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Somatically Diversified and Proliferating Transitional B Cells: Implications for Peripheral B Cell Homeostasis

Venkata A. Yeramilli and Katherine L. Knight

The peripheral B cell compartment in mice and humans is maintained by continuous production of transitional B cells in the bone marrow. In other species, however, including rabbits, B lymphopoiesis in the bone marrow abates early in life, and it is unclear how the peripheral B cell compartment is maintained. We identified transitional B cells in rabbits and classified them into T1 (CD24<sup>high</sup> CD21<sup>low</sup>) and T2 (CD24<sup>high</sup>CD21<sup>+</sup>) B cell subsets. By neutralizing B cell-activating factor in vivo, we found an arrest in peripheral B cell development at the T1 B cell stage. Surprisingly, T1 B cells were present in peripheral blood, and spleen of adult rabbits, long after B lymphopoiesis was arrested. T1 B cells were distinct from their counterparts in other species because they are proliferating and the Ig genes are somatically diversified. We designate these newly described cells as T1d B cells and propose a model in which they develop in GALT, self renew, continuously differentiate into mature B cells, and thereby maintain peripheral B cell homeostasis in adults in the absence of B lymphopoiesis. The Journal of Immunology, 2011, 186: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Ad, adenoviral/adenovirus; Apx, appendix; BAFF, B cell-activating factor; BGG, bovine γ globulin; BM, bone marrow; CR2, complement receptor 2; CVF, cervine venom factor; GC, germinal center; MLN, mesenteric lymph node; PB, peripheral blood; PP, Peyer’s patch; rBAFF, recombinant BAFF; SR, sacculus rotundus; T1, transitional type 1; T2, transitional type 2; TACI, transmembrane activator calcium modulator and cyclophilin ligand interactor; Tg, transgenic.

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cloned into the CMV-shuttle vector in frame with rabbit Fc receptor 2 (CR2) was PCR amplified from appendix (Apx) cDNA. CD21low/CD21+ or IgMlow/IgM+.

All flow cytometry data were acquired and no histology were obtained from rabbits previously described (21). The germ-free Apx tissues used for immunohistochemistry were obtained from rabbits provided by Dr. Malavasi, University of Turin, Turin, Italy. Following CFSE staining, the final contents were flushed from rabbit Apx with 1 ml PBS

class II (clone 2C4; BD Biosciences), FITC anti-rabbit C3 (Southern Biotechnology Associates, Birmingham, AL), Dylight 649 goat Fab anti-mouse IgG, and streptavidin PE/allophycocyanin (Jackson Immunoresearch Laboratories, West Grove, PA).

Rabbit recombinant BAFF (rBAFF) (20) was biotinylated using NHS-LC biotin (Pierce Biochemicals, Rockford, IL).

Cobra venom factor (CVF) was obtained from Calbiochem (San Diego, CA).

Recombinant adenosine

Adenoviral (Ad) constructs expressing transmembrane activator calcium modulus and cyclophilin ligand interactor (TACI)-Ig (extracellular portion of human TACI fused to human Fc) and mouse Fc (as control) were provided by T. Zhou (University of Alabama, Birmingham, AL). Rabbit CTLA4-Ig (provided by T. Zhou, University of Alabama, Birmingham, AL) was subcloned into CMV-shuttle vector (Invitrogen, Carlsbad, CA). For constructing CD40-Ig, the extracellular portion of human CD40 was PCR amplified from Raji cDNA (OS HuCD40XhoI, 5′-ACTGGAGGCCGCCACCATGGGCGCCGCG-3′ and ASrCD21Bam, 5′-TGGATCCCCCTTCATTGCAAGAAATGTT-3′) and cloned into the CMV-shuttle vector in frame with rabbit Fcy. Similarly, the extracellular portion of rabbit complement receptor 2 (CR2) was PCR amplified from appendix (Apx) cDNA (OsCD21XhoI, 5′-ACTGGAGGCCGCCACCATGGGCGCCGCG-3′ and ASrCD21Bam, 5′-TGGATCCCCCTTCATTGCAAGAAATGTT-3′) and cloned into the CMV-shuttle vector in frame with rabbit Fcy. Following homologous recombination and integration of CTLA4-Ig, CD40-Ig, or CR2-Ig into the Ad genome, we selected recombinant clones (Invitrogen) and Ad constructs were transfected into QBI-293A cells (Qbiogene, Carlsbad, CA). Viral particles were purified on cesium chloride gradients, titered (Qbiogene), and stored at −80 °C. Recombinant viral particles (10^{10} in 0.3 ml PBS) were injected i.p. into rabbit pups within 48 h of birth, and the rabbits were sacrificed 7–10 d later.

