and identification of a small number of T-lineage-committed TCR$^-$ lymphocytes in IEL from wild-type mice (2, 12, 19, 20, 23–25) supported the concept of localized development of IEL in the epithelial layer in situ. However, it should be pointed out that the original view of extrathymic generation of CD8αα$^+$ αβ-IEL is now inconsistent with the results of recent studies in which the thymus-dependent generation of every αβ-IEL, including the CD8αα-expressing subset, is unequivocally demonstrated (26, 27).

Our search (28) for anatomical sites of IEL generation revealed multiple tiny clusters filled with ~1000 c-Kit$^+$IL-7R$^+$Lin$^-$ (Lin, lineage markers) lymphohemopoietic cells in the lamina propria (LP) of the intestinal crypt (cryptopatches (CP)). Data obtained through a series of CP studies strongly indicated that CP were essential sites for the extrathymic development of precursor T cells destined to become TI-IEL (22, 28–30). Specifically, the presence of both TCR-γ and -β germline transcripts in the c-Kit$^+$IL-7R$^+$Lin$^-$ CP lymphocytes (30) has emphasized that various DNA recombination enzymes are able to approach these chromosomal segments to commence the region-specific recombinations (31–33). On the whole, these findings lend strong support to the idea that T lineage-committed precursors, which match the developmental stage of triple-negative c-Kit^{high}CD44$^+$CD25$^{low/-}$ thymocytes before pre-Tα gene transcription (34, 35), but after expression of CD3ε-specific mRNA (35, 36), are present in gut CP (30). One impediment to this conclusion has been the detection of a marginal level of RAG-2 transcripts for CP lymphocytes (22). However, the analysis of athymic (*nu/nu*), bone marrow (BM) chimeric mice revealed that the development of donor BM-derived TI-IEL proceeded through several consecutive steps (30). BM-derived TCR$^-$ IEL first appeared within villous epithelia overlying the regenerated CP filled with BM-derived c-Kit$^+$IL-7R$^+$Lin$^-$ cells. These TCR$^-$ IEL subsequently emerged throughout the epithelia, and thereafter, conversion of TCR$^-$ to TCR$^+$ IEL, the final

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[3] Abbreviations used in this paper: IEL, intestinal intraepithelial T cell; *aly*, alymphoplasia; BM, bone marrow; CP, cryptopatch; DN, double negative; iFABP, intestinal fatty acid-binding protein; IEC, intestinal epithelial cell; ILF, isolated lymphoid follicle; Lin, lineage marker; LNP$^-$, lymph node- and Peyer's patch deficient; LP, lamina propria; LT, lymphotoxin; LTβ-R, lymphotoxin-β receptor; MLN, mesenteric lymph node; PP, Peyer's patch; RORγt, retinoic acid-related orphan receptor; SCF, stem cell factor; Tg, transgenic; TI, thymus independent.

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step, took place very slowly. These results in conjunction with above-mentioned findings (2, 12, 19–25) have led us to conclude that TI-IEL complete their late maturational events, such as RAG-mediated TCR gene rearrangement, at a very slow rate in the epithelial layer in situ.

Recently, however, a new scenario for the extrathymic development of TI-IEL in *nu/nu* mice was described (37). By assessing RAG-2 expression in transgenic (Tg) *nu/nu* mice carrying a bacterial artificial chromosome encoding a GFP reporter instead of RAG-2, it was demonstrated that extrathymic T lymphopoiesis occurred mainly in mesenteric lymph nodes (MLN) and less in Peyer's patches (PP), but not in CP (37). To evaluate these new and important findings, we generated *nu/nu* mice that lacked all LN and PP by administration of lymphotoxin-β receptor (LTβ-R)-Ig and TNF-R55-Ig fusion proteins into pregnant *nu/+* mice (38) and double-mutant *nu/nu aly/aly* (*aly, alymphoplasia*) mice that lacked all LN, PP, as well as newly identified intestinal isolated lymphoid follicles (ILF) (39). We confirmed that these two kinds of mice harbored numerous $\gamma\delta$-IEL in the epithelial compartments of the small intestines and were found to retain gut CP. The significance of these findings is discussed from the viewpoint that all LN, PP, and ILF are dispensable anatomical sites for the generation of TI-IEL in the athymic *nu/nu* condition.

Materials and Methods

Mice

BALB/cA Jcl *nu/nu* (*nu/nu*), BALB/cA Jcl *nu/+* (*nu/+*), *aly/aly* Jcl mutant and C.B-17/Icr Jcl *scid/scid* (*scid/scid*) mice were purchased from CLEA Japan (Tokyo, Japan). TCR-$\gamma\delta$ (KN6)-Tg mice on the BALB/c background were described previously (40). Female KN6-Tg mice were crossed with *scid/scid* mice to generate KN6 *scid/+* mice, then they were backcrossed with *scid/scid* mice to obtain KN6 *scid/scid* mice. The presence of KN6-Tg was determined by PCR analysis of tail DNA with a set of primers to the KN6 Tg (5′-CAGATCCTTCCAGTTCATCC-3′ and 5′-CAGTCACTT GGGTTCCTTGTCC-3′), and the homozygous *scid/scid* genotype was determined by the absence of TCR-$\alpha\beta^+$ T cells in PBL. We generated transplacentally manipulated *nu/nu* and *nu/+* mice that lack all LN and PP according to essentially the same method described previously (38). In brief, timed-pregnant *nu/+* mice that had been mated with male *nu/nu* mice were i.v. injected with 200 μg of both LTβ-R-Ig and TNFR-55-Ig fusion proteins on gestational days 13 and 16. All mice used for experiments were between 8 and 18 wk of age, and absence of a thymus in various athymic mice was checked at necropsy. All animal procedures described in this study were performed in accordance with the guidelines for animal experiments of Keio University School of Medicine.

Production of nu/nu aly/aly *mice and genotyping of aly mutation*

Because *nu/nu* and *aly/aly* mothers are incapable of nursing the neonates, we used an in vitro fertilization technique (41) to produce (*nu/nu*×*aly/aly*)F_1 hybrid mice, then these heterozygous *nu/+ aly/+* mice were intercrossed to obtain *nu/nu aly/+*, *nu/nu aly/aly*, and *nu/+ aly/+ nu/+ aly/aly* littermates. To determine *aly/aly, aly/+*, and *+/+* alleles, the TaqMan assay of tail DNA was performed using the ABI PRISM 7000 sequence detection system (PerkinElmer) as previously described (42). The primer sequences for *aly* were 5′-GCCTACTGACATCCCGAGCTA-3′ (forward primer) and 5′-GCAGGACTGGGCTGGAAGA-3′ (reverse primer). The oligonucleotide probe corresponding mutant *aly* allele was 5′-AGACCG TACTGTTGAAG-3′ (FAM labeled), and the oligonucleotide probe corresponding wild-type allele was 5′-AGACCGTACCGTTGAA-3′ (VIC labeled). Underlining in sequences indicates point mutation. The 3′ end of each probe carried the quencher that suppressed the fluorescence of the reporter dyes. Each DNA sample was amplified with the TaqMan Universal master mixture containing AmpliTaq Gold DNA polymerase according to the manufacturer's instructions (Applied Biosystems). PCR conditions were 2 min at 50°C, 10 min at 95°C, 15 s at 95°C, and 1 min at 60°C for 40 cycles. During PCR, fluorescence developed when the oligonucleotide hybridized to perfectly matching DNA, and the exonuclease activity of *Taq* polymerase separated the quencher from the reporter dye. After PCR, the fluorescence yield for the two different dyes was measured and presented in a two-dimensional graph.

