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DEVELOPMENT AND MATURATION OF SECONDARY LYMPHOID TISSUES

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ABSTRACT

The secondary lymphoid tissues are located at strategic sites where foreign antigens can be efficiently brought together with immune system regulatory and effector cells. The organized structure of the secondary lymphoid tissues is thought to enhance the sensitivity of antigen recognition and to support proper regulation of the activation and maturation of the antigen-responsive lymphoid cells. Although a substantial amount is known about the cellular elements that compose the lymphoid and nonlymphoid components of the secondary lymphoid tissues, information concerning the signals that control the development of the tissues and that maintain the organized tissue microenvironment remain undefined. Studies over the past few years have identified lymphotoxin as a critical signaling molecule not only for the organogenesis of secondary lymphoid tissues but for the maintenance of aspects of their microarchitecture as well. Additional signaling molecules that contribute to the formation of normal lymphoid tissue structure are being identified at an accelerating pace. Analyses of mouse strains with congenital defects in different aspects of secondary lymphoid tissue development are beginning to clarify the role of these tissues in immune responses and

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host defense. This review focuses on studies defining recently identified crucial signals for the biogenesis of secondary lymphoid organs and for the maintenance of their proper microarchitecture. It also discusses new insights into how the structure of these tissues supports effective immune responses.

INTRODUCTION

The immune system is functionally compartmentalized into primary lymphoid organs and secondary lymphoid tissues. The role of primary lymphoid tissues, in which lymphocyte precursors develop into immunocompetent naive lymphocytes, has been extensively studied and is not discussed here. Secondary lymphoid tissues are the spleen, lymph nodes (LN), and organized lymphoid tissues associated with mucosal surfaces, including the tonsils, bronchial-associated lymphoid tissues, gut-associated lymphoid tissues, Peyer's patches (PP), and other less-prominent organized clusters of lymphoid cells associated with the gastrointestinal, genitourinary, and respiratory tracts. These lymphoid tissues are located at strategic sites where foreign antigens entering the body from either the skin or a mucosal surface can be trapped and concentrated. Lymphocytes, antigen transporting and presenting cells, and other regulatory cells are also located in these anatomically defined tissues and are thought to be organized into structures that optimize cellular interactions that support the efficient removal of unwanted pathogens (1–3). The structures of LN, PP, and spleen have been thoroughly reviewed (4–6). Each lymphoid tissue has a unique architecture, but they share some common features. Generally, T and B lymphocytes are segregated into distinct areas. The area of T cell predominance contains small numbers of B cells and substantial numbers of dendritic cells, which are thought to present antigens for the initial activation of the T and B cells. The B cell areas contain primary follicles that represent sites where antigen-activated B cells that have received T cell help can expand, mature, and undergo the germinal center reaction prior to becoming antibody-producing cells and memory B cells.

Packaging immune cells into secondary lymphoid tissues is thought to enhance the efficiency of immune responses by (*a*) arranging B and T cells in geographic locations that favor their interactions with antigen-presenting cells and perhaps other regulatory cells and (*b*) providing a framework to permit rapid circulation of naive cells through a space where antigens are concentrated. The efficiency generated by these organized tissue structures is shown by experiments, such as those by Kundig and coworkers (7), in which immunization with 10^6 fibrosarcoma cells was required to prime T cells when the tumor cells were injected subcutaneously, whereas only 500 cells were required when they were injected directly into the spleen. Zinkernagel et al (8) recently proposed that

both geographical localization of antigens to secondary lymphoid tissues and the dose and time of antigen exposure are key variables determining whether a regulated, productive immune response occurs (8). The ability of secondary lymphoid tissues to concentrate and retain antigens in proximity to the initially rare antigen-specific cells may be critical in order for this to occur.

STRUCTURAL FEATURES OF NORMAL SECONDARY LYMPHOID TISSUES

The microarchitecture of the LN has been the most fully characterized of all of the secondary lymphoid tissues. The infrastructure of the LN has been demonstrated at the ultrastructural level by removing cells and then analyzing fixed material by scanning electron microscopy. LN are fed by two vascular systems: the lymphatic vasculature, which delivers antigens and antigen-transporting cells from peripheral tissues to the node and returns fluid and cells to the circulation, and the blood vasculature, which brings circulating lymphocytes into the node. The afferent lymphatics empty into the marginal sinus immediately beneath the LN capsule and drain ultimately via the medullary sinus into the efferent lymphatic. Under the marginal sinus lies the LN cortex, which is separated into an outer cortex, consisting mostly of B lymphocytes, and an inner paracortex, consisting mostly of T lymphocytes. Within the outer cortex, the B cells are organized into primary follicles that support the formation of germinal centers following immunization with a T cell–dependent antigen. An important nonlymphocytic component of the primary follicles is the clusters of follicular dendritic cells (FDC). FDC with their abundant complement receptors and immunoglobulin Fc receptors are thought to focus immune complexes within the B cell follicle in a fashion that is crucial for the development of effective isotype-switched and memory B cell responses (9–12). Internal to the paracortex are the medullary cords, populated prominently by macrophages and plasma cells and which lead to the medullary sinus. The functional unit of the paracortex is the paracortical cord, which stretches from the base of a B cell follicle to an underlying medullary cord. The paracortical cord is approximately 100–1000 μm in diameter and is thought to provide a space in which antigen-presenting cells (primarily dendritic cells) can encounter rare antigen-specific T lymphocytes and favor their activation and subsequent maturation (13). Each segment of paracortex appears to be composed of hundreds of paracortical cords. In contrast, in the area of the primary B cell follicles, the tissue is penetrated by only a few fibers. This suggests that the requirements for cell trafficking change dramatically when cells leave the paracortex and enter the follicle structure. B and T lymphocytes from the blood enter the LN by crossing the specialized high endothelial venules, which are located in the paracortex near the junction with

the outer cortex (14). If they traffic through the paracortical cord and medullary cord without being activated by antigen on an antigen-presenting cell, they return to the circulation via the efferent lymphatic vessels and the thoracic duct. For a naive lymphocyte that does not encounter its cognate antigen, such a circuit through the node is thought to take fewer than 24 h.

The mucosa-associated lymphoid tissues, including the PP, bronchial-associated lymphoid tissues, gut-associated lymphoid tissues, and others, have a general structure similar to the LN, with distinct T and B cell areas and B cell follicles (5, 15, 16), but they differ prominently in the pathway by which antigens enter the lymphoid compartment. Rather than traffic through an afferent lymphatic, antigens enter the mucosa-associated lymphoid tissues across the mucosal epithelium. Often the mucosal epithelial cells immediately overlying the lymphoid tissue are specialized for uptake of antigen from the lumen of the mucosa. The M cells overlying PP in the gastrointestinal tract have been shown to transport particles as large as intact microorganisms (17). On the abluminal surface of the mucosa-associated lymphoid tissues, lymphocytes return to the blood circulation via efferent lymphatic vessels that ultimately join the thoracic duct like efferent lymphatic vessels from LN.

The spleen, the largest single lymphoid organ in mammals, contains up to 25% of the body's mature lymphocytes (18). It is separated into two major components, the red pulp and the white pulp (Figure 1). The red pulp has been thought of primarily as a filter in which aged or damaged erythrocytes are removed from the circulation. It consists of a reticular network containing stromal cells and a large population of macrophages. It also contains a variably large population of plasma cells and can be a site of substantial immunoglobulin production. The white pulp represents the organized lymphoid compartment in which regulated activation and maturation of antigen-dependent B and T cells occur. Unlike the LN, the spleen has a single vascular supply, with immune cells and antigen entering the tissue with the blood via the splenic artery. In humans, approximately 5% of the total cardiac output is directed through the spleen (19). The blood enters via the splenic artery, which branches into trabecular arteries and, ultimately, into central arterioles that penetrate the white pulp nodules. Surrounding the central arterioles is a T cell–rich compartment designated the periarteriolar lymphoid sheath (PALS). The PALS also contains abundant interdigitating dendritic cells that are thought to serve as important antigen-presenting cells early in the immune response in this tissue. The central arteriole, after it penetrates the PALS, forms a marginal sinus that is lined with a mucosal addressin cell adhesion molecule-1 (MAdCAM-1)–expressing endothelium (20). Also associated with this marginal sinus endothelium is a specialized layer of metallophilic macrophages that are thought to regulate the entry of antigen into the white pulp tissue (21). Additional venous sinuses

