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

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

Masafumi Yamamoto, Mi-Na Kwone, Paul D. Rennert, Takachika Hiroi, Kohtaro Fujihashi, Jerry R. McGhee, and Hiroshi Kiyono

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) 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), 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, genetics 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.
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 contributes 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-linked 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 half-life 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 overlaying 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 H2O2 (Moss, Pasadena, CA) was added for color development. Endpoint titers were expressed as the reciprocal log2 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 2 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% CO2 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-aminoo-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing H2O2 (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. 1f). 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. 1j).

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

FIGURE 1. Effects of TNF/LTα or LTαβ 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).

B

FIGURE 2. Effects of TNF/LTα or LTαβ 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 (J) 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. 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 TNFR55-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 LTαβ 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. 2I). 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. 4A). 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. 4B). 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. 5A). 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. 5B). These findings indicate that loss of ILF and/or alterations in the microarchitecture of MLN resulting from blockade of
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 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.

**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 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.
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 in adults. In this regard, blockade of LTαβ, but not 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 LTβ 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 LTα−/− mice (15, 37), TNFR55−/− mice (42, 43), and LTβ−/− 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 LTα+/−LTβ−/−, but not LTα+/− or LTβ+/− 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
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.

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 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.

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

**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.

**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 control-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.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total IgA (µg/ml)</th>
<th>Ag-Specific IgA (reciprocal log₂)</th>
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<tr>
<td>Control-Ig</td>
<td>52.4 ± 11.7</td>
<td>7.3 ± 0.4</td>
</tr>
<tr>
<td>LTβR-Ig/TNFR55-Ig</td>
<td>43.4 ± 14.4</td>
<td>&lt; 3</td>
</tr>
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Table I. *Intestinal mucosal IgA Ab responses*

* 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 μ- and γ-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 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 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 TNFLTLs and LTαβ pathways for the establishment of Ag-specific mucosal IgA Ab responses. Furthermore, ILF are not required for the induction of IgA Abs 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.

References