Flow cytometry and immunohistochemistry

For analysis of transitional B cells, multicolor flow cytometry (three, four, or five color) was performed by gating on CD24^{hi} cells that were either CD21^{lo}/CD21^{hi} or IgM^{hi}/IgM^{lo}. All flow cytometry data were acquired using FACSCanto or FACSCanto II or FACSaria (BD Biosciences), gated on live lymphocyte-sized cells on the basis of forward and side scatter, and analyzed using FlowJo software (Treestar, Ashland, OR). All FACS plots using fluorescent reagents are depicted on a logarithmetic scale, except where indicated. For flow cytometric analysis of commensal bacteria, luminal contents were flushed from rabbit Apx with 1× PBS and 5% FCS (FACS buffer), and after debris removal, bacteria were pelleted and resuspended in buffer. Bacteria (25–50 μl; representing a pellet of ~1–2 mm^3) were stained with the appropriate Ab and analyzed, as described above. For immunohistochemistry, acetone-fixed cryosections (7–8 μm) were blocked with goat serum and then stained with primary Ab (Table 1) and the following indirect reagents: Cy2- or Cy3-conjugated streptavidin and Cy2- or Dylight 549-conjugated goat Fab (Fab) anti-mouse IgG (Jackson Immunoresearch Laboratories). Slides were viewed under a Leica DM IRB microscope (Leica Microsystems, Bannockburn, IL), and images were captured using the MagnaFire 2.1c digital camera system (Optronics, Goleta, CA); grayscale images were edited using ImageJ software (National Institutes of Health, Bethesda, MD). The germfree Apx tissues used for immunohistochemistry were obtained from rabbits previously described (21).

Nucleotide sequence analysis of V 

Ig VH VDJ genes from genomic DNA of sorted single splenic and Apx T1 B cells from two αδαδ homozygous rabbit (1.8 y of age) were amplified by nested PCR.

First-round PCR was performed using the following primer pairs: forward, 5′-CTCTGGACAGGAGGCCTC-3′ and reverse, 5′-AGTTGAGCTACGAGAAGAGAG-3′. Aliquots (2 μl) of first-round PCR products were used as template for the second-round PCR using primers, as follows: forward, 5′-CTACCTCATGGAGACT-3′ and reverse, 5′-GAGTGTCGAAGAACCCT-3′. Products from the second-round PCR were directly sequenced, and the frequency of VH mutations was determined by comparing sequences to germline VH genes.

Immunization and ELISA

To determine the efficacy of CTLA4-Ig in vivo, rabbits neonatally injected with Ad-CTLA4-Ig were reinjected with Ad-CTLA4-Ig at 2 and 5 wk of age, and 3 d after the 2 wk injection, they received 0.5 mg bovine γ globulin (BGG) in CFA (s.c.). After the 5-wk injection of Ad-CTLA4-Ig, rabbits received a secondary immunization of 0.5 mg BGG in IFA. Serum was harvested 7 d after the primary and 10 d after the secondary immunization, and anti-BGG IgM and IgG levels were determined by ELISA using anti-rabbit IgM (clone 367; BD Biosciences)- or anti-rabbit IgG (clone 359; BD Biosciences)-coated microtiter plates. The ELISA was developed with goat anti-rabbit H&L chain-HRP (Jackson Immunoresearch Laboratories) plus ABTS (Sigma-Aldrich, St. Louis, MO) as substrate. The relative levels of serum IgM and IgG in Ad-CTLA4-Ig–treated and control PBS-treated rabbits were determined from a linear portion of the dilution curves.

