

Isolated Lymphoid Follicle Formation Is Inducible and Dependent Upon Lymphotoxin-Sufficient B Lymphocytes, Lymphotoxin β Receptor, and TNF Receptor I Function¹

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The gastrointestinal mucosa contains a complex network of lymphoid compartments that have evolved to efficiently protect the host from invading pathogens. Recently, an additional lymphoid structure resembling Peyer's patches (PP) in composition and architecture has been identified in the murine small intestine, the isolated lymphoid follicle (ILF). In this study we examine the nature and factors required for ILF formation. We observed a spectrum of structures fitting the previous descriptions of ILFs, ranging from clusters of B220⁺ cells (which we have termed immature ILFs) to well-organized lymphoid nodules (which we have termed mature ILFs). Here we demonstrate that similar to PP formation, ILF formation requires lymphotoxin (LT)- and LT β receptor-dependent events. However unlike PP formation, the LT- and LT β receptor-dependent events required for ILF formation can occur in adulthood and require LT-sufficient B lymphocytes. We demonstrate that mature ILF formation occurs in response to luminal stimuli, including normal bacterial flora, and requires TNF receptor I function. These findings suggest that ILFs are organized intestinal lymphoid structures whose formation can be induced and whose mass can be expanded in response to mucosal challenges. *The Journal of Immunology*, 2003, 170: 5475–5482.

The gastrointestinal tract is a major interface of higher organisms with the environment. The primary function of the alimentary tract is the absorption of nutrients, necessitating the exposure of this mucosal surface to a multitude of antigenic stimuli, including bacteria, bacterial products, and dietary Ags. Defending against invading pathogens while at the same time efficiently absorbing nutrients is a major challenge to the gastrointestinal tract. The mucosal immune system, a complex network of physically and phenotypically distinct lymphoid compartments, has evolved to allow these two opposing functions to coexist. Our current understanding is that this network is principally composed of immune-inductive sites (Peyer's patches (PP)³) and immune effector sites (the intraepithelial lymphocyte compartment and the lamia propria lymphocyte compartment); however, our understanding of the complexity of this system continues to progress with the descriptions of additional levels of organization in the intestine (1, 2). Recently, isolated lymphoid follicles (ILFs) were described in the murine small intestine (3). These structures

resemble PP in architecture and composition, as they possess germinal centers and an overlying follicle-associated epithelium (FAE) containing M cells, and are suggested to be inductive sites for mucosal immune responses.

The nature of ILF formation is unknown. Similar to PP, ILFs require lymphotoxin (LT) and NF- κ B-inducing kinase (NIK), a signaling molecule required for LT β receptor (LT β R) function, for their formation (3). ILF formation was not abolished by LT β R blockade given on days 14 and 17 of gestation, suggesting that, unlike PP formation, the NIK-dependent events relevant for ILF formation occur outside of this gestational window and/or are independent of the LT β R (3). ILFs were found to be present in germfree mice, suggesting that similar to PP formation, ILF formation may be independent of luminal stimuli (3).

The role of LT β R in the formation of ILF is suggested by the absence of ILF in *alymphoplasia/alymphoplasia* (*aly/aly*) mice. Other NIK-dependent events may also be important for ILF formation. However, the role of LT in the formation of ILF is well established. LT is a TNF family member, existing in two forms, a membrane-bound heterotrimer comprised of two β -chains and one α -chain (LT $\alpha_1\beta_2$) that is a ligand for the LT β R, and a soluble homotrimer (LT α_3) that is a ligand for both TNFR type I (TNFRI) and TNFRII (4). LT and the LT β R are expressed by nonoverlapping subsets of cells. LT β R expression is restricted to non-bone marrow-derived cells and a subset of monocytes, while LT (LT α_3 and LT $\alpha_1\beta_2$) is expressed on T lymphocytes, B lymphocytes, and NK cells in adult mice (5).

LT is known to contribute to the formation of organized peripheral lymphoid tissues in a least two ways. Interactions of membrane-bound LT with the LT β R during embryogenesis are essential for the formation of PP and lymph nodes (LN), as evidenced by the lack of these secondary lymphoid structures in mice deficient in LT α , LT β , and LT β R (6–8) and in mice in which these interactions have been blocked at specific times during gestation (9). The absence of PP and LN in these mice cannot be restored by

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³ Abbreviations used in this paper: PP, Peyer's patch; aly, alymphoplasia; DAB, diaminobenzidine; FAE, follicle-associated epithelium; ILF, isolated lymphoid follicle; LN, lymph node; LT, lymphotoxin; NIK, nuclear factor κ B-inducing kinase; PNA, lectin from *Arachis hypogaea*; RAG, recombinase-activating gene; TNFRI, TNFR type I.

LT/LT β R-dependent events later in life (10, 11). The cellular source of LT required for the formation of PP and LN is believed to be a bone marrow-derived, CD4⁺, CD3⁻, non-T and non-B lymphocyte precursor, which can be a progenitor of NK cells and dendritic cells (12–14). These requirements for gestational-dependent, LT/LT β R-dependent events appear to be universal for the formation of secondary lymphoid structures, although the formation of cervical LN and MLN in the absence of these signals has been reported (15).

LT may also contribute to the formation of organized tertiary lymphoid structures. These structures are induced at sites of inflammation and have a cellular composition and architecture resembling secondary lymphoid structures, suggesting that these structures are inductive sites of immune responses. Tertiary lymphoid structures are distinguished from secondary lymphoid structures by their location and their de novo formation, which has been termed lymphoid neogenesis. LT has been demonstrated to participate in this process, as ectopic LT expression may lead to lymphoid neogenesis (16).

The previous descriptions of ILFs and the requirement for LT for their formation could be consistent with either role of LT in the formation of organized lymphoid structures. Here we describe the nature and the factors contributing to ILF formation in murine small intestine. We observed that ILFs have a spectrum of appearances ranging from loosely organized clusters of B220⁺ cells at the base of villi (which we have referred to as immature ILFs) to well-organized lymphoid structures resembling PP (which we have referred to as mature ILF). Consistent with previous observations, we found that mature ILFs contain predominantly B-2 B lymphocytes and CD4⁺ T lymphocytes and have an overlying epithelium containing M cells. Similar to the formation of secondary lymphoid structures, we found that the formation of ILFs is dependent upon LT and the LT β R; however, in contrast to PP and LN formation, ILF formation does not require these LT/LT β R interactions during gestation and can occur in adults. Like the formation of PP and LN, mature ILF formation requires a LT β R-sufficient stromal cell; however, in contrast to the formation of secondary lymphoid structures, we found that LT-sufficient B lymphocytes are required for the formation of ILFs. Based upon their inducible nature, composition, and architecture, we believe that mature ILF formation represents lymphoid neogenesis of the small intestine, and we propose the progression from immature to mature ILFs occurs in response to exogenous stimuli and requires TNFRI function.