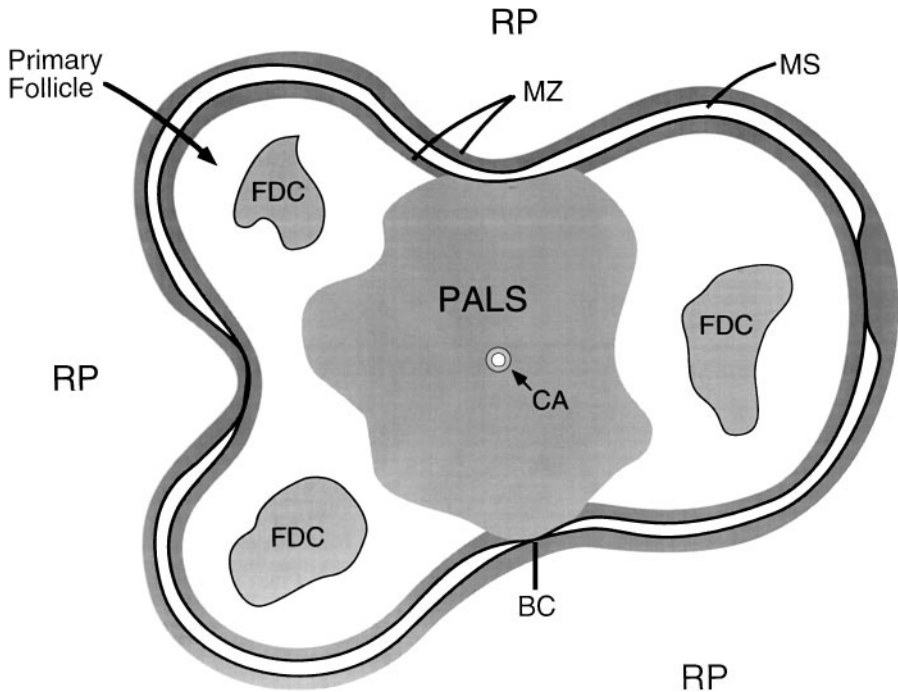

Figure 1 Structure of the spleen white pulp nodule. The white pulp nodule is separated into a central T cell–rich zone [periarteriolar lymphoid sheath (PALS)] surrounded by B cell–rich primary follicles. Within each primary follicle is a cluster of follicular dendritic cells (FDC). The white pulp nodule is separated from the red pulp (RP) by the MAdCAM-1^{+} marginal sinus (MS). The MS is embedded in a layer of marginal zone (MZ) lymphocytes. Also adjacent to the MS is a layer of metallophilic macrophages that are thought to be important to regulate antigen trafficking into the red and white pulp spaces. The bridging channels (BC) are thought to represent areas by which lymphocytes enter and leave the white pulp. CA, central arteriole.

are present within the red pulp, and these are thought to coalesce into larger segmental venules that ultimately exit the spleen as the major splenic vein. In the white pulp, the B cells are organized into two compartments (2, 22). The first consists of naive B cells and at least some memory B cells and includes the marginal zone cells that are adjacent to the MAdCAM-1–expressing marginal sinus (23, 24). The second is composed of follicle-associated cells, which in the resting state are organized into primary follicles. These primary follicle B cells surround clusters of FDC, similar to the arrangement of primary follicles in LN. Altogether, the components of the white pulp form a highly ordered structure that is thought to be critical for proper regulation of immune responsiveness in this tissue (3, 25).

Although the anatomical features of the secondary lymphoid tissues are well defined, little is known about the mechanisms that establish the normal cellular compartments within these structures during ontogeny, or that regulate trafficking of cells through these compartments during normal function of the mature tissue (1–3, 13). The general conservation of structure between the various tissues has been taken as evidence that the organized structure is critical for the function of the tissues; however, in fact, the relationship between organized tissue structure and effective immune responsiveness has not been extensively investigated (8, 22, 26). Historically, analyses of mice with inherited lack of the thymus have been highly informative for definition of the function of that structure (27); however, until recently, similar model systems for studying the consequences of congenital lack of secondary lymphoid tissues have not existed. In the last several years, studies analyzing the independent roles of lymphotoxin (LT) and tumor necrosis factor (TNF) have provided valuable models in which various aspects of secondary lymphoid tissue structure are disturbed, and the analyzing of these models is beginning to establish the requirements for normal secondary lymphoid structure in the host immune response (22, 28). This article reviews studies that identify some of the signals required for organogenesis and maturation of secondary lymphoid tissues and discusses new insights into the cellular interactions between lymphocytes and stromal cells in these lymphoid tissues.

MUTANT MOUSE STRAINS WITH ALTERED LYMPHOID TISSUE DEVELOPMENT

Three spontaneously arising mutant mouse strains have been identified that manifest disturbances of the structures of primary and/or secondary lymphoid tissues. Examination of these strains is leading to important insights into the normal mechanisms controlling development of these tissues and the biological and immunological processes that normally occur within them. The focus of this article is the secondary lymphoid tissues, but a historically important example affecting the development of lymphoid organs, the *nude* mutation, is discussed first.

The autosomal recessive *nude* mutation is phenotypically invisible in heterozygotes but leads to congenital absence of the thymus and lifelong hairlessness in homozygotes (27). Recent studies by Boehm and coworkers (29–31) have demonstrated that mutations in the *whn* gene encoding a transcription factor of the forkhead/winged-helix class causes the nude phenotype. *Whn* is expressed in thymic epithelial precursors and cells within the hair follicle.

Although *whn* expression is not required for the formation of the thymic epithelial primordium prior to the entry of lymphocyte progenitors, the *whn*

gene product is required for the subsequent differentiation of this epithelial primordium into the specialized epithelia of the subcapsular, cortical, and medullary compartments (29). Analysis of spontaneous *nude* mice and mice targeted in vitro for mutations in the *whn* locus shows that normal development of $\alpha\beta$ T cell-receptor-bearing T lymphocytes is dependent on the development of functional thymus tissue.

Less thoroughly studied is the mouse strain carrying the semidominant *Dh* (dominant hemimelia) mutation (32). In homozygous form, this mutation is embryonic lethal. Heterozygous *Dh/+* mice show skeletal and visceral abnormalities, including prominent asplenia. Studies using chimeric embryos in which *Dh/Dh* or *Dh/+* embryos were aggregated with C3H/He embryos have demonstrated that when a spleen develops (presumably under the influence of the normal C3H/He cells), its structure is grossly normal and the *Dh* cells can contribute to at least several components of the splenic tissue (33). This suggests that the *Dh* mutation affects an early step in spleen organogenesis but not the ability of cells to migrate to the spleen. Although further analysis of this strain may yield important insights into the cellular interactions that commit to spleen organogenesis, because the *Dh* mutation affects many other somatic tissues, it is unlikely that analysis of this strain will provide straightforward insights into the role of the spleen in normal immune responsiveness.

Perhaps more tractable to immunological analysis will be the recently discovered autosomal recessive *alymphoplasia (aly)* mutation (34). This mutation, which maps to mouse chromosome 11, determines in its homozygous form broad alterations in immune tissue structure and function without gross defects in other somatic tissues. *aly/aly* mice have complete absence of LN and PP. The thymus is present, but with disturbed structure. Although phenotypically mature T cells are found in blood and spleen, their numbers are reduced and they show depressed function manifested in all measures of T cell activation. The structure of the spleen is diffusely altered, with atrophic white pulp nodules and absence of detectable B cell follicle structure. Total serum immunoglobulins are low, and antigen-specific antibody responses are substantially reduced (34, 35). Comparison of the ontogeny of gut-associated lymphoid tissues in *aly/aly*, wild-type (wt), and severe combined immunodeficiency (*scid*) mice has yielded insights into the development of mouse PP (36). Adachi and colleagues (36) found that clusters of vascular cell adhesion molecule-1 (VCAM-1)–expressing cells form in the wall of the gut by day 15 after conception. Over the next 2 days, these VCAM-1^{+} clusters of apparently stromal cells become infiltrated with a homogeneous-appearing population of major histocompatibility class II^{+}, IL-7R^{+}, CD4^{+}, and CD3^{-} cells. These cells are then gradually replaced by T and B lymphocytes, as well as by the other cellular elements of the normal, mature PP.

In addition to the natural mouse mutations discussed above, targeted mutagenesis of mice using embryonic stem cells has yielded several examples in which formation of certain lymphoid tissue structures is altered or ablated. Studies by Roberts et al (37) demonstrated the absolute requirement for expression of *hox11* for the formation of the spleen. Although *hox11* is expressed in other tissues, absence of its gene leads to loss of only the spleen, with the only other detected phenotypic alterations being a modest increase in size of the stomach and pancreas (38). Disturbances in the development of secondary lymphoid tissues have also been observed in mice deficient in LT or TNF, as well as in certain intracellular signaling molecules. Defects due to absence of these molecules are discussed below.

Recent interesting studies have examined immune host defense in both the asplenic *hox11*-deficient and the *aly/aly* mice (26). *Hox11*-deficient mice showed modestly delayed antibody responses to vesicular stomatitis virus (VSV) but were otherwise resistant to infection. In contrast, *aly/aly* mice, with their profound disturbance of lymphoid tissue structure and abnormal B and T lymphocytes responsiveness, were highly susceptible to VSV. They generated a delayed and reduced immunoglobulin (Ig) M anti-VSV response and failed to generate a protective IgG response. This failure to produce an IgG response was not the manifestation of an intrinsic defect in isotype switching in the *aly/aly* lymphocytes. If *aly/aly* spleen cells were adoptively transferred into an irradiated wt mouse (with morphologically normal secondary lymphoid tissues), these cells were able to generate a protective, isotype-switched IgG response. Thus, when the structure of the secondary lymphoid tissues is disturbed, maturation of the antibody response is importantly impaired.

AN ESSENTIAL ROLE FOR LYMPHOTOXIN IN THE DEVELOPMENT OF SECONDARY LYMPHOID TISSUES

Until recently, there had been little progress in defining essential signals that supported the development of normal secondary lymphoid tissues. As described above, analyses of *aly/aly* and *scid* mice have provided clues regarding the initial steps of PP development (36). It is likely that secondary lymphoid tissue organogenesis occurs in several discrete steps and depends on the interaction of several different types of cells using several different types of cell-cell signaling processes. A full understanding of the processes that lead to the formation of normal secondary lymphoid tissue structure will require the identification of the primordial cell types that interact during the initial biogenesis of the tissues and the signals they use to communicate with each other during this organogenesis. In addition, it appears that ongoing signals are required to maintain the normal

tissue structures, even in mature animals. Recent studies using gene targeting and transgenic methods are beginning to define some of the important signals that govern these complex processes. The following section focuses on the role of LT and TNF, as recent studies of these molecules are rapidly increasing our understanding of their biological functions and are demonstrating their pivotal roles in the formation of normal peripheral lymphoid tissue structures.

