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B Cells Are Crucial for Both Development and Maintenance of the Splenic Marginal Zone

Martijn A. Nolte,*†‡ Ramon Arens,†‡ Manfred Kraus,§ Marinus H. J. van Oers, † Georg Kraal, * René A. W. van Lier,‡ and Reina E. Mebius*‡*

The splenic marginal zone is a unique compartment that separates the lymphoid white pulp from the surrounding red pulp. Due to the orchestration of specialized macrophages and B cells flanking a marginal sinus, this compartment plays an important role in uptake of blood-borne Ags and it gives the spleen its specialized function in antibacterial immunity. In this study, we demonstrate that both development and maintenance of this marginal zone is highly dependent on the presence of B cells. Spleens from B cell-deficient mice were found to lack both metallophilic and marginal zone macrophages as well as mucosal addressin cellular adhesion molecule-1⁺ sinus lining cells. Using an inducible Cre/loxP-driven mouse model in which mature B cells could be partially depleted by removal of the B cell receptor subunit Igo, we could show that the integrity and function of an established marginal zone was also dependent on the presence of B cells. This was confirmed in a transgenic model in which all B cells were gradually depleted due to overexpression of the TNF family member CD70. The loss of all cellular subsets from the marginal zone in these CD70 transgenic mice was effectively prevented by crossing these mice on a CD27⁻/⁻ or TCRα⁻/⁻ background, because this prohibited the ongoing B cell depletion. Therefore, we conclude that B cells are not only important for the development, but also for maintenance, of the marginal zone. This direct correlation between circulating B cells and the function of the spleen implies an increased risk for B cell lymphopenic patients with bacterial infections. The Journal of Immunology, 2004, 172: 3620–3627.

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e the spleen is the largest secondary lymphoid organ in the mammalian body and plays an important role in the filtration of blood and the defense against pathogens. To effectively perform its function, the spleen has been organized in different compartments, each with its own specific cell types and microarchitecture. The white pulp contains distinct areas for T and B cells, which provide an environment for the efficient initiation of Ag-specific immune responses. These lymphoid areas are confined by a marginal zone that separates the white pulp from the blood-filled sinuses of the red pulp (reviewed in Ref. 1). This marginal zone receives a substantial part of the blood flow that enters the spleen, because the sinus it comprises is connected to the arteriolar network of the organ (2). Blood-borne lymphocytes that arrive in the marginal zone are able to enter the white pulp by extravasating the marginal sinus through fenestrated mucosal addressin cellular adhesion molecule-1⁺ (MAdCAM-1⁺) sinus lining cells (3). Furthermore, specialized macrophages in the marginal zone also give this compartment an important role in Ag uptake: the inner side of the marginal zone harbors the marginal metallophilic macrophages (MMM), whereas the outer side contains the marginal zone macrophages (MZM) and the marginal zone B cells (MZB) (reviewed in Ref. 4). The marginal zone and its constituents are unique for the spleen and give this organ the ability to mount T cell-independent immune responses (5).

During ontogeny in mice, the spleen develops and becomes populated with white blood cells during embryonic life, but it is far from being fully mature at birth, as there is no clear compartmentalization and the marginal zone is still absent (6, 7). The first cells that initiate the formation of the white pulp are B cells, as they can be found even before birth as small clusters surrounding the early central arterioles (7, 8). Around day 2, the murine spleen becomes readily populated with T cells, which correlates with the large efflux of T cells from the thymus at day 2 (8, 9). During these first neonatal days, the macrophages that will later enter into the marginal zone can be found dispersed throughout the entire spleen, while the actual formation of the marginal zone starts around day 5 in mice. From this day on, these macrophages start to localize at the interface of red and white pulp and establish the marginal zone, which is then also populated by MZB (Refs. 10–12 and our unpublished observations).