Results

Identification of transitional B cell subsets

To identify B cell subpopulations in adult rabbits, we stained B cells from different tissues with Abs used to delineate immature B cell subsets in mice and humans. Because human CD24 and its murine homolog, heat-stable Ag, are expressed early in B cell development on both BM B cell progenitors and transitional B cells, but are downregulated on mature B cells (2–4, 22), we tested whether anti-CD24 can be used to identify transitional B cells in rabbits. Using anti-CD21 and anti-CD24 mAbs, we identified two subsets of CD24^{hi} B cells in the spleen (CD21^{lo} and CD21^{hi}), which we henceforth refer to as T1 and T2 B cells, respectively, and a CD24^{dim} subset, designated mature (M) B cells (Fig. 1A, upper). T1 B cells were IgM^{lo}CD62L^{lo}, whereas both T2 and mature B cells had higher levels of CD21, surface IgM, and CD62L expression (Fig. 1A, upper). CD23 was expressed at similar levels on both T1 and T2 B cells (Fig. 1A, upper), and thus, did not serve as a useful marker to distinguish between these B cell subsets. To determine whether these transitional B cells share features with human transitional B cells, which are broadly defined as CD24^{hi}/CD38^{hi}CD10^{hi}/CD20^{hi} (23), we analyzed the CD24^{hi} cells for these markers and found that the T1 B cells were CD24^{hi}/CD38^{hi}, whereas the T2 B cells were CD10^{hi}/CD20^{hi} (Fig. 1A, upper). Interestingly, CD20 was expressed on essentially all of the T1 and T2 cells, but not on the mature B cells, and thus serves as a unique marker to identify transitional B cells (Fig. 1A, lower). Furthermore, T1 and T2 B cells expressed high levels of CD90 (Fig. 1A, lower), a phenotype shared with rat immature B cells (24). Unlike in spleen, we observed only a single
subset of CD24\textsuperscript{high} cells in the peripheral blood (PB), and these had a lower expression of CD21 compared with mature B cells (Fig. 1B). These cells were IgM\textsuperscript{low}CD62L\textsuperscript{low} (Fig. 1B), suggesting that PB contains only a T1-like population of transitional B cells. We observed a similar T1-like CD24\textsuperscript{high}CD21\textsuperscript{low} subset in GALT (Appendix, sacculus rotundus [SR], Peyer’s patch [PP], and mesenteric lymph node [MLN]) (Fig. 1C). Taken together, these results demonstrate that in adult rabbits, immature B cells can be phenotypically delineated into two transitional B cell subsets, T1 and T2. The frequencies of these cells in different tissues are shown in Table II.

**Functional analysis of transitional B cells**

In vivo, murine transitional B cells require BAFF for maturation into B cells, and in the absence of BAFF, peripheral B cell development is blocked at the T1 stage (6). We investigated the role of BAFF in rabbit peripheral B cell development by neutralizing BAFF in vivo. Newborns were injected with a soluble decoy receptor (TACI-Ig) and we found a dramatic decrease in splenic T2 and mature B cells, whereas the T1 B cell population remained intact (Fig. 2A, upper right). Similarly, in the Apx, the mature B cell population was eliminated by neutralization of BAFF, but the CD24\textsuperscript{high} transitional B cell population was not reduced, and instead appeared to accumulate (Fig. 2A, lower right). These data indicate that T2 and mature B cells, but not T1 B cells, require BAFF for survival and/or maintenance. Because T1 cells are the earliest B cell precursors in the periphery, we focused our studies on T1 B cells.

### Table II. Frequency of transitional B cells in adult rabbit tissues

<table>
<thead>
<tr>
<th>Tissue\textsuperscript{a}</th>
<th>T1\textsuperscript{b}</th>
<th>T2\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen (11)</td>
<td>7.2 ± 2</td>
<td>13.6 ± 2.3</td>
</tr>
<tr>
<td>Blood (6)</td>
<td>2.9 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Appendix (6)</td>
<td>2 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>Sacculus rotundus (3)</td>
<td>3.8 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Mesenteric lymph node (4)</td>
<td>3.7 ± 0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Peyer’s patch (2)</td>
<td>9.8 ± 3.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The number in parentheses indicates the number of rabbits analyzed.

\textsuperscript{b}ND, not detectable; T1, CD24\textsuperscript{high}CD21\textsuperscript{low}CD62L\textsuperscript{low}CD10\textsuperscript{low}CD38\textsuperscript{low}; T2, CD24\textsuperscript{high}CD21\textsuperscript{low}CD62L\textsuperscript{low}CD10\textsuperscript{high}CD38\textsuperscript{low}.

