Splenic T Zone Development Is B Cell Dependent

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Abstract

The factors regulating growth and patterning of the spleen are poorly defined. We demonstrate here that spleens from B cell–deficient mice have 10-fold reduced expression of the T zone chemokine, CCL21, a threefold reduction in T cell and dendritic cell (DC) numbers, and reduced expression of the T zone stromal marker, gp38. Using cell transfer and receptor blocking approaches, we provide evidence that B cells play a critical role in the early postnatal development of the splenic T zone. This process involves B cell expression of lymphotoxin (LT)$\alpha 1\beta 2$, a cytokine that is required for expression of CCL21 and gp38. Introduction of a B cell specific LTα transgene on to the LTα-deficient background restored splenic CCL21 and gp38 expression, DC numbers, and T zone size. This work also demonstrates that the role of B cells in T zone development is distinct from the effect of B cells on splenic T cell numbers, which does not require LT$\alpha 1\beta 2$. Therefore, B cells influence spleen T zone development by providing: (a) signals that promote T cell accumulation, and: (b) signals, including LT$\alpha 1\beta 2$, that promote stromal cell development and DC accumulation. Defects in these parameters may contribute to the immune defects associated with B cell deficiency in mice and humans.

Key words: lymphotoxin • SLC • BLC • stromal cell • dendritic cell

Introduction

The spleen participates in immune responses against many types of pathogens and it is also involved in autoimmune diseases and lymphoid malignancies (1). Within the spleen, lymphocytes are organized as sheathes around arterioles, with the T zone located centrally (also called the periarteriolar lymphoid sheath or PALS) and the B cells distributed around the T zone in tightly packed follicles. The spleen contains an additional population of B cells in a compartment that surrounds the follicles, known as the marginal zone (2). Antigen-presenting dendritic cells (DCs)* are prevalent in marginal zones, T cell zones, and in the bridging channels between these two compartments (3). The T zones, follicles, and marginal zones of the spleen are commonly referred to as the white pulp cords and they account for approximately half of the splenic tissue (1). The remainder of the spleen, termed red pulp, contains large numbers of macrophages, vascular cells, and transiting blood cells. This compartment functions in red cell and immune complex clearance and leukocyte exit.

Current understanding of the factors that cause growth and patterning of lymphoid organs is limited. Within the B and T cell areas there are specialized chemokine-producing stromal cells, believed to be of mesenchymal origin, that help organize the tissue (4, 5). Follicular stromal cells produce CXCL13 (BLC), a ligand for CXCR5, and both CXCR5 and CXCL13 are necessary for formation of B cell follicles (6, 7). A subset of follicular stromal cells, known as follicular DCs (FDCs), express complement and Fc receptors and are able to trap immune complexes for presentation to B cells. Normal expression of CXCL13 and development of FDCs is dependent on signals provided by B cells (8–10). Recent studies indicated that maturation of FDCs depends on a positive feedback loop between CXCL13 and the cytokine lymphotoxin (LT)$\alpha 1\beta 2$, where CXCL13 mediates recruitment of CXCR5-expressing B cells and induces them to express LT$\alpha 1\beta 2$ (7). LT$\alpha 1\beta 2$ causes growth and maturation of the follicular stromal cells, leading to increased expression of CXCL13 and upregulation of FDC markers (7). Stromal cells within T cell areas produce the CC chemokines, CCL21 (SLC) and CCL19 (ELC) (11). In mice lacking lymphoid CCL21 and CCL19

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*Abbreviations used in this paper: AP, alkaline phosphatase; DC, dendritic cell; FDC, follicular DC; LT, lymphotoxin; RAG, recombination activating gene.

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(11, 12), or in animals deficient in the common CCL21 and CCL19 receptor, CCR7 (13), there is a severe paucity of T cells and DCs within splenic T zones, while follicles continue to develop. Expression of these chemokines is also dependent on LTα1β2 (14). However, the cell types acting as a source of LTα1β2 in T zone stromal cell development have not been defined.

We demonstrate here that splenic expression of CCL21 is dependent on the presence of B cells but not T cells, whereas expression in lymph nodes is B cell independent. By immunohistochemistry, B cell–deficient spleens have a three- to fivefold reduction in the area stained by CD3 and by the T zone stromal cell marker, gp38 (15). In addition to the role of LTα1β2 in promoting T zone chemokine expression, we find that LTα1β2 is required for T zone gp38 expression. Bone marrow transfer and LTβR-Ig blocking experiments indicate a developmentally fixed role for B cells and LTα1β2 in promoting splenic T zone development. We show that the expression of LTα1β2 on B cells is sufficient to restore splenic DC numbers in LTα-deficient mice. The presence of normal numbers of splenic T cells in LTα-deficient mice (16–19) demonstrates that B cell regulation of splenic T cell numbers is mediated by LTα1β2-independent pathways. These findings indicate a prominent role for B cells in providing LTα1β2 and other signals needed for development of T zones and accumulation of DCs and T cells in the spleen.

Materials and Methods

Mice. C57BL/6 (B6) μMT (BCR$^{-/-}$), B6 TCRβ$^{-/-}$δ$^{-/-}$ (TCR$^{-/-}$), B6/129 LTα$^{-/-}$, and B6 recombination activating gene (RAG)-1$^{-/-}$ mice were from The Jackson Laboratory. For generation of bone marrow chimeras, 1–5 × 10^6 total bone marrow cells were intravenously injected into lethally irradiated recipient mice (1,100 rads). Recipient mice were given antibiotic water (polymyxin B, 110 mg/liter and neomycin, 1.1 g/liter) during the 6–10-wk reconstitution period, before analysis. For splenocyte transfers, cell enumeration experiments and DC analysis, cells were prepared by gently pressing the spleen through a 70-μM mesh cell strainer (Fisher Scientific). Red blood cells was lysed and cells were resuspended in serum-free DMEM containing antibiotics. In some cases, spleen cell suspensions were prepared using collagenase and EDTA, as previously described (20). 2 × 10^7 splenocytes in a volume of 0.05 ml were injected into the peritoneal cavity of newborn mice with a 30-gauge needle as described previously (21). Treatment with LTβR-Ig or human LFA3-Ig was by intraperitoneal injection as described previously (14), using 100 μg per injection.