The mature spleen plays an important role in B cell development, because it is the site of terminal differentiation for developing B cells after they leave the bone marrow. So far, the following developmental stages have been defined in the spleen: transitional type 1 (T1) B cells are recent immigrants from bone marrow and can subsequently develop into transitional type 2 (T2) B cells, which in turn form the precursors for mature follicular B cells (13). The special population of MZB is thought to be derived from mature B cells, but it is also possible that these cells can develop directly from T2 B cells. Thus, B cells depend on the spleen for their development. Yet, because B cells are the first cells to enter

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3 Abbreviations used in this paper: MAdCAM-1, mucosal addressin cellular adhesion molecule-1; MMM, marginal metallophilic macrophage; MZB, marginal zone B cell; MZM, marginal zone macrophage; TG, transgenic; RPM, red pulp macrophage; LT, lymphotxin; FDC, follicular dendritic cell; BCR, B cell receptor; WPM, white pulp macrophage; CCL, CC chemokine ligand; CXCL, CXC chemokine ligand; WT, wild type.
the developing white pulp and are continuously in close contact with the cells from the marginal zone in the mature spleen, we questioned whether B cells can influence the integrity of this compartment. A direct effect of B cells on the development of the marginal zone has been suggested previously (14–16), but no data exists concerning the role of B cells in the maintenance of this compartment. Therefore, we analyzed spleens from different mouse models in which B cells were differentially affected. Our findings reveal that B cells are not only important for the development, but also for the maintenance of the marginal zone, because the marginal zone disappeared concurrent with the loss of B cells. Our data further elucidate the mechanisms that regulate the physiology of this important splenic compartment.

Materials and Methods

Mice

CD70 transgenic (TG) mice were generated by expression of the murine CD70 gene under control of the human CD19 promoter region, as previously described (17). Igα<sup>-/-</sup> Mx-Cre mice were generated by breeding mice in which exons II and III of Igα (CD79a, mb-1) are flanked by loxP sites (M. Kraus and K. Rajewsky, unpublished data) with mice in which Cre recombinase is under the control of type I IFN-inducible Mx promoter (18). Expression of Cre was induced by four i.p. injections with poly(I)/poly(C) (400 μg per injection; Amersham Pharmacia, Roosendaal, The Netherlands) during a period of 3 wk and the mice were sacrificed 5 days after the last injection. C57BL/6, CD27<sup>-/-</sup> mice (19), and TCRδ<sup>-/-</sup> mice (20) were bred and kept in the animal facility of The Netherlands Cancer Institute (Amsterdam, The Netherlands) under specific pathogen-free conditions. LTα<sup>-/-</sup> mice (21) were maintained under similar conditions at the animal facility of the Vrije Universiteit Medisch Centrum (Amsterdam, The Netherlands). μMT/μMT mice were kindly provided by Dr. M. Schreurs (The Netherlands Cancer Institute).

Immunohistochemistry

For immunohistochemical analysis, isolated spleens were frozen in Tissue-Tek (Sakura Finetek, Zoeterwoude, The Netherlands) and 7-μm cryostat sections were prepared. These sections were applied on gelatin-coated microscopic glass slides, fixed in dehydrated acetone for 10 min, air-dried, and rehydrated with 2% newborn calf serum in PBS (NBPS). For immunofluorescent staining, splenic sections were successively incubated for 45 min with unconjugated rat anti-mouse mAbs and Alexa 488-conjugated anti-rat IgG (Molecular Probes, Eugene, OR). In case of double immunofluorescence, sections were blocked for 5 min with 20% normal rat serum and subsequently incubated with biotinylated rat anti-mouse mAbs and Alexa 594-conjugated streptavidin (Molecular Probes). The following rat anti-mouse mAbs were used: 6B2 (anti-B220), KT3.1 (anti-CD3ε), FDC-M2 mAb (AMS Biotechnology, Abingdon, U.K.), SER-4 (antisialoadhesin), ER-TR9 (anti-MZM), MOMA-2, F4/80 (anti red pulp macrophages (RPM)), ED31 (anti-MARCO), MECA-367 (anti-MAdCAM-1). The mAbs 6B2 and ED31 were purified from culture supernatant from hybridoma cells with protein G-Sepharose (Amersham Pharmacia) and biotinylated in our laboratory according to standard procedures. Sections were extensively washed with PBS between each step and finally cover-slipped with Fluorostab (ICN, Zoetermeer, The Netherlands). Fluorescent stainings were analyzed using a Nikon Eclipse E800 microscope (Nikon Europe, Badhoevedorp, The Netherlands), connected to a digital camera.