Antibodies

The following mAbs, described previously (22, 28–30, 39), were used. For immunohistochemical and immunofluorescence stainings: anti-$\gamma\delta$ (GL-3), anti-c-Kit (ACK-2), anti-B220 (RA3-6B2), and anti-IgA (C10-3) were used. For flow cytometric analysis, FITC-conjugated anti-$\alpha\beta$ (H57-597), anti-$\gamma\delta$ (GL-3), anti-Vγ1 (2.11; gift from Dr. S. Tonegawa, Center for Learning and Memory, MIT, Cambridge, MA), anti-Vγ4 (UC3-10A6), anti-Vγ7 (GL-1; gift from Dr. L. Lefrancois, Department of Medicine, Division of Immunology, University of Connecticut Health Center, Farmington, CT), anti-CD4 (GK 1.5), biotinylated anti-$\gamma\delta$ (GL-3), anti-B220 (RA3-6B2), anti-CD8α (53-6.7), and anti-c-kit (ACK-2), and PE-conjugated anti-CD8β (53-5.8) and anti-CD4 (GK 1.5) were used.

Immunohistochemical procedure

Longitudinally opened small intestine, ~10 mm in length, was pasted on a filter paper to form a horizontal section and then embedded in OCT compound (Tissue-Tek; Miles) at −80°C. The tissue segments were sectioned with a cryostat at 6 μm, and sections were preincubated with Block-Ace (Dainippon Pharmaceutical) to block nonspecific binding of mAbs. The sections were then incubated with hamster (anti-$\gamma\delta$) or rat (anti-c-Kit, anti-B220, or anti-IgA) mAb for 30 min at 37°C and rinsed three times with PBS, followed by incubation with biotin-conjugated goat anti-hamster IgG Ab (5 μg/ml; Cedarlane Laboratories) or with biotin-conjugated goat anti-rat IgG (5 μg/ml; Cedarlane Laboratories). Subsequently, the sections were washed three times with PBS, then incubated with avidin-biotin peroxidase complexes (Vectastain ABC kit; Vector Laboratories). Histochemical color development was achieved with Vectastain 3,3′-diaminobenzidine substrate kit (Vector Laboratories) according to the manufacturer's instructions. Finally, the sections were counterstained with hematoxylin for microscopy. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 and 0.1% NaN_3 in distilled water for 10 min at room temperature. Tissue sections incubated with either nonimmune hamster serum or isotype-matched normal rat IgG showed only minimal background staining.

Immunofluorescence procedure

Tissue segments from thymus, spleen, inguinal LN, MLN, and PP from KN6 *scid/scid* mice were embedded in OCT compound at −80°C. The small intestine of KN6 *scid/scid* mice was longitudinally opened along the mesenteric wall, then intestine, ~10 mm in length, that had been rolled to form a vertical section was embedded in OCT compound at −80°C. Cryostat tissue sections, 6-μm thick, were fixed in acetone for 10 min at room temperature, washed three times with PBS, then pretreated with Block-Ace. Subsequently, the sections were incubated with anti-c-Kit mAb (ACK-2) for 60 min at 4°C, followed by incubation with PE-conjugated goat $F(ab')_2$ anti-rat IgG (H+L) (Invitrogen Life Technologies). The sections were then incubated with anti-$\gamma\delta$ mAb (GL-3) and counterstained with FITC-conjugated goat anti-hamster IgG (H+L) (Jackson ImmunoResearch Laboratories). Finally, the sections were examined under a fluorescence microscope (Axiovert 100; Carl Zeiss) equipped with an image analysis system (Signal Analytics).

Flow cytometry

IEL were isolated according to methods described previously (22). Lymphoid cells were incubated first with biotinylated mAb, then with streptavidin-PE (BD Biosciences) and FITC-conjugated second mAb. Stained cells were suspended in staining medium (Hanks' solution without phenol red, 0.02% NaN_3, and 2% heat-inactivated FBS) containing 0.5 μg/ml propidium iodide and analyzed using FACScan with CellQuest software (BD Biosciences). Dead cells were excluded by propidium iodide gating. Three-color analysis of IEL was also performed. IEL were incubated first with anti-CD8α mAb (biotinylated), then with streptavidin-Tri-Color (Caltag Laboratories). After washing, IEL were counterstained with two combinations of two PE-conjugated mAbs (anti-CD8β and anti-CD4 mAbs) and one FITC-conjugated mAb (anti-$\gamma\delta$), respectively. Lymphoid cells were incubated with anti-FcγR II/III mAb (2.4G2) before staining to block nonspecific binding of labeled mAbs to FcR.

Results

Development of $\gamma\delta$-IEL in nu/nu *mice is independent of all LN and PP*

To explore whether MLN are essential anatomical sites for the generation of TI-IEL in athymic *nu/nu* mice (37), we generated *nu/nu* mice that lacked all LN and PP. Pregnant *nu/+* female mice that had been mated with *nu/nu* male mice were injected with

LTβ-R-Ig and TNF-R55-Ig fusion proteins according to the protocol described by Rennert et al. (38), and the presence or the absence of LN and PP was determined in the progeny at 8 wk of age under a stereomicroscope. Although PP were absent from all treated mice, markedly attenuated remnants of MLN were present in about one-fifth of them. However, in every MLN-deficient *nu/nu* and *nu/+* offspring, the development of mandibular, axillary, inguinal, and popliteal (data not shown) LN (i.e., peripheral LN) and cervical (data not shown), iliac, and sacral LN (i.e., mucosal LN) was also ablated (LNP⁻ mice; Fig. 1).