The TNF Family of Cytokines and Receptors

TNF (also designated TNFα) and lymphotoxin-α (LTα) were first identified based on their cytotoxic activities against various cell lines and tumor cells (reviewed in 39). In supernatants of activated cultured cells, TNF and LTα are structurally related homotrimeric proteins. These homotrimeric ligands can interact with and activate each of the two defined TNF receptors, TNFR-I/p55 and TNFR-II/p75 (Figure 2). Because of the similar binding of these two ligands to the defined TNF receptors, they were generally accepted to be functionally redundant, differing only in that LTα is a conventionally secreted glycoprotein, whereas TNF is synthesized with a long N-terminal peptide that anchors it at the cell surface as a type II membrane protein (40). Secretion of TNF is the result of cleavage of the membrane protein from the cell surface by the action of the TNFα converting enzyme (TACE), a metalloproteinase produced by the same cell that synthesizes the TNF (41, 42). Studies in the early 1990s demonstrated that LT also exists in a membrane-associated form, with a single LTα chain noncovalently associated with two copies of a structurally related type II transmembrane protein designated lymphotoxin β (LTβ) (43–45). The genes encoding TNF, LTα, and LTβ are genetically linked, encoded within a 25-kb portion of the class III region of the major histocompatibility complex (46). The membrane LT heterotrimer (LT$\alpha_1\beta_2$, or mLT) is not a substrate for TACE and appears to exist only as a membrane-associated protein. It shows no detectable affinity for TNFR-I or TNFR-II but binds and signals through another receptor of the TNFR family, designated the lymphotoxin β receptor (LTβR) (47). The LTβR appears to be specific for mLT and shows no measurable affinity for the homotrimeric TNF or LTα_3 ligands. Membrane LT has also been detected in vitro as an LT$\alpha_2\beta_1$ trimer. The LT$\alpha_2\beta_1$ heterotrimer can interact with TNFR-I and TNFR-II, but its ability to interact with and activate the LTβR is not fully defined. Also unclear is whether the LT$\alpha_2\beta_1$ heterotrimer is present in biologically meaningful quantities in vivo.

Unlike TNFR-I and TNFR-II, which are expressed very broadly, the LTβR is not expressed on lymphoid cells. Rather, it is expressed on stromal cells in various lymphoid tissues (28). Because the LT$\alpha_1\beta_2$ ligand is membrane associated, it is likely that LT$\alpha_1\beta_2$-mediated responses involve physical contact between the mLT-expressing cell and its LTβR-bearing target. Thus, TNF and

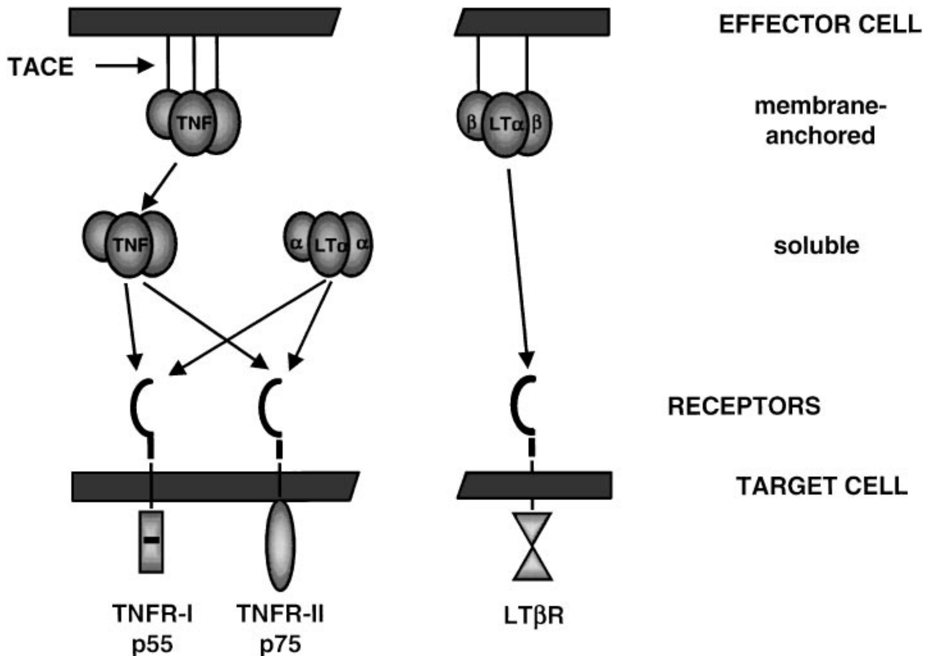

Figure 2 Ligands and receptors (R) of the lymphotoxin (LT)/tumor necrosis factor (TNF) family. The four major ligands of the LT/TNF family are shown as homo- and heterotrimeric proteins produced by the effector cell. TNF and LTβ are synthesized as type II membrane proteins, anchoring the primary translation products to the surface of the producing cell. The three major receptors for these ligands show considerable homology in their extracellular domains. Their unique intracellular domains provide mechanisms by which they can each transmit independent signals following ligand binding. The black bar in the intracellular domain of TNFR-I designates the "death domain" that is required to effect the apoptotic response to TNF or LTα_3 in sensitive target cells. Membrane TNF can bind and activate both TNFR-I and TNFR-II similarly to soluble TNF. TACE, TNFα converting enzyme.

LT encode two sets of ligands: the membrane and secreted forms of TNF and the LTα_3, which interact with TNFR-I and TNFR-II, forming one set; and the membrane LT$\alpha_1\beta_2$ heterotrimer, which interacts with the LTβR forming the other set. Acting through the different intracellular domains of their receptors, these two sets of ligands and receptors would be expected to mediate independent sets of cellular and tissue responses.

Critical Role of mLT in the Formation of LN and PP

The first experiments that linked LTα with the biogenesis of secondary lymphoid tissues used gene targeting to generate a mouse strain with homozygous

LTα deficiency (48). LT$\alpha^{-/-}$ mice showed a profound defect in formation of LN and complete absence of PP. The spleen was present, but its microarchitecture was grossly disturbed (discussed below). These observations established that LTα was a critical factor, required for the formation of these secondary lymphoid tissue structures. These results were confirmed by Banks et al (49), who independently produced an LT$\alpha^{-/-}$ mouse strain. Of interest, the congenital absence of LN and PP in the LT$\alpha^{-/-}$ mice was observed in the context of apparently normal lymphatic vasculature, with retained efficient transport of India ink injected into the footpad to the spleen (48). Thus, the development of the lymphatic vasculature is not obligately linked to the formation of intact LN. The signals required for the development of lymphatic vessels have not yet been identified, but the recent observation that mice carrying an overexpressing vascular endothelial growth factor-C transgene have hyperplasia of many lymphatic vessels suggests that vascular endothelial growth factor-C may deliver one of the important developmental signals (50).

Of interest, defective LN biogenesis was not absolute in the LT$\alpha^{-/-}$ mice. In more than 95% of the mice, LN were completely absent. In the remaining 2–4%, a mesenteric LN was detected (51). In wt mice, mesenteric LN are present as a short chain consisting of several discrete nodes. In the small fraction of LT$\alpha^{-/-}$ mice with mesenteric LN, the normal mesenteric chain was reduced to a single node. The presence of a mesenteric LN in some LT$\alpha^{-/-}$ mice indicates that some LTα-independent signal can substitute for LTα in the development of this particular node. The nature of this LTα-independent signal remains to be characterized.

That LTα was essential for the formation of LN and PP was unanticipated because mouse strains deficient in either of the two defined TNF receptors (TNFR-I or TNFR-II) had not been recognized to manifest any defects in lymphoid tissue structure (52, 53). In fact, LN appear to form with normal distribution even in mice with targeted ablation of both TNFR-I and TNFR-II (Y-X Fu, DD Chaplin, unpublished data). This suggested that disturbed LN and PP formation in LT$\alpha^{-/-}$ mice was not mediated by ablation of signals through the homotrimeric ligand/TNFR-I/TNFR-II arm of the TNF ligand/receptor family, but rather that it might represent failure of signaling through the mLT/LTβR arm of the family.

The LN and PP defect observed in LT$\alpha^{-/-}$ mice is developmentally fixed. If wt bone marrow (BM) is infused into lethally irradiated LT$\alpha^{-/-}$ mice, although the wt BM–derived cells home to and repopulate the spleen, they are unable to induce the formation of LN or PP (54). In contrast, when LT$\alpha^{-/-}$ BM was infused into lethally irradiated wt mice, the LT$\alpha^{-/-}$ cells showed apparently full potential to repopulate the wt LN, PP, and spleen. Thus, the failure to develop detectable LN or PP in LT$\alpha^{-/-}$ mice was not due to an inability of LT$\alpha^{-/-}$

BM–derived cells to traffic to the lymphoid tissues. Rather, the absence of LN and PP in the LT$\alpha^{-/-}$ mice represented a true failure of biogenesis of these organs, with some LTα-dependent developmental signal being required before adulthood in order for LN and PP structures to form.