Generation of κLTα-Transgenic Mice. A DNA fragment encoding the transmembrane and membrane-proximal (stalk) regions of the LTβ gene (22, 23), corresponding to nucleotides 1–450 (GenBank/EMBL/DDBJ accession no. U16985) was isolated by PCR from spleen cDNA using primers 5′-TAGTAGGGATCCGATATCCTGGATGGGGACACGGGGACT-3′ (containing a 5′ BamHI and EcoRV sites) and 5′-TAGTAGGTCGACATCATCATCATCTTTGTAATCGTTGAGG-3′ (containing a 3′ SalI site). A fragment containing nucleotides 124–609 (GenBank/EMBL/DDBJ accession no. M16819) of LTα, encoding for the receptor-binding domain (24), was isolated with the primers 5′-TAGTAGGTCGACCATGGCATCCTGAAACCTGCT-3′ (with SalI site) and 5′-TAGTAGATCGATGCGGCCGCCTACAGTGCAAAGGCTCC-3′ (with NotI and ClaI sites). The PCR fragments were cloned into pEF-BOS-XC (25) and an EcoRV-NotI fragment containing the LTβ-LTα chimeric gene was isolated and cloned into end-blunted ClaI and NotI sites of the pBXD1 vector. PBXD1 was generated by combining the 2.1-kb Ig κ-promoter fragment from myeloma S107 in series with: (a) a 0.24 kb 5′ chimeric intron from the pCI vector (Promega), (b) a multiple cloning site, (c) the 0.2 kb late SV40 poly A sequence, and (d) both 2.1 kb κ MAR/intron enhancer and 1.3 kb 3′ enhancer regions from the Balb/c κ gene. The BamHI and EcoRI linearized chimeric LTβ/LTα minigene was microinjected into fertilized (C57BL/6J × DBA/2J)F2 oocytes according to standard techniques. To screen for transgenic mice, ear DNA was prepared and the transgene was detected by PCR using the above indicated primer pairs. Founder #4 was crossed with B6 LTα-deficient mice (26) to generate κLT-transgenic/LTα$^{-/-}$ mice. To distinguish the LTα-targeted and wild-type alleles by PCR, a mixture of the following primers were used 5′-TAGTAGGTCGACATGACACTGCTCGGCCGTCT-3′ (LTα-specific), 5′-GCTTGCCGAATATCATGGTGG-3′ (Neo-specific), and 5′-GGACAGAAGAGAGTGGAGAGG-3′ (LTα-specific), yielding a ~180-bp fragment from the wild-type allele and a ~380-bp fragment from the targeted allele. Amplification conditions were 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, for 40 cycles. All the transgenic mice characterized were heterozygous for the transgene. The transgenic animals appeared healthy and grew equivalently to their littermate controls.

Northern Blot Analysis. 10–15 μg of total RNA from mouse spleens or lymph nodes was subjected to gel electrophoresis, transferred to Hybond N$^+$ membranes (Amersham Pharmacia Biotech), and probed using randomly primed ^{32}P-labeled mouse cDNA probes of the following types: CCL21, bases 1–848 (27) and CCL19, bases 1–755 (28). To control for RNA loading, a mouse elongation factor 1α (EF1α) probe was used. For quantitation, Northern blots were exposed to a phosphor screens for 6 h to 3 d and images were developed using a Storm860 PhosphorImager (Molecular Dynamics). Data were analyzed using ImageQuant® software (Molecular Dynamics), and chemokine mRNA levels were corrected for RNA loaded by dividing the chemokine hybridization signal by the EF-1α signal for the same sample. Relative expression levels were calculated by dividing the corrected signal for each mutant or treated sample with the mean corrected signal for the wild-type or control treated samples, as appropriate, that were included on each of the Northern blots.

Immunohistochemistry. Cryostat sections (6–7 μm) were fixed and stained as described previously (29) using the following mAbs: rat anti-B220 (CalTag); rat anti-MAdCAM-1 (BD PharMingen); goat anti-mouse 6Ckine (CCL21; R&D Systems); sheep anti-IgD (BD PharMingen); and syrian hamster anti-gp38 (provided by Andrew Farr, University of Washington, Seattle, WA). Rat IgG antibodies were detected with goat anti–rat-IgG conjugated alkaline phosphatase (AP; Southern Biotechnology Associates, Inc.) or donkey anti–rat-IgG conjugated horseradish peroxidase (HRP). Goat IgG antibodies were detected with biotinylated donkey anti–goat (Southern Biotechnology Associates, Inc.), followed by Vectastain ABC-AP (Vector Laboratories). Sheep IgG antibodies were detected with donkey anti–sheep-IgG HRP (Jackson ImmunoResearch Laboratories). Syrian hamster IgG antibodies were detected with anti-syrian hamster-AP (Jackson ImmunoResearch Laboratories). Enzyme reactions were developed with conventional substrates for peroxidases (diami-

nobenzidine/H_2O_2; Sigma-Aldrich) and AP (FAST BLUE/Naphthol AS-MX; Sigma-Aldrich). For measuring T zone and red pulp areas, cross-sections were made through central segments of the spleen, stained for gp38, CD3, or B220, analyzed at 5× objective magnification, and digital images of a fixed resolution were acquired using a cooled MDEI850 CCD camera (Optronics Engineering). Using Adobe Photoshop® software, an outline was drawn around positively stained areas using the lasso tool and the total pixels within each lassoed area were obtained using the histogram function. Data were collected from a minimum of two cross-sections per spleen. The total number of pixels was then divided by the number of spleen cross-sections analyzed to provide a measurement of pixel counts per cross-section. Pixel counts for red pulp areas were obtained by subtracting the $CD3^+$ areas (for $BCR^{-/-}$), or $B220^+$ areas (for wild-type) from the total splenic cross-sectional area obtained by lassoing the entire spleen cross-section.

Results

B Cells Regulate Splenic T Zone Chemokine Expression. To examine the cellular requirements for expression of the T zone chemokines, CCL21 (SLC) and CCL19 (ELC), we performed Northern blot analysis of spleen and lymph node tissue from B- and T cell–deficient mice. Surprisingly, expression of CCL21 in spleen was strongly dependent on B cells but not on T cells (Fig. 1, A and B). Splenic CCL19 mRNA expression was less affected by lymphocyte deficiency and showed a slightly greater dependence on B cells than T cells (Fig. 1, A and C). Lymphocytes are not a significant source of CCL19 or CCL21 (27, 28). Therefore, the absence of B and/or T lymphocyte RNA in the mutant spleens cannot explain the reduction in chemokine mRNA and instead may cause the magnitude of the reduction to be underestimated. The decrease in CCL21 in B cell deficient spleen was also evident by in situ hybridization and immunohistochemical analysis (data not shown). In contrast to spleen, CCL21 mRNA expression in LNs was not affected by B cell deficiency (Fig. 1 E). T cells were also not required for CCL21 expression in lymph nodes (Fig. 1 E). Similarly, lymph node CCL19 expression was not dependent on the presence of B cells or T cells (Fig. 1 F). Previous studies established that splenic expression of the B zone chemokine, CXCL13, is dependent on B cells but not on T cells (14) and we find here, in contrast to CCL21, that CXCL13 mRNA expression in lymph nodes is also B cell dependent (Fig. 1 D). In summary, B cells promote CCL21, and to a lesser extent CCL19, expression in spleen but not in lymph nodes, whereas they promote CXCL13 expression in both types of lymphoid tissues.