Materials and Methods

Mice

Mice used for this study, with the exception of germfree mice, were housed in a specific pathogen-free facility and fed a routine chow diet. Animal procedures and protocols were conducted in accordance with the institutional review board at Washington University School of Medicine. C57BL/6, TNFRII-deficient, recombinase-activating gene-1 (RAG1)-deficient, and B cell-deficient JH^{-/-} mice (17) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). TNFRI-deficient mice (18, 19) on the C57BL/6 background were a gift from Dr. J. J. Peschon (Immunex Corp., Seattle, WA). LT β R-deficient mice (8) on the C57BL/6 background were a gift from Dr. K. Pfeffer (Technical University of Munich, Munich, Germany). LT α -deficient mice (6) were bred onto the C57BL/6 background for >10 generations before use in experiments. Timed pregnant C57BL/6, TNFRI^{-/-}, and TNFRII^{-/-} female mice for use in experiments involving the injection of LT β R-Ig fusion protein were generated by matings with C57BL/6, TNFRI^{-/-}, and TNFRII^{-/-} male mice, respectively. Germfree C57BL/6 mice, a gift from Dr. J. Gordon (Washington University School of Medicine, St. Louis, MO), were maintained in plastic gnotobiotic isolators under a 12-h light cycle and given free access to autoclaved water and chow (B&K Universal, East Yorkshire, U.K.) (20). At the time of sacrifice, cecal cultures from germ-

free animals were obtained to confirm the absence of bacterial flora. Germ-free mice were conventionalized by exposure to cecal contents from conventionally housed C57BL/6 mice. Six- to 10-wk-old LT α ^{-/-}, LT β R^{-/-}, and RAG^{-/-} mice were used as recipients for bone marrow transfers.

Whole mounts of small intestine

Small intestines were removed intact, flushed with cold PBS, and opened along the mesenteric border. Intestines were mounted, lumen facing up, in 8-cm segments from proximal to distal and fixed with cold 10% phosphate-buffered formal saline (Fisher Scientific, Pittsburgh, PA) for 1 h at 4°C. Intestines were washed three times in cold PBS; incubated in a solution of 20 mM DTT, 150 mM Tris, and 20% ethanol at room temperature for 45 min; washed three times in cold PBS; and incubated in a solution of 1% H₂O₂ for 15 min at room temperature to block endogenous peroxidases. Intestines were washed three times in PBS, followed by incubation in PBS containing 1% BSA and 0.3% Triton X-100 for 30 min. Intestines were incubated with HRP-conjugated lectin from Ulex Europaeus (UEA-I; Sigma-Aldrich, St. Louis, MO) in PBS/BSA/Triton X-100 solution overnight at 4°C to facilitate the identification of PP and mature ILF. The following day intestines were washed three times in PBS, incubated in diaminobenzidine (DAB) metal peroxide substrate (Pierce, Rockford, IL) for 15 min, rinsed twice in distilled water, and returned to PBS for further analysis. Investigators blinded to the treatment groups determined the presence of mature ILF. Under low power microscopy ($\times 25$ – 65) the following criteria were used to determine the presence of mature ILF: 1) presence of a nodular structure with size equal to or greater than the width of one villus, 2) nodular structure possessing an overlying dome resembling the FAE of PP, and 3) nodular structures occurring singly or in groups of two (three or more nodules of approximately the same size were considered to be PP).

For B220⁺ staining of whole mounts (modified whole mounts), intestines were removed intact, flushed with PBS, opened along the mesenteric border, and mounted as described above. Intestines were then incubated three times in HBSS (BioWhittaker, Walkersville, MD) containing 5 mM EDTA at 37°C with shaking for 10 min to remove epithelial cells. Intestines were fixed in 10% phosphate-buffered formal saline and treated with 1% H₂O₂ for 15 min at room temperature as described above. Intestines were incubated in a solution of 50 mM Tris (pH 7.2), 150 mM NaCl, 0.6% Triton-X 100, and 0.1% BSA for 1 h at 4°C to block nonspecific Ab binding and then incubated with rat anti-mouse B220 Ab (BD PharMingen, San Diego, CA) diluted in the above solution overnight at 4°C. Intestines were washed three times in the above solution and incubated with a HRP-conjugated goat anti-rat IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted in the above solution at room temperature for 1 h. Intestines were washed three times and incubated in DAB/metal peroxide substrate as described above.

Cell isolation from mature ILF

Intestines from mice treated with LT β R-Ig in utero were flushed with cold PBS, opened along the mesenteric border, and mounted with the lumen facing up in cold PBS as described above. Using the dissecting microscope and a blunt-ended 26-gauge needle and syringe, the contents of multiple mature ILF were aspirated and placed in cold PBS. The single-cell suspension was then used for flow cytometric analysis as described below. Approximately four intestines were required to obtain a sufficient number of cells for each flow cytometric analysis. The average yield of viable mononuclear cells was 1×10^5 cells/intestine.

Flow cytometric analysis

Single-cell suspensions were obtained as described above, and flow cytometric analysis was performed as previously described (21). The reagents used for analysis were FITC-conjugated rat anti-mouse CD4, PE-conjugated rat anti-mouse CD19, biotin-conjugated rat anti-mouse CD11b, biotin-conjugated hamster anti-mouse TCR β , appropriate isotype control Abs, streptavidin-PE, streptavidin-FITC (all from BD PharMingen), and biotin-conjugated rat anti-mouse IgA (Southern Biotechnology Associates, Birmingham, AL). Dead cells were excluded based on forward and side light scatter. Gates for positive staining were defined such that 1% of the analyzed population stained positively with the appropriate isotype control Ab.

Immunohistochemistry

Paraffin-embedded sections containing mature ILF or PP resected from whole mount intestines (performed as described above) were deparaffinized by sequential treatments with Citrosolv (Fisher Scientific) and isopropyl alcohol, rinsed with tap water, rehydrated in PBS, treated with avidin/biotin blocking kit (Vector Laboratories, Burlingame, CA), washed three times in

PBS, and blocked for 15 min at room temperature in PBS containing 1% BSA, 0.2% powdered skim milk, and 0.1% Triton X-100. Sections were then incubated with biotin-conjugated anti-B220 (BD PharMingen) or biotin-conjugated lectin from *Arachis hypogaea* (PNA; Sigma-Aldrich) diluted in PBS containing 1% BSA, 0.2% powdered skim milk, and 0.1% Triton X-100 overnight at 4°C. For detection of B220 staining we used Cy3-tyramide signal amplification (DuPont/NEN, Boston, MA). For detection of PNA staining we used biotinyl-tyramide signal amplification (DuPont/NEN), followed by incubation with HRP-conjugated streptavidin (Jackson ImmunoResearch Laboratories) and detection with DAB metal peroxide substrate (Pierce). B220-stained sections were counterstained with Hoechst dye (Sigma-Aldrich) to visualize nuclei.

Scanning electron micrographs

Small intestines were removed intact, flushed with ice-cold PBS, opened along the mesenteric border, and examined under the dissecting microscope. PP and mature ILF were identified, removed, and treated with 0.1 M

sodium cacodylate buffer (Electron Microscopy Services, Fort Washington, PA) containing 2.5% glutaraldehyde overnight at 4°C. Sections were washed in 0.1 M sodium cacodylate buffer three times, then incubated with a solution of 20 mM DTT, 150 mM Tris, and 20% ethanol for 45 min at room temperature to remove mucin. Sections were washed in 0.1 M sodium cacodylate buffer three times, treated with alternating 1% osmium tetroxide and 1% thiocarbohydrazide (OTOTO method) (22), dehydrated in graded series of ethanol, critical point dried, and sputter-coated to produce 15-nm gold coating. Samples were examined using a Hitachi S-450 scanning electron microscope (Hialeah, FL) at an accelerating voltage of 20 kV.