The Membrane Form of LT Supports the Formation of LN and PP

Recognizing that LTα probably acted during ontogeny to signal the formation of LN and PP, Rennert et al (55) adopted a novel strategy to investigate the molecular form of LT that was active in these processes. They prepared TNFR-I- and LTβR-immunoglobulin (LTβR-Ig) fusion proteins to achieve in vivo neutralization of the LTα_3 homotrimer and the membrane LT$\alpha_1\beta_2$ heterotrimer respectively. Receptor-Ig fusion proteins have been used productively before, both to render the ligand binding domain of the receptor divalent and to enhance stability in vivo. These investigators recognized that if the Ig domain was derived from an IgG, then the receptor-Ig fusion protein could cross the placenta of a pregnant mouse and neutralize the ligand in developing embryos. When an LTβR-Ig fusion was administered to pregnant wt mice at gestational day 18, the offspring were born with unaltered LN structure, but with a total absence of PP. If the fusion protein was administered on gestational day 16, then PP and popliteal LN were ablated, although mesenteric, axillary, and inguinal nodes were retained. If the fusion protein was delivered on gestational day 12 or earlier, then PP, popliteal, axillary, and inguinal LN were all ablated, with only mesenteric LN and certain cervical LN being retained. Administration of a TNFR-I–Ig fusion protein to pregnant wt mice at any time during gestation did not interfere with the development of either LN or PP. Given that the LTβR binds to the membrane form of LT and not the homotrimer, whereas TNFR-I binds the homotrimer but not the membrane LT$\alpha_1\beta_2$ form, this study demonstrated clearly that the membrane form of LT was the active signaling molecule in LN and PP biogenesis. Furthermore, this study defined specific time windows during which mLT must act to support the development of different anatomically defined sets of LN and PP. The critical period of mLT expression varied for different sets of LN. Interestingly, the mesenteric LN were resistant to ablation by the LTβR-Ig, reminiscent of the fraction of LT$\alpha^{-/-}$ mice with retained development of the mesenteric node. This confirms that there is a fundamental difference between the mesenteric LN and other LN and suggests that there is not an absolute requirement for an mLT signal for the development of this tissue. Subsequent studies showed that several cervical, sacral, and lumbar LN, in addition to the mesenteric LN, were also resistant to ablation by the soluble LTβR-Ig fusion protein (56). This defined a subset of enteric-associated LN that have no absolute requirement for mLT to induce their development. The

selective role of mLT in LN and PP biogenesis was underscored by the observation that treatment of mice with a TNFR-I–Ig fusion protein did not interfere with formation of LN or PP.

In related studies, an alternative approach was used to neutralize the function of membrane LT (57). A mouse strain was created in which a transgene encoding a soluble LTβR-Ig fusion protein was expressed under the control of the CMV promoter. This promoter yields only very low levels of expression during embryonic development, with high-level expression beginning approximately 3 days after birth. Mice expressing the transgene showed variable loss of PP formation but no defect in LN biogenesis. This confirmed that the ligand neutralized by the soluble LTβR, mLT, gives essential signals for the formation of PP, and that these signals must be present near the time of birth. That the organogenic mLT signal must be delivered during a discrete window of time is supported by the observation that when PP did form in the LTβR-Ig transgenic animals, their overall morphology appeared normal. This was at a time when high levels of receptor-Ig fusion protein could be detected in the circulation. Thus, once commitment to development of a PP occurred, then further mLT signals appeared not to be required to sustain the tissue structure. This is consistent with earlier experiments in which BM from an LT$\alpha^{-/-}$ donor was used to reconstitute hematopoiesis in a lethally irradiated wt mouse (54). After BM reconstitution, only mLT-deficient cells were present in the circulation. Nevertheless, LN structures were retained for the remainder of the life of these animals. These experiments support the concept that during ontogeny, decisions regarding biogenesis of LN and PP are made during a specific window of time and that once a decision for or against formation of the secondary lymphoid tissue has been made, the phenotype is fixed. Expression or lack of expression of mLT outside that window of time does not alter the LN/PP phenotype.

Additional data supporting the role of mLT as a key signaling molecule in the biogenesis of LN and PP came from later studies in which the LTβ gene was inactivated by gene targeting (58). LT$\beta^{-/-}$ mice were unable to express the membrane LT$\alpha_1\beta_2$ heterotrimer but were presumed to retain expression of the LTα_3 homotrimer. The LT$\beta^{-/-}$ mice manifested absence of peripheral LN and PP but retained formation of the mesenteric LN and certain cervical LN. Because mesenteric and cervical LN were retained in the LT$\beta^{-/-}$ mice and in mice treated with the LTβR-Ig fusion protein (55, 56) and were generally absent in LT$\alpha^{-/-}$ mice (48, 49, 51), it was suggested that the development of mesenteric and cervical nodes was controlled uniquely by LTα. A subsequent study showed that crossing the LT$\beta^{-/-}$ genotype onto the TNFR-I$^{-/-}$ genotype resulted in loss of the mesenteric LN, in addition to the loss of other peripheral LN and PP (59). Similar results were obtained using

the receptor-fusion protein technology when wt pregnant mice were treated with a combination of LTβR-Ig and TNFR-I–Ig fusion proteins. In this case, in addition to the peripheral LN and PP, development of the mesenteric LN was extinguished (60). Given that the mesenteric LN is present in mice with isolated deficiency of TNFR-I, this suggests that although mLT plays an essential role in the formation of most peripheral LN and PP and a dominant role in the formation of mesenteric and enteric-associated LN, LTα_3 signaling through TNFR-I might rescue formation of the mesenteric and enteric-associated LN when LTβ is absent. Arguing against a role for LTα_3 is the observation that treatment of wt pregnant mice with a combination of LTβR-Ig fusion protein and neutralizing anti-TNF antibody blocked mesenteric LN formation similarly to treatment with the combination of LTβR-Ig and TNFR-I–Ig fusion proteins (60). This suggests synergy between mLT and TNF for the formation of mesenteric LN, rather than between mLT and LTα_3. Nevertheless, a dominant role for signals from mLT through the LTβR is generally accepted, and the role of LTβR-dependent signaling is underscored by the recent observation that LTβR$^{-/-}$ mice manifest total ablation of all LN, including the mesenteric node (61).

From a different perspective, it was recently shown that treatment of pregnant LT$\alpha^{-/-}$ mice with an agonist anti-LTβR monoclonal antibody beginning on day 12 of gestation specifically induced the genesis of both peripheral and enteric-associated LN in the LTα-deficient progeny (60). Thus, signaling through the LTβR appears to be the only essential signal delivered by LTα-containing ligands. In addition, specificity for the location of developing LN appears to be independent of the mLT-expressing cell but dependent on the location of the LTβR-bearing cell.

Although several studies have demonstrated an unequivocal and dominant role for mLT in the induction of PP formation, controversy remains regarding a potential role for signaling via TNFR-I for the development of these structures. Neumann et al (62) identified extensive disorganization of gut-associated lymphoid tissue in TNFR-I$^{-/-}$ mice, with absence of morphologically defined PP. In apparent conflict are studies by Rennert et al (55, 56), in which treatment of pregnant mice with soluble TNFR-I–Ig fusion protein failed to block PP formation, regardless of the time or duration of treatment. In related studies, it was found that PP formation was preserved in mice lacking either TNF or TNFR-I, although there were reduced numbers of PP and those that formed were smaller than in wt mice (63). These data together suggest that mLT provides the dominant signal for PP organogenesis but that signaling by TNF through TNFR-I may be required for full development of normal PP structure. It is clear that for LN and PP to develop, the mLT signal must be present during mid- to late-gestation and, for PP, during the few days after birth.

OTHER SIGNALS ESSENTIAL FOR THE ORGANOGENESIS OF SECONDARY LYMPHOID TISSUES

The studies described above have established that mLT interacting with the LTβR provides key signals that support the development of LN and PP, but they also have demonstrated that different secondary lymphoid tissue elements require different signals at specific times and that additional signals are important for the normal development of specific tissues. The careful attention being paid to the structure of experimental mouse strains with altered expression of a range of cellular signaling proteins is aiding in the identification of additional signals that contribute to the development of the normal repertoire of secondary lymphoid tissues. Integration of these signals into models of how the development and maintenance of secondary lymphoid tissue structure are controlled should occur in the context of identifying the nature of the cells that deliver and receive these signals.

BLR1

Burkitt's lymphoma receptor-1 (BLR1), recently renamed CXCR5, was identified as an orphan chemokine receptor expressed in Burkitt's lymphoma B cells. Mice with a targeted null mutation in BLR1 manifest loss of inguinal LN and severe reduction in PP biogenesis (64). Examination of BLR1$^{-/-}$ mice demonstrated that this receptor was required for trafficking of B cells to certain lymphoid tissues and to selected compartments within the spleen. Analyses of *scid* mice have shown that LN, PP, and spleen form in the absence of B and T cells. Consequently, it must be assumed that other cells besides B or T cells can express BLR1 or the BLR1 ligand that specifies BLR1-dependent induction of inguinal LN and PP development. A ligand for BLR1 designated BLC (B lymphocyte chemoattractant)/BCA-1 (B cell-attracting chemokine-1) has been recently identified (65, 66) and found to be produced constitutively in secondary lymphoid tissues by cells located within B cell follicles, perhaps the FDC. Concerning the potential roles of BLC/BCA-1 and BLR1/CXCR5 in the organogenesis of inguinal LN and PP, it is unlikely that FDC or B cells are the cells that act during ontogeny. Future studies should address the nature of cells that express BLC/BCA-1 and BLR1/CXCR5 at sites of secondary lymphoid tissue organogenesis during embryonic development.

