Role of Gut-Associated Lymphoreticular Tissues in Antigen-Specific Intestinal IgA Immunity[1]

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This study assessed the roles of the postnatal lymphotoxin-β receptor (LTβR)-mediated signals in the gut-associated lymphoreticular tissues of mice for subsequent regulation of Ag-specific intestinal IgA responses. Blockade of LTβR-dependent events by postnatal administration of the fusion protein of LTβR and IgG Fc (LTβR-Ig) reduced both the size and numbers of Peyer's patches (PP) without influencing the PP microarchitecture. Interestingly, inhibition of LTβR-dependent signaling revealed significant reductions in the formation of follicular dendritic cell clusters in mesenteric lymph nodes (MLN). Furthermore, these postnatal signaling events controlled the development of isolated lymphoid follicles (ILF) because treatment with LTβR-Ig eliminated the formation of ILF. LTβR-Ig-treated mice with altered microarchitecture of MLN and lacking ILF were still able to produce significant Ag-specific mucosal IgA responses after oral immunization; however, the levels were significantly lower than those seen in control mice. These results imply the importance of ILF for Ag-specific intestinal immunity. However, mice treated with both TNFR55-Ig and LTβR-Ig in utero, which lack PP and MLN, but retain intact ILF, failed to induce Ag-specific IgA responses after oral immunization. These findings demonstrate that ILF are not essential for induction of intestinal IgA Ab responses to orally administered Ag. Furthermore, the induction of intestinal IgA Ab responses requires the proper maintenance of the MLN microarchitecture, including a follicular dendritic cell network. *The Journal of Immunology,* **2004, 173: 762–769.**

Mucosal surfaces such as those in the gastrointestinal (GI)[3] tract provide a first line of defense against colonization by microbial pathogens (1). This area is protected by the mucosal immune system, which includes a secretory IgA (S-IgA) Ab response. This IgA constitutes greater than 80% of all Ig isotypes produced in mucosa-associated tissues in humans, and S-IgA is induced, transported, and regulated by mechanisms that are completely different from those involved in systemic Ab responses (2). Although the induction of Ag-specific S-IgA Ab responses is known to be dependent upon cognate help provided by $CD4^+$ Th cells in gut-associated lymphoreticular tissues (GALT), the exact site in which this regulatory network is formed for the subsequent development of these IgA Ab responses is only partially understood (2). In this regard, Peyer's patches (PP) have been considered to be the major inductive site for initiation of Ag-specific IgA immune responses in the GI tract (3). It has been shown that Ag-specific IgA precursors from PP repopulate the intestinal lamina propria via the common mucosal immune system and subsequently differentiate into IgA-producing plasma cells (4). Furthermore, the introduction of Ag into surgically constructed intestinal loops with PP resulted in the appearance of Ag-specific IgA Abs after immunization, whereas no mucosal IgA Ab responses were detected in the intestinal loops that lacked PP (5). These studies support the current dogma that PP are a primary inductive site for Ag-specific immune responses in the gut. However, our previous study showed that oral immunization of mice that lack PP resulted in the generation of Ag-specific IgA Ab responses in the GI tract (6), suggesting the existence of a PP-independent pathway for mucosal IgA Ab responses.

TNF, lymphotoxin (LT) α, and LTβ are members of the TNF superfamily, which also include immunoregulatory molecules such as CD30 ligand (L), CD40L, FasL, TRAIL, and LIGHT (homologous to lymphotoxins, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed by T lymphocytes) (7–9). Both TNF and LTα can be secreted as homotrimers, and TNF also exists as a cell surface homotrimer. TNF and LTα homotrimers bind and signal via either of two TNF receptors, TNFR55 or TNFR75 (10, 11). In contrast, LTβ is a type II integral membrane protein that complexes with LTα to form membrane-anchored heterotrimers (12, 13). The LTαβ complex binds to a specific receptor termed the LTβR (14). Mice genetically depleted of LTα, LTβ, or LTβR have disrupted PP and lymph node (LN) development (15–18). In contrast, TNFR55-deficient ($TNFR55^{-/-}$) or $TNFR75^{-/-}$ mice retain PP and LN (19–21). Furthermore, genesis of PP and LN was disrupted by in utero blockade of the LTαβ pathway (22, 23). These studies have shown that ligation of the LTβR by LTαβ heterotrimer during gestation is critical for the formation of PP and LN. However, the generation of the mesenteric lymph nodes (MLN) may require TNF/LTα in addition to the LTαβ signaling

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[3] Abbreviations used in this paper: GI, gastrointestinal; AFC, Ab-forming cell; CT, cholera toxin; FDC, follicular dendritic cell; GALT, gut-associated lymphoreticular tissues; GC, germinal center; ILF, isolated lymphoid follicles; L, ligand; LN, lymph node; LT, lymphotoxin; MLN, mesenteric lymph nodes; PNA, peanut agglutinin; PP, Peyer's patch; S-IgA, secretory IgA; TT, tetanus toxoid.

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cascade (24). A role in MLN organogenesis has also been postulated for the alternative LTβR-binding ligand termed LIGHT (25).

Our previous study showed that Ag-specific IgA responses could be induced in mice with a deficiency in organized PP (6). Furthermore, recent studies have reported that the LTαβ signaling pathway in the intestinal lamina propria is relevant for the production of IgA in a manner shown to be independent of the presence of PP and MLN (26, 27). These studies imply that the GALT immune system is equipped with multiple pathways, and the well-characterized PP and MLN network is just one form of the induction cascade for IgA responses in the GI tract. To this end, a recent study has demonstrated that, in addition to PP, there are small lymphoid clusters, termed isolated lymphoid follicles (ILF), in the small intestine (28, 29). ILF formation occurs postnatally in response to luminal stimuli, including normal bacterial flora (29, 30). Thus, ILF may be a component of GALT that potentially contribute to intestinal IgA immunity. To test the hypothesis that ILF contribute to the IgA responses, TNF55R- and/or LTβR-mediated signaling pathways were pre- or postnatally manipulated in vivo for the creation of ILF-compromised mice.

Materials and Methods

Mice

C57BL/6 mice were purchased from Japan Clea (Tokyo, Japan). These mice were maintained in the experimental facility under pathogen-free conditions in the Nihon University School of Dentistry at Matsudo (Chiba, Japan). The mice were free of bacterial and viral pathogens, as determined by pathogenic and serologic analysis on sentinel mice.

Fusion proteins and treatment protocols

Proteins comprised of the extracellular domain of either murine TNF receptor 55 or LTβR fused to the hinge, CH2, and CH3 domains of human IgG1 (LTβR-Ig and TNFR55-Ig, respectively) were used in our studies, as described elsewhere (22, 31, 32). Young adult mice (6 wk old) were injected i.p. with 100 μg of TNFR55-Ig or LTβR-Ig at weekly intervals during the immunization period from day −7 to day +14 relative to the day of immunization. Pharmacokinetic analysis showed that the $t_{1/2}$ of LTβR-Ig was 4–6 days, and this treatment dose provided 6–11 μg/ml fusion protein in the serum. In some experiments, pregnant mice were injected i.v. with 200 μg of both TNFR-55-Ig and LTβR-Ig on gestational days 13 and 16, as described previously, with minor modifications (6, 22).

Immunization

A vaccine grade of tetanus toxoid (TT) was kindly provided by Y. Higashi (Biken Foundation, of Osaka University, Suita, Osaka, Japan). For oral immunization, mice were deprived of food for 2 h and then given a solution of sodium bicarbonate to neutralize stomach acidity before oral immunization (33). Thirty minutes later, these mice were orally immunized by gastric intubation with 250 μg of TT in the presence of 10 μg of cholera toxin (CT) as mucosal adjuvant (34). This oral immunization procedure was conducted on days 0, 7, and 14.

Immunohistochemical analysis

Freshly isolated MLN and PP were rapidly frozen in OCT embedding medium (Tissue-Tek, Elkhart, IN) and stored at minus 80°C until processing. Cryostat sections (6 μm) were fixed in ice-cold acetone for 10 min, dried, and preblocked with anti-CD16/CD32 Fc block (BD Pharmingen, San Diego, CA) in PBS. Cells were stained with FITC-conjugated anti-B220 mAb (BD Pharmingen) and PE anti-CD3 mAb (BD Pharmingen). Reactivity with peanut agglutinin (PNA) was demonstrated using biotinylated PNA (Vector Laboratories, Burlingame, CA), followed by streptavidin-PE. Staining of sections for follicular dendritic cell (FDC) clusters used biotinylated CR1 (BD Pharmingen), followed by streptavidin PE. IgA-containing cells were visualized by FITC-labeled anti-mouse IgA mAb (BD Pharmingen). The sections were mounted and viewed under ×100 optics and a dual red/green filter. Each of the images was analyzed with Photoshop (Adobe Systems, San Jose, CA) in a consistent manner, followed by overlying of the green and red images in the screen mode (MicroRadience AG-2; Bio-Rad, Hercules, CA). The final images are representative of these sections.

Determination of numbers of ILF

The small intestine was divided into four equal parts distal from the pylorus, and segments (2 cm long) were collected from each part. The tissue segments were fixed in 4% paraformaldehyde and embedded in paraffin. The segments were sectioned consecutively at 6 μm and stained with H&E for counting the numbers of ILF. In some experiments, the segments were frozen in OCT embedding medium and the cryostat sections were stained with PE-conjugated anti-B220 mAb (BD Pharmingen).

Detection of Ag-specific Ab isotype responses

Serum and fecal extracts were obtained, and Ag-specific Ab titers were determined by an ELISA, as described elsewhere (33). Briefly, plates were coated with TT (5 μg/ml) and blocked with 10% goat serum. Analyses were performed in duplicate. Following 4 h of incubation, the plates were washed, and peroxidase-labeled goat anti-mouse μ, γ, or α H chain-specific Abs (Southern Biotechnology Associates, Birmingham, AL) were added to appropriate wells. Finally, ABTS with H_2O_2 (Moss, Pasadena, CA) was added for color development. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution, which gave an OD at 414 nm of 0.1 greater than background after 15 min of incubation.

ELISPOT for assessment of Ab-forming cells (AFCs)

Single cell suspensions were obtained from intestinal lamina propria, as previously described (33). The mononuclear cells were obtained at the interface of the 40 and 75% layers of a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Piscataway, NJ). To assess numbers of total and Ag-specific AFCs, an ELISPOT assay was performed, as previously described (35). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with goat anti-mouse Ig Ab (2 μg/ml) (Southern Biotechnology Associates) or TT (5 μg/ml), incubated for 20 h at 4°C, and then washed extensively and blocked with 10% goat serum. The blocking solution was discarded, and lymphoid cell suspensions at various dilutions were added to wells and were incubated for 4 h at 37°C in 5% CO_2 in moist air. The detection Abs consisted of goat HRP-conjugated anti-mouse α or γ H chain-specific Abs (Southern Biotechnology Associates). Following overnight incubation, the plates were washed with PBS and developed by addition of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H_2O_2 (Moss) to each well. Plates were incubated at room temperature for 15–20 min and washed with water, and AFCs were counted with the aid of a stereomicroscope (SZH-ILLB; Olympus, Tokyo, Japan).

Statistics

The data are expressed as the mean ± SEM and compared using the unpaired Mann-Whitney *U* test. The results were analyzed using the Statview II statistical program (Abacus Concepts, Berkeley, CA) adapted for Macintosh computers.

Results

Signaling pathways through TNFR55 and/or LTβR have diverse effects on the maintenance of GALT

We first evaluated the role of TNF/LTα and LTαβ signaling pathways on the maintenance of the fully developed intestinal mucosal immune system, and thus for the induction of Ag-specific mucosal IgA Ab responses. Signaling pathways through TNFR55 or LTβR in adult mice were blocked by introducing murine TNFR55-Ig or LTβR-Ig fusion proteins as soluble receptor decoys. Thus, 6-wk-old mice were injected i.p. with TNFR55-Ig or LTβR-Ig at weekly intervals. Treatment of normal adult mice with TNFR55-Ig or LTβR-Ig had no effect on the numbers or size of MLN (data not shown). Histological analysis showed that the MLN of TNFR55-Ig- as well as control-Ig-treated mice had organized and distinct B cell follicles (Fig. 1, *A* and *E*). In contrast, when mice were treated with LTβR-Ig, the follicles of the MLN were disrupted (Fig. 1*I*). The MLN of TNFR55-Ig- as well as control-Ig-treated mice retained a distinct segregation of lymphocytes into a superficial cortical B cell zone and a deep cortical T cell zone (Fig. 1, *B* and *F*). In contrast, although T and B cell areas still appeared to be segregated in LTβR-Ig-treated mice, the border of these areas became indistinct and had more diffuse edges (Fig. 1*J*).

Additional abnormalities of the MLN microarchitecture were observed in fusion protein-treated adult mice. Control, human

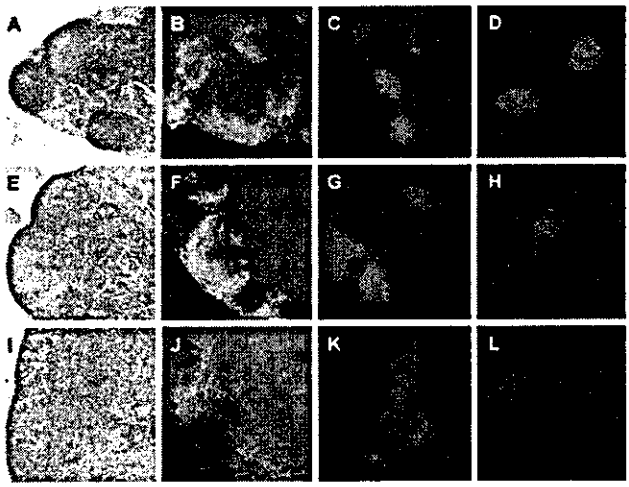

FIGURE 1. Effects of TNF/LTα or LT$\alpha\beta$ inhibition on MLN architecture. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant three times at weekly intervals. These mice were also injected i.p. with TNFR55-Ig or LTβR-Ig at weekly intervals during the immunization period from day -7 to day $+14$ relative to the day of immunization. H&E staining of mice treated with control-Ig (*A*), TNFR55-Ig (*E*), or LTβR-Ig (*I*) is shown. To compare the segregation of T and B cell zones, the sections were stained for CD3 (red) and B220 (green). Shown are the MLN from control-Ig (*B*)-, TNFR55-Ig (*F*)-, or LTβR-Ig (*J*)-treated mice. Formation of GCs in mice treated with control-Ig (*C*), TNFR55-Ig (*G*), or LTβR-Ig (*K*) was stained with PNA (red). The FDC clusters in mice treated with control-Ig (*D*), TNFR55-Ig (*H*), or LTβR-Ig (*L*) were stained with CR1 (red).