Requirement of B Cells for Splenic Stromal Cell Maturation and T Cell and DC Accumulation. Recent experiments have established that T zone stromal cells are the major source of CCL21 in the spleen (11). To test whether B cells influenced other properties of the T zone stromal cell network, we stained spleen sections for gp38 (Fig. 2), a membrane glycoprotein that is selectively expressed by these cells (15). Strikingly, there was a four- to fivefold decrease in the area of gp38 staining in B cell–deficient spleen compared with wild-type (Fig. 2 and Fig. 3 A), whereas gp38 staining in LNs was unaffected by B cell deficiency (Fig. 2). Concordant with the decreased area of $gp38^+$ T zone stromal cells, measurements of T zone cross-sectional area using anti-CD3 revealed a two- to threefold decrease (Fig. 3 A). By contrast, the red pulp cross-sectional area was equivalent in B cell–deficient and wild-type spleens (Fig. 3 B). These findings led us to quantitate T cell numbers in B cell–deficient spleens. Previous studies of B cell–deficient mice have either not reported an effect on splenic T cell numbers (30–32) or reported a decrease (33). Careful enumeration of cell numbers in spleens from specific pathogen-free, age-matched wild-type and B cell–deficient mice revealed that CD4 and CD8 T cells were reduced by threefold in B cell–deficient spleens (Fig. 3 D). The number of $CD11c^+$ DCs was also diminished (Fig. 3 F) in agreement with findings by some (34) but not others (35). Consistent with the poor lymphoid cellularity, B cell–deficient spleens were about half the weight of their wild-type counterparts (data not shown). The decreases were selective to the

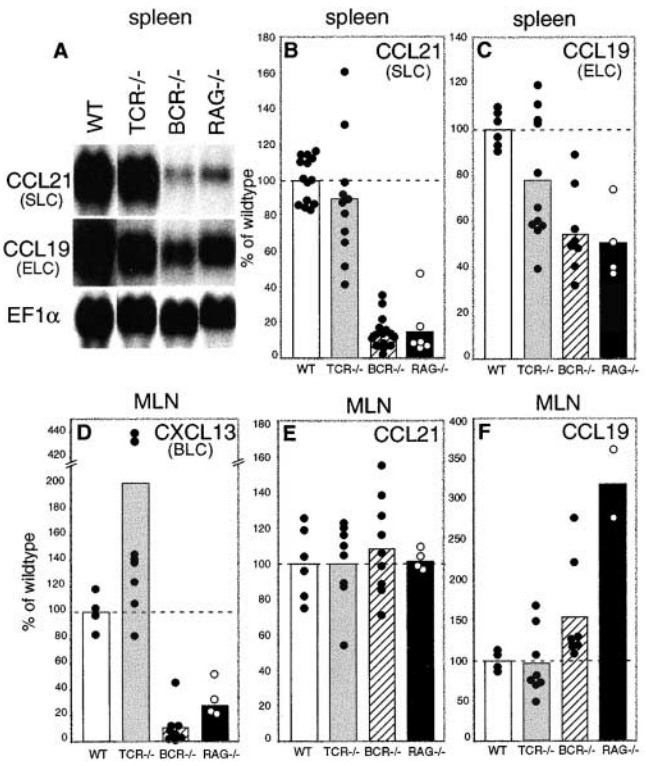

Figure 1. Reduced expression of splenic T zone chemokines in B cell–deficient mice. (A) Northern blot analysis of total RNA isolated from spleen tissue of the indicated mice, probed to detect expression of CCL21 (SLC) and CCL19 (ELC). Hybridization to EF1α was used to control for RNA loaded. WT, wild-type. (B–F) Relative chemokine mRNA levels in spleen or mesenteric lymph node (MLN) RNA, determined by PhosphorImager analysis of multiple Northern blots, including blots probed for CXCL13 (BLC). Data from individual mice are shown as circles and means as bars.

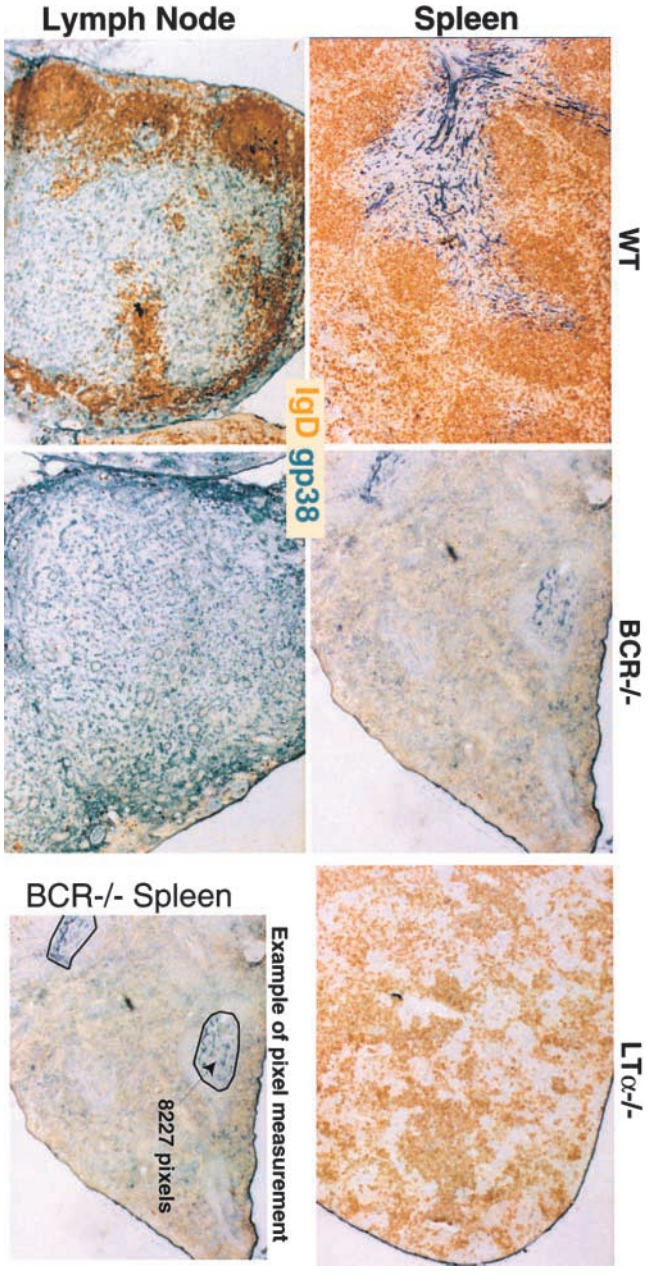

Figure 2. GP38-expressing stromal cell networks are small in B cell–deficient spleens and undetectable in LTα-deficient spleens. Spleen and lymph node tissue from wild-type (WT), B cell-deficient ($BCR^{-/-}$), and $LT\alpha^{-/-}$ mice, as indicated, was sectioned and stained to detect gp38 (blue) and IgD (brown). No lymph nodes were found in the $LT\alpha^{-/-}$ animal. The bottom left panel demonstrates the method used for measuring $gp38^+$ cross-sectional areas, showing the $BCR^{-/-}$ spleen cross-section with $gp38^+$ stained areas 'lassoed' and the number of pixels present in the single fully identified $gp38^+$ area indicated. Original objective magnification for all panels: ×5. Data are representative of tissues from more than 10 animals of each type.