Bone marrow transfers

Bone marrow transfers were performed after lethal irradiation as previously described (21). A total of 1×10^7 T lymphocyte-depleted bone marrow cells from gender-matched donors were injected i.v. into recipients

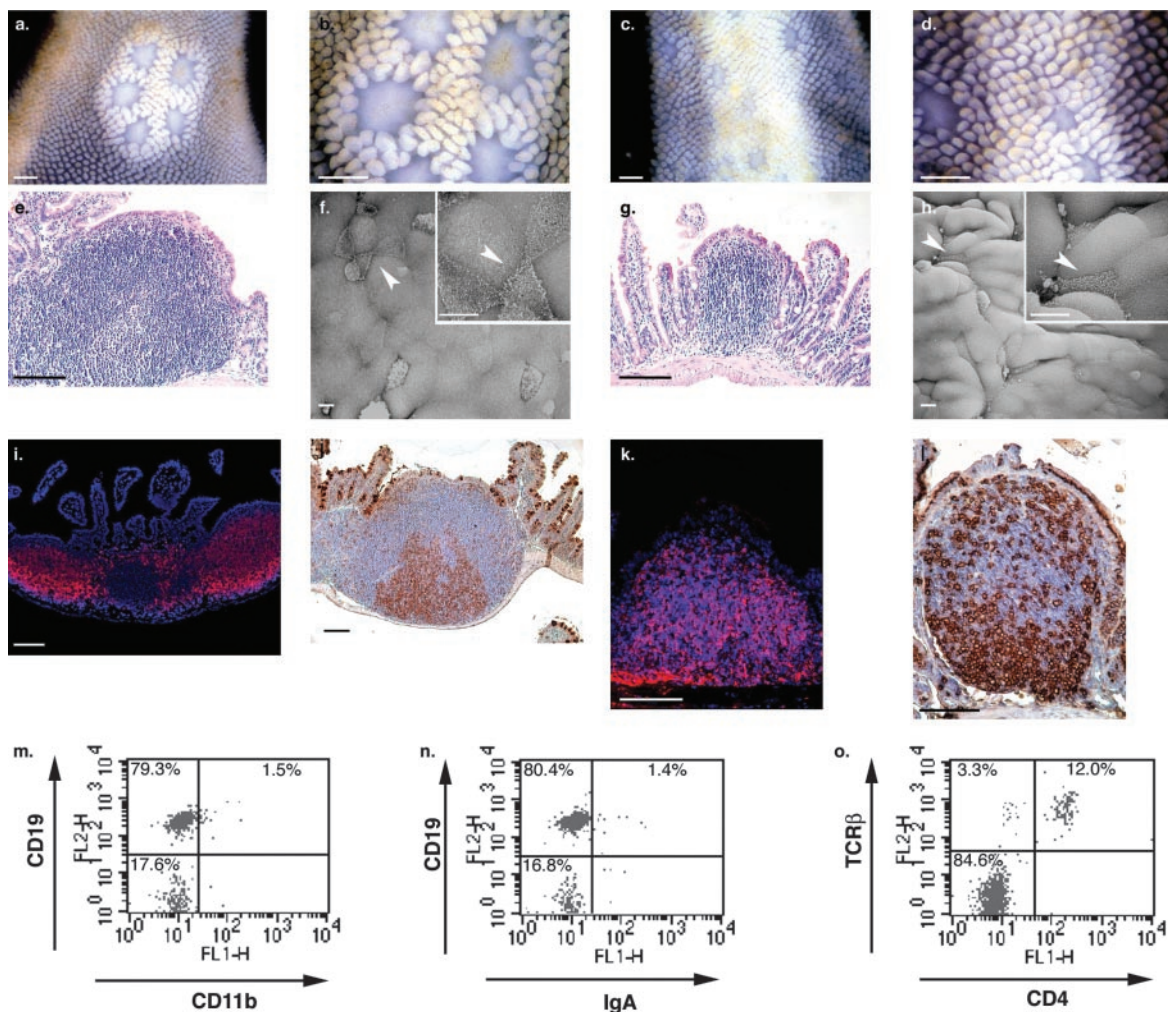


FIGURE 1. Characteristics of mature ILFs. Intestines from untreated C57BL/6 mice (*a, b, e, f, i, and j*), from $LT\alpha^{-/-}$ mice receiving C57BL/6 bone marrow (*c, d, h, and k*) and from mice receiving $LT\beta R$ -Ig in utero (*g and l*) were examined as whole mounts (*a–d*), by H&E staining (*e and g*), by scanning electron microscopy (*f and h*), and by immunohistochemical analysis for B220 (red, *i and k*) and PNA lectin staining (brown, *j and l*). Sections from *i and k* were counterstained with Hoechst dye (blue) to visualize nuclei. Intestines from untreated C57BL/6 mice contained PP (*a and b*) with an overlying FAE containing M cells (denoted by arrowheads, *f*). Intestines from $LT\alpha^{-/-}$ mice receiving C57BL/6 bone marrow and C57BL/6 mice treated with $LT\beta R$ -Ig in utero lacked PP, but possessed multiple nodules randomly oriented with respect to the mesenteric border (*c and d*). These nodules have architecture resembling PP by H&E staining (compare *e and g*), contain an FAE with cells fitting the morphologic criteria of M cells by scanning electron microscopy (denoted by arrowheads, *h*), and, like PP, contain B220⁺ and PNA⁺ cells consistent with the presence of germinal centers (compare *i and j* with *k and l*). Flow cytometric analysis of nodules from mice treated with $LT\beta R$ -Ig in utero (*m–o*) demonstrates that these nodules contain predominantly IgA-negative B-2 (CD19⁺, CD11b[−], IgA[−]) B lymphocytes and a smaller population of CD4⁺ TCRβ⁺ T lymphocytes. The numbers in *m–o* represent the percentage of the total lymphocyte population from mature ILF as determined by forward and side scatter. Scale bars: *a–d*, 1 mm; *e, g, i, j, k, and l*, 100 μm; and *f and h*, 5 μm. Original magnification: *a and c*, $\times 25$; *b and d*, $\times 45$; *e and g*, $\times 200$; *f and h*, $\times 1200$ (*inset* at $\times 5000$); *i and j*, $\times 100$; *k and l*, $\times 200$. Nodules from $LT\alpha^{-/-}$ mice receiving C57BL/6 bone marrow and from C57BL/6 mice treated with $LT\beta R$ -Ig in utero were identical in all examined criteria; therefore, a representative section from either group is shown for simplicity.