In vivo Ag uptake

The functional capacity of the marginal zone was examined by the ability to take-up fluorescently labeled polysaccharides. Therefore, mice were injected i.v. in the tail vein with 200 μl of 500-kDa lysine-fixable dextran-FITC (Molecular Probes) at 2 mg/ml in sterile saline. After 40 min, the mice were sacrificed and their spleens were frozen in Tissue-Tek and subjected to immunohistochemical analysis.

Flow cytometric analysis of lymphotixin (LT) expression

Single cell suspensions from freshly isolated spleen and lymph nodes (axillary, brachial, inguinal, and mesenterical) were obtained by mincing and gently pressing the tissue through a fine nylon mesh, while frequently rinsing with NBPS. Erythrocytes from spleen were lysed by incubation for 5 min at room temperature in ACK lysis buffer (150 mM NH4Cl, 1.0 mM KHCO3, and 0.1 mM Na2EDTA, pH 7.4) and lymphocytes were washed and resuspended in NBPS.

For four-color analysis of developing B cell subsets, 1 × 10<sup>6</sup> cells per sample were pretreated on ice with Fc-block (clone 2.4G2) and subsequently incubated for 30 min with CD21-FITC (clone 7G6; BD Pharmingen, San Diego, CA), IgD-PE (clone 11.26; Southern Biotechnology Associates, Birmingham, AL), IgM-biotin (clone R6-60.2; BD Pharmingen), and B220-allophycocyanin (clone RA3-6B2; BD Pharmingen). Streptavidin-PerCP-Cy5 (BD Pharmingen) was used as second-step reagent and cells were analyzed by flow cytometry on a FACScalibur (BD Biosciences, Mountain View, CA) using CellQuest software (BD Biosciences).

LT expression on B cells was examined on either freshly isolated cells or on lymphocyte suspensions that had been cultured for 40 h in the presence of 10 μg/ml anti-mouse CD40 (clone 1C10, 23); kindly provided by Dr. F. Lund, Trudeau Institute, Saranac Lake, NY) at 3 × 10<sup>6</sup> cells/ml in RPMI 1640 supplemented with 10% FCS. Membrane-bound LTαβ<sub>2</sub> was detected by pretreating the cells with Fc-block supplemented with 5% normal mouse serum for 30 min and subsequently incubating them for 60 min with a LTβR-IgG fusion protein (24) (a kind gift of Dr. P. Rennert, Biogen, Cambridge, MA). Anti-human-PE (Jackson ImmunoResearch Laboratories, West Grove, PA) was used as a second step conjugate and B cells were detected with Alexa 488-conjugated anti-B220 (6B2). Splenocytes isolated from LTαβ<sub>2</sub> mice were used as negative controls. Dead cells were excluded by using 7-aminoactinomycin D (Molecular Probes). Cells were analyzed by flow cytometry on a FACScan (BD Biosciences).

Results

The developing marginal zone is dependent on the presence of B cells

To determine whether the absence of B or T cells affects the development of the marginal zone, we examined this compartment in

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**FIGURE 1.** B cell-deficient, but not T cell-deficient, mice lack a normal marginal zone. Spleens from WT (left), TCRα<sup>-/-</sup> (middle), and BCR<sup>-/-</sup> mice (right) were analyzed for the expression of SER-4<sup>+</sup> MMM (upper panel in red) and ER-TR9<sup>+</sup> MZM (lower panel in red). Sections were also stained for B cells (in WT and TCRα<sup>-/-</sup> mice) or T cells (in BCR<sup>-/-</sup> mice) in green.
both B cell-deficient (BCR−/−; μMT/μMT) and T cell-deficient (TCR−/−) mice. TCR−/− mice were found to have normal populations of both MMM and MZM (Fig. 1), as well as MAdCAM-1 expression, while other macrophage populations in the spleen and follicular dendritic cell (FDC) clusters were also normally present (Table I). In contrast, in the absence of B cells neither MMM nor MZM could be found (Fig. 1), while MAdCAM-1 expression and FDC clusters were also absent (Table I). This was not due to a general defect in myeloid development, because F4/80 macrophages in the red pulp and MOMA-2 macrophages in the white pulp were normally present in BCR−/− mice (Table I). These findings demonstrate that B cells, but not T cells, are a prerequisite for normal development of the marginal zone.