Soluble rBAFF binds to most freshly isolated murine B cells (25), but not to most B cells in rabbit due to occupied receptors (20). Instead, in rabbits, rBAFF binds to a small subset of IgM\textsuperscript{low} cells in spleen, Apx, and PB, which we previously described as putative transitional B cells (20). In this study, we show that these B cell subsets are CD24\textsuperscript{+} B cells (Fig. 2B) and that all T1 B cells in the spleen and Apx are Ki-67\textsuperscript{+} (Fig. 2C), indicating that they are proliferating.

To further characterize T1 B cells, we tested how they responded to anti-Ig stimulation in vitro. Following anti-Ig treatment of sorted splenic T1 and mature B cells, T1 cells underwent apoptosis, whereas the mature B cells did not (Fig. 2D). We also tested whether the Ig genes in T1 B cells were somatically diversified. Murine transitional B cells are constantly replenished from the BM and consequently have unmutated Ig genes even in adults (26). Because new B cells are not made in BM of adult rabbits (18, 19), we predicted that the T1 B cells would be diversified. We isolated T1 B cells from spleen and Apx of adult rabbits, and PCR amplified and sequenced the Ig VDJ genes. As expected, we found the IgH genes had undergone somatic diversification (Fig. 2E, Supplemental Fig. 1), suggesting that the B cells had been through a germline (GC)-like reaction and were not recent emigrants from the BM.

### Tissue localization of transitional B cells

To localize transitional B cells in tissues, we performed immunohistology on tissue sections. Because anti-CD24 did not stain frozen tissue sections effectively, we used anti-CD20, which binds all CD24\textsuperscript{+} B cells both in the spleen (Fig. 1A, lower) and Apx (Fig. 3A, upper), but does not bind mature B cells or non-B cells (Fig. 3A, lower). By using anti-CD23 to label the follicular zone, we found that CD20\textsuperscript{+} transitional B cells in spleen were located near the margins of the follicles and also in the red pulp (Fig. 3B). These data are similar to the localization of splenic transitional B cells in mouse (2). We also identified T1 B cells (CD24\textsuperscript{high}CD21\textsuperscript{low}IgM\textsuperscript{low}CD62L\textsuperscript{low}) in the BM of young rabbits (Fig. 4A) and a CD24\textsuperscript{low}CD21\textsuperscript{low} population that we thought might include proB and preB cells (arrow in Fig. 4A). To test whether proB and preB cells are CD24\textsuperscript{+}, we stained BM cells for MHC II and cytoplasmic IgM (19) and found that proB cells and also cells in the preB and B cell gate (presumably preB cells) were CD24\textsuperscript{+}. 

![Flow cytometric identification of transitional B cell subsets.](http://www.jimmunol.org/)

**FIGURE 1.** Flow cytometric identification of transitional B cell subsets. A, Staining of splenic B cells for T1 (CD24\textsuperscript{high}CD21\textsuperscript{low}) (dashed), T2 (CD24\textsuperscript{high}CD21\textsuperscript{low}), and mature (M) (CD24\textsuperscript{low}CD21\textsuperscript{low}) (black) for IgM, CD62L, CD23, CD10, CD38, CD20, and CD90. B, Staining of PB T1 (dashed) and M (black) for IgM and CD62L. C, IgM\textsuperscript{+} B cells from Apx, SR, PP, and MLN stained for CD24 and CD21. The gray-filled histograms in A and B represent staining with an appropriate isotype control mAb. The plots are representative of staining obtained from at least three rabbits.
During the time of ongoing B lymphopoiesis in young rabbits, B cells leave the BM and migrate to GALT, where they undergo extensive proliferative expansion and somatic diversification of Ig genes (28, 29). To study the early stages of peripheral B cell development in rabbits, we examined the Apx from an IgH transgenic (Tg) rabbit. Using flow cytometry, we found luminal bacteria were characterized by increased expression of the complement C3 (Fig. 5B). These findings prompted us to investigate whether complement plays a role in promoting the proliferative expansion of B cells in GALT. To inhibit signaling via CR2/CD21, we injected newborn rabbits with a rAd expressing soluble CD21 (CD21-Ig) and analyzed the Apx by immunohistochemistry after 7–10 d. Upon CD21-Ig treatment, we found little to no Ki-67 expression in the Apx (Fig. 5C), indicating that signaling through C3 is required for B cell expansion in GALT. Additionally, we depleted C3 in vivo by i.p. injection of CVF (0.5 mg/kg body weight) 24 and 48 h after birth. Rabbits were sacrificed at 6–7 d of age, and we found, similar to CD21-Ig treatment, B cell proliferation in the Apx was inhibited (Fig. 5C). We conclude that complement is required for B cell proliferation in GALT.