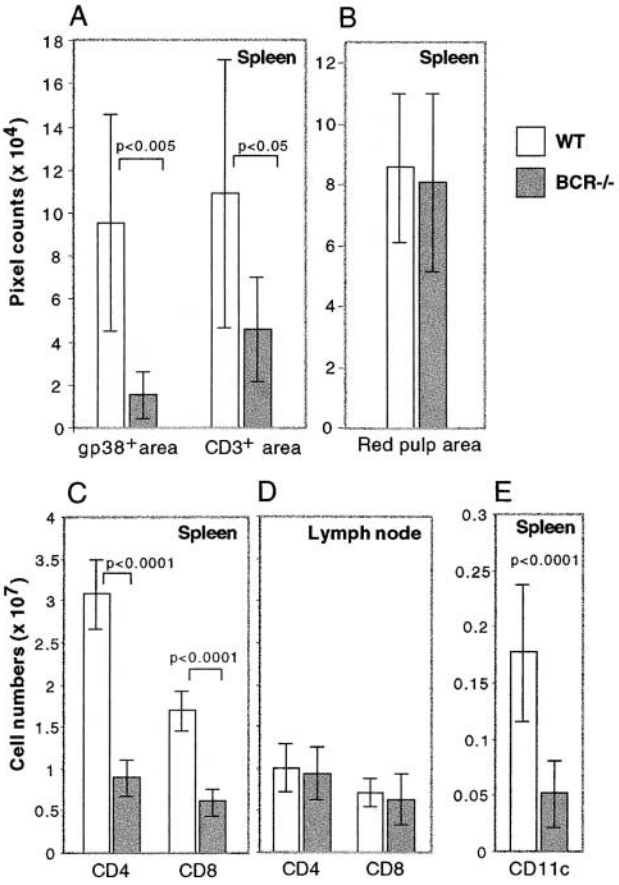

Figure 3. B cells promote splenic T zone stromal cell development and accumulation of splenic T cells and DCs. (A) Cross-sectional areas of gp38- and CD3 staining in B cell–deficient and wild-type (WT) spleens. Positively stained areas were determined as indicated in Fig. 2 and the numbers of pixels per splenic cross-section was calculated using Adobe Photoshop® software as described in Materials and Methods. Data were collected from seven wild-type and seven $BCR^{-/-}$ spleens. (B) Cross-sectional area of red pulp in B cell–deficient and wild-type spleens. Pixel counts were determined as in A, and red pulp areas were obtained by subtracting the white pulp areas (identified as the outer boundaries of CD3 and B220 staining) from the total splenic cross-sectional area. Data were collected from 12 spleens of each type. (C–E) Cell numbers in spleen, peripheral lymph nodes (pool of brachial, axillary, and inguinal) and blood. Cells were identified by flow cytometry as $CD3^+CD4^+$, $CD3^+CD8^+$, or MHC $classII^+CD11c^+$. Data are from 8–11 mice per group. In all panels, open bars correspond to wild-type and shaded bars to B cell deficient. Bars represent means and error bars, standard deviations. Statistical comparison is by Student's *t* test.

spleen as B cell–deficient mice had normal numbers of T cells in lymph nodes (Fig. 3 E).

LT Is Required for Splenic T Zone Development. Mice lacking the genes for LTα or LTβ, that are unable to express LTα1β2, are severely deficient in CCL21 and CCL19 expression (14). Analysis of gp38 revealed that expression of this T zone stromal cell marker is strongly dependent on LTα (Fig. 2) and LTβ (data not shown). Collectively, these findings establish that LTα1β2 is required for splenic T zone stromal cell development or maturation. In agreement with this role, T cells fail to accumulate normally in T cell areas in LTα-deficient mice (14, 36). However, many T cells are distributed in the red pulp and, in contrast to B cell–deficient spleens, T cell numbers in $LT\alpha^{-/-}$ spleens are in the range of wild-type (16–18). Therefore, separate signals appear to be involved in regulating T cell numbers in the spleen versus induction of T zones within the white pulp cords.

B cells have previously been established to function as a source of LTα1β2 necessary for FDC development (9, 10, 37). To test whether B cell–derived LTα1β2 is required for splenic T zone development, we first asked whether constitutive LTα1β2 signaling was important for maintaining CCL21 expression. Previous studies indicated that blocking LTα1β2 signaling in adult mice using LTβR-Ig treatment reduced CCL21 levels by ~30% (14); however, it was unclear in these studies whether all LTα1β2 function had been blocked. Therefore, we took a further approach to test for the requirement for LTα1β2, reconstituting lethally irradiated adult mice with LTα-deficient bone marrow. Despite efficient reconstitution by donor-derived cells (data not shown), CCL21 expression was not strongly affected compared with animals reconstituted with wild-type bone marrow (Fig. 4 A). Similar findings were made when lethally irradiated mice were reconstituted with BCR$^{-/-}$ or RAG1$^{-/-}$ bone marrow (data not shown). By contrast, CXCL13 levels were reduced to ~40% of wild-type (Fig. 4 A). Reciprocally, when lymphocyte-deficient mice were reconstituted with wild-type bone marrow, they failed to upregulate CCL21 expression (Fig. 4 B), whereas substantial upregulation of CXCL13 was observed (Fig. 4 B). Taken together, these results suggest that the strong deficit in CCL21 in mice congenitally deficient in LTα1β2 or B cells reflects a role for B cells and LTα1β2 in inducing CCL21 during a fixed window in spleen development.

B lymphocytes begin seeding the spleen soon after birth (38, 39), and consistent with an ability of B cells to have early postnatal effects, intraperitoneal transfer of TCRβ$^{-/-}$δ$^{-/-}$ spleen cells to RAG1$^{-/-}$ mice shortly after birth was sufficient to lead to an increase in CCL21 expression, measured in the spleen 5 wk later (Fig. 4 C). B cell numbers were only restored to between 1 and 10% of wild-type by this approach, and this may account for the incomplete upregulation of CCL21 expression. Importantly, if the transferred cells lacked LTα, they failed to cause an upregulation in CCL21 (Fig. 4 C).

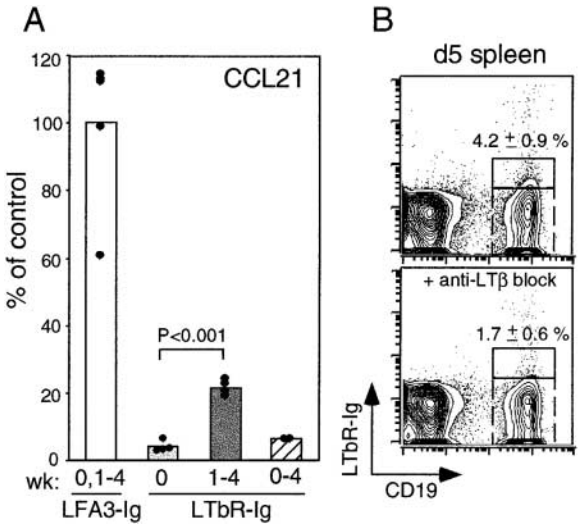

Figure 5. LTα1β2 antagonism in newborn mice inhibits CCL21 upregulation. (A) Relative CCL21 mRNA levels in spleen of 5-wk-old mice that were treated at day 0.5 (wk 0), days 7, 14, 21, and 28 (wk 1–4) or days 0.5, 7, 14, 21, and 28 (wk 0–4) after birth with LFA3-Ig or LTβR-Ig, as indicated. Each circle represents an individual animal and bars represents means. (B) Representative flow cytometric profiles of spleen cells from 5-d-old mice stained to detect B cells (CD19) and LTβR ligands (LTβR-Ig). In the bottom panel, the cells were preincubated with unconjugated anti-LTβ antibody that inhibits LTβR-binding to LTα1β2 as a staining control. The mean percentage of B cells falling within the gate indicated by the filled box is indicated (± SD) for cells from three 5-d-old mice.