Flow cytometric analysis of IEL isolated from *nu/nu*, *nu/nu* LNP⁻, *nu/+*, and *nu/+* LNP⁻ mice was performed using anti-TCR-αβ and anti-TCR-γδ mAbs. Consistent with well-established findings (3, 4, 8, 43), the proportion of αβ-IEL to γδ-IEL was sharply reduced, and the composition of IEL not expressing either type of TCR was expanded in athymic *nu/nu* conditions regardless of the presence or the absence of all LN and PP (Fig. 2*A*). In contrast, no significant differences in absolute numbers of IEL were observed between LNP⁻ and control LNP⁺ mice (data not shown). Notably, although the population size was slightly smaller by a factor of ~1.5 compared with that in IEL from control *nu/nu* mice, a large number of γδ-IEL was detected in IEL from *nu/nu* LNP⁻ mice (Fig. 2*A*). Compartmentalization of γδ-IEL within the epithelial layer of small intestine in *nu/nu* LNP⁻ mice was also verified by immunohistochemistry (Fig. 2*B*). These results indicate

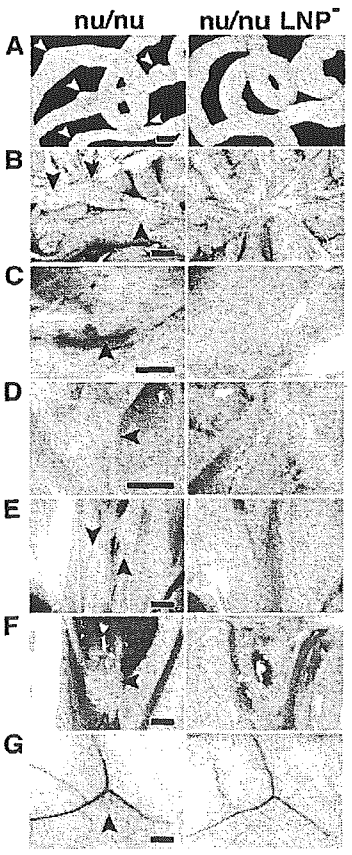

FIGURE 1. Development of PP and all LN in athymic *nu/nu* mice is ablated by in utero treatment with both LTβ-R-Ig and TNF-R55-Ig fusion proteins. PP (*A*), MLN (*B*), mandibular LN (*C*), axillary LN (*D*), iliac LN (*E*), sacral LN (*F*), and inguinal LN (*G*) are present in untreated *nu/nu* mice (arrowheads), but are undetectable in *nu/nu* mice that were treated in utero with both LTβ-R-Ig and TNF-R55-Ig fusion proteins (*nu/nu* LNP⁻ mice). Bar, 2 mm.

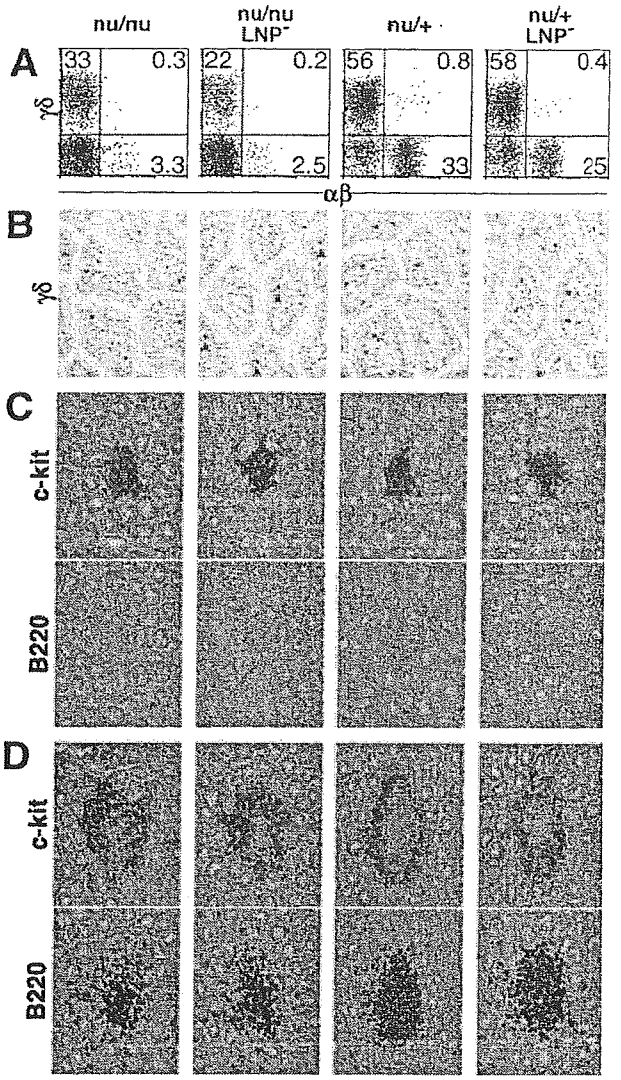

FIGURE 2. Flow cytometric analysis of IEL and immunohistochemical examination of small intestines from *nu/nu*, *nu/nu* LNP⁻, *nu/+*, and *nu/+* LNP⁻ mice. *A*, Although the population size of γδ-IEL in *nu/nu* LNP⁻ mice that lack all LN and PP is smaller than that of *nu/nu* mice, a substantial number of γδ-IEL are present in the epithelial compartment of *nu/nu* LNP⁻ mice, indicating that γδ-IEL are capable of developing in the absence of thymus, all LN including MLN, and PP. Note that the composition of αβ-IEL is reduced drastically in the athymic *nu/nu* condition compared with that in the euthymic *nu/+* condition. *B*, Representative immunohistochemical visualization of γδ-IEL in the small intestines of *nu/nu*, *nu/nu* LNP⁻, *nu/+*, and *nu/+* LNP⁻ mice (magnification, ×200). Although numbers of γδ-IEL in *nu/nu* LNP⁻ mice are decreased compared with those in *nu/nu* mice, colonization of γδ-IEL takes place in the absence of thymus, all LN including MLN, and PP (arrowheads). *C*, Representative immunohistochemical verification of CP in the small intestines of *nu/nu*, *nu/nu* LNP⁻, *nu/+*, and *nu/+* LNP⁻ mice (magnification, ×200). An average number and an approximate mass of CP filled with c-Kit⁺B200⁺ lymphocytes in these four different mice remain almost the same. *D*, Representative immunohistochemical verification of ILF in the small intestines of *nu/nu*, *nu/nu* LNP⁻, *nu/+*, and *nu/+* LNP⁻ mice (magnification, ×200). Note that a cluster of B220⁺ B cells that reside in the central region of ILF is surrounded by the layer of cells expressing c-Kit molecules.

that the development of γδ-IEL per se is independent of thymus, all LN, and PP.

With these findings in mind, we examined whether CP and ILF were present in these in utero manipulated LNP⁻ mice, because, in

contrast to PP that are already microscopically well developed just before birth (44), organogenesis of CP (28) and ILF (39) commences in early postnatal life. In fact, it was corroborated that the development of CP filled with closely packed c-Kit$^+$ lymphocytes (Fig. 2*C*) and ILF containing B220$^+$ B cell aggregation (Fig. 2*D*) remained intact in these *nu/nu* LNP$^-$ and *nu/+* LNP$^-$ mice.