Table 1. Splenic phenotype of several genetically deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lymphocytes</th>
<th>Cells in Red and White Pulp</th>
<th>Cells in the Marginal Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B cells</td>
<td>T cells</td>
<td>FDC (FDC-M2)</td>
</tr>
<tr>
<td>WT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TCRα−/−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>BCR−/−</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>CD27TG × CD27−/−</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD70TG × TCRα−/−</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

The respective molecule or mAb that was used to detect these cells is indicated in parentheses. At least three mice were analyzed for each of the indicated genotypes. Mφ, macrophage; MARCO, macrophage receptor with collagenous structure; MZ, marginal zone.

B cell depletion results in loss of the marginal zone

Because development of the marginal zone could depend on cellular interactions that are subsequently not required for its maintenance, we investigated the fate of a well-developed marginal zone during gradual decline of B cell numbers. Ablation of mature B cells was achieved in IgαTmd×TmMx-Cre mice in which exons II and III of the Igα gene (CD79a, mb-1), that encode its extracellular and transmembrane region, are flanked by loxP sites and Cre expression is under the control of a type I IFN-inducible Mx promoter (18). Because mature B cells require the B cell receptor (BCR) complex for their survival (25) and Igα is required for BCR surface expression, treatment of these mice with the IFN-α inducer...
poly(I)/poly(C) results in deletion of Igα and consequently in a systemic B cell depletion (M. Kraus and K. Rajewsky, unpublished data).

We investigated the spleens from IgαTmf/ΔTmMx-Cre mice after repetitive treatment with poly(I)/poly(C) and used similarly treated mice that did not carry the Cre transgene (IgαTmf/ΔTm−/) as controls. FACS analysis showed a decrease of B cells in the spleen up to 70% (data not shown). A substantial reduction was also evident on splenic sections (Fig. 2A). The reduction in B cells was manifest in the red pulp and in a strongly reduced size of several follicles, although intact follicles could still be found. Subsequent analysis of the marginal zone in IgαTmf/ΔTmMx-Cre mice revealed that both MMM and MZM were also lacking in those areas where B cells were depleted (Fig. 2B). However, both subsets of macrophages were detectable in those areas where B cells were still present. Spleens from similarly treated IgαTmf/ΔTm−/ mice were normal, indicating that the poly(I)/poly(C) treatment itself did not affect the B cells or the marginal zone (Fig. 2B). These findings suggest that not only the development but also the maintenance of the marginal zone is dependent on the presence of B cells.

Concomitant loss of B cells and marginal zone in CD70TG mice

Although the IgαTmf/ΔTmMx-Cre mice show a local disappearance of the marginal zone, many MMM and MZM could still be detected at those sites where B cells were present. Therefore, we also examined the spleens of a mouse model in which all B cells were depleted. Previously, we have shown that overexpression of the TNF family member CD70 leads to a gradual and complete depletion of the entire B cell pool due to massive expansion of IFN-γ-producing effector-type T cells (17). Analysis of developing B cell subsets in these CD70TG mice, as previously defined by the differential expression of surface markers IgM, IgD, and CD21 (see Ref. 13 and Fig. 3A), revealed that both T1 and mature B cells were significantly reduced at 4 wk of age and gradually declined over time (Fig. 3B). T2 B cells and MZB were maintained at normal numbers up to 6–8 wk, but finally also declined and disappeared in older mice (Fig. 3B).

Therefore, we examined whether this gradual loss of B cells affected the integrity of the spleen in general and the marginal zone in particular. Constitutive interaction between CD27 and CD70 was found to have no effect on the development of the splenic compartments, because CD70TG mice of 3- to 4-wk-old had normal white pulp areas with distinct T and B cell compartments and an obvious marginal zone (Fig. 4A). Macrophages expressing the marginal zone markers sialoadhesin (MMM) and MARCO (MZM and MMM), as well as the MAdCAM-1+ sinus lining cells were clearly present in this compartment, although the number of ER-TR9+ MZM was reduced (Fig. 4A). Furthermore, other macrophage subsets in red and white pulp, like F4/80+ RPM and

![FIGURE 3. All developmental stages of splenic B cells are gradually depleted in CD70TG mice.](http://www.jimmunol.org/)