As a further approach to test the requirement of LTα1β2 early in life for T zone chemokine expression, newborn or 1-wk-old wild-type mice were treated with LTβR-Ig and then the treatment was either stopped or continued as the animals grew to become adults. Analysis of spleen tissue from 5-wk-old animals given continuous injections of LTβR-Ig from birth showed a striking inhibition of CCL21 expression (Fig. 5 A), an effect markedly greater than the ~30% decrease observed after treating adult ani-

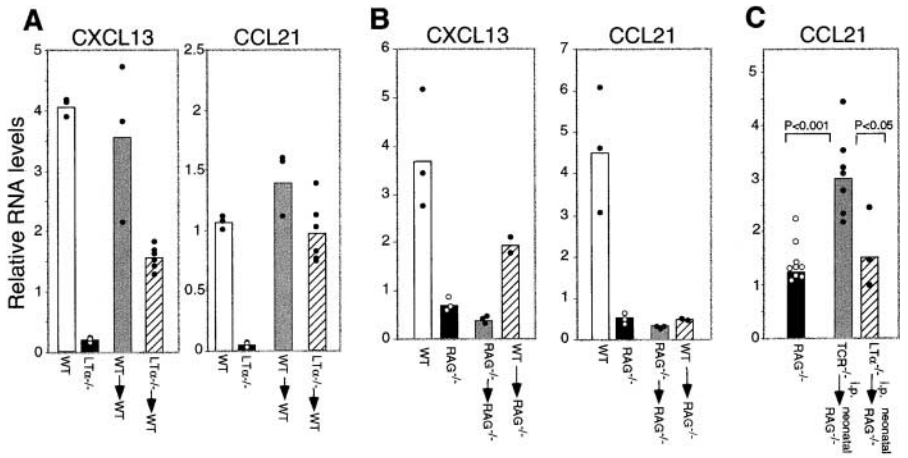

Figure 4. B cell and LTα dependence of splenic T zone stroma is developmental. (A) Relative CXCL13 or CCL21 mRNA levels in spleen, as determined by Northern blot, of wild-type (WT) or LTα-deficient mice, or irradiated wild-type mice 6 wk after reconstitution with WT or LTα$^{-/-}$ bone marrow. (B) Relative CXCL13 or CCL21 mRNA levels in spleen of WT or RAG1-deficient mice, or irradiated RAG1-deficient mice 6 wk after reconstitution with WT or RAG1-deficient bone marrow. (C) Relative CCL21 mRNA levels in spleen of 6-wk-old RAG1-deficient mice or RAG1$^{-/-}$ mice that were injected intraperitoneally (i.p.) with 2 × 10^7 splenocytes from adult T cell-deficient (TCR$^{-/-}$) or LTα-deficient mice at day 0.5 after birth. B cell numbers in spleens at isolation were between 0.7–3.10^6 (TCR$^{-/-}$ recipients) or 1.0–2.5 × 10^6 (LTα$^{-/-}$ recipients). Each circle represents an individual recipient and bars represent means. White bars, wild-type, black bars, LTα$^{-/-}$ or RAG1$^{-/-}$, shading, mice reconstituted with wild-type bone marrow or TcR$^{-/-}$ spleen cells, hatched bars, mice reconstituted with mutant bone marrow or LTα$^{-/-}$ spleen cells. Statistical comparison is by Student's *t* test.

mals with the antagonist for a similar period (14). In further agreement with a role for LTα1β2 early in spleen T zone development, CCL21 levels were strongly reduced in animals treated with a single injection of LTβR-Ig at day 0.5 of life (Fig. 5 A). Although the LTβR-Ig is likely to have persisted in the serum of these animals for several weeks (40), the importance of LTα1β2 signaling during the first week after birth is supported by the lesser effect on CCL21 levels when the treatment was delayed until week one (Fig. 5 A). Strikingly, immunohistochemical analysis of spleens from mice treated at day 0.5 revealed a severe disruption in organization and failure to express gp38 (Fig. 6 A), a phenotype similar to congenic LTα-deficiency (see Figs. 2 and 6 B). When the antagonist treatment was delayed until day 7, the effect was much less severe as white pulp cords with $gp38^+$ T cells areas could be identified (Fig. 6 A). In agreement with B cells serving as a source of LTα1β2 in the early postnatal period, flow cytometric analysis of B cells from day 5 spleens showed that, despite having the expected $IgD^{lo}CD21^{lo}$ immature phenotype (data not shown), they expressed measurable surface LTα1β2 as assessed by staining with LTβR-Ig (Fig. 5 B), although the fraction of positively stained B cells was two- to threefold lower than in adult spleen (7). In summary, these findings suggest that B cells promote CCL21 upregulation and splenic T zone development at least in part by acting as a source of LTα1β2 in the neonate.

Selective Expression of LTα1β2 in B Cells Is Sufficient to Promote Splenic CCL21 Expression, White Pulp T Zone Development, and Splenic DC Accumulation. The developmentally fixed requirement for LTα1β2 made it difficult to use adoptive transfer approaches to test whether B cells