Ikaros

Ikaros, a member of the kruppel family of zinc finger DNA-binding proteins, is recognized as a key regulator for the specification and development of all lymphoid lineages (67). A mutation that deletes the N-terminal zinc finger

DNA-binding domain from the Ikaros proteins blocks lymphocyte development at its earliest recognizable stage (68). Mice carrying this mutation lack mature T and B lymphocytes and also natural killer (NK) cells, as well as their earliest described precursors. The production of subsets of dendritic cells is also defective (69, 70). Interestingly, these Ikaros mutant mice show defective formation of LN and PP (67). When a different mutation of the Ikaros gene was targeted to the C terminus of the protein, thymocyte development was considerably restored, with the numbers of $\alpha\beta$ T cells substantially recovered; however, development of secondary lymphoid tissues was still grossly disturbed (69).

Common Cytokine Receptor γ Chain and JAK-3

The common γ chain (γc) of the interleukin (IL)-2, IL-4, IL-7, IL-9, and IL-15 receptors is defective in humans with X-linked severe combined immunodeficiency. Mice lacking γc expression manifested a profound defect in the development of T and B cells and had no detectable NK cells. These mice lack peripheral LN and have mesenteric LN that are small (71, 72). The Janus family tyrosine kinase JAK3 is the key signaling molecule known to be associated with γc, so it was hypothesized that defects in JAK3 might cause an XSCID-like phenotype in mice (73). JAK-$3^{-/-}$ mice have profoundly reduced numbers of T cells and lack B and NK cells (74–76). Similar to mice lacking γc, JAK-$3^{-/-}$ mice have a broad defect in the formation of peripheral LN and PP (76) and show no recovery of these lymphoid tissues after reconstitution with BM from wt mice (JW Verbsky, Y-X Fu, & DD Chaplin, unpublished data). Both γc and JAK-3 targeted mice have defects in the development of several hematopoietic cell lineages, in addition to their defective peripheral LN and PP development. Experiments have not yet been performed to determine whether it is the lack of certain BM-derived cell lineages or the lack of specific signaling via γc or JAK-3 that leads to the impaired secondary lymphoid tissue development in these mutant mouse strains.

LYMPHOTOXIN IS REQUIRED FOR DEVELOPMENT OF NORMAL SPLENIC WHITE PULP STRUCTURE

Membrane LT Establishes White Pulp B Cell/T Cell Segregation and B Cell Follicle Structure

Not all elements of the secondary lymphoid system are lost in LT$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice. Although LN and PP are dramatically deficient in mice lacking mLT, the spleen is retained. Together with the observation that *Hox11*-deficient mice have isolated absence of the spleen without impairment of LN or PP development, this observation confirms that the organogenesis of the LN and PP is controlled by signals different from those that control the organogenesis of

the spleen. In mLT-deficient mice, although the spleen forms, the organization of its white pulp compartment is grossly disturbed (48, 51, 58, 77–79). The overall size of the spleen is retained, but the white pulp nodules are generally reduced in size and their margins are blurred. The organized MAdCAM-1$^+$ marginal sinus that normally separates the white pulp from the red pulp cannot be detected and appears to be absent. Similarly undetectable is the population of MOMA-1$^+$ metallophilic macrophages that normally associates with the marginal sinus endothelium and that is thought to participate in the trafficking of antigen and cells from the blood circulation into the lymphoid compartment. The altered white pulp structure affects essentially all aspects of the normal organization, including disturbance of the normal segregation of the distinct T and B cell compartments. In LT$\alpha^{-/-}$ mice, there is nearly complete loss of segregated B and T cell zones and apparent dissociation of the central arteriole from the T cell zone (48, 80). The B cells and T cells appear equally scattered throughout the white pulp nodule and B cell follicle structure is lost, including disappearance of the network of FDC that is normally located near the center of each follicle. Although the marginal sinus structure seems equally disturbed in the LT$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice, the extent of ablation of normal T and B cell segregation appears more extreme in the LT$\alpha^{-/-}$ mice.

Recent studies by Alexopoulou et al (81) have shown that the grossly disturbed T cell/B cell segregation in LT$\alpha^{-/-}$ mice can be repaired by breeding into the strain a TNF-expressing transgene. The ability of the TNF transgene to restore segregated B and T cell zones correlated with reduced TNF expression in the LT$\alpha^{-/-}$ mice (81; A-S Johannson, DD Chaplin, unpublished data). Although the mechanism underlying this reduced TNF expression in the LT$\alpha^{-/-}$ strain has not been definitively established, it is manifested at the level of TNF mRNA expression and may be a consequence of the retention in the LTα locus of the *neo*^{r} expression cassette used for gene targeting. Other investigators have observed effects of transgenes used for insertional gene targeting on loci upstream or downstream of the targeted locus (82). In the Alexopoulou et al study, the expression of the TNF transgene did not restore B cell follicle structure, but rather its action appeared limited to the formation of independent B and T cell zones. Although there is no loss of T and B zones in mice singly deficient in TNF (79, 83), this finding demonstrates that there can be interactions between LT and TNF in the formation of lymphoid tissue structure, and it underscores the general problem that gene targeting can alter the expression of loci adjacent to the intentionally targeted locus. Support for the coordinated action mLT and TNF also comes from studies showing that treatment of developing mouse embryos with soluble LTβR-Ig together with soluble TNFR-I-Ig had effects on the formation of lymphoid tissue structure that were not seen with treatment with either soluble receptor alone (60).

Disturbance of white pulp T and B cell segregation similar to that seen in LT$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice was observed in LTβR$^{-/-}$ mice, which suggests that the LT-dependent signal for this segregation was delivered by the mLT heterotrimer (61). Consistent with this interpretation was the earlier finding that transgenic expression of a soluble LTβR-Ig fusion protein beginning shortly after birth resulted in similar loss of T cell/B cell segregation and follicle structure (57).

Lymphotoxin-Dependent Establishment of Splenic T Cell and B Cell Zones

Experiments in which either mixtures of lymphoid cells, purified cell populations, or BM are transferred from one strain of mice to another have been highly informative for defining rules that govern the compartmentalization of cells within the splenic white pulp. For example, mice carrying the severe combined immunodeficiency mutation (*scid*), a result of mutation of the DNA-dependent protein kinase that functions during T and B cell antigen receptor rearrangement, have spleen white pulp nodules that are small, that are bordered by a marginal sinus, and that contain primarily NK and dendritic cells. It is interesting that when wt splenocytes were transferred into a *scid* recipient, within 1 day after infusion the transferred cells had partitioned into periarteriolar T cell and marginal B cell zones (84). In contrast, when wt splenocytes were transferred to a LT$\alpha^{-/-}$ mouse, B cell/T cell segregation did not occur (80). These data suggest that in a B cell– and T cell–independent but LTα-dependent fashion, the underlying addressing mechanism that specifies segregated B and T cell zones could be laid down. In the *scid* mouse system, this maintenance of the addressing system appeared to require ongoing mLT expression, as pretreatment of the *scid* recipients for 1 week prior to transfer with soluble LTβR-Ig fusion protein ablated the ability of the transferred cells to segregate (84). This was not due to neutralization of mLT on the transferred splenocytes, because splenocytes from an LT$\alpha^{-/-}$ donor could segregate effectively in an untreated *scid* recipient.

Other investigators have found less plasticity in the ability of T and B cells to segregate into discrete zones in the white pulp. In earlier studies using BM from either a wt or a LT$\alpha^{-/-}$ donor to reconstitute lethally irradiated wt recipients, it was found that similar to wt cells, the LTα-deficient cells segregated into discrete T and B cell zones (80). When BM from an LT$\alpha^{-/-}$ donor was used, the crisp distinction between the T and B cell zones in the normal white pulp was partially lost, which suggests that LTα expression is required to retain complete T cell/B cell segregation; however, even 6 months after BM transfer, considerable segregation of B and T cells was still retained. This indicated that it was not necessary for B or T lymphocytes to express LTα in order to target existing white pulp B and T cell zones. In contrast, when BM from either wt or LT$\alpha^{-/-}$ donors was used to reconstitute lethally irradiated LT$\alpha^{-/-}$ recipients,

segregation into discrete T and B cell areas did not occur. This suggested that the ability to form discrete white pulp B cell and T cell zones is a fixed feature of the microenvironment, imprinted by the time mice reach maturity. In contrast, if mice underwent blockage of mLT either during prenatal development by transplacental exposure to an LTβR-Ig fusion protein (55) or immediately after birth by transgenic expression of an LTβR-Ig fusion protein under the control of the cytomegalovirus promoter (57), then B cell/T cell segregation was ablated.

Partial Plasticity of B Cell Follicle Structure

The studies described above showed that once the splenic white pulp has become populated with lymphocytes, the T cell/B cell segregation phenotype becomes relatively fixed. If segregated T cell and B cell zones have formed under the influence of mLT, then removal of mLT-expressing lymphocytes does not ablate segregation. If B and T cells are not segregated, because of congenital absence of LTα, then introduction of LTα-expressing cells cannot induce segregation. In dramatic contrast, cell transfer studies show that B cell follicle structure is highly plastic and dependent on the ongoing presence of LTα-expressing cells (80). When LT$\alpha^{-/-}$ BM was used to reconstitute irradiated wt mice, B cell follicle structure, including the FDC network, was lost. In contrast, when irradiated LT$\alpha^{-/-}$ mice were reconstituted with wt BM, the transferred cells were able to induce the formation of a robust FDC network. These FDC networks were competent to support the formation of functional germinal centers (GC) with isotype switching. Thus, FDC networks and B cell follicular structure are plastic, requiring ongoing expression of LTα for their maintenance, and responsive to the introduction of LTα-expressing cells into an LT$\alpha^{-/-}$ environment. Similar results were obtained using mice doubly deficient in TNF and LTα (85).