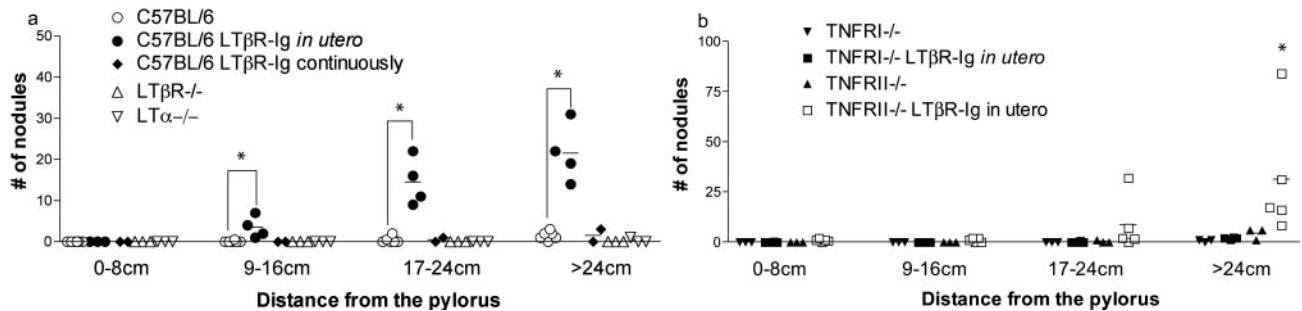


FIGURE 2. Mature ILFs are formed in the distal small intestine in an $LT\beta R$ - and $TNFRI$ -dependent manner. Small intestines from untreated C57BL/6, $TNFRI^{-/-}$, $TNFRII^{-/-}$, $LT\alpha^{-/-}$, and $LT\beta R^{-/-}$ mice; C57BL/6, $TNFRI^{-/-}$, and $TNFRII^{-/-}$ mice receiving $LT\beta R$ -Ig in utero; and C57BL/6 mice receiving $LT\beta R$ -Ig in utero and after birth were scored for the presence of mature ILF as outlined in *Materials and Methods*. The progeny of untreated C57BL/6 mothers have few or no mature ILF, while progeny from C57BL/6 mothers treated during gestation alone had mature ILF preferentially located in the distal small intestine (a). The formation of mature ILF in these mice could be inhibited by the continuous administration of $LT\beta R$ -Ig after birth. Consistent with the requirement for $LT\beta R$ in the formation of mature ILFs, we observed an absence of mature ILFs in the intestine of $LT\beta R^{-/-}$ mice. Mature ILFs were absent from the intestine of $TNFRI^{-/-}$ and $TNFRII^{-/-}$ mice, but could be induced in $TNFRII^{-/-}$, but not $TNFRI^{-/-}$, mice by treatment with $LT\beta R$ -Ig in utero (b). Each point represents the average number of nodules present in the specified intestinal segment. *, $p < 0.05$ compared with the number of nodules in untreated C57BL/6 mice (a).

on the second day of irradiation. Mice receiving bone marrow from multiple donors ($LT\alpha^{-/-}$ and $RAG^{-/-}$ or $LT\alpha^{-/-}$ and $JH^{-/-}$; Fig. 4), received 5×10^6 cells from each donor. Mice were allowed 12 wk for reconstitution with donor bone marrow before use in experiments. Flow cytometric analysis was performed on splenocytes from recipients at the time of sacrifice to document appropriate lymphocyte reconstitution.

LTβR-Ig treatment

$LT\beta R$ -Ig production and treatment were performed as previously described (21). Timed pregnant female C57BL/6 mice, $TNFRI^{-/-}$ mice, and $TNFRII^{-/-}$ mice were injected with 100 μ g of $LT\beta R$ -Ig via the tail vein on day 16 pc. For C57BL/6 mice receiving $LT\beta R$ -Ig continuously, offspring from timed pregnant female C57BL/6 mice injected with 100 μ g of $LT\beta R$ -Ig on day 16 pc received 20 μ g of $LT\beta R$ -Ig i.p. weekly after birth for 5 wk. Mice receiving $LT\beta R$ -Ig in utero and their controls were analyzed for the presence of mature ILF at 6–7 wk of age (Figs. 2 and 3).

Statistical analysis

Data analysis using one-way ANOVA, followed by Dunnett's multiple comparison post-test, was performed using GraphPad PRISM (GraphPad, San Diego, CA).

Results

Mature ILFs are formed in the small intestine in an $LT\beta R$ - and $TNFRI$ -dependent manner

Following treatment with $LT\beta R$ -Ig in utero to ablate the formation of PP, we noted the presence of nodules in the small intestine of adult mice (Figs. 1 and 2). In contrast to PP, these nodules were not visible macroscopically, but could be adequately visualized using low power microscopy. The basic characteristics of these nodules are outlined in Fig. 1. These nodules contain an overlying epithelium resembling the FAE of PP. By scanning electron microscopy the FAE of these nodules contain cells fitting the morphologic criteria of M cells (23). Light and fluorescence microscopy revealed that these nodules have architecture similar to that of PP and contain $B220^+$ and PNA^+ cells consistent with the presence of germinal centers. Flow cytometric analysis of lymphocytes isolated from these nodules revealed that they contain predominantly IgA-negative B-2 B lymphocytes, with a smaller population of $CD4^+$ T lymphocytes. These lymphocyte populations are similar to those seen in flow cytometric analysis of PP (3). Based upon these observations we believe that these nodules are similar to the recent description of ILF and have termed them mature ILFs.

Under low power microscopy ($\times 25$ – 65) we examined the distribution of these mature ILFs in mice treated with $LT\beta R$ -Ig. Ma-

ture ILF were prominent in the distal small intestine of mice treated with $LT\beta R$ -Ig in utero and were rare or absent from the intestines of untreated C57BL/6 mice in our colony (Fig. 2a). We noted an average of two mature ILFs per intestine in untreated C57BL/6 mice in our colony. In every situation in which mature ILF were observed, they were preferentially located in the distal small intestine; therefore, for clarity, further analysis only displays the numbers of mature ILFs in the distal small intestine (sections >24 cm from the pylorus; Figs. 3 and 4). To assess the requirement for $LT\beta R$ -dependent events for the development of these mature ILFs, we treated mice with $LT\beta R$ -Ig both in utero and through adulthood. As shown in Fig. 2a, this treatment blocked the formation of mature ILFs. Consistent with this we noted that mature ILFs were absent from the intestine of $LT\alpha^{-/-}$ and $LT\beta R^{-/-}$

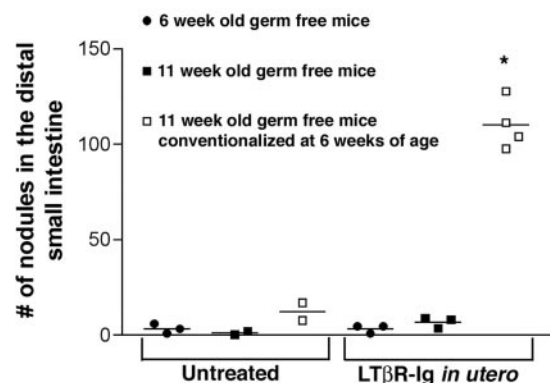


FIGURE 3. Mature ILFs are formed in response to normal gut flora. Six- and 11-wk-old germfree mice, 11-wk-old germfree C57BL/6 mice that had been conventionalized with normal cecal contents at 6 wk of age, as well as identical groups of mice that received $LT\beta R$ -Ig in utero were examined for the presence of mature ILF in the distal small intestine. Six- and 11-wk-old germfree mice had few or no mature ILF regardless of $LT\beta R$ -Ig therapy. Germfree mice that had been conventionalized by exposure to normal cecal contents developed a small number of mature ILF; however, this was significantly augmented by $LT\beta R$ -Ig therapy. These findings document that luminal stimuli, including normal gut flora, induce the formation of mature ILF. Each point represents the average number of nodules for one mouse, determined as described in *Materials and Methods*. *, $p < 0.05$ compared with the number of nodules in the distal small intestine of untreated C57BL/6 mice (Fig. 1a).