For analysis of developing B cell populations, B220+ splenocytes are separated into IgD+ and IgD− cells and further defined by their expression of CD21 and IgM (13). The cells that are not included in the indicated regions have not been previously defined. B. The relative number of T1, T2, MZB, and mature follicular B cells was analyzed in spleens of WT and CD70TG mice at various ages. *Significant difference (*p < 0.05*) as determined by Student’s t test.
white pulp macrophages (WPM), were normally present, as were FDC clusters in the follicles (Table II). When 8-wk-old CD70TG mice were examined, a strong reduction in the size of the B cell follicles could be seen (Fig. 4A), which agrees with our previous report (17). In addition, we observed that macrophages from the marginal zone were gradually depleted: MZM were absent after 5 wk of age, whereas the population of MMM declined more slowly and was almost undetectable at 12 wk (Fig. 4A). The few MMM that were still present in 10- to 12-wk-old mice generally colocalized with residual B cells, as seen in Fig. 4A. Staining for acid-phosphatase, a macrophage-related enzyme, which is normally abundantly expressed in MMM and MZM, was not detectable in the marginal zones of older CD70TG mice (data not shown), confirming the loss of these macrophages, rather than down-regulation of their distinctive cell surface markers. In contrast, the F4/80<sup>+</sup> macrophages in the red pulp, as well as MOMA-2<sup>+</sup> macrophages and CD11c<sup>+</sup> dendritic cells in the white pulp of CD70TG mice remained present throughout life, indicating that the myeloid populations outside the marginal zone remained unaffected (Fig. 4A and Table II). Furthermore, the cellular depletion in these mice was not restricted to MMM and MZM, because MAdCAM-1<sup>+</sup> sinus lining cells and FDC clusters gradually disappeared as well and were finally absent after 24 wk (Table II). Staining for endothelial cells with anti-ICAM-1 and MECA-32 Abs indicated that the vasculature of the marginal sinus was still intact (data not shown). It is therefore likely that the disappearance of the MAdCAM-1<sup>+</sup> sinus lining cells is rather a result of down-regulation of the MAdCAM-1 molecule than a complete disappearance of these cells.

We have previously shown that B cell depletion in CD70TG mice was not due to an intrinsic defect of these cells, but was mediated by CD27 and T cells, because normal B cell numbers were found in CD70TG × CD27<sup>−/−</sup> mice, as well as CD70TG × TCRα<sup>−/−</sup> mice, respectively (17). Examination of the spleens of...
these mice revealed that both CD70TG × CD27−/− and CD70TG × TCRα−/− mice expressed a well-developed marginal zone, as did nontransgenic CD27−/− and TCRα−/− mice (Table I). Together, these data demonstrate that overexpression of LTαβ2 of CD70TG B cells was comparable with WT B cells (Fig. 5A), irrespective of the age of the mice (data not shown). In addition, when these B cells were stimulated with anti-CD40 mAb, which is a potent inducer of LTαβ2 expression (29), CD70TG B cells could adequately up-regulate this molecule (Fig. 5B). It can therefore be concluded that CD70TG B cells are intrinsically normal and have normal expression and regulation of LTαβ2.

In summary, our data clearly demonstrate that the marginal zone is phenotypically as well as functionally lost when B cells solely are depleted from the spleen, thereby revealing an important role for B cells in maintaining the integrity of the splenic microenvironment.

**Discussion**

The spleen harbors a large variety of different cellular subsets from erythroid, lymphoid, as well as myeloid, origin and most of these populations are confined to their own well-organized compartment. Interactions between various subsets and compartments might occur, but the nature, frequency, and relevance of such contacts is not known. This study shows that B cells not only play an important role in the development, but also in the maintenance of the various cell types that constitute the marginal zone. Mice lacking all mature B cells from birth do not have MMM, MZM, nor MAdCAM-1− sinus-lining cells (Fig. 1), which is in accordance with previous findings (14–16). Thorough analysis of CD70TG mice, in which B cells are gradually depleted, revealed that all cells from the marginal zone disappeared concurrently (Fig. 4A).