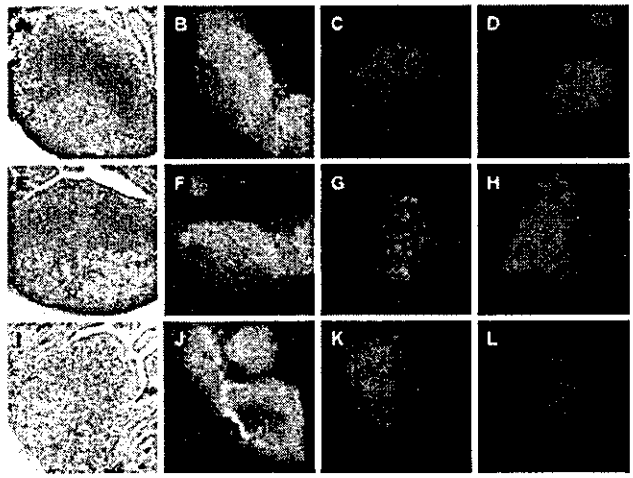

FIGURE 2. Effects of TNF/LTα or LT$\alpha\beta$ inhibition on PP architecture. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. H&E staining of mice treated with control-Ig (*A*), TNFR55-Ig (*E*), or LTβR-Ig (*I*) is shown. To compare the segregation of T and B cell zones, the sections were stained for CD3 (red) and B220 (green) expression. Shown are the MLN from control-Ig (*B*)-, TNFR55-Ig (*F*)-, or LTβR-Ig (*J*)-treated mice. GC formation in mice treated with control-Ig (*C*), TNFR55-Ig (*G*), or LTβR-Ig (*K*) was stained with PNA (red). The FDC clusters in mice treated with control-Ig (*D*), TNFR55-Ig (*H*), or LTβR-Ig (*L*) were stained with CR1 (red).

IgG-, or TNRF55-Ig-treated mice formed clearly defined PNA^+ germinal centers (GCs) and $CR1^+$ FDC clusters in MLN (Fig. 1, *C*, *D*, *G*, and *H*). In contrast, the intensity of FDC clusters was markedly reduced in LTβR-Ig-treated adult mice, although PNA^+ B cells resembling GCs were detected (Fig. 1, *K* and *L*). These results indicate that signaling provided via the LTβR plays an important role in the maintenance of the MLN microarchitecture.

Previous studies have shown that blockade of LT$\alpha\beta$ signaling in utero results in the failure to generate PP (6, 22); it was important to assess the contribution of the TNFR55- and LTβR-dependent events in the maintenance of the organized PP structure. There was no discernable change in numbers or size of PP when TNFR55-Ig was administered to adult mice to inhibit TNF/LTα signaling. Furthermore, the segregation of T and B cell areas was maintained, and clear GC formation and FDC clusters were also detected in PP of mice treated with TNFR55-Ig (Fig. 2, *E–H*) just as was seen in the PP of control-Ig-treated mice (Fig. 2, *A–D*). In contrast, the size of PP was reduced in LTβR-Ig-treated mice, as reported earlier (36) (Fig. 2*I*). Furthermore, the numbers of visible PP were reduced to an average of 2–4 per mouse in LTβR-Ig-administered mice when compared with an average of 6–10 visible PP in control-Ig-treated mice. Immunohistological analysis of LTβR-Ig-treated PP showed the maintenance of segregated T and B cell areas and GC formation (Fig. 2, *J* and *K*); however, FDC clusters were slightly less intense than those seen in the control, IgG-treated mice (Fig. 2, *D* and *L*). Thus, the cellular microarchitecture appears to be maintained in the visible PP remaining in LTβR-Ig-treated adult mice.

Previous studies showed that the treatment of mice in utero with LTβR-Ig ablated PP development, but did not impact the development of ILF, recently identified in the mouse small intestine (28, 29). These findings suggested that ILF developed postnatally, and were not affected by the in utero blockade of LTβR-mediated signals. We therefore assessed the effects of TNFR- or LTβR-dependent signaling on the established ILF in adult mice. TNFR55-Ig-treated mice possessed significantly decreased numbers of ILF when compared with those seen in control-Ig-treated mice (Fig. 3, *A*, *B*, and *D–F*). Furthermore, essentially no ILF were seen in the small intestine of adult mice treated with LTβR-Ig (Fig. 3, *C* and *F*).

Influence of postnatal treatment with TNFR55-Ig or LTβR-Ig on the generation of IgA Ab responses

Based upon the alteration of the mature GALT network by blockade of TNFR55- or LTβR-dependent signaling, it was important to examine the influence of these cytokine-mediated signals for the generation of mucosal IgA Ab responses in the intestinal lumen. Immunohistochemical analysis revealed that significant numbers of IgA^+ plasma cells were seen in the intestinal lamina propria of TNFR-Ig- or LTβR-Ig-treated, adult mice (Fig. 4*A*). Analysis of total numbers of IgA AFC supported the immunohistochemical study and showed that identical numbers of total IgA-producing cells were detected in TNFR55-Ig- or LTβR-Ig-treated, adult mice when compared with those seen in the control, IgG-treated mice (Fig. 4*B*). These findings suggest that neither TNFR55-Ig nor LTβR-Ig treatment influenced the maintenance of IgA-producing cells in the intestinal lamina propria.

In contrast, blockade of selected inflammatory cytokine signals showed some influence on Ag-specific IgA responses. Mice that received TNFR55-Ig during oral immunization with TT plus CT had TT-specific IgA Ab titers that were comparable to those seen in control, IgG-treated mice. In contrast, oral TT plus CT elicited significant TT-specific IgA Ab responses in adult mice treated with LTβR-Ig; however, the levels were significantly lower than those induced in control mice (Fig. 5*A*). The presence of TT-specific IgA Abs was further confirmed by the analysis of Ag-specific IgA AFCs where significant, but lower numbers of TT-specific IgA AFCs were detected in mononuclear cells isolated from intestinal lamina propria of LTβR-Ig-treated adult mice when compared with control mice (Fig. 5*B*). These findings indicate that loss of ILF and/or alterations in the microarchitecture of MLN resulting from blockade of

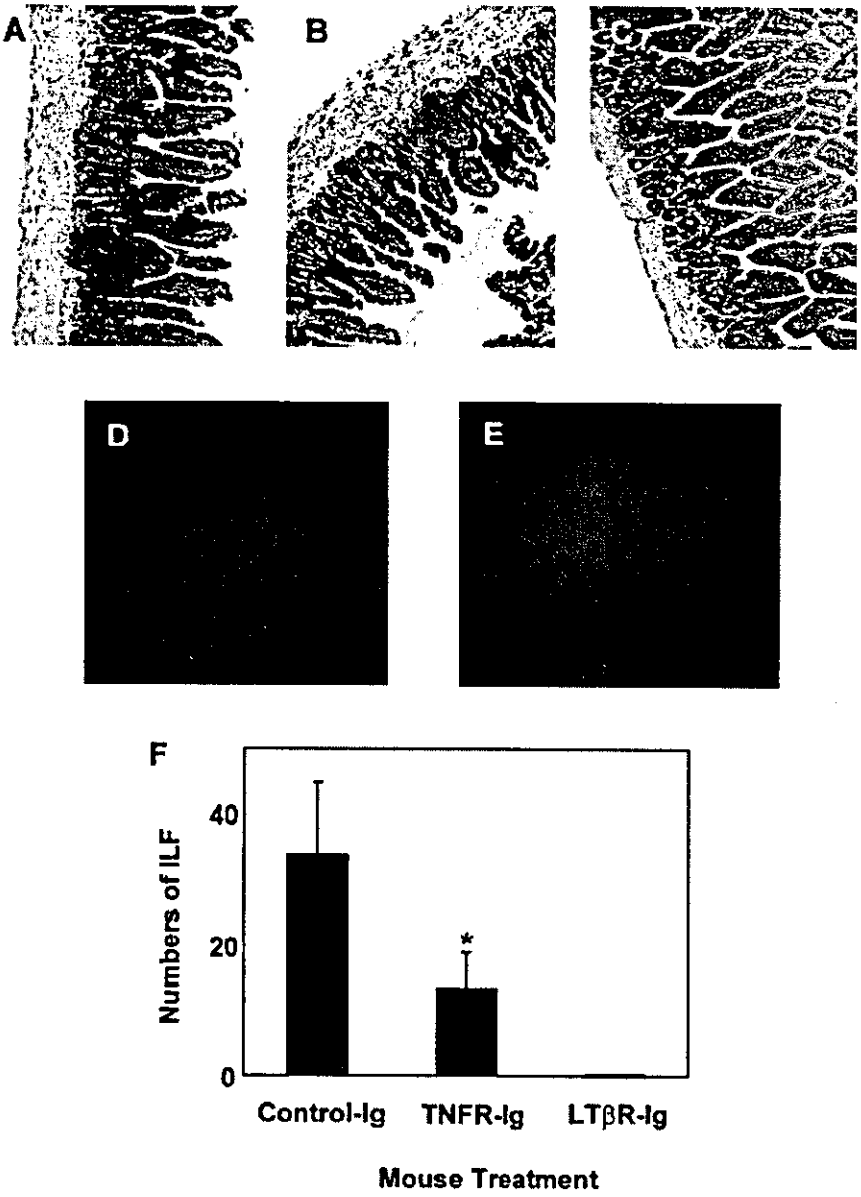

FIGURE 3. Numbers of ILF in the small intestine of mice treated with TNFR55-Ig or LTβR-Ig. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. The small intestine was divided into four equal parts from the pylorus downward, and segments (2 cm long) were collected from each part. The segments were stained by H&E (*A–C*) or PE anti-B220 mAb (*D* and *E*). Shown are ILF (pointed out by arrowheads) in control-Ig (*A* and *D*)-, TNFR55-Ig (*B* and *E*)-, or LTβR-Ig-treated mice (*C*). Total numbers of ILF from four segments are shown (*F*). The results are representative of three separate experiments containing four to six mice in each group/experiment. *, $p < 0.05$ when compared with control-Ig-treated mice.

LTβR-dependent signaling can directly influence Ag-specific intestinal IgA Ab responses.

ILF are not the essential inductive sites for initiation of the Ag-specific intestinal IgA response

Our previous study had shown that oral immunization of PP-null mice elicited intestinal mucosal IgA Ab responses after oral immunization (6). Other studies have shown that the structure of ILF resembles that of PP (28, 29). Taken together, these studies imply that ILF may act as an additional and compensatory inductive site for regulation of Ag-specific intestinal IgA responses in the absence of other GALT such as PP. Thus, we next investigated the possible role of ILF in the regulation of Ag-specific intestinal IgA Ab responses. For this purpose, mice were treated with both TNFR55-Ig and LTβR-Ig in utero, because the treatment of pregnant mice with both TNFR55-Ig and LTβR-Ig resulted in the lack of both PP and MLN in the progeny, whereas the fusion protein treatment in the gestation period did not influence the development of ILF in the offspring of treated dams (24, 28, 29). Treatment of pregnant mice with both TNFR55-Ig and LTβR-Ig resulted in elimination of both PP and MLN in the progeny, although some mice still possessed MLN-like tissues (data not shown). When numbers of ILF were examined in mice that completely lack both MLN and PP, higher numbers of ILF were detected when compared with control-Ig-treated mice (Fig. 6). Furthermore, total IgA levels were normal in mice treated in utero with TNFR55-Ig and LTβR-Ig (Table I). However, no Ag-specific IgA Ab responses were detected (Table I). These results indicate that ILF are unlikely to be the alternative inductive sites for the induction of intestinal Ag-specific IgA Ab responses following oral immunization.

Discussion

Our previous studies have shown that Ag-specific IgA Ab responses are induced in the small intestinal lumen, despite the lack of an organized PP (6). In the present study, we have further analyzed the involvement and role of the GALT network represented by PP, ILF, and MLN in the induction and regulation of Ag-specific intestinal IgA Ab responses when a protein Ag is given orally with mucosal adjuvant. Our results provide direct evidence for several roles of TNF/LTα and LTαβ signaling pathways in the

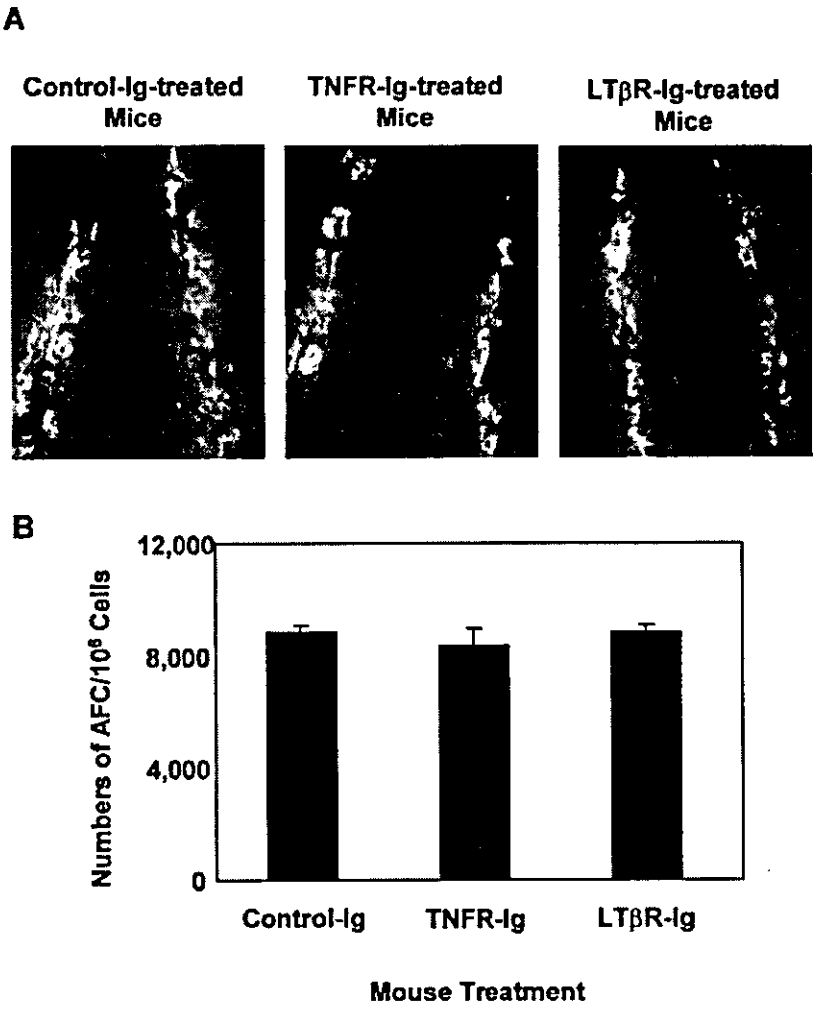

FIGURE 4. Immunofluorescence staining of IgA^+ plasma cells (*A*) and IgA AFCs (*B*) in the small intestinal lamina propria of mice treated with TNFR55-Ig or LTβR-Ig. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant and were treated with TNFR55-Ig or LTβR-Ig during the immunization period, as described in Fig. 1 legend. One week after the last immunization, mononuclear cells were isolated from the small intestinal lamina propria, and total IgA AFCs were determined by ELISPOT. The results are representative of three separate experiments containing four to six mice in each group/experiment.