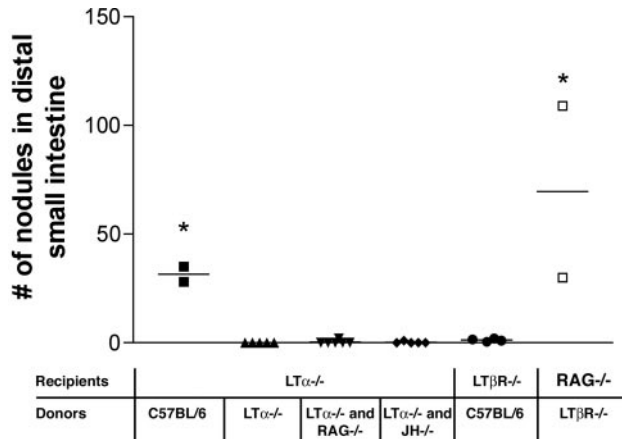


FIGURE 4. LT-sufficient B lymphocytes and LT β R-sufficient stromal cells are required for the formation of mature ILFs. Intestines from irradiated LT $\alpha^{-/-}$, LT β R $^{-/-}$, and RAG $^{-/-}$ mice receiving bone marrow from gender-matched donors were examined for the presence of mature ILF as outlined in *Materials and Methods*. LT $\alpha^{-/-}$ recipients of C57BL/6 bone marrow developed mature ILF, while LT $\alpha^{-/-}$ recipients of LT $\alpha^{-/-}$ bone marrow, a combination of LT $\alpha^{-/-}$ and RAG $^{-/-}$ bone marrow, or a combination of LT $\alpha^{-/-}$ and JH $^{-/-}$ bone marrow failed to develop mature ILF, thus documenting a requirement for LT-sufficient B lymphocytes in the formation of mature ILF. In contrast to LT $\alpha^{-/-}$ recipients of C57BL/6 bone marrow, LT β R $^{-/-}$ recipients of C57BL/6 bone marrow failed to develop mature ILF, while RAG $^{-/-}$ recipients of LT β R $^{-/-}$ bone marrow developed mature ILF, thus documenting a requirement for an LT β R-sufficient, non-bone marrow-derived (stromal) cell for the development of mature ILF. Each point represents the average number of nodules for one mouse determined as described in *Materials and Methods*. *, $p < 0.05$ compared with the number of nodules in the distal small intestine of LT $\alpha^{-/-}$ mice receiving LT $\alpha^{-/-}$ bone marrow.

mice (Fig. 2*a*). Treatment of mice with LT β R-Ig in utero induced the formation of mature ILFs in TNFR2 $^{-/-}$ mice, but failed to induce mature ILF formation in TNFR1 $^{-/-}$ mice (Fig. 2*b*). These observations suggest that TNFR1-dependent events are required for the formation of mature ILFs.

Mature ILF are formed in response to gut flora

The architecture, distribution, and requirement for LT in the formation of mature ILFs suggest that mature ILFs may be tertiary lymphoid structures formed in response to luminal stimuli. To assess the requirement for bacterial stimuli in the formation of these mature ILFs, we examined the formation of mature ILFs in germfree mice. As shown in Fig. 3, germfree mice do not spontaneously develop mature ILFs. Following treatment with LT β R-Ig a small number of mature ILFs can be induced in germfree mice. If germfree mice are conventionalized with normal cecal contents, a modest number of mature ILFs can develop; however, optimal development of mature ILFs requires both treatment with LT β R-Ig and exposure to luminal stimuli.

LT-expressing B lymphocytes and LT β R-expressing stromal cells are required for the formation of mature ILFs

To compare the formation of mature ILFs with that of PP, we performed bone marrow transfers into adult LT $\alpha^{-/-}$ mice. Adult LT $\alpha^{-/-}$ mice are unable to form PP or LN after receiving wild-type bone marrow (Refs. 10 and 11 and our unpublished observations), as LT-dependent events during embryogenesis are required for PP and LN formation. However, transfer of wild-type bone marrow to LT $\alpha^{-/-}$ mice resulted in the formation of mature ILFs (Fig. 4). Mature ILFs were not present in recipients of LT $\alpha^{-/-}$

bone marrow. This demonstrates that LT-dependent events occurring during embryogenesis are not required for the formation of mature ILFs, and that LT-expressing bone marrow cells are essential for this process. To determine the LT α -sufficient bone marrow cell lineage required for the formation of mature ILFs, we performed mixed bone marrow transfers. LT $\alpha^{-/-}$ recipients of a combination of RAG $^{-/-}$ and LT $\alpha^{-/-}$ bone marrow or a combination of JH $^{-/-}$ and LT $\alpha^{-/-}$ bone marrow failed to develop mature ILFs, thus demonstrating the requirement for an LT α -sufficient B lymphocyte in the development of mature ILFs.

Stromal cells and a subset of monocytes express the LT β R (5). To determine the relevant population of LT β R-expressing cells for the development of mature ILFs we performed transfers of wild-type and LT β R $^{-/-}$ bone marrow into LT β R $^{-/-}$ and RAG $^{-/-}$ mice and evaluated these recipients for the presence of mature ILFs. LT β R-deficient mice receiving wild-type bone marrow failed to develop mature ILFs, while RAG $^{-/-}$ recipients of LT β R $^{-/-}$ bone marrow developed mature ILFs. These findings demonstrate that LT β R expression by a radioresistant stromal cell is essential for the development of mature ILFs.

Immature ILF (B220⁺ clusters) are inducible structures requiring LT β R, but not TNFR1, for their formation

Recently, ILFs in the murine small intestine have been described (3). The characteristics of mature ILFs described here are similar to this initial description of ILFs in the murine small intestine; however, our observations have significant differences. First, we did not observe mature ILFs in the small intestine of untreated

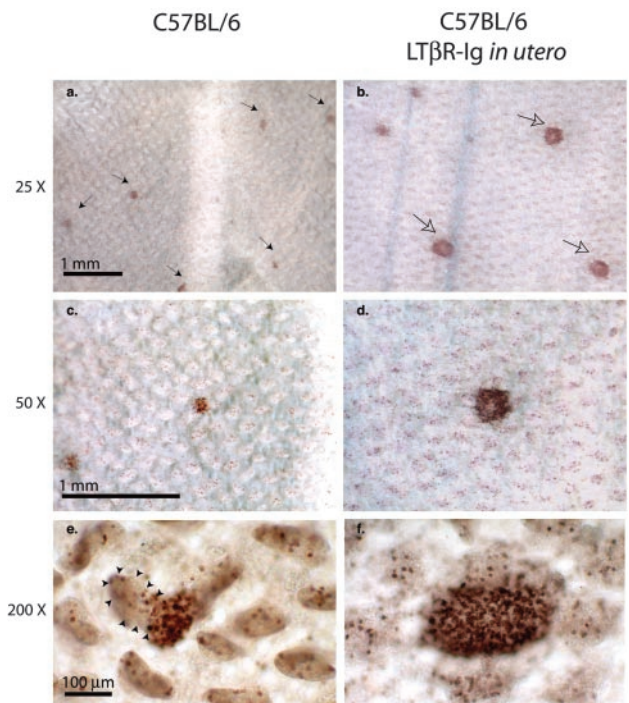


FIGURE 5. Characteristics of immature ILFs (B220⁺ clusters). Intestines from untreated adult C57BL/6 mice (*a*, *c*, and *e*) and adult C57BL/6 mice receiving LT β R-Ig in utero (*b*, *d*, and *f*) were examined using a modified whole mount technique described in *Materials and Methods*. Clusters of B220⁺ cells were seen in the small intestine of untreated and treated C57BL/6 mice. B220⁺ clusters in untreated C57BL/6 mice were preferentially located in the distal small intestine at the base of villi. Closed arrows denote B220⁺ clusters (immature ILFs) (*a*); open arrows denote lymphoid nodules (mature ILFs) (*b*); closed arrowheads delineate a villus projecting out of a B220⁺ cluster (*e*).