Role of complement in the proliferative expansion of Apx B cells

To determine whether T cell help is required for the proliferative expansion of B cells, we inhibited T cell activation by injecting newborn rabbits with a rAd expressing soluble CTLA4 (CTLA4-
After 7–10 d, we analyzed the Apx by immunohistochemistry for the presence of follicles with proliferating (Ki-67+) B cells and found that CTLA4-Ig did not inhibit B cell proliferation (Fig. 5D). To confirm that the absence of a phenotype in the CTLA4-Ig–injected rabbits was not due to insufficient or nonfunctional CTLA4-Ig, we immunized two rabbits with a T-dependent Ag (BGG) and found, as expected, a dramatic reduction in both primary IgM and secondary IgG (anti-BGG) Ab titers (Fig. 5E), indicating that CTLA4-Ig was functional in vivo.

To determine whether CD40–CD40L interaction is required for the proliferative expansion of B cells in the Apx, we injected newborn rabbits with rAd-expressing soluble CD40 (CD40-Ig) and found that it inhibited B cell proliferation in the Apx (Fig. 5D). We conclude that activation of T cells via the B7–CD28 pathway is not required for the proliferative expansion of B cells in GALT, whereas CD40–CD40L interaction is required.

Discussion

Studies in mice and humans indicate that transitional B cells play a key role in the peripheral stages of B cell development (4). However, in rabbits and other species (such as sheep, pigs, and cattle) that use GALT to develop their B cell repertoire (12–17), essentially nothing is known about transitional-like B cells and their development. Due to the paucity of rabbit-specific Abs, we used cross-reactive Abs to identify subsets of rabbit B cells. In general, the phenotype of transitional B cells in rabbit was more similar to transitional B cells in humans than in mice. Using anti-CD24 and anti-CD21 mAbs, we identified two subsets of CD24high transitional B cells: T1 (CD21low = T1; CD21+ = T2), as described recently in humans (11). Anti-CD23, which distinguishes T1 (CD23−) and T2 (CD23+) B cells (2, 5) in mice, but not humans (11), also did not distinguish rabbit T1 and T2 B cells. Like in humans, CD10 and CD38 can be used to identify transitional B cells. Another marker, CD20, is a pan human B cell marker and is expressed at high levels on transitional B cells (10, 23). Interestingly, in rabbits, CD20 is expressed only on transitional B cells; if CD20 is expressed on mature B cells, it is at a level below the limits of detection. As with all the other cross-reactive Abs used in this study, we cannot be certain that the Ags recognized by the Abs are the rabbit homologs of the respective mouse and human proteins. Nevertheless, these cross-reactive Abs provide a means to identify B cell subsets.

Functionally, transitional B cells in rabbits exhibit similarities with their counterparts in other species. Neutralization of BAFF in neonatal rabbits arrested B cell development at the T1 stage, confirming that T2 and mature B cells like in mice depend on BAFF.
for their development (33). Consistent with results in mice and humans (3, 10), T1 B cells underwent apoptosis upon anti-Ig stimulation. T1 B cells in rabbit also exhibited some unique characteristics. For example, essentially all T1 B cells in adult rabbits were proliferating in vivo and had somatically diversified Ig genes. A diversified repertoire indicates that these cells had undergone a GC-like reaction and were not recent emigrants from the BM. The presence of diversified T1 B cells in adults, long after the arrest of B lymphopoiesis, suggests that T1 B cells are maintained in the periphery, possibly because they are long-lived and/or self-renewing.

B cell maturation in the mouse proceeds in a T1 → T2 → M pathway (2). It remains to be determined whether rabbit T1 B cells give rise to T2 and mature B cells in a BAFF-dependent manner. Mature and transitional (T1 and T2) B cell subsets were readily detected in the spleen of neonatal rabbits, with transitional B cells being present at the frequencies similar to those in adults. In contrast, in neonatal mice, B cells in the periphery are mostly HSA^{high} immature/T1 B cells, and reach adult levels in the spleen (5–10% of all B cells) only after 6–8 wk of age (2, 3, 26). Because B lymphopoiesis occurs only early in life, rabbits may have evolved a strategy to rapidly differentiate their immature B cells and generate a functional B cell compartment before the arrest of B lymphopoiesis.