maintenance of the GALT network that subsequently influence the induction of Ag-specific intestinal IgA Ab responses. First, this study has shown that the signaling pathway through LTβR, but not through TNFR55, plays a key role in the maintenance of MLN microarchitecture, including the segregation of T and B cell areas as well as the presence of FDC clusters in adults. In this regard, blockade of LTαβ, but not the TNF/LTα, pathway after birth resulted in alterations in the splenic white and red pulp (15, 18, 22, 37, 38). These studies, together with our present results, indicate that the LTαβ signaling pathway plays a critical role in the maintenance of the MLN microarchitecture, including the segregation of T and B cell areas, as well as the presence of FDC clusters. Other studies have reached somewhat conflicting conclusions as to the role of TNFR55 and LTβR signaling in maintaining the LN cellular organization. For example, blockade of the TNF/LTα signaling pathway with TNFR55-Ig fusion protein in adult mice only resulted in the dissolution of B cell follicles if the LN were in a quiescent state, that is, not under Ag challenge (24, 39). Similarly, in this study, postnatal LTβR-Ig treatment was found to inhibit the FDC network in LN, as well as disrupt the organization of T and B cell zones, whereas a previous study failed to demonstrate disruption of T and B cell zones in LN (24). The basis for this discrepancy is not known. Despite this, it now appears that FDC networks are particularly sensitive to LTβR antagonism, both in the spleen and LN. Collectively, these results suggest that maintenance of the MLN architecture, e.g., T and B cell segregation and FDC clustering, is not programmed during development, but is dependent upon stimuli provided by the LTαβ pathway.

The GCs, with their prominent clusters of FDCs, are thought to provide a primary venue for the development of Ag-specific Ab responses. However, in this study, the formation of GCs was detected in the MLN of LTβR-Ig-treated, adult mice, despite a reduction in FDC clusters. In this regard, previous studies have shown that LTβ-deficient mice form GC clusters, despite the absence of an FDC network (40, 41). Thus, although the LTαβ pathway is required for generation of the FDC network, PNA^+ B cells resembling GCs can be formed in the absence of this type of FDC network.

Previous studies have shown that $LT\alpha^{-/-}$ mice (15, 37), $TNFR55^{-/-}$ mice (42, 43), and $LT\beta^{-/-}$ mice (16, 18) lack PP development. Furthermore, mice treated with LTβR-Ig in utero lack PP (6, 22). In contrast, others have reported that $TNFR55^{-/-}$ mice have PP; however, these mice only have, on average, 2–4 PP when compared with 8–10 PP in normal mice (44). Furthermore, those PP appear flattened due to a lack of B cell follicle structures (44). Finally, an interesting study has shown that $LT\alpha^{+/-}LT\beta^{+/-}$, but not $LT\alpha^{+/-}$ or $LT\beta^{+/-}$ mice specifically lack PP (45). These studies suggest that both TNFR55 and LTβR pathways are involved in the development of PP. However, the PP seen in adult mice treated with TNFR55-Ig or LTβR-Ig have a normal follicular structure. Immunohistochemical analysis revealed that T cell areas were clearly segregated from B cells, and GC formation and FDC clustering were shown to be normal. These results indicate that the

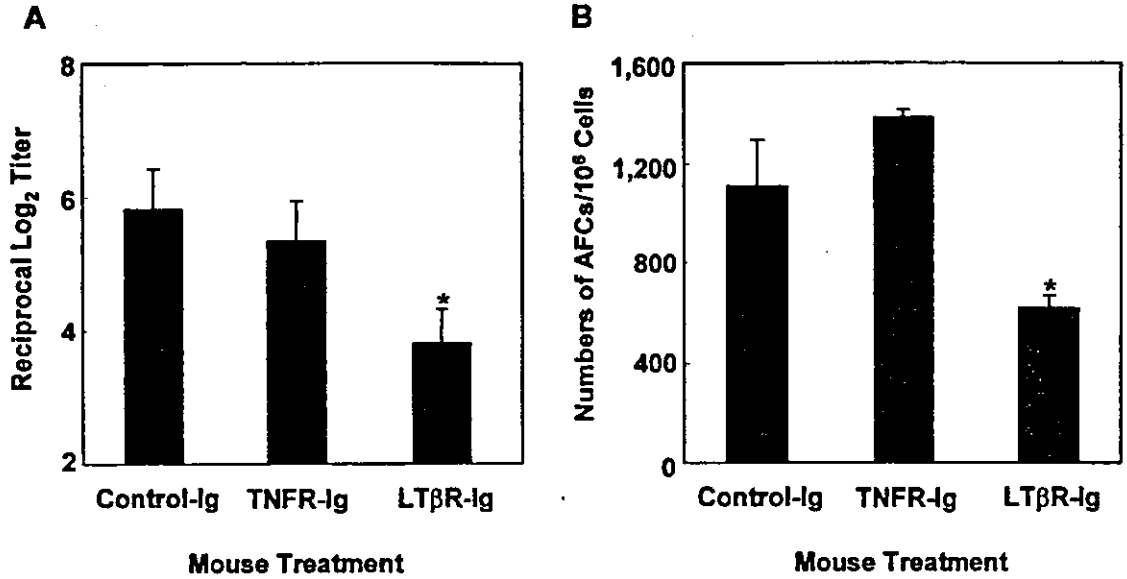

FIGURE 5. The TT-specific fecal IgA Ab titers (*A*) and IgA AFCs in lamina propria (*B*) are shown. Groups of mice were orally immunized with TT plus CT as mucosal adjuvant, and were also treated with LTβR-Ig or both TNFR55-Ig and LTβR-Ig during the immunization period, as described in Fig. 1 legend. One week after the last immunization, fecal samples were collected and examined for TT-specific Abs by ELISA. Mononuclear cells were isolated, and TT-specific IgA AFCs were examined by ELISPOT. The results are representative of three separate experiments containing four to six mice in each group/experiment. *, $p < 0.05$ when compared with control-Ig-treated mice.

TNFR55 and LTβR pathways are not essential for the maintenance of a PP microarchitecture. Importantly, however, in adult mice treated with LTβR-Ig, but not TNFR55-Ig, the PP were small in appearance, and their numbers were reduced. This finding further confirms a previous study (36). In this regard, the PP have been shown to reach their fully developed size and appearance only after stimulation with Ags that initiate a chronic GC reaction within a follicular B cell compartment (46). In this study, although mice were orally immunized with TT plus the strong mucosal adjuvant, CT, the size and numbers of PP were reduced after treatment with LTβR-Ig during the immunization period. Thus, the appearance of a reduced size and numbers of PP may be explained by their inability to undergo expansion of their B cell compartment after oral Ag challenge.

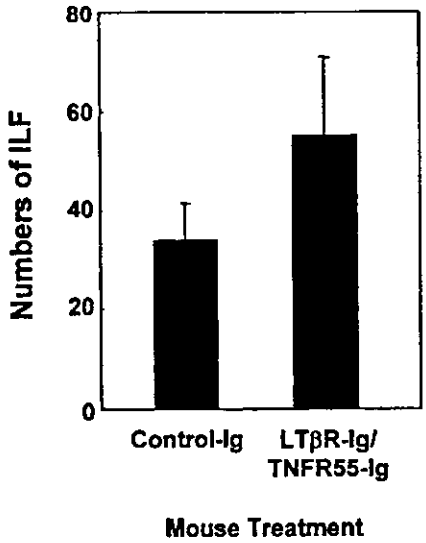

FIGURE 6. Numbers of ILF in the small intestine of mice treated in utero with TNFR55-Ig and LTβR-Ig. Groups of mice were treated with conrtol-Ig or both LTβR-Ig and TNFR55-Ig in utero. The small intestine was divided into four equal parts from the pylorus downward, and segments (2 cm long) were collected from each. The segments were opened longitudinally and sectioned consecutively, and then stained by H&E or PE anti-B220, and numbers of ILF from four segments were counted. The results are representative of three separate experiments containing four to six mice in each group/experiment.

The small intestine has a large number of lymphoid follicles, termed ILF, throughout the length of the antimesenteric wall (28, 29, 47–49). Our previous study showed that ILF consist of a large B cell area, including a GC, and epithelia overlying these ILF contain M cells (28). Thus, ILF are similar to the follicular units that comprise the PP. However, the ILF are not detectable until postnatal life, while PP genesis is already initiated before birth (28, 50). Furthermore, the present results and other studies showed that in utero treatment with LTβR-Ig or both TNFR55-Ig and LTβR-Ig abrogates the development of PP, leaving the development of ILF unaffected (28). Conversely, blockade of TNFR55-mediated signaling in adult mice resulted in significant reductions in ILF formation. Furthermore, blockade of LTβR-dependent events led to a complete ILF deficiency, while the PP remained intact with a normal microarchitecture, although their overall size was reduced. In this regard, it has been shown that while prenatal treatment with LTβR-Ig enhanced the formation of ILF, mice treated pre- and postnatally failed to develop those lymphoid follicles (29). Furthermore, TNFR55$^{-/-}$ mice lacked ILF (29). These studies together with our results indicate that ILF and PP have a different developmental program, although both types of lymphoid tissues

Table I. *Intestinal mucosal IgA Ab responses*

Mice[a]	Total IgA (μg/ml)	Ag-Specific IgA (reciprocal $\log_2$)
Control-Ig	52.4 ± 11.7	7.3 ± 0.4
LTβR-Ig/TNFR55-Ig	43.4 ± 14.4	< 3

[a] Pregnant mice were injected i.v. with LTβR-Ig and TNFR55-Ig on gestational days 13 and 16.

require the LTβR signaling pathway. Thus, LTβR-dependent events in embryonic intestine are essential for organogenesis of PP, whereas postnatal LTβR signaling is required for development and maintenance of ILF. Furthermore, TNFR55-dependent events in adulthood are also important for ILF formation.

The structural similarity of ILF and PP led us to query whether ILF could serve as inductive sites for intestinal IgA Ab responses. In this regard, a previous study reported that isotype switching from $B220^+$ IgM^+ B cells to IgA^+ plasma cells may occur in situ via direct interactions with lamina propria stromal cells (26). Furthermore, LTβR signaling on the intestinal lamina propria stromal cells has been shown to be important for IgA production (27). Moreover, our separate studies have demonstrated that the activation-induced cytidine deaminase- and Iμ-Cα-specific mRNA were detected in lymphocytes isolated from ILF, but not in diffuse connective tissue of the intestinal lamina propria (51). Thus, ILF could be an additional IgA inductive tissue in the GI tract. However, our present study showed that oral immunization of TNFR55-Ig-treated mice, which possess significantly lower numbers of ILF, induced identical levels of Ag-specific IgA Ab responses when compared with control-Ig-treated mice. Furthermore, LTβR-Ig-treated, ILF-null mice elicited significant Ag-specific IgA Ab responses after oral immunization. These results indicate that ILF are not required for the induction of intestinal IgA Abs. In this regard, a recent study has shown that in utero LTβR-Ig-treated, PP-null mice have mature ILF, which contain an overlying epithelium, M cells, GCs, and $CD4^+$ T cells resembling PP, while nontreated mice have immature ILF formed by $B220^+$ cells only (29). This study implies that ILF are alternative inductive tissues that compensate for the function of PP. Thus, ILF may act as inductive sites for IgA Ab responses to orally administered Ag in the absence of PP, whereas these lymphoid follicles do not fully develop and are not strictly required for Ag-specific intestinal IgA responses in the presence of PP. However, our present results showed that mice treated with both TNFR55-Ig and LTβR-Ig in utero that retain ILF, but not PP and MLN, failed to induce TT-specific IgA responses after oral immunization. These findings indicate that ILF are not essential inductive sites for initiation of IgA Ab responses to orally administered Ag, although these lymphoid follicles could be an important source of IgA B cell development.

Alternatively, development of ILF may be induced by luminal bacterial Ag in the small intestine. In this regard, it has been shown that alterations in the bacterial flora by antibiotic treatment abolished ILF hyperplasia, which was provoked by the deficiency of activation-induced cytidine deaminase (30). Furthermore, mature ILF were not found in in utero LTβR-Ig-treated germfree mice; however, the ILF were reorganized when those mice were conventionalized (29). These studies, together with our results, suggest that formation of ILF is induced in response to microenvironmental bacterial Ags, but not to an orally administered protein. An interesting study has shown that IgA can be generated in peripheral LN of μMT mice lacking Ig μ and γ gene expression (52). Furthermore, those IgA Abs bind the protein of commensal intestinal bacteria and are induced by *Salmonella* infection (52). Because ILF can be formed in response to commensal intestinal bacterial Ag, IgA produced without μ- or δ-chain expression in developing B cells may be induced in ILF. These issues are currently under investigation in our laboratories.

It is important to note that postnatal treatment with LTβR-Ig results in the alteration of an MLN microarchitecture, including disorganized T and B cell zones and a diminished FDC network. Furthermore, although Ag-specific mucosal IgA responses were induced in mice treated with LTβR-Ig, the levels were significantly lower than those induced in control-Ig-treated mice. In contrast, ILF alone are not sufficient for the induction of Ag-specific mucosal IgA responses after oral immunization. These results suggest that the MLN microarchitecture at least in part plays an important role in the regulation of intestinal IgA Ab responses to orally administered Ag. In this regard, the MLN is considered to be one of the important components of GALT (2, 53, 54). Indeed, Ag-specific $CD4^+$ T cells are induced in the MLN with subsequent intestinal IgA Ab responses in the absence of PP (6), implying that the MLN is somewhat more important than PP for the generation of Ag-specific intestinal IgA immunity. In the lymphoid tissues, the presence of primary and secondary lymphoid follicles that contain FDC is thought to be required for a mature T cell-dependent, B cell response, and is associated with B cell isotype switching, affinity maturation, and development of Ab-secreting cells (55–57). Thus, it is likely that the disorganized follicular structure, T and B cell zones, and diminished FDC clusters in MLN of LTβR-Ig-treated, adult mice negatively impact Ag-specific mucosal immunity and lead to reductions in IgA Ab responses.