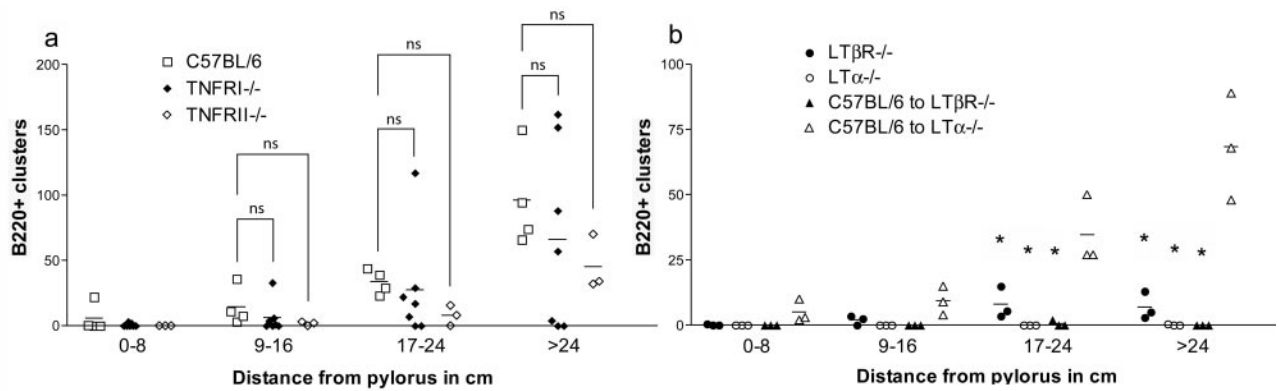


FIGURE 6. Immature ILFs (B220⁺ clusters) can be formed de novo in adult mice in an LTβR-dependent and TNFR1-independent fashion. Intestines from C57BL/6, TNFR1^{-/-}, TNFR2^{-/-}, LTβR^{-/-}, and LTα^{-/-} mice as well as LTα^{-/-} and LTβR^{-/-} mice receiving bone marrow from C57BL/6 mice were examined for the presence of B220⁺ clusters using a modified whole mount technique. B220⁺ clusters were present in C57BL/6, TNFR1^{-/-}, and TNFR2^{-/-} mice (a) and were absent in LTα^{-/-} and LTβR^{-/-} mice (b). The formation of B220⁺ clusters could be induced in LTα^{-/-} mice, but not in LTβR^{-/-} mice by reconstitution with C57BL/6 bone marrow (b), thus documenting the inducible nature of these B220⁺ clusters and the requirement for an LTβR⁺ non-bone marrow-derived cell for their formation. *, *p* < 0.05 compared with B220⁺ clusters in C57BL/6 mice (a).

animals in our colony; second, the numbers of mature ILFs we observed are significantly less than that noted in this initial description; third, the location of the mature ILFs is different from that noted in this initial description; and fourth, we did not see mature ILFs in the intestine of germfree mice. The initial description of ILF used immunohistochemistry for B220 performed on sections of intestine cut on an axis perpendicular to the villus structure. Using this technique it is difficult to accurately assess the architecture of these B220⁺ clusters. To resolve the differences between our observations and to better understand the relationship of the mature ILFs we observed to the B220⁺ clusters found by others, we used a modification of the intestinal whole mount method that allowed us to assess the number, position, and macroscopic architecture of B220⁺ clusters in the small intestine. In untreated C57BL/6 mice in our colony (lacking mature ILFs), we noted the presence of clusters of B220⁺ cells (Fig. 5). Similar to our observations of mature ILFs, these clusters were more prominent in the distal small intestine. These clusters were generally smaller than the mature ILFs we observed, were present at the base of villi (Fig. 5e), and were not visible on standard whole mounts of the small intestine. Using this modified whole mount technique, we examined the intestines of mice deficient in LTα, LTβR, TNFR1, and TNFR2. We observed that similar to mature ILFs, these

B220⁺ clusters required LT and LTβR for their formation. However, unlike mature ILFs, these B220⁺ clusters did not depend upon TNFR1 for their formation (Fig. 6a). Using bone marrow transfers, we were able to assess the formation of new B220⁺ clusters in the adult mouse small intestine. We observed that like mature ILFs, the B220⁺ clusters could be formed de novo in the adult mouse intestine, and their formation required LTβR-sufficient radioresistant stromal cells (Fig. 6b). Unlike PP, both mature ILFs and B220⁺ clusters are inducible in adult animals and do not require LT-dependent events during gestation for their formation. Based upon these observations and the previous descriptions of others we believe that the B220⁺ clusters described by others and the lymphoid nodules we describe here represent a spectrum in the formation of ILF and have termed the B220⁺ clusters immature ILFs.

Discussion

ILFs were recently described in the murine small intestine (3). In their description, Hamada et al. (3) noted that these structures were absent from the intestine of LTα^{-/-} and *aly/aly* mice, suggesting that ILF may share with secondary lymphoid structures a requirement for gestational LT/LTβR-dependent events in their formation. They also noted that ILFs were present in the intestine of

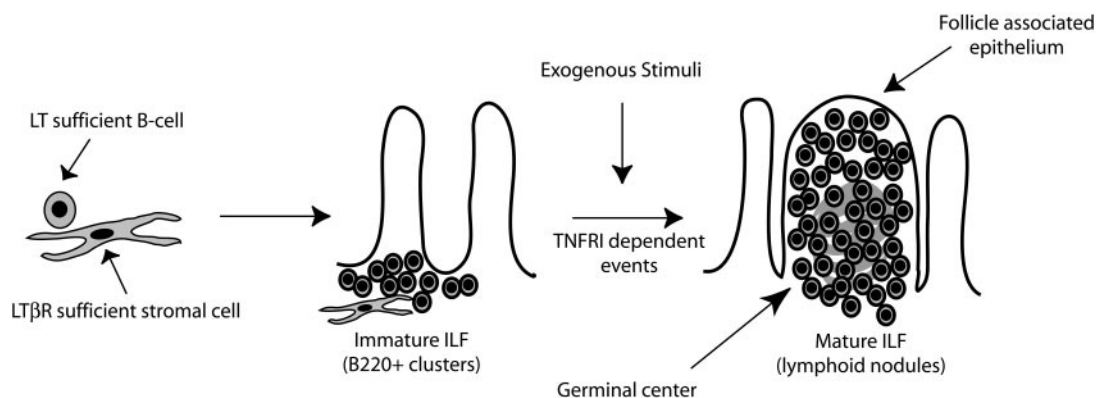


FIGURE 7. Putative sequence of events in the formation of ILFs. LT-expressing B lymphocytes interact with LTβR-expressing stromal cells within the intestinal lamina propria to induce the formation of B220⁺ clusters (immature ILFs) at the base of villi. The progression of immature ILFs to mature ILFs possessing germinal centers and a FAE containing M cells can occur in response to exogenous stimuli, including normal bacterial flora, and requires TNFR1 function.

germfree mice, further suggesting that the formation of these structures occurred independently of environmental stimuli. Contrasting with these observations, ILFs have been described to hypertrophy and regress in response to environmental stimuli (24), suggesting that ILF formation may have an inducible component in response to environmental challenges. In this study we sought to define the nature of ILF formation.