During development, B cells leave the BM and migrate to GALT, where they expand in numbers and somatically diversify the Ig genes (29). Using an IgH Tg rabbit, which was B cell deficient at birth and in which few B cells accumulated over a span of several months (30), we found that the first B cells to appear in GALT were CD20^{+} transitional B cells. These cells were localized predominantly in the domes and villi. A similar distribution of transitional B cells was found in conventional neonatal rabbits, suggesting that CD20^{+} transitional B cells first migrate to the domes and villous regions of GALT before differentiating into mature follicular B cells.

What could be the significance of this unique pattern of localization of transitional B cells in the domes and villi of GALT? B cells in the domes are in close proximity to M cells, which are known to translocate bacteria and sample luminal Ags (34). The T1 B cells in the domes and villi may interact directly with commensal bacteria or with bacterial-derived products and promote further differentiation of transitional B cells into mature B cells. In support of this idea, rabbits, which had either limited or no microbiota in the Apx, had reduced numbers of peripheral B cells (31). In germfree mice, the number of mature B cells is strongly reduced; the number of T2 B cells was reportedly normal, indicating that commensal bacteria are required for the development of transitional B cells into mature B cells (2).

Following the appearance of T1 B cells in the Apx, organized follicles with proliferating B cells begin to form. These follicular B cells most likely arise from the incoming T1 B cells and expand to form GC-like structures, where they somatically diversify the Ig genes. We think that these processes occur in an Ag- and T cell-independent manner because interference of B7–CD28 costimulation by CTLA4-Ig did not inhibit B cell proliferation. However, CD40–CD40L interactions are required for B cell proliferation. Although the source of CD40L would be expected to be activated T cells, we predict that one of several cell types, such as dendritic cells, macrophages, NK cells, and epithelial cells, all of which can express CD40L is the source of CD40L (35). We predict...
this because we found CD40L transcripts and protein localized throughout the Apx tissue, rather than limited to the T cell areas (V. Yeramilli and K. Knight, unpublished observations).

By inhibiting the interaction of CD21 and its ligand (CD21L), and also by depleting C3, we found that complement is required for B cell expansion in GALT. Many commensal bacteria are coated with C3 and IgA, and it may be that immune complexes of IgA and microbial Ags are trapped by follicular dendritic cells and presented to B cells, resulting in stimulation through cross-linking of BCR and its coreceptor, CD21. This possibility is suggested by the presence of C3 in the Apx follicles, which could be due to deposition of complement C3-containing fragments on follicular dendritic cells and/or B cells.

**Transitional B cells in adult rabbits: implications for peripheral B cell maintenance**

We designate the transitional B cells in adult rabbits as T1d B cells because the Ig genes are somatically diversified. These T1d cells are most likely generated in GALT during the first few weeks of life, a time during which the Ig genes of essentially all B cells somatically diversify (12, 18). Following the arrest of B lymphopoiesis a few weeks after birth, the T1d B cells, which are proliferating, are most likely maintained by self-renewal. In the absence of new B cell formation in the BM after 2–4 mo of age, we propose that it is the self-renewing T1d B cells that are responsible for maintaining the peripheral B cell compartment. We think the self-renewing T1d B cells in adults continuously develop into mature B cells in a BAFF-dependent manner, and thereby maintain B cell homeostasis, although we cannot rule out the possibility that a few B cells are generated in the BM in adults under conditions such as infection and inflammation (36–38).

Moreover, the BAFF receptors of most mature B cells are occupied by endogenous BAFF (20), and we think that this chronic occupancy of BAFF receptor(s) allows them to remain long-lived by providing a tonic and/or survival signal (20). Additionally, IL7RI, a novel isoform of IL-7, may provide a survival signal to mature B cells (39). Thus, we think that together T1d B cells and long-lived mature B cells regulate peripheral B cell homeostasis in adult rabbits.