Our results clearly indicated that LTβR-Ig-treated mice that lack ILF induced intestinal IgA Ab responses to orally administered Ag. Thus, we at the least can conclude that ILF are not essential for induction of Ag-specific IgA responses. However, because the responses induced in the mice lacking ILF were significantly lower than those seen in the control mice, ILF may contribute to the generation of maximum IgA responses. In this regard, our previous study has shown that Ag-specific intestinal IgA Abs were induced after oral immunization in mice made deficient in PP, but not ILF and MLN, by in utero treatment with LTβR-Ig; however, the responses were also significantly lower than those seen in the control mice, suggesting a contribution by PP for maximum IgA responses (6). This finding together with our present results suggest that aggregated lymphoid follicles residing in the intestinal lumen, e.g., PP and ILF, and draining MLN may comprise an integrated regulatory network for the induction of IgA Ab responses to orally administered Ag. This interesting possibility is currently under investigation in our laboratories.

In summary, our study has demonstrated that LTβR-dependent events contribute importantly to the maintenance of a normal MLN microarchitecture and to the size and numbers of PP. In contrast, the microarchitecture of the PP was not controlled by LTβR signaling. Furthermore, postnatal blockade of the LTβR pathway resulted in a lack of ILF. Oral immunization of LTβR-Ig-treated mice with TT plus CT elicited significant TT-specific mucosal IgA Abs; however, the responses were significantly lower than those of control mice. In contrast, in utero TNFR55-Ig- and LTβR-Ig-treated mice that have ILF, but not PP and MLN, failed to induce intestinal IgA Abs to orally administered Ag. Taken together, our findings have addressed the roles of TNF/LTα and LTαβ pathways for the establishment of Ag-specific mucosal IgA Ab responses. Furthermore, ILF are not required for the induction of IgA Ab responses to orally administered protein Ag. Finally, the microarchitecture of the MLN plays a critical role in the induction and regulation of IgA Ab responses to orally administered Ag.

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IgA Class Switch Occurs in the Organized Nasopharynx- and Gut-Associated Lymphoid Tissue, but Not in the Diffuse Lamina Propria of Airways and Gut[1]

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Secretory IgA plays a crucial role in the host immune response as a first line of defense. A recent demonstration of in situ IgA class switching in intestinal lamina propria provided an opportunity to reconsider the model for the homing of IgA-committed B cells characterized by distinctive trafficking patterns to effector sites. Those effector sites depend on the organized mucosa-associated lymphoid tissues as their site of induction. In this report we show the preferential presence of IgM^+B220^+ and IgA^+B220^+ cells belonging to pre- and post-IgA isotype class-switched cells in the organized mucosa-associated lymphoid tissues, such as nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer's patches, and the defect of those populations in the diffuse effector tissues, such as the nasal passage and intestinal lamina propria. Consistent with these findings, the expressions of a series of IgA isotype class switch recombination-related molecules, including activation-induced cytidine deaminase, Iα-Cμ circle transcripts, and Iα-Cμ circle transcripts, were selectively detected in these organized mucosa-associated lymphoid structures, but not in the diffuse mucosal effector sites. Taken together, these findings suggest that IgA isotype class switching occurs only in the organized mucosa-associated lymphoid organs (e.g., nasopharynx-associated lymphoid tissues, isolated lymphoid follicles, and Peyer's patches), but not in the diffuse effector tissues of the upper respiratory and gastrointestinal tracts. ***The Journal of Immunology*****, 2004, 172: 6259–6264.**

The mucosal immune system provides the first line of defense against the ingress of microbial pathogens during the physiological processes of inhalation and ingestion. One of the major components of the mucosal immune system is secretory IgA (S-IgA),[3] which is produced by the mucosal interaction of epithelial cells, IgA-committed B cells, and Th cells in the respiratory and gastrointestinal tracts (1, 2). Thus, mucosal effector sites such as the lamina propria of the upper respiratory and intestinal tract contain high numbers of IgA blast and plasma cells derived from postswitched IgA^+ B cells.

IgA-committed B cells that have undergone μ to α isotype class switching in nasopharynx-associated lymphoid tissue (NALT), Peyer's patches (PPs), and other mucosal inductive lymphoid organs are generally believed to migrate to diffuse mucosal effector tissues, including the nasal passage (NP) and intestinal lamina propria (i-LP) (3, 4). PPs show a high frequency of IgM^+ $B220^+$ B cells, which are a prerequisite for isotype class switching to IgA^+B220^+ B cells after antigenic or mitogenic stimulation in the presence of the isotype switch-inducing cytokine TGF-β (1, 5–8). PPs are an example of organized gut-associated lymphoid tissue (GALT) with germinal centers (GCs) and are thus considered to be a major site for the μ to α class switch recombination (CSR) involving activation-induced cytidine deaminase (AID). Iα-Cμ circle transcripts (αCTs), and Iμ-Cα transcripts (9, 10). Recent results, generated from analysis of AID-deficient mice, however, have suggested another pathway for the development of intestinal IgA plasma cells (11). IgA class switching was shown to occur in i-LP without the involvement of PPs or other organized mucosa-associated lymphoid tissues (MALT) containing GCs. In this study, IgM^+ $B220^+$ B cells in i-LP switched to IgA^+ B cells under the influence of TGF-β derived from i-LP stromal cells (11). These findings suggest that i-LP could have the immunological function of both effector and inductive sites. However, the concerns with that study were that the researchers did not have control of the compartment from which the B cells were harvested, either the diffuse lamina propria or isolated lymphoid follicles (ILFs) embedded in the lamina propria, due to the nature of AID-deficient mice. Because $AID^{-/-}$ mice exhibited enormous hyperplasia of ILFs, there was little of the diffuse lamina propria region left (11).

We recently identified ILFs as part of the organized GALT in mouse small intestine (12). ILFs are comprised of a single B cell

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[3] Abbreviations used in this paper: S-IgA, secretory IgA; AID, activation-induced cytidine deaminase; CSR, class switch recombination; CT, cholera toxin; αCT, Iα-Cμ circle transcript; GALT, gut-associated lymphoid tissue; GC, germinal center; ILF, isolated lymphoid follicle; i-LP, intestinal lamina propria; MC, mononuclear cell; MLN, mesenteric lymph node; NALT, nasopharynx-associated lymphoid tissue; NP, nasal passage; PP, Peyer's patch.

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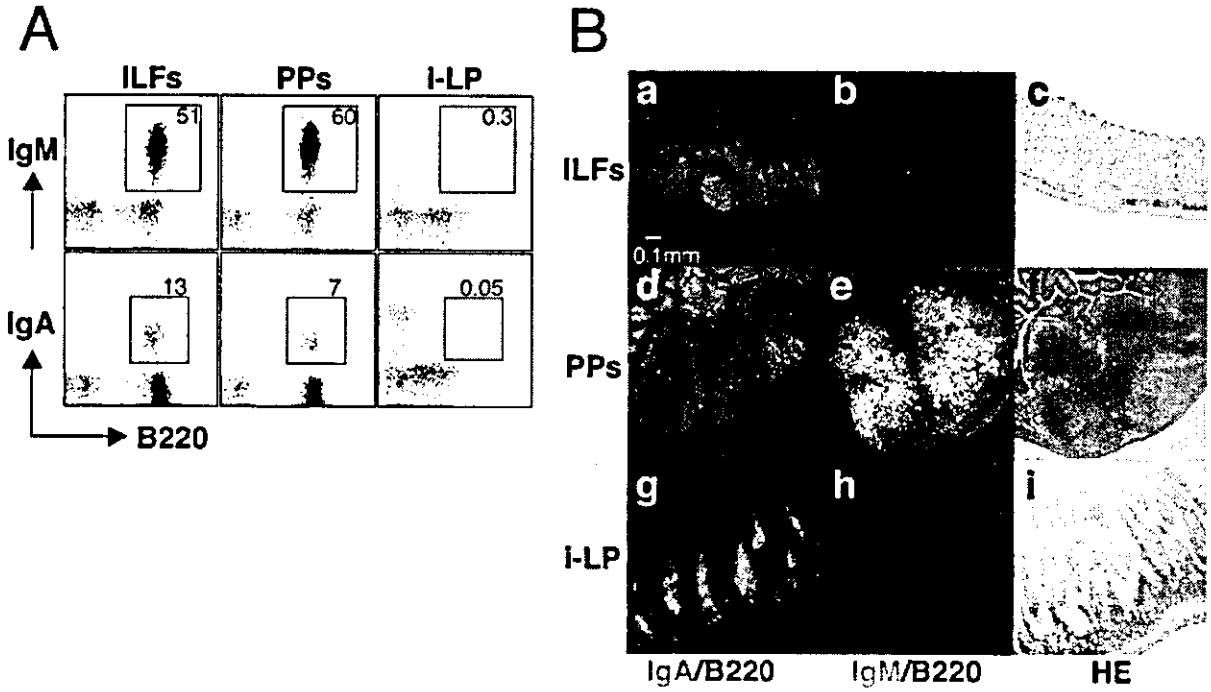

FIGURE 1. The accumulation of IgM^+B220^+ and IgA^+B220^+ cells in ILFs and PPs and their absence in the i-LP of mouse small intestine. *A*, MCs isolated from ILFs, PPs, and i-LP were stained with a panel of mAbs specific for IgM, IgA, and B220. The i-LP MCs were prepared after complete removal of ILFs and PPs. *B*, Consecutive tissue sections were stained with FITC-conjugated anti-IgA (*a*, *d*, and *g*), anti-IgM (*b*, *e*, and *h*), and PE-conjugated anti-B220 (*a*, *b*, *d*, *e*, *g*, and *h*). These tissues were also stained with HE (*c*, *f*, and *i*). Although cells expressing IgA or IgM (green) as well as those expressing B220 (red) were found in ILFs and PPs (*a*, *b*, *d*, and *e*), double-positive cells were hardly detected in i-LP (*g* and *h*), where IgA^+B220^- blast/plasma cells (green) were abundant (*g*). These sections were analyzed by confocal microscopy using a ×25 magnification. Results are representative of three separate experiments.

follicle with GCs overlaid by a follicle-associated epithelium containing Ag up-taking M cells that are similar to the follicle-associated epithelium of PPs. The presence of ILF tissue could provide additional mucosal inductive sites for the generation of IgA-committed B cells in the gastrointestinal tract (13). In fact, looking at the data overall, it is not possible to exclude the possibility that ordinary preparations of i-LP samples may contain IgM^+ $B220^+$ B cell fractions undergoing in situ class switching inside the GCs of these newly discovered murine ILFs (12).

For the upper respiratory tract, NALT has been shown to contain all the necessary molecular and cellular environments for the initiation of IgA B cell responses (2, 14, 15). NALT has also been identified as a region in which both IgA-committed B cells and memory-type IgA^+ B cells are generated (15). Nasal immunization has been shown to effectively induce Ag-specific IgA Ab responses via NALT for the upper respiratory tract, including the NP (2, 16). Thus, in the respiratory tract, NALT, with its inductive sites, and NP, with its effector sites, are considered to be part of the IgA-committed B cell-homing pathway analogous with PPs and i-LP in the gastrointestinal tract.

In this study, using the molecular and cellular analyses of transcription as well as immunocytochemistry and immunohistochemistry, NALT and GALT, including ILFs and PPs as well as NP and i-LP, were examined as examples of organized inductive and diffuse effector tissues, respectively. In this paper we report evidence that organized MALT, but not the diffuse lamina propria of the respiratory and intestinal tracts, play an essential role in the generation of IgA-committed B cells, especially in the μ to α isotype class switching for the mucosal immune system.

Materials and Methods

Mice

BALB/c mice were purchased from CLEA Japan (Tokyo, Japan) and used at 6–12 wk of age. In some experiments mice were nasally immunized with

Table I. *Distribution of different B cell subsets in organized mucosa inductive vs diffuse effector tissues*[a]

B Cell Subsets		Respiratory Tract		Intestinal Tract		
				Inductive		
sIg	B220	Inductive, NALT	Effector, NP	ILFs	PPs	Effector, i-LP
IgM	+	46.75 ± 0.19	0.53 ± 0.1	49.6 ± 5.8	63.9 ± 4.9	0.31 ± 0.11
IgA	+	0.79 ± 0.59	0.06 ± 0.01	11.3 ± 3.1	8.2 ± 1.7	0.06 ± 0.04
IgA	−	0.20 ± 0.14	7.69 ± 1.2	1.9 ± 0.8	0.5 ± 0.2	21.7 ± 5.3

[a] MCs were isolated from the organized inductive and effector tissues of the respiratory and intestinal tracts for flow cytometric analysis with fluorochrome-conjugated mAbs anti-μ, anti-α, and PE-conjugated mAb anti-B220. The percentages of the B cell subset indicated in the *left column* in various tissues are shown in each row. The data are presented as the mean ± SD from three separate experiments. sIg, surface Ig.

1 μg of cholera toxin (CT; Sigma-Aldrich, St. Louis, MO), a potent immunogen with strong adjuvanticity (17), once a week for 3 consecutive wk (16).

Cell preparation

Mononuclear cells (MCs) were isolated from spleen, PPs, NP, and NALT as described previously (16, 18). MCs from spleen and NALT were obtained using a mechanical dissociation procedure (16, 18). MCs from PPs and NP were prepared using the enzymatic dissociation protocol with collagenase D (Roche, Mannheim, Germany) (16, 18). MCs from ILFs were prepared as described previously (12). In brief, the small intestine was opened longitudinally along the mesenteric wall. After removal of mucus and feces, an intestinal fragment ~3 cm in length was pasted on a culture dish. Under a transillumination stereomicroscope (TH3; Olympus, Tokyo, Japan), a tiny fragment of the small intestine containing a single ILF was amputated by a sharpened needle. After removal of PPs and ILFs, i-LP lymphocytes were isolated from the small intestine by the enzymatic dissociation procedure with collagenase D (Roche) (18).

Flow cytometry

FITC-conjugated anti-mouse IgA and IgM, and R-PE-conjugated anti-B220 were used for staining (BD PharMingen, San Jose, CA) (18). MCs isolated from different tissues were preincubated with Fc Block (2 μg/ml; BD PharMingen) before fluorochrome-conjugated mAbs. MCs were then incubated with optimal concentrations of FITC-conjugated anti-mouse IgA (2 μg/ml) or IgM (2 μg/ml) together with PE-conjugated anti-B220 (2 μg/ml). Flow cytometric analysis was then performed using FACSCalibur (BD Biosciences, San Jose, CA) (18).