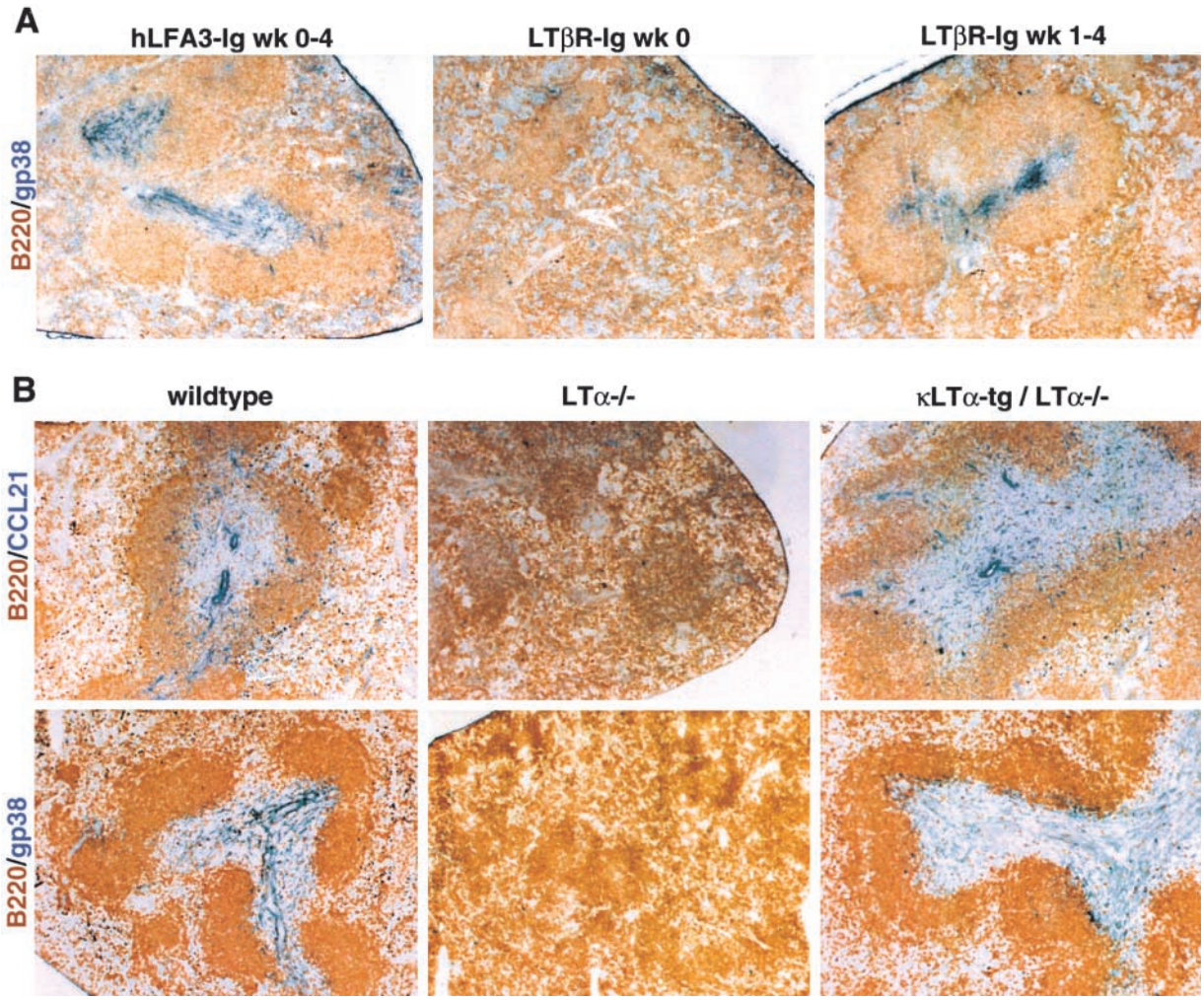

Figure 6. LTα1β2 antagonism in newborn mice inhibits T zone stromal cell development, and transgenic expression of LTα1β2 on B cells rescues T zone development in LTα-deficient mice. (A) Spleen tissue from mice treated with control (hLFA3-Ig) on days 0.5, 7, 14, 21, and 28 (left panel), or with LTα1β2 antagonist (LTβR-Ig) on day 0.5 (center panel) or days 7, 14, 21, and 28 (right panel), stained to detect B220 (brown) and gp38 (blue). (B) Spleen tissue from wild-type, LTα-deficient or LTα-deficient carrying the κLTα transgene (κLTα-tg/LTα−/−) stained in brown to detect B220 (all panels) and in blue to detect CCL21 (top panels) and gp38 (bottom panels). Data are representative of more than four animals of each type. Note that the faint blue signal in the red pulp of the panels in A and the upper $LT\alpha^{-/-}$ panel in B was due to background staining and was also observed in sections stained with control antibodies.

were a sufficient source of this cytokine for T zone development. As a further strategy to test this issue, we produced mice that selectively express LTα1β2 on B cells (Fig. 7). Transgenic animals were made that carry the coding region of the LTα gene fused to the segment of the LTβ gene encoding the membrane and spacer domains (23). We chose to make a membrane bound form of LTα to reduce the likelihood of soluble LTα being released and functioning at distal sites, either as LTα3 homotrimer or by restoring LTα1β2 in trans, phenomena that appeared to occur in mice overexpressing soluble LTα in the pancreas (41). Expression of the chimeric LTα gene was placed under control of the Ig κ enhancer and promoter elements to provide B cell–specific expression (Fig. 7 A).

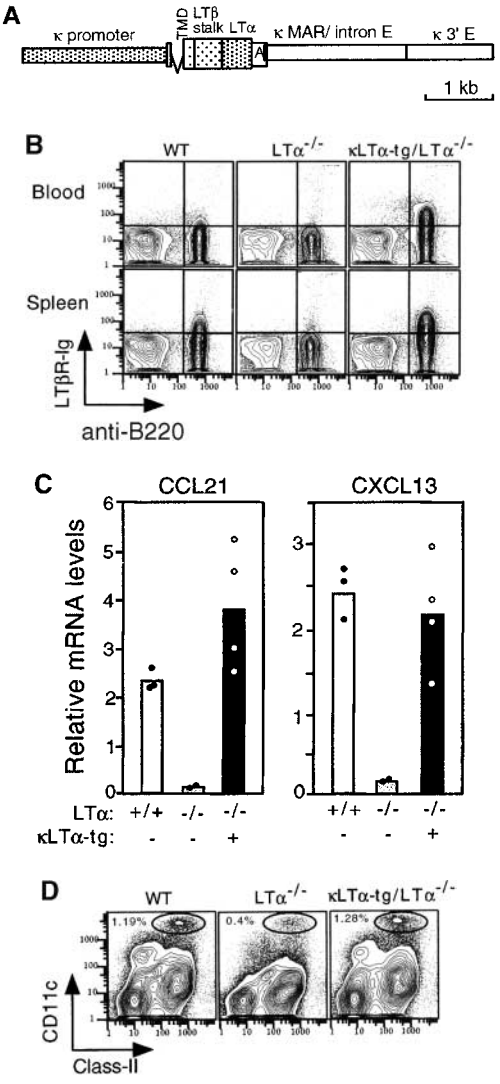

Figure 7. Transgenic restoration of LTα1β2 on B cells in LTα-deficient mice promotes splenic CCL21 and CXCL13 expression and DC accumulation. (A) A schematic diagram of the membrane LTα transgenic construct. See Materials and Methods for details. TMD, transmembrane domain; MAR, matrix-attachment region; E, enhancer. (B) Representative flow cytometric profiles of LTβR-Ig binding of $B220^+$ B cells from blood and spleen of WT, $LT\alpha^{-/-}$, and κLTα-transgenic/$LT\alpha^{-/-}$ mice as indicated. (C) Relative CCL21 and CXCL13 mRNA levels in spleen tissue from the indicated mice. Each circle represents an individual animal and bars represents means. (D) Flow cytometric profiles of CD11c staining in the indicated mice. Profiles are representative of four mice of each type and are for spleen cells prepared in the absence of collagenase. Analysis of two further spleen samples that had been prepared with collagenase revealed greater frequencies of $CD11c^+$ cells, and continued to show restoration of $CD11c^+$ cell frequency in the κLTα-tg/$LT\alpha^{-/-}$ animals.