**Model of T1d B cell development and maintenance**

Based on our current and previous findings, we propose a model (Fig. 6A) in which CD24<sup>high</sup> immature B cells exit the BM early in ontogeny as transitional (T1) B cells (IgM<sup>low</sup>CD21<sup>low</sup>CD62L<sup>low</sup>) and traffic to GALT (Apx), where they enter the dences and villi (Fig. 6A, inset). In this article, (after birth) they interact with commensal bacteria or bacterial-derived products, such as superantigens (40) and become activated. Following activation, T1 B cells proliferate and somatically diversify the Ig genes to become T1d B cells. The follicular B cells, which early in ontogeny are most likely derived from BAFF-stimulated BM-T1 (or T1 B cells in GALT), expand in a B7-CD28–independent, CD40-CD40L–, and CD21-CD21L–dependent manner to form GC-like structures. We propose that the follicular B cells as well as T1d B cells leave the Apx and seed other peripheral tissues and, upon BAFF stimulation, the T1d B cells give rise to mature B cells. It may well be that some of the BM-derived T1 B cells also directly traffic from BM to other sites, such as spleen, and develop into mature B cells. We propose that in adults, in the absence of newly-formed B cells from the BM, the T1d B cells that are presumably maintained through self-renewal continually develop into mature B cells and thus maintain peripheral B cell homeostasis (Fig. 6B, left). Mature B cells have occupied BAFF receptors (20), and we propose that BAFF provides tonic signaling to these B cells and thereby also contributes to B cell homeostasis (Fig. 6B, right).

**FIGURE 6.** Model of T1d B cell development and maintenance. A. Development of T1d B cells. T1 B cells leave the BM and enter GALT (Apx) through the high endothelial venules and traffic to the dences and villi, where they are stimulated by BAFF and commensal bacteria or bacterial-derived products. The activated T1 B cells proliferate and somatically diversify the Ig genes to become T1d B cells. The follicular B cells, derived from BAFF-stimulated BM-T1 or GALT T1 B cells, undergo a proliferative expansion to form organized follicles in a CR2-CR2L– and CD40-CD40L–dependent manner. After undergoing the GALT GC-like reaction, mature and T1d B cells enter the circulation where T1d B cells further differentiate into mature B cells. Some of the GALT-derived T1d B cells traffic to the spleen and differentiate into T2 and mature B cell subsets. Alternatively, some T1 B cells from the BM may directly traffic to the spleen and develop into mature B cells (data not shown). B. Maintenance of peripheral B cells. In adult rabbits, in the absence of ongoing lymphopoiesis, the B cell compartment is maintained by the proliferating T1d B cells, which self renew and continually differentiate into mature B cells. Additionally, the BAFF receptor(s) on mature B cells in the periphery is bound by endogenous BAFF, and this chronic engagement of BAFF receptors may provide a tonic/survival signal for the B cells to remain long-lived.

Our model leads one to ask the question how and where T1d B cells mature into B cells; how this is regulated; how are T1d B cells maintained; and if T1d cells participated in GC reactions, as evidenced by somatic diversification of the Ig genes, then how do they remain transitional-like B cells that proliferate and yet die in response to BCR stimulation? As reviewed by Alitheen et al. (41), different species have distinct strategies to maintain the B cell compartment, and rabbits may have evolved a new B cell type for this purpose. Alternatively, T1d-like B cells may be present in most mammals, but have not yet been identified. The finding that essentially all T1d B cells are proliferating may suggest that they are constantly being stimulated, perhaps because they have
a restricted repertoire to specific bacterial and/or self-Ags and that continuous stimulation by these Ags promotes self-renewal, thereby maintaining a constant source of transitional B cells in the absence of ongoing B lymphopoesis. Experiments to address these questions will elucidate some of the mechanisms by which rabbits and presumably other species develop and maintain their B cell compartment.

In summary, our study is the first, to our knowledge, to characterize transitional B cells in rabbit and, by extension, in mammals that use GALT to develop their B cell repertoire. Remarkably, transitional B cells are maintained in the periphery of adult rabbits when there is no evidence for ongoing lymphopoesis in the BM. The finding(s) that these cells have a diversified repertoire and are undergoing proliferation confirms that these cells are not newly made, and instead, leads to the idea that the T1d B cells are maintained by self-renewal and are responsible for maintaining the B cell compartment in the absence of detectable B lymphopoesis. Many of the markers expressed on rabbit transitional B cells are also found on human transitional B cells, suggesting that rabbits can be used as a model to study human B cell biology. Finally, we suggest that similar to rabbits, subsets of human transitional B cells may contribute to the maintenance of peripheral B cells as B lymphopoesis in the BM decreases in the elderly.

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Disclosures
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