Immunohistochemical analysis

Vertically oriented sections of the small intestine that included PPs and/or ILFs as well as NP and NALT were prepared as previously described (2, 12). Briefly, the small intestine was longitudinally opened along the mesenteric wall, rolled up, and frozen in OCT compound (Sakura Finetechnical, Tokyo, Japan) in liquid nitrogen. Serial frozen sections were then incubated with FITC-conjugated anti-IgA (2 μg/ml) or FITC-conjugated anti-IgM (2 μg/ml) and PE-conjugated anti-B220/CD45R (2 μg/ml; all from BD PharMingen) for 1 h at room temperature. The slides were then examined using confocal microscopy (Bio-Rad, Hercules, CA). After confocal fluorescence microscopic analysis, the sections were counterstained with H&E. The sections used for examination were prepared from at least three individual mice.

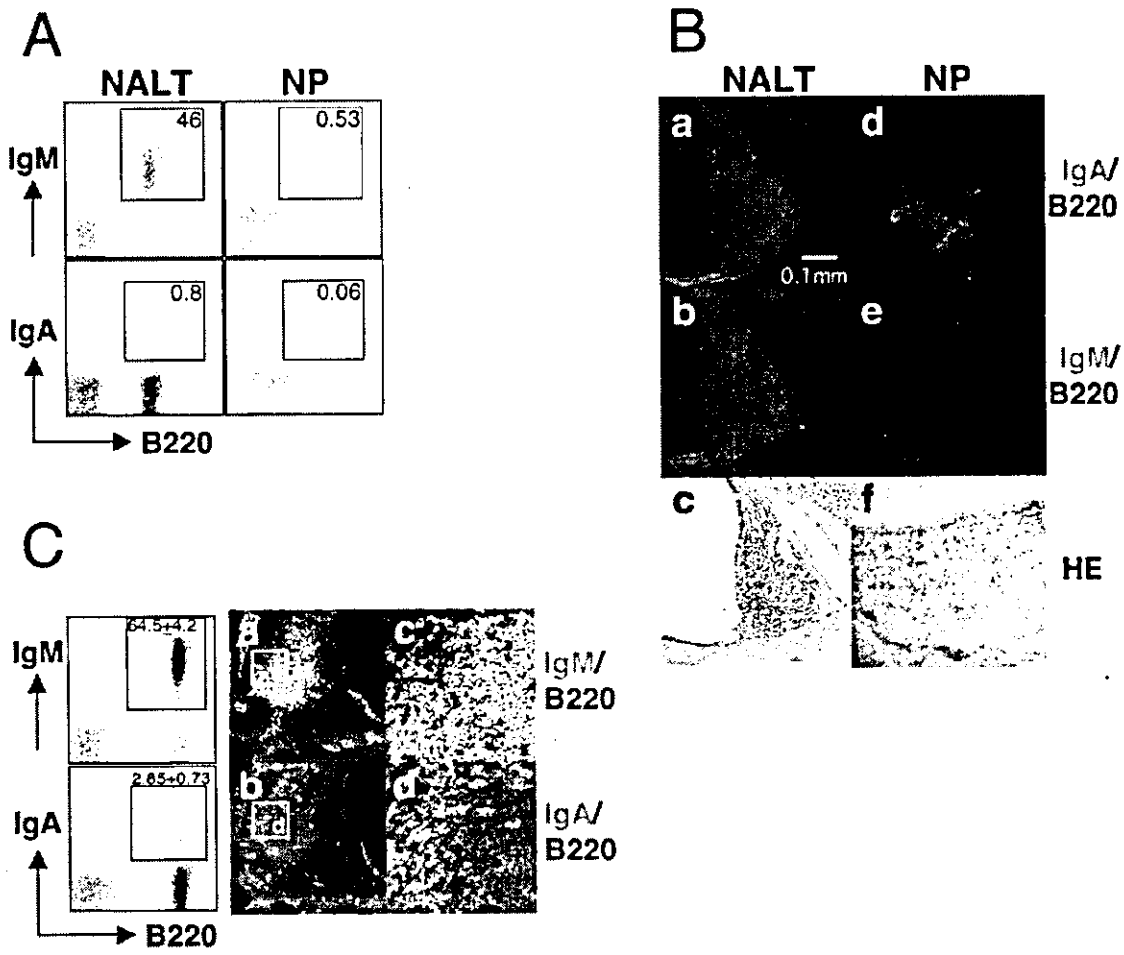

FIGURE 2. Selective localization of IgM^+B220^+ cells in NALT, but not in NP. *A*, MCs isolated from NALT and NP were stained with a panel of mAbs specific for IgM, IgA, and B220. *B*, Consecutive tissue sections were stained with FITC-conjugated anti-IgA (*a* and *d*), anti-IgM (*b* and *e*), and PE-conjugated anti-B220 (*a*, *b*, *d*, and *e*). Samples of each tissue were also stained with HE (*c* and *f*). Cells expressing IgA or IgM (green) as well as those expressing B220 (red) were found only in NALT (*a* and *b*). The numbers of double-positive cells (yellow) of IgA or IgM and B220 were negligible (*d* and *e*), although IgA^+B220^- blasts and plasma cells were plentiful (*d*). *C*, IgA^+ $B220^+$ B cells increased in NALT after nasal immunization with CT. MCs isolated from NALT were stained with a panel of mAbs specific for IgM, IgA, and B220. Consecutive tissue sections were stained with FITC-conjugated anti-IgM and PE-conjugated anti-B220 (*a*) or FITC-conjugated anti-IgA and PE-conjugated anti-B220 (*b*). Higher magnification of sections *a* and *b* are shown in *c* and *d*, respectively. Double-positive cells (yellow) for IgA (green) and B220 (red) were increased in NALT (*C-b* or *C-d*) after nasal immunization with CT (0.8–2.9%). In contrast, double-positive cells (yellow) for IgM (green) and B220 (red) were found in both naive and immunized NALT (*B-b* and *C-a* or *C-c*). These sections were analyzed by confocal microscopy using ×35 (*B*, *C-a*, and *C-b*) and ×200 (*C-c* and *C-d*) magnifications. Results are representative of three separate experiments.

RT-PCR

Total RNA isolated from mouse tissues was extracted, following the manufacturer's instructions, using TRIzol (Invitrogen, Carlsbad, CA). cDNA was prepared by reverse transcriptase (Invitrogen) with oligo(dT) primer. AID transcripts, αCTs, Iμ-Cα, and β-actin transcripts were amplified as described previously (9–11). The oligonucleotide primers specific for AID transcripts (5'-GGCTGAGGTTAGGGTTCCATCTCAG-3' and 5'-GAGG GAGTCAAGAAAGTCACGCTGGA-3'), Iα-Cμ circle transcripts (5'-CCAGGCATGGTTGAGATAGAGATAG-3' and 5'-AATGGTGCTGGG CAGGAAGT-3'), Iμ-Cα transcripts (5'-CTCTGGCCCTGCTTATTGT TG-3' and 5'-GAGCTGGTGGGAGTGTCAGTG-3'), and β-actin transcripts (5'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGC TCAGTAACAGTCCG-3') were prepared according to previously described methods (9–11). The experiments were conducted on three separate occasions.

Results

Focal accumulation of IgM$^+$B220$^+$ and IgA$^+$B220$^+$ cells in organized GALT structures, but not diffuse effector tissues

To investigate the exact class-switching sites in different parts of the wall of the mouse small intestine, we initially examined and compared the localized presence of IgM$^+$B220$^+$ B cells, which are considered to be prerequisite for class switching recombination (19, 20). To accomplish this goal, careful separation of the organized lymphoid tissue and the diffuse effector tissue was required. GALT, including PPs and especially ILFs, was carefully identified and removed. After the removal of GALT, but before enzymatic dissociation, the intestine was further re-examined, using microscopic analysis, to ensure and confirm the complete removal of organized lymphoid tissue. Screening for IgM$^+$B220$^+$ B cells revealed their presence in ILFs and PPs, but their almost complete absence from i-LP (Fig. 1*A* and Table I). Furthermore, because IgA$^+$B220$^+$ B cells are believed to result from recent class switching involving the expression of CSR molecules such as AID and looped-out circular DNA (11), we next screened for the presence of IgA$^+$B220$^+$ B cells in these different intestinal mucosa-associated tissues. Although FACS analysis revealed the presence of IgA$^+$B220$^+$ B cells in ILFs and PPs, hardly any were detected in i-LP (Fig. 1*A* and Table I). Furthermore, the preferential localization of both IgM$^+$B220$^+$ cells and IgA$^+$B220$^+$ B cells in the organized lymphoid tissues was also revealed by immunohistochemical analysis using fluorescence confocal microscopy (Fig. 1*B*). By contrast, both populations were absent in diffuse i-LP regions. Instead, larger numbers of IgA$^+$B220$^-$ B cells, corresponding to the blast and plasma stages, were found in diffuse effector tissues (Table I). Taken together, these observations suggest that the isotype class switching of B cells from μ- to α-chains occurs selectively in the organized lymphoid structure of ILFs and PPs, but not in the diffuse effector tissues of i-LP of the small intestine.

In the upper respiratory tract, IgM$^+$B220$^+$ and IgA$^+$B220$^+$ B cells are present in NALT, but not in NP

In the generation of IgA-committed B cells, NALT has been shown to be as important an inductive site as GALT (1, 15, 16). When similar evaluation of the respiratory mucosal immune system for IgM$^+$B220$^+$ B cell presence was conducted, the tissue localization pattern was similar to that we described for the intestinal tract. Thus, IgM$^+$B220$^+$ B cells were preferentially localized in the organized NALT, but were virtually absent from the diffuse effector tissues of the NP (Fig. 2, *B-b* or *B-e*, and Table I). In contrast to PPs and ILFs, extremely few IgA$^+$B220$^+$ B cells were found in NALT (Fig. 2*B-a* and Table I). Ag stimulation by bacterial flora of the upper respiratory tract is weaker than that by gut flora surrounding GALT, and we hypothesized that this was the reason for the lack of IgA$^+$B220$^+$ B cells in NALT. To test this hypothesis we performed nasal immunization with CT, which is known to possess a strong antigenicity with potent mucosal adjuvant activity (17). After nasal immunization, the presence of IgA$^+$B220$^+$ B cells increased in NALT (Fig. 2*C* and Table II). The formation of GCs and the accumulation of IgA$^+$B220$^+$ B cells were also seen in the NALT of these nasally immunized mice (Fig. 2*C*). These results demonstrate that in the upper respiratory tract, IgA isotype class switching occurs in the organized NALT, but not in the diffuse lamina propria of the NP.

Table II. *Influence of nasal CT on the development of IgA$^+$ B cells in NALT*[a]

B Cell Subsets		Nasal Immunization	
sIg	B220	Before	After
IgM	+	54.8 ± 6.90	64.5 ± 4.2
IgA	+	0.93 ± 0.72	2.85 ± 0.73
IgA	−	0.04 ± 0.02	0.48 ± 0.09

[a] After nasal immunization with CT, which is known to possess a strong antigenicity with potent mucosal adjuvant activity, MCs were isolated from NALT and then analyzed by flow cytometry with fluorochrome-conjugated mAbs anti-μ, anti-α, or PE-conjugated mAb anti-B220. Percentages of the B cell subset indicated in the *left column* in NALT before and after nasal immunization with CT are shown in each row. The data are presented as the mean ± SD from three separate experiments. sIg, surface Ig.

Expression of CSR-associated mRNA observed in the organized MALT structure, but not in diffuse effector tissues

To test for CSR from the μ-chain to the α-chain at the molecular level, three molecular markers, AID, αCTs, and Iμ-Cα, were selected. AID was essential for CSR and completely regulated stimulated B cells undergoing class switching (9–11). αCTs were produced from circular DNA that is looped out and lost after CSR (9–11). Iμ-Cα transcripts were produced from α germline transcripts after looping out of αCTs (9–11). Because the expression of AID and αCTs is strictly up-regulated during and is quickly down-regulated after isotype class switching, such expression is considered to characterize the class switching of B cells from μ to α (10). Meanwhile, Iμ-Cα transcripts were seen to be expressed immediately after completion of IgA-specific CSR (11). To further confirm that IgA isotype class switching occurs in vivo at the organized MALT, we next used RT-PCR analysis to test for the expression of CSR-associated molecules, including AID, αCTs,

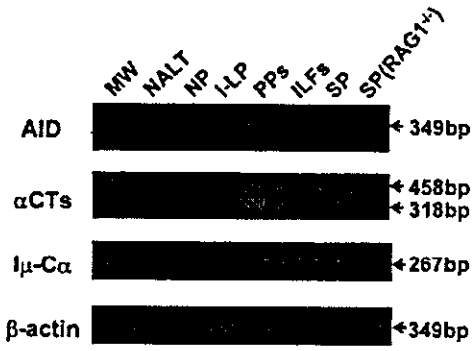

FIGURE 3. Preferential presence of AID, αCT, and Iμ-Cα transcripts in the organized lymphoid tissues of NALT, PPs, and ILFs, but not in the diffuse effector tissues of NP and i-LP. RT-PCR analysis revealed AID, αCT, and Iμ-Cα transcripts in NALT, PPs, and ILFs, but not in i-LP and NP. As a negative control for RT-PCR, the total RNA of MCs isolated from spleen of recombinase-activating gene-1(RAG1)$^{-/-}$ mice was used. The experiments were conducted on three separate occasions.

and Iμ-Cα transcripts, in the organized lymphoid and diffuse effector tissues of the gastrointestinal and respiratory tracts. The results were consistent with our finding of histological localization of IgM^+B220^+ B cells and IgA^+B220^+ B cells (Figs. 1 and 2, and Tables I and II). mRNA expressions of AID, αCTs, and Iμ-Cα transcripts were restricted to the organized MALT, such as ILFs, PPs, and NALT, but were not found in the diffuse effector tissues of i-LP and NP (Fig. 3). These results indicate that IgA isotype class switching selectively occurs in the organized MALT, but not in the diffuse effector tissues.