In our studies we observed a spectrum of structures fitting the previous descriptions of ILFs. In mice receiving $LT\beta R$ blockade in utero to ablate PP formation, we observed the presence of nodular lymphoid structures in the distal small intestine. These structures were rare or absent from untreated C57BL/6 mice in our colony. Consistent with previous description of ILFs by Hamada et al. (3), the mature ILFs we observed were predominantly composed of B-2 B lymphocytes and $CD4^+$ $TCR\beta^+$ T lymphocytes and possessed a FAE containing M cells. The formation of these structures was dependent upon $LT\beta R$ and TNFRI function, as we could block their formation by continuous $LT\beta R$ -Ig administration, and these structures could not be formed in TNFRI-deficient mice. We found that optimal formation of these mature ILFs required environmental stimuli. Using bone marrow reconstitution, we demonstrated that the formation of these mature ILFs could be induced in adult animals and required an LT-sufficient B lymphocyte and an $LT\beta R$ -sufficient non-bone marrow-derived cell.

Unlike the mature ILFs we describe here, Hamada et al. (3) noted that ILFs were present in both germfree mice and unmanipulated wild-type mice and were much more numerous than the mature ILFs we observed. We used a modified whole mount method to examine the presence, position, and macroscopic architecture of $B220^+$ clusters in the small intestine and to resolve the differences between our observations. We found that these $B220^+$ clusters were more numerous than the mature ILFs we observed, were preferentially located at the base of villi, and did not share the macroscopic architecture of mature ILFs. However, like mature ILFs, $B220^+$ clusters were preferentially located in the distal small intestine, were absent from the intestine of $LT\beta R^{-/-}$ and $LT\alpha^{-/-}$ mice, required $LT\beta R$ expressing non-bone marrow-derived cells for their formation, and were inducible in adult animals. Based upon our observations and the description of ILFs by Hamada et al., (3) we believe that these $B220^+$ clusters and the lymphoid nodules we observed represent a spectrum in the formation of ILF and have tentatively termed the $B220^+$ clusters immature ILFs.

In this study we demonstrate that mature ILFs are formed in C57BL/6 mice in response to luminal stimuli in the absence of PP. Based upon their architecture and composition it is reasonable to assume that mature ILFs are inductive sites for the immune response and that their formation in response to luminal stimuli in the absence of PP may be a compensatory response for the absence of PP and PP functions, including fecal IgA production. This hypothesis is consistent with the recent observations of others showing that ILFs may hypertrophy or regress in response to changes in luminal bacterial flora (24). Also consistent with this hypothesis we have noted that BALB/c mice have significantly lower fecal IgA levels compared with C57BL/6 mice and have mature ILFs in the absence of manipulations to prevent PP formation (our unpublished observations). These observations suggest that loss of the protective effects of fecal IgA may allow an inflammatory response to luminal stimuli, leading to the formation of mature ILF. Based upon these observations we propose the following sequence of events in the formation of ILFs (Fig. 7). LT-expressing B lymphocytes and $LT\beta R$ -expressing stromal cells in the intestine induce the formation of clusters of $B220^+$ cells within the intestine at the base of villi. Under the influence of exogenous stimuli and

TNFRI-dependent events, $B220^+$ clusters develop into lymphoid nodules with germinal centers and FAE containing M cells.

The pathways leading to intestinal IgA production are complex. Our understanding of this process continues to evolve, as recent studies indicate that events occurring within the intestinal lamina propria (outside of PP) play important roles in the production of intestinal IgA (reviewed in Ref. 23). Stromal cells within the lamina propria mediate some of these events. Lamina propria stromal cells produce factors favoring Ig class switch to IgA and the differentiation of IgA-producing plasma cells (25). Recent studies demonstrated that $LT\beta R$ -sufficient lamina propria stromal cells are required for the production of intestinal IgA (26). The findings presented here suggest an additional role for $LT\beta R$ -sufficient lamina propria stromal cells in the formation of ILFs. These studies suggest that stromal cells intrinsic to the intestine play a crucial role in the production of intestinal immune responses. Whether the same stromal cell type or different stromal cell types mediate each of these events is unknown.

The de novo formation of ectopic lymphoid structures resembling LN has been termed lymphoid neogenesis (16). This process is seen in infections and autoimmune diseases and is believed to play a role in the production of autoreactive lymphocytes and enhancing the neoplastic potential of lymphocytes by providing an unregulated environment for the interaction of lymphocytes, Ags, and APC (27). Studies of the factors involved in lymphoid neogenesis largely rely upon the induction of tertiary lymphoid structures by the ectopic expression of cytokines and chemokines. Transgenic mice expressing $LT\alpha$ in the pancreatic islets develop tertiary lymphoid structures in a TNFRI-dependent, but $LT\beta$ -independent, manner (28), while mice over expressing B lymphocyte chemoattractant in the pancreatic islets develop tertiary lymphoid structures in a B lymphocyte-dependent, $LT\alpha$ -dependent, $LT\beta R$ -dependent, but TNFRI-independent, manner (29). The formation of mature ILFs shares the requirements demonstrated in both these models; mature ILF formation requires $LT\alpha$ -sufficient B lymphocytes, $LT\beta R$ -sufficient stromal cells, and TNFRI function. Based upon these requirements and the inducible nature of mature ILF formation, we believe that the formation of mature ILF represents lymphoid neogenesis in the adult small intestine. The presence of ectopic lymphoid structures has been observed in a number of inflammatory settings within the gastrointestinal tract, including inflammatory bowel disease, gastritis, and animal models of intestinal inflammation (30–33). While obvious parallels can be drawn between these observations and the findings we present here, there is reason to believe that the requirements for the formation of ectopic lymphoid structures may differ from organ to organ as we have noted that $B220^+$ cluster formation is preserved in the colon of LT-deficient mice (our unpublished observation).

In this study we investigated the nature of ILF formation. In contrast to secondary lymphoid structures (PP and LN), we observed that ILFs do not require gestational $LT\beta R$ -dependent events during gestation for their formation, and in contrast to secondary lymphoid structure formation, we observed that ILFs can be formed de novo in adult animals. The cellular source of LT for ILF and secondary lymphoid structure formation also differs; LT-sufficient B lymphocytes are required for ILF formation, but are not necessary for the formation of LN and PP (34) (our unpublished observations). In addition, we found that the formation of immature ILFs was independent of TNFRI function; however, TNFRI function was essential for the development of mature ILFs. Our findings suggest that the intestinal immune system is not only complex in the variety of lymphoid structures it contains, but has an additional capacity to form and expand the mass of some lymphoid compartments in response to mucosal challenges.