Previously, we have observed that membrane LTα1β2 on B cells is unstable and is lost as cells enter circulation (7). We expected that the membrane form of LTα encoded by the transgene would increase the stability of the LTα1β2 and, consistent with this, we were able to identify transgenic founders by elevated LTα1β2 expression on blood B cells (Fig. 7 B, and data not shown). One transgenic line was selected that exhibited a level of LTβR-Ig staining on blood B cells that was similar to the level of staining of splenic B cells in wild-type animals and this line was intercrossed with LTα-deficient mice. Analysis of splenocytes from these mice showed surface LTα1β2 expression on B cells at levels ~2–3-fold above wild-type controls (Fig. 7 B) and no measurable expression on T cells, $CD11c^+$ cells, or $gran\text{-}1^+$ cells (Fig. 7 B, and data not shown). The transgenic cells did not stain detectably with TNFR1-Ig, indicating that there was little or no generation of functional surface LTα3 homotrimers (data not shown). Consistent with B cell–restricted expression, the transgene failed to restore lymph node or Peyer's patch development in the LTα-deficient animals (data not shown). However, there was a striking restoration of spleen architecture compared with LTα-deficient animals. T cells accumulated in well developed T cell areas, and these zones showed strong expression of CCL21 (Fig. 6 B, top panels). The T cell areas also exhibited extensive networks of $gp38^+$ T zone stromal cells, although the distribution of staining often differed from wild-type spleen, with a higher concentration of $gp38^+$ cells near the boundary of B and T zones (Fig. 6 B, middle panels). B cell regions were also present in the transgenic animals (Fig. 6 B) and they contained $CD35^+$ follicular stromal cells (data not shown). By Northern blot, expression of LTα1β2 selectively on B cells restored CXCL13 to wild-type levels and caused CCL21 to be expressed somewhat above wild-type controls (Fig. 7 C). Furthermore, expression of LTα1β2 on B-lineage cells was also sufficient to restore splenic DC numbers (Fig. 7 D). The DCs appeared to have a wild-type localization, with cells distributed in the marginal zone, marginal zone bridging channels, and in T cell areas, although in some cases clusters were also detected in the red pulp (data not shown). In summary, these findings provide strong evidence that B cells can act as a sufficient source of LTα1β2 to promote development of the splenic T zone and to cause accumulation or development of splenic DCs.

Discussion

In the above studies, we demonstrate that B cells play a critical role in splenic, but not lymph node, T zone development. In mice lacking B cells, splenic $gp38^+$ T zone

stromal cells fail to develop into their normal extensive network, and T zone CCL21 expression is markedly reduced. Our findings indicate that B cells promote splenic T zone stromal cell development and chemokine expression at least in part by acting as a source of LTα1β2. This function of B cells is largely a fixed, developmental role. We also find that B cell–derived LTα1β2 promotes splenic DC accumulation or development. Furthermore, B cells are needed for accumulation of normal numbers of T cells in the spleen. Previous studies have indicated that LTα1β2 and T zone chemokines, CCL19 (ELC) and CCL21 (SLC), are not required for T cell accumulation in the spleen (16–18, 42). Therefore, we conclude that B cells provide at least two types of signals necessary for normal spleen organogenesis: (a) signals that promote CD4 and CD8 T cell accumulation in the spleen, and: (b) signals, including LTα1β2, that regulate properties of white pulp stromal cells and promote development of T zones and development or accumulation of DCs.

T zone stromal cells have been characterized as a population of mesenchymal cells that are distributed throughout lymphoid organ T zones (15, 43). The recent finding that these cells are a major source of CCL21 and CCL19 has suggested that they play a central role in bringing CCR7-positive T cells and DCs together (11). Previously, using the white-pulp stromal cell marker BP3, we provided evidence that T zone stromal cell maturation was dependent on LTα1β2 (14). Other studies have indicated that there are alterations in the expression pattern of the T zone and red-pulp stromal marker, ER-TR7, in LT-deficient or depleted mice (18, 44, 45). We show here that expression of gp38, a marker that is thought to stain the majority of T zone stromal cells (15), is also LTα1β2-dependent. Therefore, LTα1β2 appears to be critical for development of T zone stromal cells and, in turn, for the development of organized splenic T cell zones (Fig. 8).

Several lines of evidence indicate a requirement for LTα1β2 in splenic T zone development that is fixed during the first few weeks after birth. First, in contrast to the severely defective splenic T zones of mice congenitally deficient in LTα1β2, in studies where LTα1β2 function was blocked in adult animals, effects on T zone organization were minimal (45) and T zone chemokine expression was only mildly diminished (14). Second, when adult mice are depleted of LTα-expressing cells by irradiation and reconstitution with LTα-deficient bone marrow, T zones remain visible (data not shown and see reference 46) and there is little reduction in CCL21 expression (Fig. 4). Therefore, once the splenic CCL21-expressing T zone stromal network has developed, it has only a weak requirement for continued LTβR-signaling to be maintained. By contrast, treatment of newborn mice with LTα1β2 antagonist had a marked inhibitory effect on subsequent CCL21 and gp38 expression in the adult (Figs. 5 and 6). Interestingly, the effect of LTβR-Ig treatment on the first day after birth was also more severe than in utero treatment at day 14 and 17 of embryogenesis (40, 47). This suggests that, in contrast to the critical embryonic function of LTα1β2 in lymph node development (36), most of the organizing activity of LTα1β2 in the spleen occurs after birth. Although experiments involving treatment with LTβR-Ig do not rule out involvement of the second known LTβR ligand, LIGHT, the similarities between the effect of LTβR-Ig treatment and LTα- and LTβ-deficiency (36) argue that the principal pathway involved in promoting splenic white pulp organization is the LTα1β2-LTβR pathway.

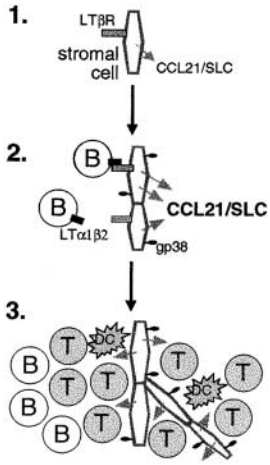

Figure 8. Model of splenic T zone development in the mouse. Step 1, occurring in the days before and around the time of birth, LTβR$^+$ mesenchymal cells proximal to splenic arterioles acquire some features of T zone stromal cells by unknown mechanisms. These cells produce low levels of CCL19 (not shown) and CCL21 in a LTα1β2-independent manner. Step 2, occurring in the first days to a few weeks after birth, newly produced B cells arrive in the spleen and express surface LTα1β2, possibly in response to CCL19 and CCL21 (reference 7), but likely also in response to other signals, and induce LTβR-signaling in the primitive stromal cells. These signals may act directly to promote growth (possibly involving cell division) of the stromal cell network and to increase expression of CCL21, gp38 and, to a lesser extent, CCL19. Alternatively, the signals may act indirectly, through intermediary LTβR$^+$ cell types to cause these outcomes. It is also possible that the effects of LTα1β2 and B cells on chemokine and gp38 expression do not reflect direct induction but are secondary to growth of the T zone stromal network. Furthermore, cells in addition to B cells may contribute LTα1β2, especially for induction of gp38 as this marker is expressed at higher amounts in B cell–deficient animals than in LTα-deficient animals. Step 3, T cells and DCs are recruited to the network of CCL21 and CCL19 producing stromal cells and the mature T zone forms.

The finding that T zone development is dependent on both LTα1β2 and B cells, together with the previous demonstration that B cells are a source of LTα1β2 (7, 9, 10, 37), led to the prediction that B cells act as the major source of this cytokine for splenic T zone development. Consistent with this, B cells enter the spleen in the first few days after birth and during this period the cells are located around central arterioles, in regions destined to become T cell areas (38, 39). Later, B cells continuously migrate through the outer T zone as they traffic to B cell areas (4). Immature B cells in the developing spleen were found to have low but measurable surface LTα1β2 (Fig. 5) and transfer of wild-type B cells, but not LTα-deficient cells, to newborn lymphocyte-deficient mice was sufficient to upregulate splenic CCL21 (Fig. 4). Furthermore, transgenic expression of LTα under control of Ig-kappa promoter and enhancer elements restored splenic CCL21 (and also CXCL13) expression and led to development of gp38$^+$ T zone stromal cell networks in LTα-deficient mice (Figs. 6 and 7). Therefore, several different findings support the conclusion that B cells act as a critical source of LTα1β2 in splenic T zone development. A model incorporating these findings is shown in Fig. 8.