Discussion

PPs have been shown to contain all the cellular and microarchitectural environments (e.g., B cell follicle, including GCs, follicular dendritic cells network, and interfollicular T cell area) needed for the generation of IgA-committed B cells (1). A large number of IgM^+B220^+ B cells in the GCs of PP follicles express AID and undergo the molecular event of μ to α isotype class switching (9). Results have shown that the incubation of IgM^+ IgA^- B cells isolated from PPs together with TGF-β results in the induction of IgA isotype class switching, which leads to the generation of IgM^-IgA^+ B cells (5–8). After generation in PPs, these postswitched IgA^+ B cells (or IgA-committed B cells) migrate to effector tissues, such as i-LP, where they become IgA blast and plasma cells under the influence of IgA-enhancing cytokines IL-5, IL-6, and IL-10 (1). Therefore, it is generally accepted that organized MALT, such as PPs, acting as inductive sites, play a major role in the initiation of the IgA Ab response, while diffuse i-LP tissues provide the effector sites (1). This conjecture was further confirmed by our current results, which show that IgM^+B220^+ B cells capable of undergoing μ to α isotype class switching were exclusively located in PPs and were absent from diffuse i-LP. In contrast, large numbers of IgA blast and plasma cells were found in these diffuse i-LP. Furthermore, evaluation of CSR-associated molecules for the μ to α gene rearrangement revealed that AID, αCTs, and Iμ-Cα transcripts were selectively expressed in PPs with the organized lymphoid structure, but not in the diffuse i-LP.

In addition to testing PPs, we tested ILFs, which we recently characterized as part of the organized GALT on the antimesenteric wall of the mouse small intestine (12). This cell formation is composed of a large B cell area, including GCs. A large fraction of the B cells in ILFs are B-2 cells, similar to those found in PPs (12). In this study our findings further demonstrate that ILFs are a rich source of IgM^+B220^+ B cells that can undergo μ to α CSR. Thus, CSR-associated transcripts of AID, αCT, and Iμ-Cα were present in the mRNA preparation obtained from ILF as well as PP samples. After careful removal of ILFs and PPs, MCs that had been isolated from the diffuse effector tissues of i-LP were evaluated for the IgA-associated CSR-associated transcripts. No AID, αCT, or Iμ-Cα transcripts were found in the diffuse tissue of i-LP. Taken together, these findings indicate that ILFs and PPs, both of which contain B cell follicles with GCs, constitute the organized GALT and behave as key inductive sites for μ to α isotype class switching when IgA-committed B cells are generated.

When we evaluated the presence and population density of IgM^+ B cells and/or IgA^+ B cells in different histological locations of the upper respiratory tract, accumulations of IgM^+B220^+ B cells were always observed only in the organized MALT such as NALT. These cells were absent in the NP, a representative diffuse effector site for the upper respiratory tract (Fig. 2 and Table I). In addition, AID, αCT, and Iμ-Cα transcripts were expressed in NALT, but not in NP, paralleling expression by ILFs and PPs, but not i-LP, in the intestinal tract (Fig. 3). Previously we have shown that NALT B cells belong to a subset of B-2 cells (18). That study also showed that IgA isotype class switching of B-2 cells in the upper respiratory tract occurs only in the organized MALT, such as NALT, and not in the diffuse lamina propria region of the NP.

Comparisons of NALT and GALT (e.g., ILFs and PPs) for the population density of switched IgA^+B220^+ B cells revealed a dramatic difference. Much higher numbers of IgA^+B220^+ B cells were found in GALT (e.g., 6.5–14.4%; Table I) than in NALT (e.g., 0.2–1.4%; Table I). That finding may be explained by the different microbial environments found in NALT and GALT. In comparison with NALT, GALT structures are situated in a part of the gut where there is an enormous load of microbial Ags and mitogens to continuously stimulate immunocompetent cells located in the organized lymphoid tissue for the mucosal immune system. Thus, administration of CT possessing potent immunogenicity and adjuvanticity via the nasal route resulted in a 3- to 10-fold increase in the population (e.g., 2.9 ± 0.7%; Fig. 2). This increase points to the importance of antigenic stimulation in the initiation of IgA class isotype switching for the generation of IgA-committed B cells in NALT. Elsewhere, we have shown that the tissue organogenesis of NALT is also accelerated by nasal exposure to Ag (21).

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Intestinal villous M cells: An antigen entry site in the mucosal epithelium

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M cells located in the follicle-associated epithelium of Peyer's patches (PP) are shown to be the principal sites for the sampling of gut luminal antigens. Thus, PP have long been considered the gatekeepers of the mucosal immune system. Here, we report a distinct gateway for the uptake of gut bacteria: clusters of non-follicle-associated epithelium-associated *Ulex europaeus* agglutinin (UEA)-1$^+$ cells, which we have designated intestinal villous M cells. Interestingly, villous M cells are developed in various PP [or gut-associated lymphoid tissue (GALT)]-null mice, such as *in utero* lymphotoxin β receptor (LTβR)-Ig-treated, lymphotoxin α (LTα)$^{-/-}$, tumor necrosis factor/LT$\alpha^{-/-}$, and inhibition of differentiation 2 (Id2)$^{-/-}$ mice. Intestinal villous M cells have been observed to take up GFP-expressing *Salmonella*, *Yersinia*, and *Escherichia coli*-expressing invasin, as well as gut bacterial antigen for subsequent induction of antigen-specific immune responses. Thus, the identified villous M cells could be an alternative and PP-independent gateway for the induction of antigen-specific immune responses by means of the mucosal compartment.

The huge intestinal surface area is physically protected by a layer of tightly joined epithelial cells, which prevent most enteric environmental antigens from penetrating the host (1). However, entry into the host is made possible by a special gateway, comprised of M cells, located over organized mucosal lymphoid follicles such as Peyer's patches (PP). The M cells, characterized by an irregular brush border and reduced glycocalyx, efficiently take up and transport a wide variety of macromolecules and microorganisms from the gut lumen to the inside of the PP (2–6), which contain all of the necessary lymphoid cells for the induction and regulation of antigen-specific IgA responses (7). However, the origin of M cells and the regulation of their development are not understood. A previous study (8) showed that i.v. injection of PP lymphocytes into severe combined immunodeficient mice resulted in formation of new lymphoid follicles and follicle-associated epithelium (FAE) with typical M cells. A similar phenomenon was seen by using *in vitro* studies in which coculture with B lymphocytes triggered the conversion of enterocyte cell lines into M cell-like cells (9). Further, B cells have recently been proposed to play a role in the organogenesis of the mucosal immune barrier (10). Two different B cell-null mice, lacking expression of either μ membrane exon or the J_H segment of Ig genes, showed drastic reduction of FAE size and M cell numbers (10). In contrast, a recent study (11) demonstrated that the absence of mature T and B cells does not prevent the formation of FAE and M cells, and signaling of lymphotoxin (LT) α/β from non-B and non-T cells plays a critical role in formation of M cells in FAE of PP.

The common mucosal immune system (CMIS), which connects the inductive (e.g., PP) and effector (e.g., lamina propria; LP) sites, has been shown to be a central pathway for the induction of antigen-specific IgA immune responses in the gastrointestinal tract (7). For example, oral administration of *Salmonella typhimurium* leads to the transport of the bacterial antigen from the lumen of the intestinal tract into the PP by means of M cells for the initial priming of antigen-specific CD4$^+$ T cells and IgA-committed B cells (12). These antigen-sensitized cells leave the PP and contribute to the subsequent induction of *Salmonella*-specific IgA response in the distant intestinal LP by means of CMIS. In addition to the well-characterized CMIS-dependent IgA induction pathway, recent evidence suggests the presence of an additional IgA induction pathway that is independently operated from the PP-originated CMIS (13–15). Interestingly, it also has been reported that induction of intestinal mucosal IgA against the commensal bacteria was independent from T cell help and organized lymphoid tissue (16). Further, our recent study (17) has demonstrated that antigen-specific IgA antibody responses can be induced in the absence of PP. These studies imply the existence of a PP-independent mucosal immune pathway for dietary antigen and bacteria uptake.

A recent study (18) has suggested that the invasion gene (SPI1)-deficient *S. typhimurium* can be disseminated from the intestinal epithelium to the systemic compartment in the absence of PP-associated M cells by means of the CD18-dependent pathway. Further, dendritic cells in the lamina propria of the small intestine expressing tight junction protein offer another possible antigen uptake site (19). Thus, intestinal DCs are capable of extending dendrites to the lumen side by opening the tight junction. However, the exact mechanism for inducing Ag-specific immune responses independently of PP requires further elucidation.

In this study, we have discovered intestinal villous M cells, which serve as an antigen gateway for the sampling of gut bacteria and subsequent induction of Ag-specific immune responses in a PP-independent manner. These lines of study are crucial for understanding the mechanisms of antigen uptake from the gut lumen, and for the rational design of effective mucosal vaccines and optimal drug delivery across the gut.

Experimental Procedures

Mice. BALB/c and C57BL/6 mice were purchased from CLEA Japan (Tokyo). LTβR-Ig fusion protein-treated and tumor necrosis factor (TNF) and LTα double knockout (TNF/LT$\alpha^{-/-}$; 129 × C57BL/6) mice were generated as described (20, 21). LT$\alpha^{-/-}$ mice (C57BL/6) were obtained from The Jackson Laboratory. Inhibi-

Abbreviations: PP, Peyer's patches; FAE, follicle-associated epithelium; LT, lymphotoxin; TNF, tumor necrosis factor; WGA, wheat germ agglutinin; UEA, *Ulex europaeus* agglutinin; TRITC, tetramethylrhodamine B isothiocyanate; IEC, intestinal epithelial cell; ILF, isolated lymphoid follicle; GALT, gut-associated lymphoid tissue; Id2, inhibition of differentiation 2.

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tion of differentiation 2 $(Id2)^{-/-}$ mice (129/Sv) were generated as described (22).

M Cell Staining. A standard lectin staining procedure was used for the detection of murine M cells (23). Mucus-free small intestine of naive BALB/c or C57BL/6 mice, with or without PP, was fixed in 4% paraformaldehyde for 1 h, washed, and then blocked with 10% FBS in PBS containing 0.1% glycine. A lectin-labeling experiment was performed with *Ulex europaeus* agglutinin (UEA) conjugated with tetramethylrhodamine B isothiocyanate (TRITC) (UEA-1-TRITC, Vector Laboratories) and wheat germ agglutinin (WGA) conjugated with FITC (WGA-FITC) at a concentration of 20 μg/ml for 2 hr. After being rinsed in PBS, samples were stored in a Tris-buffered solution containing 30% glycerol and 0.1% NaN_3. The specimens were examined in a Bio-Rad MRC-600 confocal imaging system (Bio-Rad). Alkaline phosphatase activity and alcian blue staining were assessed on whole fixed small intestine as described (11). In addition, scanning and transmission electron microscopy analyses were performed for the characterization of M cells (see *Supporting Experimental Procedures*, which is published as supporting information on the PNAS web site).

Antigen Uptake *in Situ*. *S. typhimurium* PhoPc strain transformed with the pKKGFP plasmid was kindly provided by F. Niedergang (24, 25). Further, GFP-expressing *Yersinia pseudotuberculosis*, *Escherichia coli*-invasin, and *E. coli* were prepared by the method described (26, 27). Mice were anesthetized by i.p. injection of 2 mg of ketamine (Sigma) per mouse. Segments ≈10 cm long of the small intestine of $TNF/LT\alpha^{-/-}$ mice and wild-type mice were ligated at both ends with surgical thread. GFP-expressing bacteria (5×10^8) were suspended in 1.0 ml and inoculated into the loop and incubated *in situ*. Ten minutes later, PP and the intestinal segments (without PP) were removed and extensively washed with cold PBS and RPMI medium 1640 including gentamycin (100 μg/ml). Intestinal epithelial cells (IECs) were isolated from PP and the intestinal segments as described (28), then fixed in 4% paraformaldehyde, washed with 10% FBS in PBS, and labeled with UEA-1-TRITC. The percentage of double-positive IECs was analyzed on a FACSCalibur flow cytometer (Becton Dickinson). In selected mice, whole-mounted small intestinal segments were processed for confocal microscopy as described above. To remove weakly adhered and/or extracellular bacteria, vigorous washing with cold PBS and RPMI medium 1640 containing gentamycin were adopted during the process of isolation of villous epithelium including M cells and epithelial cells after infection with bacteria. Gentamycin was selected as the antibiotic due to its lethal effects on *Salmonella* (29). Therefore, our present data include only *Salmonella* that had strongly adhered and was intracellular but not *Salmonella* that was weakly adhered and extracellular.

Immunization. The recombinant *S. typhimurium* BRD 847 strain used in the immunization study is a double *aroA aroD* mutant that expresses the nontoxic, immunogenic 50-kDa ToxC fragment of tetanus toxin from plasmid pTET*nir*15 under the control of the anaerobically inducible *nirB* promoter (r*Salmonella*-ToxC) (30). For the control, r*Salmonella* that are not expressing ToxC were adopted. Recombinant *Salmonella* organisms were resuspended in PBS to a concentration of 2.5×10^{10} bacteria per ml. Bacterial suspensions were orally administered by gavage (0.2 ml per mouse). Ab titers in serum were determined by ELISA, as described elsewhere (17).

Data Analysis. Data were expressed as mean ± SD and evaluated by the Mann–Whitney *U* test. *P* values of <0.05 were assumed to be statistically significant.

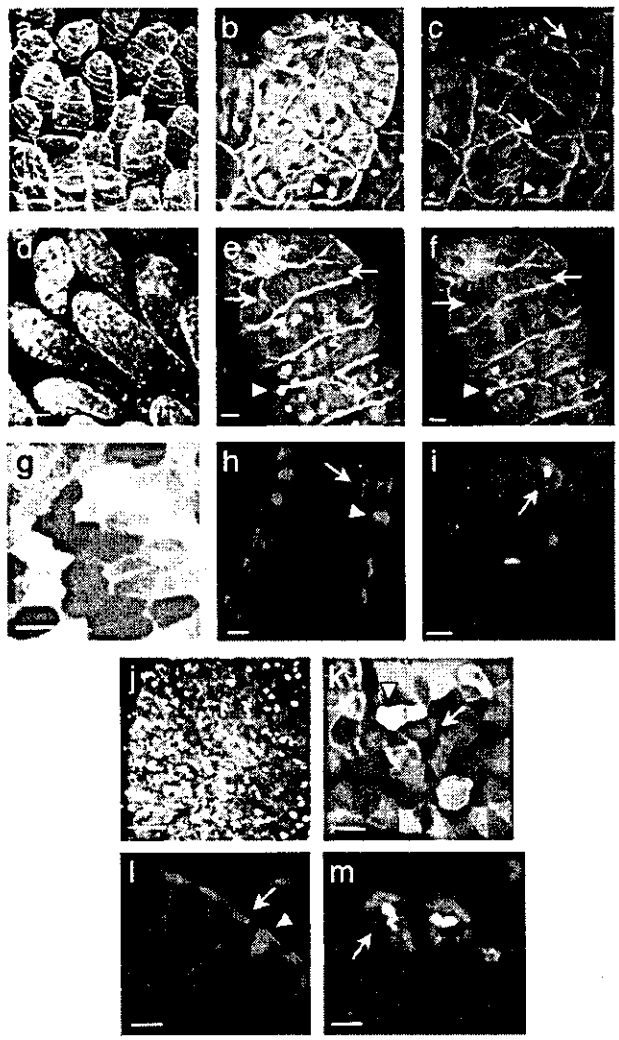

Fig. 1. Confocal view of UEA-1^+ cells in villous epithelium (*a–i*) and FAE of PP (*j–m*) isolated from naive BALB/c mice. M cell- and columnar epithelial cell-specific UEA-1-TRITC and WGA-FITC, respectively, were applied to the whole-mount preparation of the small intestine (*a–f*, *j*, and *k*). M cells were stained by UEA-1 (red, arrow), enterocytes by WGA (green), and goblet cells by UEA-1 and WGA (yellow, arrowhead). Villous M cells were found as two different distribution forms, dense (*a* and *b*) and diffuse (*d* and *e*) types. In contrast to the epithelial and goblet cells, M cells in the villous epithelium were completely negative to the WGA staining (*c* and *f*). Frozen sections were prepared and stained with UEA-1-TRITC alone (*h* and *l*) or with UEA-1-TRITC and B220 mAb-FITC (*i* and *m*) and the M cells were shown to have a pocket membrane and pocket lymphocytes (arrow) whereas the goblet cells do not (arrowhead). M cells were doubly negative cells for alkaline phosphatase activity demonstrated by red/pink color substrate, and alcian blue staining (white; *g*). The scale bar for *a*, *d*, and *j* is 50 μm; for *b*, *c*, *e*, *f*, *h*, and *l* is 20 μm; and for *g*, *i*, *k*, and *m* is 10 μm.