Immune Dysfunction in LT$\alpha^{-/-}$ Mice

With their disturbed B cell and T cell compartmentalization and loss of normal B cell follicle structure, LT$\alpha^{-/-}$ mice provide a model to examine the role of normal secondary lymphoid tissue structure on immune responsiveness. When LT$\alpha^{-/-}$ mice were immunized with sheep red blood cells (SRBC), they produced high levels of antigen-specific IgM but low or no antigen-specific IgG in either primary or secondary responses (80). To test whether this failure to produce class-switched serum IgG antibody was due to the altered splenic microarchitecture in these mice, or perhaps to a requirement for LTα expression by lymphocytes cooperating in the antibody response, reciprocal splenocyte and BM transfers were performed. When irradiated LT$\alpha^{-/-}$ mice were reconstituted with wt splenocytes and immunized immediately with SRBC, splenic

Table 1 Correlation between the presence of LTα-expressing cells and the appearance of FDC, GC, and IgG responses[a]

Donor[b]	Cells transferred[c]	Recipient	Altered spleen: Microarchitecture	Altered spleen: T cell/B cell zones	FDC[d]	GC	IgG
Short-term reconstitution							
+/+	Spleen	+/+	No	No	++	++	++
+/+	Spleen	−/−	Yes	Yes	—	+/−	—
−/−	Spleen	+/+	No	No	+	++	++
−/−	Spleen	−/−	Yes	Yes	—	—	—
Long-term reconstitution							
+/+	BM	+/+	No	No	++	++	++
+/+	BM	−/−	Yes	Yes	++	++	++
−/−	BM	+/+	No	Slight	—	—	—
−/−	BM	−/−	Yes	Yes	—	—	—

[a]LT, Lymphotoxin; FDC, follicular dendritic cells; GC, germinal centers; Ig, immunoglobulin; BM, bone marrow.
[b]+/+ and −/−, Wild-type and LT$\alpha^{-/-}$ mice, respectively.
[c]For transfer of spleen cells, a suspension of viable cells was prepared from a single donor spleen and infused intravenously with 10^8 sheep red blood cells (SRBC) 3 h after the recipient had been irradiated with 750 rad. For BM transfer, the recipients were lethally irradiated (1050 rad) and reconstituted with BM, then immunized with SRBC 6–8 weeks later. Tissue and sera were collected 10 days after immunization. Anti-SRBC IgG was determined by ELISA. Anti-Thy1.2 and anti-B220 were the markers for T cells and B cells, anti-CR1/2 or FDC-M1 for FDC, and peanut agglutinin for GC.
[d]++, Similar to unirradiated wild-type mice; +, weak staining or IgG response; —, no response.

microarchitecture remained disturbed and there was no IgG response. This suggests that merely providing mature immune cells, including T cells, B cells, NK cells, and macrophages, is not sufficient to generate a productive IgG response. In contrast, when irradiated wt animals received splenocytes from LT$\alpha^{-/-}$ mice, follicle structure and a strong IgG response were retained (Table 1). Thus, LTα-deficient B cells and T cells have no intrinsic defect in their ability to generate an IgG response. Rather, the altered microenvironment characteristic of LT$\alpha^{-/-}$ mice appears to impair the ability to switch to a productive IgG response. Further studies using BM transfer, as discussed above, showed that when wt BM was transferred to LT$\alpha^{-/-}$ recipient mice, B cell follicles were, over time, restored, and the ability to sustain an isotype-switched IgG response was also, over time, restored (80).

MULTIPLE SIGNALS ARE REQUIRED FOR THE FORMATION OF MATURE B CELL FOLLICLES

The studies reviewed above indicate that LTα expression is needed inductively for the formation of functional B cell follicles, and also tonically for the

maintenance of mature follicles with FDC clusters that can support the formation of a high-affinity, isotype-switched Ig response. Absence of FDC networks and B cell follicles is also seen in LT$\beta^{-/-}$ mice (58, 79). The similar primary follicle phenotype of LT$\alpha^{-/-}$ and LT$\beta^{-/-}$ mice suggests that the form of LT required to develop and maintain the FDC network is the membrane LT$\alpha_1\beta_2$ heterotrimer. Support for this comes from experiments in which specific neutralization of membrane LT in mice expressing transgenic LTβR-Ig fusion protein (57) or treated with soluble LTβR-Ig fusion protein (55, 86) shows loss of B cell follicles. Final confirmation that membrane LT provides essential signals for the development of the FDC network has come from analyses of mice deficient in LTβR that also show absence of morphologically defined FDC networks, GC, and IgG responses (61).

In addition to signals through the LTβR, signals via TNFR-I are also required for the formation of FDC networks and GC (77, 87). The signals that are delivered by TNFR-I are likely delivered by TNF as the ligand, because mice deficient in TNF also show absence of FDC clusters (83, 88). Thus, signaling via both the TNF/TNFR-I axis and the mLT/LTβR axis appears to be required for development of FDC networks. Consistent with this, both LTβR and TNFR-I are expressed on FDC (89, 90).

Although LT$\alpha^{-/-}$, LT$\beta^{-/-}$, and TNF$^{-/-}$ mice all show failure to form splenic FDC networks, this does not mean that mLT and TNF deliver identical tissue morphogenic signals. LT$\alpha^{-/-}$, LT$\beta^{-/-}$, and LTβR$^{-/-}$ mice manifest profound disturbances of splenic T cell/B cell segregation (58, 61, 80), whereas TNF$^{-/-}$ and TNFR-I$^{-/-}$ mice retain segregated T cell/B cell zones (83, 87, 91). Thus, in the spleen, the dominant role of TNF appears to concern the formation of primary B cell follicles. Both TNF$^{-/-}$ and TNFR-I$^{-/-}$ mice have impaired development of primary B cell follicles, whereas the distribution of B cells in the marginal zone appears intact. We have observed that the formation of morphologically defined and functionally defined primary B cell follicles is linked to the formation of FDC clusters (Table 1). It remains an interesting question whether a lack of organized FDC prevents the formation of primary B cell follicles or whether some other developmental failure leading to a lack of primary B cell follicles prevents the formation of clusters of FDC.

The studies outlined above indicate clearly that signaling through both the LTβR and TNFR-I is required for the formation of FDC networks within splenic primary follicles. These findings must be reconciled with observations comparing the ability of soluble LTβR-Ig and TNFR-I–Ig fusion proteins to disrupt spleen primary follicle structure (86). When LTβR-Ig was administered to wt mice in an intraperitoneal dose of 100 μg once a week, after 2–4 weeks extensive disruption of the white pulp structures was observed, with loss of T cell/B cell organization, loss of expression of several markers of marginal sinus

structure, and failure to form morphologically detectable GC. These changes were accompanied by an inability to generate isotype-switched Ig responses following immunization with SRBC. Although the distribution of FDC was not specifically studied, it can be assumed that the FDC network was ablated following treatment with the soluble receptor because, in other studies, withdrawal of membrane LT signaling resulted in loss of the FDC reticulum (80). In contrast, when wt mice were injected twice a week for up to 3 weeks with up to 300 μg of TNFR-I–Ig fusion protein, there was no detectable interference with either the GC reaction or the generation of an isotype-switched antibody response (86). An explanation for the apparent discrepancy between these data and those obtained using genetically deficient mice may relate to the time courses of the different experiments. It may be that in order to affect FDC function and the maturation of the immunoglobulin response, TNF blockade must be more prolonged than blockade of membrane LT. Perhaps TNF signaling is required for the induction but not the maintenance of an FDC network, whereas mLT signaling appears to be required for both induction and maintenance. In this case, if FDC have a relatively long half-life, then blocking TNF signaling would have a much less dramatic impact compared with blocking mLT signaling. Or, more trivially, it may be impossible to block the presumed cell-cell interactions signaled by TNF/TNFR-I using this kind of soluble receptor reagent. This may be particularly true because biological potency of the soluble TNFR-I–Ig fusion proteins has generally been verified for the neutralization of soluble TNF. The ability of the soluble reagent to block the biological actions of membrane TNF is less well defined. Of relevance here are studies that have demonstrated that membrane TNF signals with selectivity through TNFR-II (92). This may be important because other studies have shown that the disturbed spleen follicle phenotype of TNF$^{-/-}$ mice can be restored by selective transgenic expression of the membrane form of TNF (83). Thus, treatment of mice with soluble TNFR-I–Ig might fail to block signals mediated by membrane TNF, and administration of soluble TNFR-II–Ig might produce a different result compared with administration of soluble TNFR-I–Ig.