Development of γδ-IEL in nu/nu aly/aly *double-mutant mice*

To ascertain the universality of the above findings, we explored the development of IEL in a mutant mouse that inherently lacked thymus, all LN, and PP, because fusion protein-treated LNP$^-$ mice might possess a minute and stereomicroscopically invisible MLN, even though this possibility appeared to be remote (38). With this purpose in mind, we generated *nu/nu aly/aly* double-mutant mice. In accordance with the earliest description (45), *nu/nu aly/aly* mice were devoid of all LN and PP (data not shown) as well as thymus. Importantly, substantial colonization of γδ-IEL in the small intestine of *nu/nu aly/aly* mice was verified by flow cytometric (Fig. 3*A*) and immunohistochemical (Fig. 3*B*) analyses. Furthermore, as inferred from our previous observations (28, 39), histogenesis of CP was detected (Fig. 3*C*), whereas development of ILF was completely blocked (Fig. 3*D*), in these double-mutant animals. Taking all of these results together (Figs. 2 and 3), neither thymus, all LN including MLN, PP, nor ILF is an absolute requirement for the development of γδ-IEL.

In this context, it is important to determine T and B cells and IgA$^+$ B cells that sojourn, respectively, in the spleen and LP of *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice, because spleen is most likely the sole organized peripheral lymphoid tissue remaining in these animals, and *nu/nu aly/aly* mice lack intestinal IgA$^+$ B cell-producing plants such as PP and ILF. In contrast to abundant B220$^+$ B cells, mature T cells were virtually absent in the spleens of *nu/nu* LNP$^-$ (Fig. 4*A*) and *nu/nu aly/aly* (Fig. 4*C*) mice. In the small intestines, however, *nu/nu* LNP$^-$ mice possessed IgA$^+$ B cells (Fig. 4*B*), γδ-IEL, CP, and well-developed ILF (Fig. 2), whereas *nu/nu aly/aly* mice possessed γδ-IEL (Fig. 3, *A* and *B*) and CP (Fig. 3*C*), but lacked IgA$^+$ B cells (Fig. 4*C*) and ILF (Fig. 4*D*). These results indicate that all LN, including MLN, PP, and ILF, are not an absolute requirement for the generation of γδ-IEL in *nu/nu* mice and that the *aly* mutation interferes the formation of PP and ILF, resulting in the impaired development of IgA$^+$ B cells in villous LP.

Phenotypic and Vγ gene usage analyses of γδ-IEL in nu/nu *LNP$^-$ and* nu/nu aly/aly *mice*

The data reported to date indicate that γδ-IEL are potentially capable of developing in *nu/nu* mice lacking all LN, PP, and ILF. In this regard, however, it is reasonable to consider the possibility that γδ-IEL generated under such harsh conditions might differ from those generated in *nu/nu* mice possessing all LN, PP, and ILF. To address this issue, we conducted flow cytometric analysis of γδ-IEL isolated from *nu/nu*, *nu/nu* LNP$^-$, and *nu/nu aly/aly* mice. Although absolute numbers of γδ-IEL were lower by a factor of 2 compared with those in *nu/nu* mice (Fig. 5*A*), the composition of the major γδ-IEL subset expressing CD8αα homodimer (Fig. 5*A*) and the Vγ1, Vγ4, and Vγ7 gene segment used by such γδ-IEL (Fig. 5*B*) remained the same in both *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice. Collectively, these results indicate that the absence of all LN, PP, and ILF exerts only a small effect on the phenotypic configuration of γδ-IEL in *nu/nu* mice.