Results

Identification of Clusters of UEA-1^+ Cells in the Intestinal Villous Epithelium. M cells have been thought to be associated with, and to develop only in, the dome epithelium (or FAE) of mucosa-associated lymphoid tissues, e.g., PP. However, using confocal image analysis of whole-mount murine intestine stained with TRITC-conjugated UEA-1 and FITC-labeled WGA, we have found UEA-1^+WGA^- cells not only in the FAE region of PP (Fig. 1 *j–l*), but also in the villous epithelium (Fig. 1 *a–i*). UEA-1, which possesses specificity for carbohydrate structures containing α(1–2)-fucose, selectively binds to the entire plasma membrane of PP M

cells but not to WGA^+ columnar epithelial cells (23). To further confirm the specificity of UEA-1 staining, we have performed a blocking experiment using 50 mM soluble fucose. Preincubation of the UEA-1 with soluble fucose for 1 hr clearly blocked UEA-1 staining in fluorescence-activated cell sorter (FACS, Becton Dickinson) and immunohistochemistry analyses further indicating the specificity of the UEA-1 staining method (data not shown). Interestingly, two forms of villous $UEA\text{-}1^+WGA^-$ cells, i.e., dense and diffuse, may be distinguished on the basis of the density of $UEA\text{-}1^+WGA^-$ cells (Fig. 1 *a* and *b* vs. *d* and *e*).

Our study revealed that these newly identified villous $UEA\text{-}1^+WGA^-$ cells share features with PP M cells but differ from goblet and columnar epithelial cells. Analysis of frozen sections of intestinal villi stained with TRITC-UEA-1 reveals that the villous $UEA\text{-}1^+$ cells possess the characteristic feature of M cells in PP FAE, i.e., a unique subdomain of the basolateral membrane, also known as the pocket membrane (Fig. 1*h*). The pocket lymphocytes were further confirmed by the staining with TRITC-UEA-1 and FITC-B220 mAb in villous $UEA\text{-}1^+$ cells (Fig. 1*i*) as well as in PP M cells (Fig. 1*m*).

Although they possess some affinity for UEA-1, goblet cells, unlike M cells, are capable as well of binding to WGA, making them doubly positive cells (UEA^+ WGA^+; Fig. 1 *b*, *e*, and *k*). In contrast to the epithelial and goblet cells, $UEA\text{-}1^+$ cells in the villous epithelium were completely negative for WGA staining (Fig. 1 *c* and *f*). Further, goblet cells are morphologically distinguished from M cells in that they do not possess the characteristic pocket membrane (Fig. 1 *h* and *i*). Intestinal columnar epithelial cells have high alkaline phosphatase (ALP) activity demonstrating with red or pink color, as do goblet cells stained with alcian blue, but M cells have neither of these features (11). Like PP M cells, villous $UEA\text{-}1^+$ cells in whole-mount intestinal samples were found to be negative for ALP activity and alcian blue staining (Fig. 1*g*). Thus, the $UEA\text{-}1^+$ cells, shown by our study to be analogous to PP M cells, have been designated villous M cells.

Development of Clusters of Villous $UEA\text{-}1^+$ M Cells in the Various PP-Null Mice. To further support this view, we examined whether villous M cells can develop in PP [or gut-associated lymphoid tissue (GALT)]-deficient mice, such as *in utero* LTβR-Ig-treated (21), $LT\alpha^{-/-}$ (31), $TNF/LT\alpha^{-/-}$ (20), and $Id2^{-/-}$ mice (22). We found M cells with the characteristic $UEA\text{-}1^+$ WGA^- staining in the tip regions of intestinal villi of all PP-deficient mice (Fig. 2), thus documenting the presence of an FAE-independent M cell developmental pathway. This view was further supported by the presence of M cells in $TNF/LT\alpha^{-/-}$ mice lacking newly described isolated lymphoid follicle (ILF) in addition to PP (data not shown). To define the distribution and number of villous M cell population in wild-type mice and GALT-null mice, we determined the frequency of the dense type of villous M cells in whole small intestine using the confocal imaging system. Approximately 40–50 dense-type villous M cell clusters were found per whole small intestine of wild-type mice. Similarly, ≈50–60 villous M cell clusters were found in the whole small intestine of $TNF/LT\alpha^{-/-}$ mice, one of the representative GALT-null mice. The finding of similar numbers of villous M cells in the GALT-null and wild-type mice could suggest that the development of villous M cells is completely independent of GALT and FAE.

Attachment and Internalization of Bacteria by Villous M Cells. A further experiment was performed to gauge the ability of villous M cells to take up pathogenic microorganisms. Ligated small intestinal loops from wild-type mice were inoculated with r*Salmonella typhimurium* expressing green fluorescence (r*Salmonella*-GFP), *Yersinia pseudotuberculosis* (*Yersinia*-GFP), *E. coli*-expressing *Yersinia* invasin (*E. coli*-invasin-GFP), and wild-type *E. coli*-GFP. After a 10-min incubation with each bacteria *in situ*, sequential immunohistologic analyses of ligated small intestinal loops directly demonstrated the

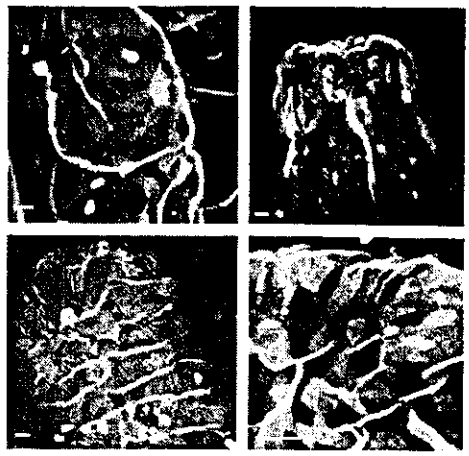

Fig. 2. The presence of villous M cells in PP-null mice, such as *in utero* LTβR-Ig-treated C57BL/6 mice (*Upper Left*), $LT\alpha^{-/-}$ mice of C57BL/6 background (*Upper Right*), $TNF/LT\alpha^{-/-}$ mice of 129 × C57BL/6 background (*Lower Left*), and $Id2^{-/-}$ mice of 129×Sv background (*Lower Right*). The scale bar for all pictures is 10 μm. The whole-mount preparations of small intestine were stained with FITC-WGA and TRITC-UEA-1.

presence of r*Salmonella*-GFP in $UEA\text{-}1^+$ cells in the villous epithelium of wild-type mice (Fig. 3 *Aa* and *Ab*) and $TNF/LT\alpha^{-/-}$ mice (Fig. 3 *Ae* and *Af*). In addition, *Yersinia*-GFP was also specifically adhered to villous $UEA\text{-}1^+$ cells of wild-type mice (Fig. 3 *Ac* and *Ad*) and $TNF/LT\alpha^{-/-}$ mice (Fig. 3 *Ag* and *Ah*). Immunohistologic analyses using frozen sections showed that r*Salmonella*-GFP was located in the apical membrane regions of villous $UEA\text{-}1^+$ cells (Fig. 3 *Ai* and *Aj*). To show the ability of villous $UEA\text{-}1^+$ cells to take up bacteria, we performed an ileal loop infection experiment using *rSalmonella*-GFP and analyzed the localization of bacteria with sequential confocal microscopy (Fig. 3*B*). Sequential Z plans of whole mount staining revealed the localization of *rSalmonella*-GFP in the intracellular region (Fig. 3 *B a–c*). In addition, *rSalmonella*-GFP was found in the intracellular region of villous $UEA\text{-}1^+$ cells prepared by cytospin (Fig. 3*Bd*).

Intestinal epithelial cells were further isolated from villous epithelium and PP after ileal loop injection of the microorganism expressing GFP, and then counterstained with TRITC-UEA-1 for flow cytometry analysis. We found a higher frequency of r*Salmonella*-GFP-, *Yersinia*-GFP-, or *E. coli*-invasin-GFP-containing cells in the fraction of $UEA\text{-}1^+$ cells than in the $UEA\text{-}1^-$ cells isolated from villous epithelium (Fig. 3*C*), and similar patterns were noted for the $UEA\text{-}1^+$ and $UEA\text{-}1^-$ cells isolated from the dome region of PP. In addition, high numbers of r*Salmonella*-GFP-, *Yersinia*-GFP-, or *E. coli*-invasin-GFP-containing cells were also recovered from $UEA\text{-}1^+$ but not $UEA\text{-}1^-$ cells isolated from the villous epithelium of $TNF/LT\alpha^{-/-}$ mice lacking GALT (Fig. 3*C*). Taken together, these results indicate that villous M cells have the ability to take up several different bacteria from the lumen known to be taken up by FAE-M cells.

Scanning and Transmission Electron Microscope Analysis of Villous M Cells. Scanning electron microscopy (SEM) of the villous M cells revealed a hallmark feature of M cells, i.e., a depressed surface with short and irregular microvilli (Fig. 4 *a*, *b*, and *c*), seen also in the M cells of PP (Fig. 4 *d* and *e*). Transmission electron microscopy analysis also showed the binding of gold particle-conjugated $UEA\text{-}1^+$ cells in the villous M cells (Fig. 4 *f* and *g*). Further, the presence of infiltrating mononuclear cells was also seen in the pocket of villous M cells (Fig. 4*h*). The SEM also demonstrated the binding of bacteria to the membrane of villous M cells in the small intestine of FAE-null $TNF/LT\alpha^{-/-}$ mice after intestinal exposure