THE LT-DEPENDENT SIGNALS THAT SUPPORT FORMATION OF THE FDC NETWORK ARE DELIVERED BY B CELLS

The studies described above indicate clearly that the LTα expressing cells that are required for the formation and maintenance of the FDC network are BM derived. Although the full repertoire of LTα-expressing cells has not been rigorously defined, the major cell lineages known to produce LTα are all BM derived and include T, B, and NK cells (28, 93). Recent studies have begun to dissect

the nature of the cell lineage that delivers the LTα-dependent signal leading to the apparently de novo induction of splenic FDC networks (94). Immunohistochemical analyses of the spleens of T cell–deficient mice (with targeted null mutations of the genes encoding both the T cell receptor β and δ chains) show white pulp nodules consisting primarily of B lymphocytes, NK cells, and dendritic cells, with the majority of nodules containing clusters of FDC. This established that neither $\alpha\beta$ nor $\gamma\delta$ T cells are required for either the induction or maintenance of FDC clusters. In contrast, the spleens of B cell–deficient mice (with targeted null mutation of the Ig heavy-chain locus) contained white pulp nodules consisting primarily of T lymphocytes, NK cells, and dendritic cells and devoid of detectable FDC. These results indicated that B cells were absolutely required for the development of FDC clusters (Table 2). Within 3 weeks of intravenous infusion of purified wt splenic B cells into recombination activating gene-1 (RAG-1)–deficient mice, FDC clusters were induced in both spleen and LN. Treatment of the recipient mice with LTβR-Ig fusion protein at the time of B cell transfer blocked the development of the FDC clusters, establishing a requirement for mLT in the reconstitution process. To exclude the possibility that NK cells (present in the T cell–deficient and RAG-1$^{-/-}$ mice) might play an essential role in the induction or maintenance of FDC networks, CD3ε transgenic mice lacking both mature T and mature NK cells (95) were investigated. Robust FDC clusters were found in the splenic white pulp of the T cell– and NK cell–deficient mice (Table 2). Results supporting a key role for LTα-expressing B cells have been obtained elsewhere (84). Together these studies show that B cells, in a LTα-dependent fashion, provide a signal that induces

Table 2 B cells, but not T cells or NK cells, are required for the development of the splenic white pulp FDC network[a]

Mice[b]	Lymphocytes[c]	B cell follicles[d]	FDC	MAdCAM-1
wt	T, B, NK	+	+	+
RAG-1$^{-/-}$	NK	—	—	+/−
BCR$^{-/-}$	NK, T	—	—	+/−
TCR$^{-/-}$	B, NK	+	+	+
CD3ε trangene	B	+	+	+
JAK3$^{-/-}$	Few	—	—	—

[a]NK, Natural killer; FDC, follicular dendritic cells; MAdCAM-1, mucosal address in cell adhesion molecule-1.

[b]Mouse strains tested: wt, wild type; RAG-1$^{-/-}$, recombination activating gene 1 deficient; BCR$^{-/-}$, B cell receptor deficient (immunoglobulin H targeted); TCR$^{-/-}$, T cell receptor deficient (TCRβ and δ targeted); CD3ε transgene, overexpressing CD3ε (95); JAK3$^{-/-}$, Janus kinase 3 deficient (75).

[c]Lymphocyte populations present in the spleen of the indicated mouse strain.

[d]+, Detectable at a level similar to wt mice; +/−, weakly positive staining; —, not detectable.

and maintains clusters of FDC in spleen and LN. Mature T cells and NK cells are not required for this to occur. It is important, however, to keep in mind that transgenic and gene targeted mice that manifest congenital absence of selective cell lineages may continue to be populated by immature cells from the affected lineage. So, for example, the CD3ε transgenic mice that have no detectable NK cell activity and no mature circulating or tissue NK cells may continue to contain NK precursors. Although no biological functions of such precursors have yet been defined, it remains possible that they may, during their own maturation, contribute to the maturation of other lineages and tissue environments.

Identifying the B cell as the cell responsible for delivering an mLT signal that induces the formation of FDC clusters expands the repertoire of important B cell functions. The role of the B cell is not limited solely to the presentation of antigen to T cells and the production of antibodies to neutralize and eliminate these antigens. B cells also act by delivering key signals that support the development of the lymphoid tissue structure that is itself required for the B cell to express its mature functions. Whether B cells express mLT or not, and whether they gain access to sites appropriate for the induction of FDC clusters, will have a profound impact on the quality of the immune response. The target cell with which the B cell interacts to induce mature FDC structure has not been defined. Given that LTβR and TNFR-I are both expressed on FDC themselves (89, 90), it is reasonable to assume that the B cell delivers its LT signal directly to the FDC precursor. The differentiation pathway that leads to the formation of mature FDC is poorly defined. The elusive FDC precursor has been thought not to be derived from hematopoietic stem cells but rather to belong to a fibroblast-like cell (96–101). Recently, however, it was demonstrated that FDC can be transferred to newborn *scid* recipients using either BM or fetal liver (10). Whether the FDC developed from a hematopoietic precursor or a stromal precursor remains undefined.

With the understanding that B cells provide key signals supporting the development of primary lymphoid follicle structure, we can begin to make predictions about the types of molecules whose function will be required for normal primary follicles to form. Any molecule that is required for normal B cell development or to render B cells competent to deliver an mLT signal will also be required for the development of primary follicle structures. Although many studies have identified molecules that are required for normal B cell development and activation, most of them have used as their functional readout the ability to form GC and serum isotype-switched antibodies. The requirements for the formation of a GC can be anticipated to be different from the requirements to form a mature FDC network. GC are thought to depend for their formation on productive interactions between B cells, T cells, FDC, and perhaps other antigen-presenting cells (25, 102). Loss of any molecule essential for these interactions is expected

to lead to loss of secondary B cell follicles and GC, without a priori disrupting the FDC network.

Molecules that are now known to support the development of GC structures include the intercellular signaling proteins CD40 and CD40L (103), CD19 (104), CD28 (105), and B7-2 (106), among others. These molecules have the potential to act directly at the level of the B cell/T cell interactions that are required for maturation of B cells into GC cells. That additional intracellular signaling molecules are required for the development of the GC reaction has also been recognized but without a clear understanding of which cell lineage(s) they affect. These additional molecules include members of the NF-kB/rel family, with p52 (107, 108) and Bcl-3 (109, 110) both required for the formation of GC. Mice deficient in the transcriptional repressor Bcl-6 also show failure to form GC (111, 112). Alterations in the formation of GC are not always absolute. For example, mice deficient in the complement receptors CR1 and CR2 (encoded in mice as alternatively spliced products of a single gene) show underdeveloped GC and manifest reduced but not absent primary and secondary IgG responses (113). Because the complement receptors encoded by this gene are expressed on both FDC and B cells, their absence could affect GC formation at multiple levels. For many of these factors, it remains unclear whether they are required to support cellular interactions necessary for the activation of GC B cells or rather to support the development of follicular structure (such as the FDC network) that is required for GC to develop.

WHAT CELLS DELIVER AND RECEIVE LT SIGNALS DURING SECONDARY LYMPHOID TISSUE ORGANOGENESIS?

We know that B lymphocytes provide a crucial membrane LT signal that is required for the formation of the FDC network in primary and secondary B cell follicles. We know little about the nature of the cell type(s) that deliver mLT signals that specify the initial organogenesis process itself. Similarly, we have little information about the nature of the mLT-expressing cells that support the formation of segregated T and B cell zones in the splenic white pulp. For these processes, we do not know whether the mLT-dependent signals are required for the earliest stages of lymphoid organ biogenesis, or whether they are required to nurture nascent lymphoid organs. Regardless of when the mLT-dependent signal leads to commitment for LN, PP, and organized splenic white pulp structure, it is clear that two of the major known mLT-expressing cell lineages, mature B and T lymphocytes, cannot be essential for delivering this mLT-dependent signal. This is based on the observation that both *scid* and *RAG-1*$^{-/-}$ mice have intact development of LN and are able to segregate

transferred B and T cells within their splenic white pulp, in spite of their absence of mature B and T cell lineages. In addition, the initial steps of the organogenesis of secondary lymphoid tissues appear to precede the maturation of T and B lymphocytes during ontogeny. This suggests again that mature T and B cells may not be required for the formation of these tissues.

NK cells, one of the non-T and non-B cell lineages that are retained in *scid* and RAG-$1^{-/-}$ mice, are also able to express LT, and cells of the NK lineage should certainly be considered as cells that might use mLT as one of the signals for the initiation of development of normal secondary lymphoid tissue structure. The possibility that an immature cell of one of the hematopoietic lineages (B cell, T cell, NK cell, or uncommitted) might provide a critical mLT-dependent signal should be kept in mind. Precursor B and T lineage cells are thought to be present in normal or greater than normal numbers in *scid* and RAG-$1^{-/-}$ mice.

A potential role for NK cells in LN and PP biogenesis was suggested not only by the recognition that NK cells represent an LT-expressing lineage that is retained in scid and RAG-$1^{-/-}$ mice, but also by the finding that LN and PP are absent or present in reduced numbers in mice carrying mutations that lead to loss of mature NK cells [$\gamma c^{-/-}$ (71, 72), JAK-$3^{-/-}$ (74–76), and CD3ε transgenic (95); Y-X Fu, JW Verbsky, G Huang, DD Chaplin, unpublished data]; however, we have been unable to block the development of LN or PP by depletion of NK cells in the developing fetus by administering various anti-NK cell antibodies to pregnant mice. Because these antibodies only bound to mature NK cells, it remains possible that immature NK cell precursors might contribute to delivering the inductive signals for LN and PP formation. In this regard, the recent observation of $CD4^{+}CD3^{-}LT\alpha\beta^{+}$ cells in the LN of developing mouse embryos may be of particular significance (114). These cells express $\alpha 4\beta 7$ integrin and appear to differentiate into NK cells, dendritic antigen-presenting cells, and follicular cells but not into T or B lymphocytes. Although they have only been identified within already developing LN structures, they have many of the characteristics of the cell type that might deliver the mLT-dependent signal for the earliest stages of secondary lymphoid tissue commitment.