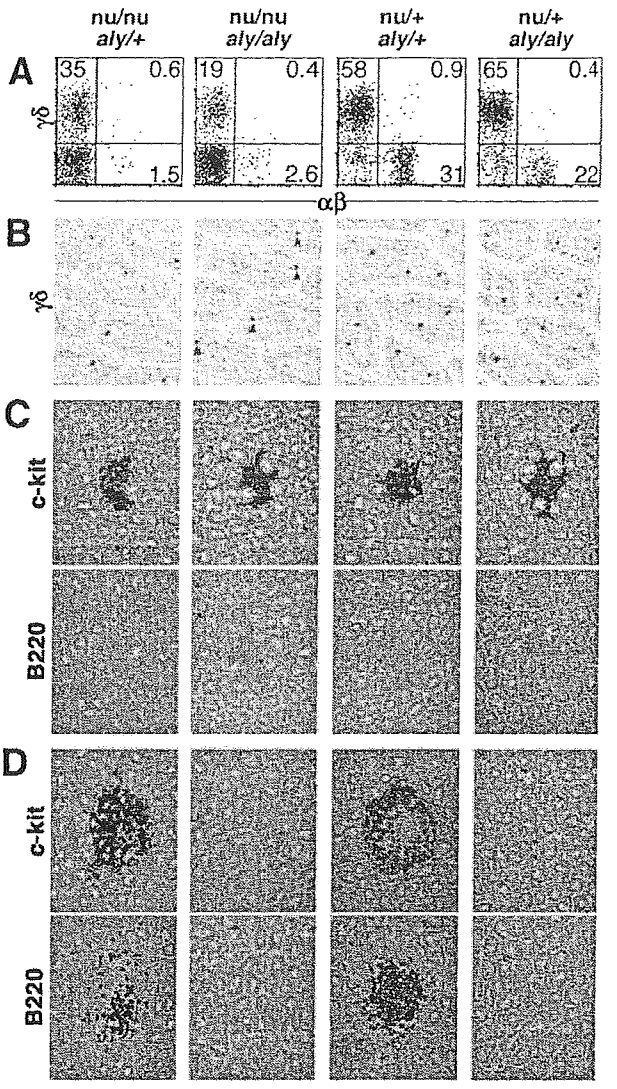

FIGURE 3. Flow cytometric analysis of IEL and immunohistochemical examination of small intestines from *nu/nu aly/+*, *nu/nu aly/aly*, *nu/+ aly/+*, and *nu/+ aly/aly* mice. *A*, Although the population size of γδ-IEL in *nu/nu aly/aly* mice that lack all LN, PP, and ILF is smaller than that in *nu/nu aly/+* mice, a significant number of γδ-IEL are present in the epithelial compartment of *nu/nu aly/aly* mice, indicating that γδ-IEL are capable of developing in the absence of thymus, all LN including MLN, PP, and ILF. Note that the composition of αβ-IEL is reduced drastically in the athymic *nu/nu* condition compared with that in the euthymic *nu/+* condition. *B*, Representative immunohistochemical verification of γδ-IEL in the small intestines of *nu/nu aly/+*, *nu/nu aly/aly*, *nu/+ aly/+*, and *nu/+ aly/aly* mice (magnification, ×200). Although numbers of γδ-IEL in *nu/nu aly/aly* mice are lower by a factor of 1.5–2 compared with those in *nu/nu aly/+* mice, a significant colonization of γδ-IEL takes place in the absence of thymus, all LN including MLN, PP, and ILF (arrowheads). *C*, Representative immunohistochemical verification of CP in the small intestines of *nu/nu aly/+*, *nu/nu aly/aly*, *nu/+ aly/+*, and *nu/+ aly/aly* mice (magnification, ×200). Although an average number of CP filled with c-Kit$^+$B220$^-$ lymphocytes in these four different mice remain almost the same, an approximate mass of CP present in *nu/nu aly/aly* and *nu/+ aly/aly* mice is slightly reduced compared with that of CP present in *nu/nu aly/+* and *nu/+ aly/+* mice. *D*, Representative immunohistochemical verification of ILF in the small intestines of *nu/nu aly/+*, *nu/nu aly/aly*, *nu/+ aly/+*, and *nu/+ aly/aly* mice (magnification, ×200). Note that ILF are undetectable throughout the small intestinal mucosa of *nu/nu aly/aly* and *nu/+ aly/aly* mice.

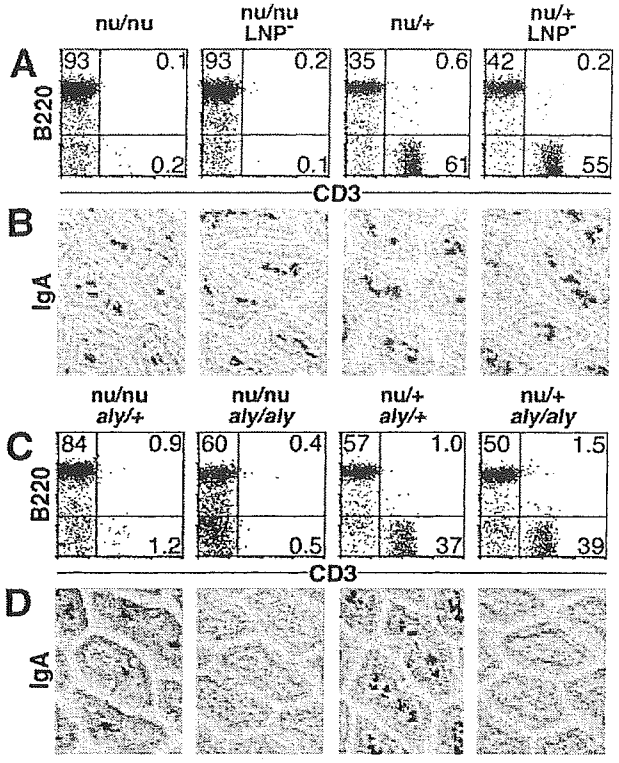

FIGURE 4. Flow cytometric analysis of splenic T and B cells and immunohistochemical verification of intestinal IgA$^+$ B cells in *nu/nu*, *nu/nu* LNP$^-$, *nu/nu aly/+*, and *nu/nu aly/aly* mice. *A*, Although mature CD3$^+$ T cells are nearly absent from the spleens of *nu/nu* and *nu/nu* LNP$^-$ mice, abundant B220$^+$ B cells are detected in the spleens of these athymic animals. Colonization of IgA$^+$ B cells in the villous LP of *nu/nu*, *nu/nu* LNP$^-$, *nu/+*, and *nu/+* LNP$^-$ mice is comparable. *B*, Only a marginal number of mature CD3$^+$ T cells are detected in the spleens of *nu/nu aly/+* and *nu/nu aly/aly* mice, and a large fraction of the remaining lymphoid cells consists of B220$^+$ B cells. In contrast, villous LP of *nu/nu aly/aly* and *nu/+ aly/aly* mice has no IgA$^+$ B cells.

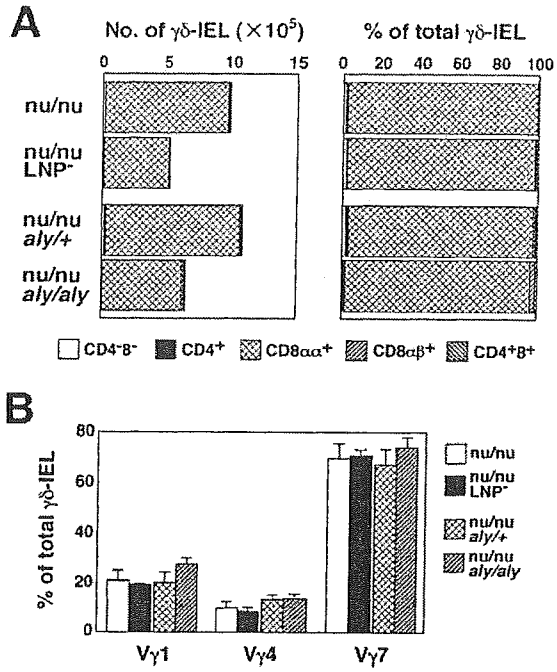

FIGURE 5. Flow cytometric analysis of γδ-IEL from *nu/nu*, *nu/nu* LNP$^-$, nu/nu *aly/+*, and *nu/nu aly/aly* mice and Vγ gene segments used by γδ-IEL in these athymic *nu/nu* mice. *A*, Three-color analysis was performed. Absolute numbers of double-negative (CD4$^-$8$^-$), single-positive (CD4$^+$, CD8αα$^+$, or CD8αβ$^+$), and double-positive (CD4$^+$8$^+$) subsets in the γδ-IEL population were calculated on the basis of total numbers of γδ-IEL. Data are the mean values from five mice per group. *B*, Two-color analysis was performed. IEL isolated from these *nu/nu* mice were incubated first with anti-TCR-γδ mAb (biotinylated), then with streptavidin-PE and FITC-conjugated anti-Vγ1, anti-Vγ4, or anti-Vγ7 mAb. The results are the mean ± SD of data obtained from four mice per group.