**Table II.** *Splenic phenotype of CD70TG mice at different weeks after birth*.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Age (weeks)</th>
<th>B cells (B220)</th>
<th>T cells (CD3)</th>
<th>FDC (FDC-M2)</th>
<th>RPM (F4/80)</th>
<th>WPM (MOMA-2)</th>
<th>Sinus-lining cells (MAdCAM-1)</th>
<th>MZM (ER-TR9)</th>
<th>MMM (Sialo-adhesin)</th>
<th>Mφ in MZ (MARCO)</th>
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<tbody>
<tr>
<td>Lymphocytes</td>
<td>3</td>
<td>+</td>
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*Presence of several splenic cell types was represented as +, normal; ±, (severely) reduced; −, absent. Mφ, macrophage; MARCO, macrophage receptor with collagenous structure; MZ, marginal zone.*

**FIGURE 5.** CD70TG B cells have normal expression and regulation of membrane-bound LTαβ. A. Freshly isolated lymphocytes were gated for B220+ B cells and the expression of LTαβ was analyzed using a LTβR-IgG fusion protein. Dead cells were excluded with 7-aminoactinomycin D. Shown are cells from WT mice (filled gray graph) and CD70TG mice (thick black line). Cells from LTαβ−/− mice (dashed black line) served as negative controls. B. Freshly isolated lymphocytes were cultured for 40 h with anti-CD40 mAb, to induce B cell activation and subsequent up-regulation of LTαβ. Cells were gated for B220+ B cells, excluded for dead cells, and analyzed for LTαβ expression using the LTβR-IgG fusion protein. Data are representative for two mice per experiment and the experiment has been performed three times with similar results.
Although we cannot formally exclude that activated T cells in these mice negatively affect the marginal zone, we also found concomitant loss of B cells and the marginal zone in the inducible B cell knockout model (Fig. 2). In fact, the presence of MMM or MZM without surrounding B cells could not be detected in either model. Furthermore, the observation that the marginal zone is normal in both CD70TG × CD27−/− and CD70TG × TCRα−/− mice excludes the possibility that loss of this compartment was caused by an intrinsic effect of CD70 overexpression or a direct effect of CD70-expressing B cells on CD27-expressing hematopoietic stem cells in the bone marrow (30). In conclusion, the findings presented here demonstrate that the disappearance of the marginal zone in CD70TG mice is caused by the concurrent loss of B cells, thereby revealing a so far unknown dependency of this compartment on the continuous presence of B cells.

More insight in the ongoing B cell depletion in the CD70TG mice was obtained by detailed analysis of the various B cell populations. As we found that both T1 B cells in the spleen (Fig. 3B) and immature B cells in the bone marrow (17) were strongly reduced, it can be concluded that the generation of B cells is disturbed in CD70TG mice. Still, the gradual decline of the mature follicular B cells and MZB suggests that depletion of B cells in the periphery occurs as well, because these long-lived populations can survive under normal circumstances for several months in the absence of influx from the bone marrow (31). Based on the slow decline of the MZB (Figs. 3B and 4A, and data not shown) and their specific location, it could be anticipated that MZB are more important for the marginal zone than follicular B cells. However, the fact that Pyk-2−/− mice, which lack only the MZB and not the follicular B cell population, express normal MMM and MZM subsets (32) indicates that the presence of these macrophages is not (solely) dependent on MZB, but also on follicular and recirculating B cells.

The dependency of the marginal zone on the presence of B cells is most likely associated with the TNF family member LT. Mice deficient not only for the expression of LTα or LTβ, but also for the LTβ receptor, are not only exhibiting defects in splenic organization, such as nonsegregated T/B cell areas and absence of germinal centers and FDC, but also completely lack all cells normally present in the marginal zone (21, 33–36). Additionally, inhibition of LTβ receptor signaling in adult mice also leads to loss of a well-developed marginal zone (28). Strong evidence for the importance of LT on B cells during ontogeny comes from a recent study in which the LTβ gene was specifically deleted from B cells, resulting in a defective marginal zone (37). In our study, we found that B cells from CD70TG mice expressed normal levels of LTα1β2 at all ages and were able to up-regulate it upon stimulation (Fig. 5). Therefore, we conclude that CD70TG mice lost their most important source of LTα1β2 when B cells were depleted, which consequently resulted in loss of the marginal zone. This was supported by the observation that the last few MPM present in older CD70TG mice were always found adjacent to residual B cell clusters (Fig. 4A).