A question that arises from these findings is whether B cells function as the only source of LTα1β2 required for splenic T zone development. Arguing against this is the Northern blot analysis of CCL21, which indicates that mRNA levels are reduced 5–10-fold in B cell–deficient spleen compared with ~20-fold in LTα-deficient spleen (14; Figs. 1 and 4). In addition, Gonzalez et al., found that pretreatment of SCID mice with LTβR-Ig influenced the subsequent ability of transferred B and T cells to organize within the white pulp (10). Recently, a hematopoietic cell type, characterized as $CD4^+CD3^-IL7R\alpha^+LT\alpha1\beta2^+$, has been implicated in the earliest steps in lymph node and Peyer's patch development (48–50). As we have not found a role for B cells or T cells in lymph node T zone stromal cell development (Figs. 1 and 2), we favor a model where $CD4^+CD3^-$ cells provide the necessary LTα1β2 signals. By contrast with lymph nodes, $CD4^+CD3^-$ cells are very infrequent in embryonic spleen and they have so far not been implicated in spleen development (48, 50, 51). In addition, we have shown that T cells are not needed as a source of LTα1β2 for induction of splenic CCL21. Future studies need to investigate what other cell types express LTα1β2 in the developing spleen. However, our studies are also consistent with the possibility that in normal animals, B cells act as a sufficient source of LTα1β2 for spleen development and the contributions of other cell types only become evident when B cells are absent. Interestingly, in the transgenic animals constitutively expressing LTα1β2 on B cells, the pattern of $gp38^+$ T zone stromal cell networks is well developed but differs from wild-type by showing higher amounts of staining at B/T-zone boundaries (see Fig. 6). In addition, although B cell areas develop, they fail to form the highly polarized clusters typical of wild-type follicles (Fig. 6, and data not shown). A likely explanation for these differences is the altered regulation of LTα1β2 expression in B cells. In wild-type animals, we have provided evidence that LTα1β2 expression on B cells is cyclical, being induced by CXCL13 to maximal levels as B cells migrate into the FDC networks and then being downregulated as cells pass into the blood (7). By contrast, in the transgenic animals the expression is constitutive and divorced from normal regulatory controls, and B cells at the B/T boundary are likely to express the same high levels of LTα1β2 as cells within the CXCL13-producing FDC networks. While further study of the transgenic animals is needed, these observations provide support for the conclusion that cyclical expression of LTα1β2 on migratory B cells is important for the formation and maintenance of pattern in lymphoid tissue.

The ability of B cells to promote development of T zones in a LTα1β2-dependent manner was previously suggested by the finding that ectopic expression of CXCL13 in the pancreas caused recruitment of B cells and development of lymph node like structures, with T cell areas containing stromal cells, DCs and CCL21 (52). Such ectopic T cell areas did not develop in mice lacking B cells and antagonism of LTα1β2 function was sufficient to block T zone development (52). However, in contrast to the ectopic system, CXCL13 is not required for promoting splenic T zone development as these areas develop normally in CXCL13-deficient mice (7), and splenic CCL21 levels are unaffected by CXCL13 deficiency (unpublished observations). Perhaps another lymphoid tissue–expressed chemokine that is capable of upregulating LTα1β2 on B cells (7) plays this role during spleen development. The findings here and in the RIP-BLC system indicate that there are several pathways by which B cells can be induced to express LTα1β2 and to promote T zone development.

In addition to their requirement for development of T zone stromal cells, B cells (34; Fig. 2) and LTα1β2 (19) are required for normal accumulation of DCs in the spleen. Our findings provide evidence that B cells can function as a sufficient source of LTα1β2 for promoting normal DC accumulation in the spleen. Consistent with an important role for B cells in regulating splenic DC development, a recent report found that DCs from B cell–deficient spleens were functionally distinct from DCs taken from wild-type spleens (53). The origin of splenic DCs is not well defined. While some may derive directly from precursors in the blood or from DCs that have entered the blood, for example, from the heart or liver, there is also evidence that splenic DCs can develop locally from precursors in the spleen (54–56). LTβR expression has been reported in myeloid-lineage cells (57, 58), making it possible that B cell LTα1β2 acts directly on DC precursors. Alternatively, B cell–mediated induction of white pulp stromal cell maturation may indirectly promote DC development or may allow the survival of more DCs to be supported. It is unlikely that the effect of B cells on DC numbers is through effects on CCL21 or CCL19 as mice that lack these chemokines in lymphoid tissues have normal numbers of spleen DCs (11, 12), but it remains possible that B cells promote DC homing to the spleen through effects on other chemokines or trafficking molecules.

The finding that B cells were needed in the spleen for normal T zone stromal cell development and chemokine expression, and for normal accumulation of CD4 and CD8 T cells initially led us to consider that these processes were linked. However, this does not appear to be the case as animals that lack LTα or LTβ contain approximately normal numbers of T cells in the spleen (16–18). The *plt* mouse strain, lacking lymphoid CCL19 and CCL21 expression, also has normal numbers of splenic T cells (12). In both types of animal, the T cells are distributed mostly in the red pulp. A complication with these studies is that these animals either lack or have a severe paucity of T cells in lymph nodes, possibly leading to an indirect effect on T cell numbers in the spleen. However, we have found that when B cell–deficient mice are reconstituted with wild-type bone marrow or are restored as adults with a transfer of B cells, spleen size grows and T cell numbers reach normal levels (data not shown) despite the failure to upregulate CCL21 expression in spleen (Fig. 4). Therefore, the findings suggest a dissociation between B cell effects on T zone stromal cell development, CCL21 expression and DC accumulation, which involves B cell–derived LTα1β2, and B cell

promotion of T cell accumulation in the spleen, which does not. We suggest that B cells provide signals, other than LTα1β2, that allow the spleen to accommodate the adult number of T cells. In this regard it is important to note that B cells have been shown to provide signals necessary for full development of Peyer's patches (59, 60), and at least some of these signals are suggested to be independent of LTα1β2 (59).

Our findings establish an important role for B cells in controlling spleen T zone development and provide a possible additional mechanism for how B cell deficiency may cause defects in immunity unrelated to a lack of antibody production. Such defects include reduced T cell responses to *Salmonella typhimurium* (61), *Bordatella pertussis* (62), *Chlamydia trachomatis* (63), *Francisella tulaensis* (64), *Plasmodium chabaudi* (65), and *Leishmania major* (66). B cells are also required for development of auotreactive T cells in MRL-lpr mice (33) and they facilitate T cell–mediated destruction of pancreatic β-cells in NOD mice (67). While B cell antigen-presenting function may be a critical parameter in these diseases, we propose that some of the defects of B cell deficiency may reflect the role of B cells in promoting CCL21 expression, T zone stromal cell development and DC maturation or accumulation in spleen, in other B cell rich organs, and in B cell-rich inflammatory lesions. Futures studies need to investigate the contributions of these parameters to disease susceptibility in B cell–deficient humans, and to human inflammatory diseases involving B cells (68, 69).

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