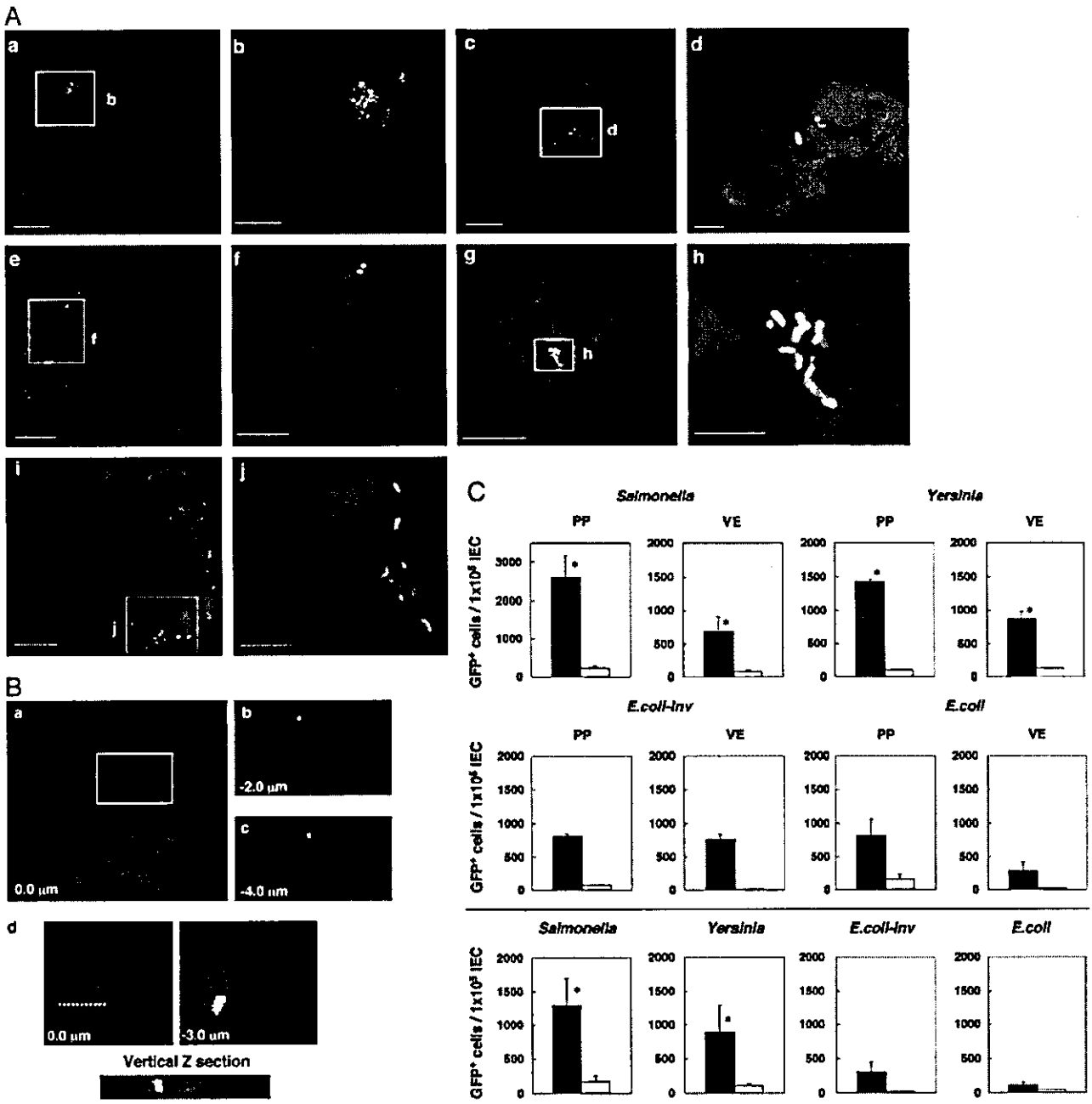

Fig. 3. (*A*) Immunohistochemistry for antigen uptake by UEA-1$^+$ villous M cells. Each panel shows histological features for sampling of GFP-expressing *Salmonella* (*a*, *b* and *e*, *f*) and *Yersinia* (*c*, *d* and *g*, *h*) by UEA$^+$ cells in the small intestine of wild-type (*a–d*) and PP-null TNF/LT$\alpha^{-/-}$ mice (*e–j*). Whole mount (*a–d* and *e–h*) and frozen sections of small intestine after exposure of GFP-expressing *Salmonella* were prepared and stained with UEA-1-TRITC (*i* and *j*). The scale bars are as follows: for *a*, *c*, and *g*, 50 μm; for *b*, *e*, and *i*, 20 μm; and for *d*, *f*, *h*, and *j*, 10 μm. (*B*) Localization of GFP-expressing *Salmonella* in the intracellular region of UEA-1$^+$ villous M cells. An ileal loop infection experiment using *rSalmonella*-GFP was performed for 30 min, and whole-mount tissues and UEA-1$^+$ IEC cells were analyzed by sequential confocal planar microscopy. Sequential Z plans of whole-mount staining revealed the localization of *rSalmonella*-GFP in the intracellular region of villous UEA-1$^+$ cells (*a*, *b*, and *c*). Further, the cytospin analysis revealed that *rSalmonella*-GFP also existed in the intracellular region of villous UEA-1$^+$ cells (*d*). (*C*) Antigen uptake by UEA-1$^+$ villous M cells. Cells (5×10^8) of GFP-expressing *S. typhimurium* PhoPc (*Salmonella*), *Y. pseudotuberculosis* (*Yersinia*), *E. coli*-invasin (*E. coli*-Inv), and *E.-coli* were administered into a 10-cm loop of the small intestine of naive wild-type mice (*Top* and *Middle*) or TNF/LT$\alpha^{-/-}$ mice (*Bottom*). After 10 min of incubation *in situ*, IECs were isolated from PP and villous epithelium. After being fixed with 4% paraformaldehyde, IECs were stained by UEA-1-TRITC, and uptake efficiency was analyzed by fluorescence-activated cell sorter (FACS). Data demonstrate the frequency of GFP$^+$ cells in the UEA$^+$ (filled bar) and UEA$^-$ (open bar) cells isolated from PP and villous epithelium (VE). The results represent the mean values ± SD from three separate experiments (three mice per group). *, $P < 0.05$ vs. the UEA$^-$ IEC group.

of *Salmonella* (Fig. 4 *i–l*). These findings provide supportive evidence that the newly identified villous M cell formed an alternative gateway for antigen sampling and/or entry from the lumen of intestinal villous epithelium. These findings provide evidence that M cells are developed and localized in the villous epithelium as well as in the FAE of PP.

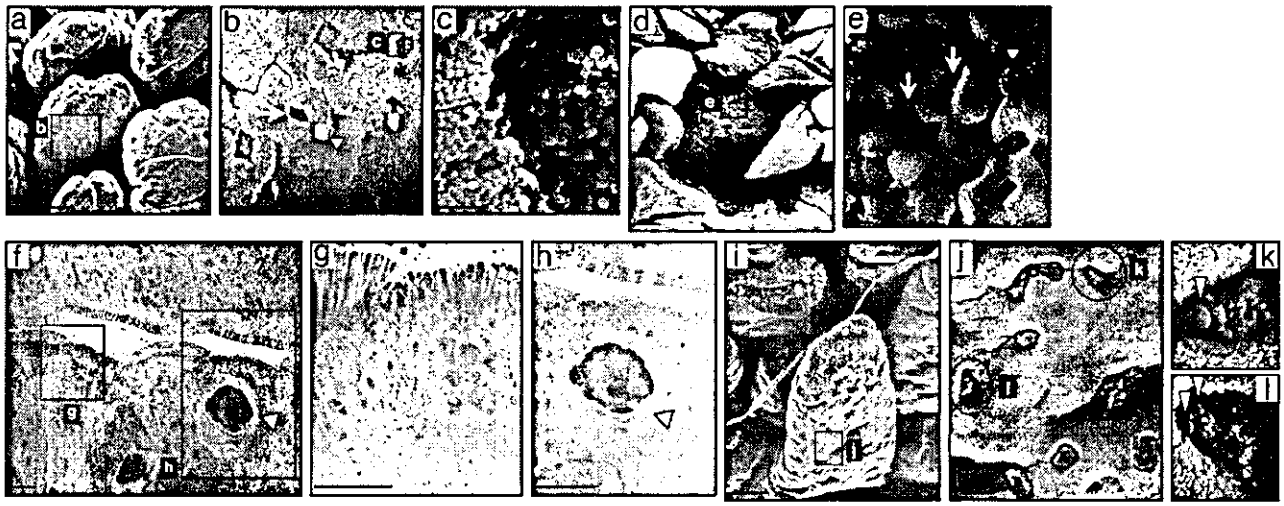

Fig. 4. Scanning and transmission electron microscopy of M cells in villous epithelium and FAE. Scanning electronic microscopy demonstrates that the M cells (arrow) in villous epithelium (*a–c*) and PP (*d* and *e*) are distinguished from enterocytes and goblet cells (arrowhead) by their relatively depressed and dark brush border. A transmission electron microscopy view of villous M cells shows short stub-like microvilli (*f*, *g*, and *h*) and the presence of infiltrating mononuclear cells in the pocket of villous M-cells (*h*; arrowhead). (*i–l*) The presence of villous M cells and the uptake of bacteria in the villous epithelium (arrowhead) after intestinal exposure of *Salmonella* (see Fig. 3 legend) in PP-deficient TNF/LT$\alpha^{-/-}$ mice. The scale bars are as follows: for *a*, *d*, and *i*, 50 μm; for *b*, *e*, *f*, and *j*, 5 μm; for *g* and *h*, 1.0 μm; and for *c*, *k*, and *l*, 0.5 μm.

Induction of Ag-Specific Immune Responses in PP-Deficient Mice. Our next experiments sought to examine whether antigen-specific antibody responses could be induced in GALT-deficient mice by means of the villous M cells. When GALT-null mice with TNF/LTα gene deficiency and wild-type mice were immunized orally with r*S. typhimurium* BRD 847 expressing a 50-kDa ToxC fragment of tetanus toxin (r*Salmonella*-ToxC), titers of tetanus toxoid (TT)-specific serum IgG antibodies were as high in the serum of the TNF/LT$\alpha^{-/-}$ mice as in orally immunized wild-type mice (Fig. 5). Expectedly, levels of TT-specific serum IgG antibody titers were not detectable when wild-type and TNF/LT$\alpha^{-/-}$ mice were orally immunized with r*Salmonella* not expressing ToxC (under the 5 of reciprocal $\log_2$ titer in Fig. 5). These findings suggest that the villous M cells are an important antigen-sampling site for the induction of antigen-specific immune responses to gastrointestinal environmental antigens.

Discussion

Because there is currently no reliable identified gene and corresponding antigen marker that positively identified M cells, the phenotype of M cells is defined by a combination of criteria including (3, 4) (*i*) the presence of the fucose epitope defined by the lectin UEA on M cell membrane, (*ii*) short and irregular microvilli, (*iii*) endocytic activity and ability to take up bacteria as well as macromolecules, and (*iv*) an intraepithelial pocket that allows a cluster of lymphocytes to be located in the epithelium. Based on our present results, villous M cells share all of the identifying features necessary to identify M cells found in the FAE of PP. Although M cell development has been thought to depend on FAE in organized mucosal lymphoid tissues, our results provide evidence that it can occur in the villous epithelium even in the absence of FAE. Further, these villous M cells are a gateway for entry or sampling of bacteria (e.g., *Salmonella*-, *Yersinia*-, and *E.-coli*-expressing invasin) for the subsequent induction of antigen-specific immune responses.

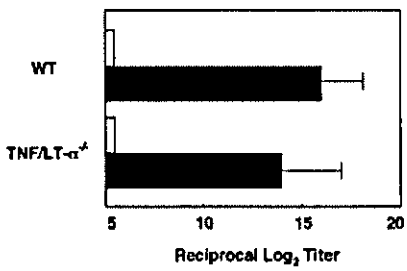

Fig. 5. Induction of Ag-specific immune responses in PP-deficient mice. Shown are PP-deficient (TNF/LT$\alpha^{-/-}$) and wild-type mice, which were orally immunized with r*Salmonella*-ToxC (filled bar) or r*Salmonella* alone (open bar). Serum samples were obtained 21 days after oral immunization for the assessment of tetanus toxoid (TT)-specific antibody responses by ELISA. The results represent the mean values ± SD from three separate experiments (three mice per group). There is no statistically significant difference between TNF/LT$\alpha^{-/-}$ and wild-type mice analyzed by unpaired Mann–Whitney *U* test.

M cells have been identified and documented only in the FAE-associated epithelium and occasionally on villi immediately adjacent to the lymphoid follicle (4, 32). A previous study (32) indicated that isolated M cells were found in the villous epithelium near the PP of the rabbit small intestine. In addition, clusters of UEA-1$^+$ cells in the small intestinal villi of conventional mice have been reported (33). These two studies suggested the existence of UEA-1$^+$ cells in the small intestinal villi of rabbit and mouse but did not address their identity or biological function. In this regard, our present study provides evidence of the existence of M cells in the villous epithelium away from PP of not only wild-type mice but also GALT-null mice. Further, our present results directly demonstrate the functional aspect of the villous M cells as a gateway for bacteria. Thus, we have substantially advanced the case that villous M cells are distinct from FAE-associated M cells in PP and have further shown that these villous M cells are a biologically important component of the mucosal immune system.

As discussed above, M cells reportedly are occasionally found in the villous epithelium adjacent to rabbit PP (32); however, we stress that villous M cells are located quite a distance from PP. Although the exact source of M cells has yet to be pinpointed, it is widely held that their development and localization are always associated with the organized lymphoid tissue of mucosal surfaces (e.g., PP). Our findings presented here, however, challenge this common assumption by providing evidence that M cells can be developed in villous epithelium in the absence of the FAE thought to be necessary to

their development in the organized mucosal lymphoid tissue. The diverse cellular phenotypes in the intestinal epithelium arise from crypt stem cells whose differentiation pathways can be modified by endogenous and exogenous influences (33–37). A previous study (34) demonstrated that *Streptococcus pneumoniae*-treated FAE tissues showed a marked increase in both IEL and epithelial cells with morphological and functional features of M cells. Further, enterocytes located in the peripheral of the FAE were converted into operational M cells as early as one hour after *in vivo* exposure to *S. pneumoniae* (32). Interestingly, expression of α1,2-linked fucosylated glyconjugates in the ileal epithelium was induced by the flora (33). Further, our unpublished data indicate that significantly increased numbers of UEA-1$^+$ cells in the villous epithelium of both wild-type and PP-null mice were detected after *in vivo* exposure to *S. typhimurium*. Therefore, it is possible that newly identified villous M cells can be developed from epithelial cells in response to foreign antigens and/or pathogens in the gut lumen. An interesting possibility would be that these UEA-1$^+$ crypt cells could be programmed to develop into the villous UEA-1$^+$ M cells after exposure to the exogenous microorganisms.

M cells in the FAE provide an entry site for pathogens, such as *S. typhimurium*, *Mycobacterium bovis*, *Shigella flexneri*, *Y. enterocolitica* and retroviruses (4, 38–40). It is well known that the invasion genes of the *Salmonella* pathogenicity island (SPI1) are necessary for the entry of *S. typhimurium* into FAE-M cells and epithelial cells (38, 41). However, SPI1-deficient *Salmonella* is transported from the gastrointestinal tract to the blood stream by CD18-expressing phagocytes, and CD18-deficient mice were shown to be resistant to orally administered *Salmonella* (18). Overall, it seems likely that several cell types, including M cells, epithelial cells, and CD18-expressing macrophages, are involved in permitting the penetration of *Salmonella*. On the other hand, previous studies have showed that *Y. enterocolitis* selectively and specifically invades the FAE of PP by means of M cells but not by means of other cells (42, 43). Interestingly, it has been suggested $\alpha_4\beta_1$ integrin is expressed on the apical membranes of M cells but not on villous or dome epithelial enterocytes, implying that this integrin may be exploited by *Yersinia* to attach to and invade the M cells (44). Further, invasin mediates uptake of *Y. pseudotuberculosis* into mammalian cells through binding with β1-chain integrins with high affinity (45, 46). In light of these complexities, the fact that villous M cells and FAE-associated M cells in PP sampled GFP-expressing *Salmonella*, *Yersinia*, and *E. coli*-invasin suggests that villous M cells likely possess a capacity of playing as professional bacteria translocating cells.

A recent study (13) has provided new evidence that IgA-specific B cell responses including isotype-switching can be induced in intestinal lamina propria without the influence of PP. In addition, our recent study showed that ILF in the small intestine are structurally and functionally similar to the PP and contain M cells on their FAE region (47). To eliminate the possible role of M cells associated with the ILF for antigen sampling, we used TNF/LT$\alpha^{-/-}$ mice, which lack both PP (17) and ILF (unpublished data). Interestingly, high numbers of GFP-expressing *Salmonella*, *Yersinia*, and *E. coli*-invasin were recovered from UEA-1$^+$ but not UEA-1$^-$ cells isolated from the villous epithelium of TNF/LT$\alpha^{-/-}$ mice although the total uptake of GFP$^+$ eukaryotic cells was less pronounced than in wild-type mice (Fig. 3*C*). Together with the data for the induction of antigen-specific antibody responses after oral immunization in these PP- and ILF-deficient mice (Fig. 5), our results provide a strong case that villous M cells are an alternative gateway of antigen entry for the mucosal immune system. On the other hand, a recent study (19) showed that antigen sampling occurs across the non-FAE by mucosal intraepithelial dendritic cells. Thus, it is still possible that antigen-specific immune responses seen in TNF/LT$\alpha^{-/-}$ mice could be initiated by means of these intraepithelial dendritic cells.

In summary, our observations indicate that typical M cells can develop without the influence of the FAE associated with mucosal lymphoid tissue such as PP and in fact are present in villous epithelium. Moreover, villous M cells may be an alternative gateway for the penetration of pathogenic microorganisms as well as an additional antigen-sampling site for the induction of antigen-specific immune responses by means of the mucosal tissues.

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