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References

- Kanamori, Y., K. Ishimaru, M. Nanno, K. Maki, K. Ikuta, H. Nariuchi, and H. Ishikawa. 1996. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit⁺ IL-7R⁺ Thy1⁺ lympho-hemopoietic progenitors develop. *J. Exp. Med.* 184:1449.
- Moghaddami, M., A. Cummins, and G. Mayrhofer. 1998. Lymphocyte-filled villi: comparison with other lymphoid aggregations in the mucosa of the human small intestine. *Gastroenterology* 115:1414.
- Hamada, H., T. Hiroi, Y. Nishiyama, H. Takahashi, Y. Masunaga, S. Hachimura, S. Kaminogawa, H. Takahashi-Iwanaga, T. Iwanaga, H. Kiyono, et al. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168:57.
- Chaplin, D. D., and Y. Fu. 1998. Cytokine regulation of secondary lymphoid organ development. *Curr. Opin. Immunol.* 10:289.
- Browning, J. L., I. D. Sizing, P. Lawton, P. R. Bourdon, P. D. Rennert, G. R. Majeau, C. M. Ambrose, C. Hession, K. Miatkowski, D. A. Griffiths, et al. 1997. Characterization of lymphotoxin- $\alpha\beta$ complexes on the surface of mouse lymphocytes. *J. Immunol.* 159:3288.
- De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan, S. C. Smith, R. Carlson, L. P. Shormick, J. Strauss-Schoenberger, et al. 1994. Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. *Science* 264:703.
- Alimzhanov, M. B., D. V. Kuprash, M. H. Kosco-Vilbois, A. Luz, R. L. Turetskaya, A. Tarakhovskiy, K. Rajewsky, S. A. Nedospasov, and K. Pfeffer. 1997. Abnormal development of secondary lymphoid tissues in lymphotoxin β -deficient mice. *Proc. Natl. Acad. Sci. USA* 94:9302.
- Futterer, A., K. Mink, A. Luz, M. H. Kosco-Vilbois, and K. Pfeffer. 1998. The lymphotoxin β receptor controls organogenesis and affinity maturation in peripheral lymphoid tissues. *Immunity* 9:59.
- Rennert, P. D., J. L. Browning, R. Mebius, F. Mackay, and P. S. Hochman. 1996. Surface lymphotoxin $\alpha\beta$ complex is required for the development of peripheral lymphoid organs. *J. Exp. Med.* 184:1999.
- Mariathasan, S., M. Matsumoto, F. Baranyay, M. H. Nahm, O. Kanagawa, and D. D. Chaplin. 1995. Absence of lymph nodes in lymphotoxin- α (LT α)-deficient mice is due to abnormal organ development, not defective lymphocyte migration. *J. Inflamm.* 45:72.
- Fu, Y. X., H. Molina, M. Matsumoto, G. Huang, J. Min, and D. D. Chaplin. 1997. Lymphotoxin- α (LT α) supports development of splenic follicular structure that is required for IgG responses. *J. Exp. Med.* 185:2111.
- Yoshida, H., H. Kawamoto, S. M. Santee, H. Hashi, K. Honda, S. Nishikawa, C. F. Ware, Y. Katsura, and S. I. Nishikawa. 2001. Expression of $\alpha_4\beta_7$ integrin defines a distinct pathway of lymphoid progenitors committed to T cells, fetal intestinal lymphotoxin producer, NK, and dendritic cells. *J. Immunol.* 167:2511.
- Mebius, R. E., P. Rennert, and I. L. Weissman. 1997. Developing lymph nodes collect CD4⁺CD3⁻LT β ⁺ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* 7:493.
- Cupedo, T., G. Kraal, and R. E. Mebius. 2002. The role of CD45⁺CD4⁺CD3⁻ cells in lymphoid organ development. *Immunol. Rev.* 189:41.
- Koni, P. A., R. Sacca, P. Lawton, J. L. Browning, N. H. Ruddle, and R. A. Flavell. 1997. Distinct roles in lymphoid organogenesis for lymphotoxins β and β revealed in lymphotoxin β -deficient mice. *Immunity* 6:491.
- Kratz, A., A. Campos-Neto, M. S. Hanson, and N. H. Ruddle. 1996. Chronic inflammation caused by lymphotoxin is lymphoid neogenesis. *J. Exp. Med.* 183:1461.
- Gu, H., Y. R. Zou, and K. Rajewsky. 1993. Independent control of immunoglobulin switch recombination at individual switch regions evidenced through Cre-loxP-mediated gene targeting. *Cell* 73:1155.
- Matsumoto, M., S. Mariathasan, M. H. Nahm, F. Baranyay, J. J. Peschon, and D. D. Chaplin. 1996. Role of lymphotoxin and the type I TNF receptor in the formation of germinal centers. *Science* 271:1289.
- Peschon, J. J., D. S. Tarrance, K. L. Stocking, M. B. Glaccum, C. Otten, C. R. Willis, K. Charrier, P. J. Morrissey, C. B. Ware, and K. M. Mohler. 1998. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J. Immunol.* 160:943.
- Hooper, L. V., J. C. Mills, K. A. Roth, T. S. Stappenbeck, M. H. Wong, and J. I. Gordon. 2002. Combining gnotobiotic mouse models with functional genomics to define the impact of the microflora on host physiology. In *Methods in Microbiology: Molecular Cellular Microbiology*. P. J. Sansonetti and A. Zychlinsky, eds. Academic Press, London, p. 559.
- Newberry, R. D., J. S. McDonough, K. G. McDonald, and R. G. Lorenz. 2002. Postgestational lymphotoxin/lymphotoxin β receptor interactions are essential for the presence of intestinal B lymphocytes. *J. Immunol.* 168:4988.
- Malick, L. E., and R. B. Wilson. 1975. Modified thiocarbonylhydrazide procedure for scanning electron microscopy: routine use for normal, pathological, or experimental tissues. *Stain Technol.* 50:265.
- Golovkina, T. V., M. Shlomchik, L. Hannum, and A. Chervonsky. 1999. Organogenic role of B lymphocytes in mucosal immunity. *Science* 286:1965.
- Fagarasan, S., M. Muramatsu, K. Suzuki, H. Nagaoka, H. Hiai, and T. Honjo. 2002. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. *Science* 298:1424.
- Fagarasan, S., K. Kinoshita, M. Muramatsu, K. Ikuta, and T. Honjo. 2001. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 413:639.
- Kang, H. S., R. K. Chin, Y. Wang, P. Yu, J. Wang, K. A. Newell, and Y. X. Fu. 2002. Signaling via LT β R on the lamina propria stromal cells of the gut is required for IgA production. *Nat. Immunol.* 3:576.
- Hjelmstrom, P. 2001. Lymphoid neogenesis: de novo formation of lymphoid tissue in chronic inflammation through expression of homing chemokines. *J. Leukocyte Biol.* 69:331.
- Sacca, R., C. A. Cuff, W. Lesslauer, and N. H. Ruddle. 1998. Differential activities of secreted lymphotoxin- α_3 and membrane lymphotoxin- $\alpha_1\beta_2$ in lymphotoxin-induced inflammation: critical role of TNF receptor 1 signaling. *J. Immunol.* 160:485.
- Luther, S. A., T. Lopez, W. Bai, D. Hanahan, and J. G. Cyster. 2000. BLC expression in pancreatic islets causes B cell recruitment and lymphotoxin-dependent lymphoid neogenesis. *Immunity* 12:471.
- Dohi, T., K. Fujihashi, P. D. Rennert, K. Iwatani, H. Kiyono, and J. R. McGhee. 1999. Hapten-induced colitis is associated with colonic patch hypertrophy and T helper cell 2-type responses. *J. Exp. Med.* 189:1169.
- Kaiserling, E. 2001. Newly-formed lymph nodes in the submucosa in chronic inflammatory bowel disease. *Lymphology* 34:22.
- Yeung, M. M., S. Melgar, V. Baranov, A. Oberg, A. Danielsson, S. Hammarstrom, and M. L. Hammarstrom. 2000. Characterisation of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TcR- $\gamma\delta$ expression. *Gut* 47:215.
- Eidt, S., and M. Stolte. 1993. Prevalence of lymphoid follicles and aggregates in *Helicobacter pylori* gastritis in antral and body mucosa. *J. Clin. Pathol.* 46:832.
- Banks, T. A., B. T. Rouse, M. K. Kerley, P. J. Blair, V. L. Godfrey, N. A. Kuklin, D. M. Bouley, J. Thomas, S. Kanangat, and M. L. Mucenski. 1995. Lymphotoxin- α -deficient mice: effects on secondary lymphoid organ development and humoral immune responsiveness. *J. Immunol.* 155:1685.