Development of c-Kit$^+$ γδ-IEL in scid/scid *mice expressing Tg TCR-γδ*

We have shown that IL-7 produced by intestinal epithelial cells (IEC) is important for intraintestinal development of γδ-IEL and is crucial for organization of intestinal mucosal lymphoid tissues, such as PP and CP (18). It has also been shown that c-Kit and stem cell factor (SCF) are expressed by γδ-IEL and IEC, respectively, and signaling through c-Kit/SCF is indispensable for normal development of γδ-IEL (46, 47). Thus, these previous (46, 47) and the present findings in conjunction with results obtained with BM chimeric mice (30), reinforce the idea that the development of γδ-IEL takes place in the intestinal mucosa in situ. In an attempt to confirm and visualize directly the cellular events that proceed toward gut-oriented γδ-IEL generation, i.e., c-Kit$^+$ CP cells→c-Kit$^+$ TCR-γδ T cells→c-Kit$^-$ γδ-IEL, we generated *scid/scid* mice expressing KN6-Tg TCR-γδ (48). Double-immunofluorescence analysis of small intestinal tissues containing CP highlighted these presumptive cellular events. Thus, a representative picture of jejunal tissue sections from KN6 *scid/scid* mice (Fig. 6*A*) shows that a cluster of c-Kit$^+$ cells in a CP (red) does not express TCR-γδ, and that large numbers of γδ-IELs (green) are present in the epithelial layer, especially in the epithelium adjacent to CP. Notably, quite a large number of lymphocytes expressing both c-Kit and TCR-γδ molecules (yellow or orange) is also present in the epithelial and LP compartments of villi (Fig. 6*A*). Neither clustering c-Kit$^+$ cells nor lymphocytes stained yellow/orange were detected in other lymphoid tissues, such as thymus, LN, PP, and spleen (data not shown). Flow cytometric analysis of cell surface c-Kit molecules on the gated γδ T cells confirmed that a large fraction of γδ-IEL was c-Kit positive, namely, double-positive TCR-γδ$^+$c-Kit$^+$ cells (Fig. 6*B*, *upper panel*). In contrast, abundant Tg γδ T cells residing in MLN, spleen, and thymus did not include such c-Kit-expressing, double-positive cells (Fig. 6*B*, *lower three panels*). Overall, these results support the above-described basic premise that the development of γδ-IEL takes place in the intestinal mucosa in situ. However, because KN6-Tg TCR-γδ-expressing cells have not been detected in the CP of KN6 *scid/scid* mice (Fig. 6*A*), whether CP are essential and indispensable anatomical sites in generating γδ-IEL remained highly contentious.

Discussion

Because most of the numerous lymphocytes residing in the murine IEC compartment unexpectedly turned out to be T cells (IEL), additional revelation toward the end of the last century indicated that they display phenotypic and functional characteristics distinct from those of other T cell populations and that their development does not necessarily depend on the thymus (TI-IEL) (1–18). Likewise, several lines of information have illuminated the distinctive T cell facets of human fetal intestine (49, 50), and on the basis of RAG expression, there may also be TI-IEL that develop in human (51, 52) and rat (53) intestines. Although evidence for the vestigial lymphocyte-producing function of gut mucosa is substantial, as mentioned above (2, 5, 7, 11, 12, 18–25, 49–53), recent studies

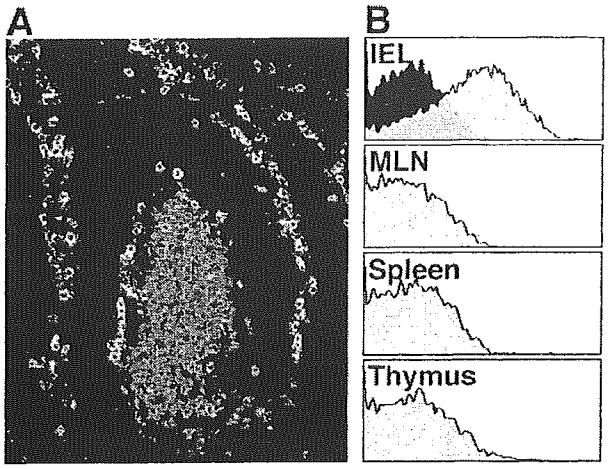

FIGURE 6. Double immunofluorescence analysis of small intestinal tissues containing CP and flow cytometric analysis of lymphocytes from KN6 *scid/scid* mice. *A*, Intestinal birthplace of intraepithelial γδ T cells (magnification, ×400). A cluster of lymphocytes in a CP is positively stained with anti-c-Kit mAb (PE), but not with anti-γδ mAb (FITC), resulting in a red color. Conversely, many IEL, especially those adjacent to CP, are positively stained with anti-γδ mAb (FITC), but not with anti-c-Kit mAb (PE), resulting in a green color. Note that a large number of lymphocytes present in the epithelial and LP compartments of villi are positively stained with anti-c-Kit mAb (PE) and anti-γδ mAb (FITC), resulting in a yellow or orange color. Neither the clusters filled with c-Kit$^+$ cells nor lymphocytes stained yellow or orange are detected in the other lymphoid organs, such as thymus, LN, and spleen. *B*, Expression of c-Kit molecules by Tg γδ T cells that colonize in the intestinal epithelial, MLN, and splenic and thymic compartments of KN6 *scid/scid* mice. IEL, MLN cells, spleen cells, and thymocytes were incubated first with anti-TCR-γδ mAb (biotinylated), then with streptavidin-PE and FITC-conjugated anti-c-Kit mAb. Profiles of c-Kit expression by the gated TCR-γδ$^+$ cell population are presented. The *dark area* in the *upper panel* depicts a FITC-positive cell profile of IEL that were stained simply with biotinylated anti-TCR-γδ mAb and streptavidin-PE (negative control). It is evident that IEL contain a large number of double-positive (TCR-γδ$^+$ c-Kit$^+$) cells, but that MLN, spleen, and thymus have no such double-positive cells.

have made it clear that CD8αα does not serve as a marker for TI development of αβ-IEL (26, 27). In contrast, because positive and/or negative selection of TCR-γδ T cells in the thymus is not as evident (26, 27), thymic dependency for functional TCR-γδ T cells is less obvious.