It is important to keep in mind that the commitment to the formation of different lymphoid tissues may occur at different times, may require the interactions of different types of cells, and may involve different intercellular and intracellular signaling molecules. The temporal distribution of commitment events has been shown in the development of PP and different sets of LN, which was blocked depending on the time of administration of the LTβR-Ig fusion protein (55). Additional studies in which LTα was expressed in LT$\alpha^{-/-}$ mice under the control of the rat insulin promoter showed that the development of some but not all LN was restored (115, 116). The molecular basis for partial restoration of LN structures in this model remains undefined, but it underscores the hypothesis

that selective spacial or temporal expression of LT determines which secondary lymphoid tissues are formed. In addition, different secondary lymphoid tissues may require a different complex of signals for their development. For example, mice deficient in BLR1 have selective loss of inguinal LN (64), and absence of mature NK cells correlates with a more extensive but still partial failure to form LN (71, 72, 74–76, 95; Y-X Fu, JW Verbsky, G Huang, DD Chaplin, unpublished data). Finally, lack of LTα leads to an essentially complete failure to form LN and PP (48, 49). Further definition of the cellular interactions required during the early steps of secondary lymphoid tissues biogenesis will be difficult because of the small numbers of cells that are likely to be involved, and the general inaccessibility of embryonic mammalian tissues to provocative manipulation. Nevertheless, identification of candidate cell lineages such at the $CD4^+CD3^-LT\alpha\beta^+$ cells seen in embryonic LN (114) may provide new leverage to track the formation of the tissues.

INDEPENDENT SIGNALS REGULATE DEVELOPMENT OF PRIMARY AND SECONDARY FOLLICLE STRUCTURE IN SPLEEN AND LN

Additional data demonstrating the complexity of signals controlling secondary lymphoid tissue structure come from studies comparing the structures of mesenteric LN and spleen in both $LT\alpha^{-/-}$ and TNFR-I$^{-/-}$ mice (51). Consistent with data described above, the splenic white pulp of the $LT\alpha^{-/-}$ mice showed loss of discrete T and B cell zones, loss of the MAdCAM-1–staining marginal sinus, loss of discrete B cell follicles and FDC networks, and loss of the ability to generate peanut agglutinin$^+$ (PNA$^+$) GC B cell clusters after immunization with T cell–dependent antigens. The spleens of TNFR-I$^{-/-}$ mice (and TNF$^{-/-}$ mice) (Y-X Fu, MW Marino, DD Chaplin, unpublished data) showed a similar absence of marginal sinus MAdCAM-1 staining, lack of discrete B cell follicles and FDC networks, and lack of splenic GC, but they retained substantial segregation of T and B cell zones. A small fraction of the $LT\alpha^{-/-}$ mice have a single mesenteric LN. It is interesting that although the spleens in these mice show grossly disturbed B cell/T cell segregation, the mesenteric LN, when it develops, shows segregation of B cell and T cell zones similar to that seen in the mesenteric LN of wt mice. In stark contrast, in TNFR-I$^{-/-}$ mice, although the spleens show preserved B cell/T cell segregation, the mesenteric LN is totally disordered, with no distinct B and T cell areas (Table 3). Of additional interest, although both $LT\alpha^{-/-}$ and TNFR-I$^{-/-}$ mice fail to form GC in the spleen following intraperitoneal or intravenous immunization with SRBC, they both manifested prominent GC-like clusters of PNA$^+$ cells in their mesenteric LN. To study further the role of LTα in the formation of these PNA$^+$

Table 3 LTα and TNF independently regulate the development of primary and secondary follicle structure in spleen and lymph nodes[a]

Mice	T/B zone		FDC clusters		GC	
	Spleen	MLN	Spleen	MLN	Spleen	MLN
wt	+	+	+	+	+	+
LT$\alpha^{-/-}$	—	+	—	—	+	+
wt to RAG-1$^{-/-}$	+	+	+	+	+	+
LT$\alpha^{-/-}$ to RAG-1$^{-/-}$	+/–	+	—	—	—	+
TNF$^{-/-}$	+	—	—	—	—	+
TNFR-I$^{-/-}$	+	—	—	—	—	+

[a]LT, Lymphotoxin; TNF, tumor necrosis factor; FDC, follicular dendritic cells; GC, germinal centers MLN, mesenteric lymph node; +, similar to wt; +/–, modestly disorganized; —, disorganized or undetectable.

clusters of GC-like cells in the mesenteric LN, RAG-1$\alpha^{-/-}$ mice were treated with an infusion of spleen cells from either LT$\alpha^{-/-}$ or wt mice and immediately immunized with SRBC. Ten days later, the RAG-1$^{-/-}$ mice reconstituted with wt splenocytes showed typical PNA$^+$ GC in their reconstituted spleens, whereas RAG-1$^{-/-}$ mice reconstituted with LT$\alpha^{-/-}$ splenocytes did not. RAG-1$^{-/-}$ mice reconstituted with either wt or LT$\alpha^{-/-}$ splenocytes showed robust PNA$^+$ clusters in their reconstituted mesenteric LN (Table 3). Comparison of the LT$\alpha^{-/-}$ and the TNFR-I$^{-/-}$ mice shows that the signals that regulate the development of T and B cell zones are different in the spleen and the mesenteric LN. They show further that the signals that regulate B cell activation to produce clusters of PNA$^+$ cells differ between the spleen and mesenteric LN. The nature of the PNA$^+$ clusters that are induced in the mesenteric LN of these targeted mice following immunization with SRBC remains unclear. They form in the absence of detectable clusters of FDC and are not associated with the production of antigen-specific serum IgG. They, therefore, probably do not represent functionally intact GC but rather are likely to represent partially activated clusters of proliferating cells. Further analysis of these structures may provide insight into the activation steps required for the generation of the normal GC response.

Comparison of the LT$\alpha^{-/-}$ and the TNFR-I$^{-/-}$ mice provides strong evidence that the rules governing the development of organized structure in the spleen and mesenteric LN are different, and that the signals required for activation of B cells in these two compartments are also distinct. This may be one of the morphological correlates of the prior observations that different lymphoid tissues express distinct cytokine profiles following antigen stimulation (117), and that the quality of the immune response may be quite different depending on whether the responding lymphocytes are activated in the LN or in the spleen (118).

CONCLUDING REMARKS

The membrane form of LT is now recognized as providing crucial signals required for the biogenesis of secondary lymphoid tissues and primary B cell follicles within these structures. TNF mediates independent signals for primary B cell follicle structure and can also cooperate with mLT contributing to other aspects of secondary lymphoid tissue development. Additional molecules participating in the development of normal secondary lymphoid tissue structure are being identified (summarized in Table 4). Some of the actions of mLT lead to the development of fixed characteristics of the lymphoid tissues, but some other structural features are plastic, indicating a need for ongoing expression of mLT to sustain normal tissue elements. This is particularly true for the formation of FDC clusters throughout the secondary lymphoid tissues. Withdrawal of mLT-expressing cells results over a period of days to weeks in loss of recognizable FDC and loss of primary B cell follicle structure. Of special interest, B cells have been identified as the lineage that delivers the mLT signal for formation and maintenance of the FDC network. Thus, B cells use mLT as an inductive signal for the formation of the lymphoid tissue structure that is

Table 4 Phenotypic effects of LTα, LTβ, and TNF on lymphoid tissue development[a]

Mutant strain or treatment with soluble receptor	Lymph nodes	Peyer's patches	Segregated splenic B and T zones	Splenic marginal zones	Primary B cell follicles	FDC networks	References
LT$\alpha^{-/-}$	Absent[b]	Absent	Disorganized	Disorganized	Absent	Absent	48, 49, 51, 77, 80, 88, 94, 101
LT$\beta^{-/-}$	Absent[c]	Absent	Disorganized	Disorganized	Absent	Absent	58
TNF$^{-/-}$	Present	Reduced	Retained	Enlarged	Absent	Absent	83, 88
TNFR-I$^{-/-}$	Present	Reduced	Retained	ND	Absent	Absent	62, 63, 77, 87, 101
TNFR-II$^{-/-}$	Present	Present	Retained	ND	Present	Present	53, 77
LTβR$^{-/-}$	Absent	Absent	Disorganized	Disorganized	Absent	Absent	61
LTβR-Ig	Partially absent[e]	Absent[d]	Mildly disorganized	Disorganized	Absent	Absent	55–57, 86
TNFR-I–Ig	Present	Present	Mildly disorganized	Disorganized	Absent[f]	Absent[f]	57, 86
LTβR-Ig+ TNFR-I–Ig	Absent	Absent	ND	ND	Absent	Absent	60
aly/*aly*	Absent	Absent	Disorganized	Disorganized	ND	ND	34
BLR1$^{-/-}$	Partially absent[g]	Present	ND	ND	ND	ND	64

[a]LT, Lymphotoxin; TNF, tumor necrosis factor; FDC, follicle dendritic cells; R, receptor; ND, not determined; Ig, immunoglobulin; BLR1, Burkitt's lymphoma receptor-1.
[b]Mesenteric lymph nodes (LN) retained in a small fraction of LT$\alpha^{-/-}$ mice.
[c]Mesenteric and cervical LN retained in all LT$\beta^{-/-}$ mice.
[d]Peyer's patches ablated if LTβR-Ig Fc administered at birth or earlier.
[e]Mesenteric, cervical, lumbar, and sacral LN resistant to ablation.
[f]FDC networks and primary follicles sensitive during embryonic development.
[g]Absence of inguinal LN.

required for the B cell to express its fully differentiated functions. The actions of mLT and TNF in signaling the formation of secondary lymphoid tissues are mediated primarily through the LTβR and TNFR-I, but the participation of additional, recently defined receptors in this family has not yet been excluded (119). Analyses of mice with selective abnormalities of components of secondary lymphoid tissue structures are providing a unique opportunity to relate lymphoid tissue structure and immune responsiveness. Detailed definition of the signals that sustain and modulate lymphoid macro- and microarchitecture may identify fruitful new targets for immunomodulating drug therapy.

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