Although it is not yet known how LTα1β2 expressing B cells interact with cells in the marginal zone to preserve its integrity, local stromal cells could be involved in this process. This is in accordance with the development and maintenance of T and B cell areas, where it has been shown that interaction of LTα1β2 on B cells with the LTβ receptor on stromal cells in the white pulp is required for normal production by these cells of lymphocyte-specific chemokines, such as CC chemokine ligand (CCL)19, CCL21, and CXC chemokine ligand (CXCL)13 (38–41). In splenic sections from 12-wk-old CD70TG mice, we could still detect significant protein expression for both CCL21 and CXCL13 (data not shown), which indicates that, once the production of these chemokines has been established, B cell-derived LTα1β2 is no longer required for the maintenance of their expression. This is consistent with previous experiments that showed that CCL21 and CXCL13 were still expressed in spleens of adult WT mice that had received bone marrow from LTα−/− mice (40).

Although CCL21 and CXCL13 are not involved in the micro-architecture of the marginal zone, it could be that a similar process underlies the formation, as well as the maintenance, of the marginal zone: interaction of LTαβ-expressing B cells with local stromal cells could elicit the production of marginal zone-specific chemokines, to attract specific macrophages or their precursors. Alternatively, stromal cells could produce growth factors that influence the final maturation of macrophage precursors locally in the marginal zone, although a direct cell-cell contact between macrophages and B cells might also be of critical importance. Further research will be required to address these questions.

As our study indicates that B cells are important for the maintenance of the marginal zone, similar defects could be expected in other mouse models in which gradual B cell depletion has been reported. Mice that overexpress costimulatory molecules, such as CD80 (42, 43), CD86 (43, 44), and CD137L (45), are of special interest in this respect, because they resemble the phenotype of CD70TG mice. In addition, loss of B cells was also manifest in mice that overexpress IFN-γ (46) as well as in SOCS-1−/− mice, which are hyperresponsive to this cytokine (47). Detailed information on the condition of the marginal zone in these mice could potentially contribute to the understanding of their phenotype.

The splenic phenotype of CD70TG mice illustrates that the marginal zone is susceptible to major changes in lymphocyte number and their activation state. As this compartment gives the spleen its important function in host defense against bacterial infections, this implies that impaired immune responses during B cell deficiencies could also be the result of lacking populations of phagocytic cells. Indeed, we demonstrated that mice that have lost most B cells and as a consequence also their marginal zone, are unable to take up blood-borne neutral polysaccharides (Fig. 4B), which serve as model Ags for bacterial capsules. Furthermore, it has also been reported that lack of mature B cells and macrophages in the marginal zone correlates with susceptibility to viral infections (48, 49), because these macrophages are capable of controlling virus spread during the acute phase (50). In this respect, our findings may also be important from a clinical point of view. It can be anticipated that treatment of B cell lymphomas (51) or autoimmune disorders like rheumatoid arthritis (52) with B cell-specific mAbs has destructive side effects for the architecture of lymphoid organs. Therefore, it should be taken into consideration that B cell-ablative therapy could have additional disadvantages for the antipathogenic defense mechanism of the patient.

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References

27. van den Eertwegh, A. J., J. D. Laman, M. M. Schellekens, W. J. Boersma, and
22. Kitamura, D., J. Roes, R. Ku
21. De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan,¨
27. van den Eertwegh, A. J., J. D. Laman, M. M. Schellekens, W. J. Boersma, and
22. Kitamura, D., J. Roes, R. Ku
21. De Togni, P., J. Goellner, N. H. Ruddle, P. R. Streeter, A. Fick, S. Mariathasan,¨
20. Alexander, W. S., R. Starr, J. E. Fenner, C. L. Scott, E. Handman, N. S. Sprigg,
17. Crawley, M., T. C. Reilly, and D. Lo. 1999. Influence of lymphocytes on the
12. Loder, F., B. Matschier, R. J. C. P. Pauger, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1998. B cell development in the spleen takes place in
discrete steps and is determined by the quality of B cell receptor-derived signals. J. Exp. Med. 190:75.