In an assessment of the expression of Tg-encoded GFP in place of RAG-2 protein, no evidence was obtained for a lymphopoietic process involving CP cells migrating into gut epithelium to undergo TCR gene rearrangement and maturation into αβ- and γδ-IEL even in the athymic *nu/nu* condition (37). Instead, MLN and, less efficiently, PP have been identified as the major extrathymic T cell-producing plants in athymic *nu/nu* mice, and the newly generated T cells migrate from MLN into thoracic duct lymph to reach the gut epithelia, indicating that MLN and PP are the pivotal sites in generating TI-IEL, mostly γδ-IEL (37). In this context, the development of γδ-IEL should be hampered in *nu/nu* mice that simultaneously lack MLN and PP. Our present findings argue against this scenario by showing that not only transplacentally manipulated *nu/nu* LNP$^-$ mice lacking all LN and PP, but also genetically defined *nu/nu aly/aly* mice lacking all LN, PP, and ILF harbor a substantial population of γδ-IEL (Fig. 2, *A* and *B*, and Fig. 3, *A* and *B*), although these two different mouse models may share the same confounding factors. In contrast to what was observed in *nu/nu* mice, the extrathymic pathway of IEL generation was shown to be totally repressed in the euthymic condition using the same GFP RAG-2 Tg mouse model (37). The authors proposed that all IEL, including CD8αα$^+$ IEL, in normal mice were the likely progeny of double-negative (DN) TCR-αβ$^+$ and -γδ$^+$ thymocytes and noted that extremely complex and unusual T cell characteristics of murine IEL with respect to their expression of various accessory, costimulation, activation, and adhesion markers (4, 5) might be brought about by the distinctive microenvironment of gut epithelium (37). In this context, for instance, many DN thymocytes somehow down-regulate cell surface expression of Thy-1 molecules, because a substantial fraction of IEL is Thy-1 negative, whereas most of them must up-regulate c-Kit molecules on the way to becoming IEL (46, 47) (Fig. 6). Even if all of those transfigurations (>20) are attributable to the inherent properties of gut epithelium, the biological significance as well as the molecular level of the mechanisms underlying such enigmatic cellular events remain highly contentious. It should also be pointed out that our recent findings (18) are inconsistent with this idea (37). γδ T cells are absent in IL-7$^{-/-}$ mice due to the selective blockade of TCR-γ gene rearrangements (54). Using the intestinal fatty acid-binding protein (iFABP) promoter, we reinstated the expression of IL-7 to mature IEC of IL-7$^{-/-}$ mice (iFABP-IL7) (18). Although γδ-IEL were restored in iFABP-IL7 mice as well as CP and PP, γδ T cells remained absent from all tissues, including thymus, spleen, and skin. These results clearly indicate that γδ-IEL generated in iFABP-IL7 mice are not of DN TCR-γδ$^+$ thymocyte origin and that the recombination of TCR-γ genes in TCR-precursor T cells takes place in situ with the assistance of IL-7 produced locally by IEC.

Our present findings provide compelling evidence for the development of γδ-IEL within the intestine of *nu/nu* mice that lack the thymus, all LN, PP, and ILF. It should be pointed out, however, that the population size and absolute numbers of γδ-IEL from *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice are smaller than those from the corresponding control *nu/nu* mice (Figs. 2*A*, 3*A*, and 5*A*). These features would indicate that LN and PP, in fact, contribute to γδ-IEL numbers in the *nu/nu* condition. In contrast to the results obtained in GFP RAG-2 Tg mouse model (37), our previous RT-PCR analysis of lymphocytes from normal euthymic mice showed that under conditions in which mRNA from 50 thymocytes displayed a strong signal for RAG-2 transcripts and mRNA from 6250 RAG-2$^{-/-}$ thymocytes failed to display any detectable signals, very low levels of RAG-2 transcripts were constantly detected in an amount of mRNA equivalent to 6250 IEL and CP cells (22). These findings suggest that a small minority of IEL and possibly CP cells also is undergoing TCR gene rearrangement, that they are able to do so with a minimum amount of RAG-2 transcripts, or both. Actually, we still do not know how many RAG-1 and -2 molecules per nucleus are required to drive the region-specific V(D)J recombinations of TCR genes. It is possible that the amount of mRNA encoding RAG-1 and -2 molecules required by thymocytes for the successful recombination of TCR genes may not be that large. It should also be pointed out that using cell fate mapping, almost all αβ-IEL have recently been shown to be the progeny of immature CD4$^+$CD8$^+$ thymocytes (55). Furthermore, by using elegant and sophisticated approaches (55), it has been revealed that the retinoic acid-related orphan receptors (RORγt) detected in fetal lymphoid tissue-inducer cells are also expressed in cells within gut CP, and that γδ-IEL are not the progeny of such RORγt-positive CP lymphocytes. Specifically, however, it has remained an open question whether a small, but significant, fraction of lymphocytes in CP does not retain RORγt molecules or whether almost all CP lymphocytes express RORγt (55). In any event, to substantiate the intraintestinal development of γδ-IEL in these *nu/nu* LNP$^-$ and *nu/nu aly/aly* mice, clear identification of T cells

undergoing TCR gene rearrangement in the gut mucosa appears to be of critical importance.

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Triggering of TLR3 by polyI:C in human corneal epithelial cells to induce inflammatory cytokines

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Abstract

Epithelial cells of the ocular surface are key in the first-line defense as a part of the mucosal immune system against pathogens. We investigated whether polyI:C induces the production by human corneal epithelial cells (HCEC) of pro-inflammatory cytokines and IFN-β, and whether Toll-like receptor (TLR)-3 expression is amplified by polyI:C. TLR3 was expressed on the surface of HCEC. Stimulation with polyI:C elicited the elevated production and mRNA expression of IL-6 and IL-8 in HCEC. While polyI:C induced IFN-β, far stronger than human fibroblasts, and TLR3 gene expression in HCEC, LPS stimulation did not. Similarly, polyI:C, but not LPS, induced the gene expression of IκBα and MAIL, members of the IκB family, in HCEC. The innate immune response of HCEC is distinct from that of immune-competent cells, and we suggest that this is indicative of the symbiotic relationship between corneal epithelium and microbes inhabiting the ocular surface.

Keywords: Human corneal epithelial cells; PolyI:C; TLRs; Inflammation; LPS

On the ocular surfaces as in the intestine, the surface epithelium serves a critical function in the front-line defense of the mucosal innate immune system [1–3]. Upon challenge, epithelial cells lining mucosal surfaces play a pivotal role in innate immunity by secreting chemokines and other immune mediators. The ability to detect microbes is arguably the most important task of the immune system. Exaggerated host defense reaction of the epithelium to endogenous bacteria may induce the initiation and perpetuation of inflammatory mucosal responses [4–6].

The ability of cells to recognize microbial motifs and pathogen associated molecular patterns (PAMPs) rests on pattern recognition receptors (PRRs) [7–9]. Signal transduction depends on the expression of a family of type I transmembrane receptors, Toll-like receptors (TLRs). To date, 11 TLRs have been identified in humans; they are expressed primarily on cell types that are mammalian host immune-competent cells such as dendritic cells and macrophages. These are the cells that are most likely to come into direct contact, via the mucosal epithelia, with pathogens from the environment [10]. TLR expression is not restricted to phagocytic cell types, rather, it appears that the majority of cells in the body including mucosal epithelial cells express at least a subset of TLRs [11]. Some PRRs are located on the cell membrane and respond to extracellular PAMPs; others exist in the cytosol and respond to PAMPs that cross the plasma membrane [12–14]. Signaling through TLRs results in the activation of IKK, NF-κB, and NF-κB target genes, and the coordinated activation of several transcription factors that regulate the expression of antimicrobial genes, cytokines, chemokines, and co-stimulatory molecules [7–9].

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Although the eye is relatively impermeable to microorganisms [1,3,15,16], if corneal integrity is compromised by trauma or contact lens wear, sight-threatening bacterial infection may occur [17,18]. Uniquely, human corneal epithelial cells (HCEC) are in constant contact with bacteria and bacterial products; they form a structural and functional barrier against numerous bacteria both pathogenic and nonpathogenic. Factors normally pro-inflammatory for other cell types do not induce epithelial cells to initiate a defensive response [19]. This is especially important with respect to the epithelial cells of the avascular and transparent cornea, where the formation of scar tissue in response to a host inflammatory reaction results in opacification and loss of vision. We previously reported that human corneal epithelial cells failed to respond functionally to PAMPs such as peptidoglycan (PGN) and lipopolysaccharide (LPS) because they lack TLR2 and TLR4 on their surface [20]. Despite the existence of TLR2 and TLR4 in the cytoplasm of HCEC, the experimental translocation of LPS to the cytoplasm did not elicit an immune response [20].

Among the TLRs, TLR4 which recognizes LPS, and TLR3 which recognizes the viral double-stranded RNA-mimic polyI:C have received the greatest attention [21–23]. TLR3-mediated responses are unique because TLR3 activation elicits lower levels of inflammatory cytokines than the activation of other TLR family members, although TLR3 activation induces the very robust secretion of IFN-β [21]. The remarkable similarities in the cellular responses to bacterial and viral infection after pathogen recognition are indicative of cross-talk between virus- and bacteria-induced signaling [24]. Although there were two reports by the same group describing the inhibitory effect of polyI:C against herpetic keratitis in rabbits, nothing is yet known on the reproducibility of their experiments or the effect on the innate immune response [25,26].

Here we demonstrate that HCEC express TLR3 at the cell surface and thus respond to polyI:C to generate pro-inflammatory cytokines and IFN-β. We also show that the surface expression of TLR3 on HCEC was amplified in an autocrine/paracrine manner by polyI:C.

Materials and methods

All experimental procedures were conducted in accordance with the principles set forth in the Helsinki Declaration. The purpose of the research and the experimental protocols were explained to all participants and their prior written informed consent was obtained.

Human corneal epithelial cells. For RT-PCR, human corneal epithelial cells (HCEC) were obtained from corneal buttons of patients undergoing corneal transplantation for early-stage bullous keratopathy (one eye) or keratoconus (two eyes) at the affiliated hospital of Kyoto Prefectural University of Medicine.

Primary HCEC, obtained from Kurabo (Osaka, Japan), were cultured at 37 °C under 95% humidity and 5% CO_2 in serum-free medium consisting of EpiLife (Kurabo) supplemented with HCEC growth supplement (HCGS) containing 1 ng/ml murine epidermal growth factor (mEGF), 5 μg/ml insulin from bovine pancreas, 0.18 μg/ml hydrocortisone, and 0.4% v/v bovine pituitary extract (Kurabo), 0.2% PSA solution, and antibiotic–antimycotic solution (5000 U/ml penicillin, 50 mg/ml streptomycin, and 12.5 μg/ml amphotericin B) (Kurabo) [20]. For assays, 2×10^6 primary HCEC were plated in 25 cm^2 flasks. After reaching sub-confluence, they were either left untreated, exposed to 1 μg/ml LPS from *Pseudomonas aeruginosa* (Sigma, St. Louis, MO), or exposed to 25 μg/ml polyI:C (Invivogen, San Diego, CA) for 1-, 3- or 6 h. The culture time of polyI:C-treated cells was adjusted to be optimal for the maximum induction of IL-6, IFN-β, IκBα, MAIL, and TLR3, it was 6 h for IL-6, IL-8, and TLR3, and 3 h for IFN-β, IκBα, and MAIL.

Purification of human peripheral mononuclear cells. Venous blood samples from healthy volunteers were anti-coagulated with 2Na-EDTA, placed in sterile 50-ml polypropylene tubes, mixed with 1 volume of Ca^{2+}-free PBS (PBS(−)), overlaid with Ficoll–Paque Plus (Amersham Biosciences AB, Uppsala, Sweden), and centrifuged for 20 min at 2000 rpm at 20 °C. Human peripheral mononuclear cells (HPMC) were gently aspirated from the interface and washed with PBS(−). For stimulation with LPS or polyI:C, isolated HPMC were cultured for 1-, 3- or 6 h in RPMI medium (Gibco-BRL Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco).

Human conjunctival fibroblasts. Human conjunctival fibroblasts (HCFB) were obtained from redundant subconjunctival tissues of patients undergoing cataract surgery at the affiliated hospital of Kyoto Prefectural University of Medicine. Primary HCFB were cultured in DMEM (Gibco) supplemented with 10% fetal calf serum (Gibco) and 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco). For assays, all procedures were the same as those described above for HCEC.

MRC-5 and HeLa cells. MRC-5 and HeLa, expressing TLR3 on the cell surface and producing IFN-β upon polyI:C stimulation, were the gift of Dr. T. Seya (Hokkaido University). MRC-5, normal human lung fibroblasts, and HeLa cells were maintained in MEM (Gibco) supplemented with 1% antibiotic–antimycotic solution (100 U/ml penicillin, 100 mg/ml streptomycin, and 250 ng/ml amphotericin B) (Gibco), and 10% or 5% fetal calf serum (Gibco). For assays, all procedures were the same as those described above for HCEC.

RT-PCR. Total RNA was isolated from human corneal epithelium and HPMC using Trizol Reagent (Life Technologies, New York, NY) according to the manufacturer's instructions. For the RT reaction, we used the SuperScript Preamplification kit (Invitrogen). PCR amplification was performed with DNA polymerase (Takara; Shiga, Japan) for 38 cycles at 94 °C for 1 min, annealing for 1 min, and 72 °C for 1 min on a commercial PCR machine (GeneAmp; PE Applied Biosystems). The primers we used are listed in Table 1. The integrity of the RNA was assessed by electrophoresis in ethidium bromide-stained 1.5% agarose gels.

Flow cytometric analysis. Human primary corneal epithelial cells were treated with 0.02% EDTA. The cell-surface expression of TLR2, TLR3, and TLR4 was examined by flow cytometry. For TLR3 expression, cells were incubated with mouse anti-human TLR3 monoclonal antibody (mAb; Imgenex, San Diego, CA) or isotype control mouse IgG1 (DakoCytomation, Kyoto, Japan) for 30 min at 4 °C. Alexa Fluor 488 goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR) was used as the secondary antibody. For TLR2 and TLR4 expression, cells were incubated for 30 min at 4 °C with PE-conjugated mouse anti-human TLR2 (TL2.1), TLR4 (HTA125) monoclonal antibody (eBioscience, San Diego, CA), or isotype control mouse IgG2a (BD PharMingen). Stained cells were analyzed with a FACSCalibur (Becton–Dickinson, San Jose, CA); data were analyzed using Cellquest software (Becton–Dickinson). Moreover, HCFB, MRC-5, and HeLa were examined for their cell-surface expression of TLR3.