Peripheral B Cell Homeostasis

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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 (CD24highCD21low) and T2 (CD24highCD21+) 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 normal B lymphopoiesis and spleen of adult rabbits. After B lymphopoiesis was arrested, T1 B cells differentiated into 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: 6437–6444.

During B cell development, immature B cells in the bone marrow (BM), designated transitional B cells, exit the BM and migrate to the spleen, where they develop into mature B cells (1, 2). Transitional B cells are identified by several cell surface markers expressed on newly formed B cells in the BM. One such marker, CD24, is expressed at high levels on both human and murine transitional B cells and is downregulated on mature B cells (3, 4). Using CD24 and AA4 (C1qR), several subsets of transitional B cells have been identified in mice. Loder et al. (2) classified the CD24high transitional B cells into two stages, as follows: transitional type 1 (T1) and type 2 (T2) based on the differential expression of CD21, CD23, and IgD. Using AA4, a type I transmembrane protein, Allman et al. (5) identified three populations of transitional B cells, as follows: AA4+CD23−IgMlow (T1), AA4−CD23+IgMhigh (T2), and AA4−CD23−IgMlow (transitional type 3). Adoptive transfer experiments revealed that T1 cells give rise to T2 and mature B cells (2), and Schieman et al. (6) demonstrated that this maturation is dependent on B cell-activating factor (BAFF). T1 B cells are found in the BM, blood, and spleen, whereas T2 B cells are restricted to spleen (7). Transitional B cell subsets exhibit distinct functional characteristics. For example, T2 B cells proliferate upon BCR cross-linking, whereas T1 B cells die (7). Transitional B cells in humans are largely described as a single subset that is CD10+ (8) or CD24++CD38++ (4), although some investigators have classified these cells as T1-like and T2-like based on the differential expression of CD24 and CD38 (9) or IgD and CD38 (10). Recently, Suryani et al. (11), using CD21 as a marker, identified two transitional B cell subsets (CD21low and CD21high) in peripheral blood and demonstrated that the CD21low subset is the precursor to the CD21high B cells.

Transitional B cells mark a crucial link between immature BM B cells and mature peripheral B cells. Whereas many studies of peripheral B cell development have been performed in mice and humans, essentially no such studies are available in rabbits or other mammals that use GALT for B cell expansion and somatic diversification of Ig genes (12–17). The mechanism by which B cells undergo proliferative expansion in GALT is not known. Furthermore, in rabbits, and most likely in other species, B lymphopoiesis in primary lymphoid organs abates early in life (18, 19), and it is unclear how the peripheral B cell compartment is maintained in the absence of ongoing B lymphopoiesis. Weill and Reynaud (14) proposed that the GALT-derived B cells in these species might serve as transitional-like B cells.

In this study, we used several cross-reactive Abs to identify transitional B cells (T1 and T2) in rabbit. Using anti-CD24 and anti-CD21 mAb, we identified transitional B cell subsets in blood, spleen, and GALT of adult rabbits, long after the arrest of B lymphopoiesis in the BM. Using soluble decoy receptors that inhibited cell–cell and cell–cytokine interactions, we identified several signals required for the proliferative expansion of B cells in GALT. We describe a model for peripheral B cell development and maintenance in which proliferating and somatically diversified transitional B cells in adults develop in GALT, continuously differentiate into mature B cells, and thereby maintain peripheral B cell homeostasis.

Materials and Methods

Rabbits and reagents

Rabbits were from the colony maintained by K. L. Knight at Loyola University Chicago. Adult rabbits used in this study ranged from 4 mo to 2 y of age. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of Loyola University Chicago (Maywood, IL).

We tested commercially available Abs for cross-reactivity to rabbit B lineage cells. A list of Abs that cross-reacted and were used in this study is shown in Table I. Rabbit-specific Abs and secondary reagents were as follows: anti-IgM (clone 367; BD Biosciences, San Jose, CA), anti-IgA...
Table I. Cross-reactive Abs used for flow cytometry and immunohistochemistry

<table>
<thead>
<tr>
<th>Ab</th>
<th>Specificity</th>
<th>Clone</th>
<th>Vendor</th>
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<tbody>
<tr>
<td>CD10</td>
<td>Human</td>
<td>CB-CALLA</td>
<td>eBiosciences, San Diego, CA</td>
</tr>
<tr>
<td>CD20</td>
<td>Human</td>
<td>B9E9</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA; Immunotech, Marseille Cedex, France</td>
</tr>
<tr>
<td>CD21</td>
<td>Human</td>
<td>BL13</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD23</td>
<td>Human</td>
<td>HP2</td>
<td>Immunotech</td>
</tr>
<tr>
<td>CD24</td>
<td>Mouse</td>
<td>M1/169</td>
<td>eBiosciences; BD Biosciences, San Jose, CA</td>
</tr>
<tr>
<td>CD38</td>
<td>Human</td>
<td>IB6</td>
<td>Miltenyi Biotec, Auburn, CA; also provided by Dr. Malavasi, University of Turin, Turin, Italy</td>
</tr>
<tr>
<td>CD62L</td>
<td>Human</td>
<td>LAM-1</td>
<td>Provided by Dr. Tedder, Duke University, Durham, NC</td>
</tr>
<tr>
<td>CD90</td>
<td>Human</td>
<td>SE10</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Human</td>
<td>B56</td>
<td>BD Biosciences</td>
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Recombinant adenovirus

Adenoviral (Ad) constructs expressing transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI)-Ig (extracellular portion of human TACI fused to human Fcy) and mouse Fcy (as control) were provided by T. Zhou (University of Alabama, Birmingham, AL). Rabbit CTLA4-Ig (provided by D. Dicker, University of California) was subcloned into CMV-shuttle vector (Invitrogen, Carlsbad, CA). For constructing CD40-Ig, the extracellular portion of human protein CD40 was PCR amplified from Raji cDNA (OS HuCd40XhoI, 5’- ACTGCAGACCATGTTCTGTCG-CTCTGCAG-3’ and AS HuCd40BamHI, 5’- TGATCCGACCCAGCCTGG-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 (Osr Cd21XhoI, 5’- ACTGCAGACCATGTTCTGTCG-CTCTGCAG-3’ and ASr Cd21BamHI, 5’- TGATCCGACCCAGCCTGG-3’) and cloned into the CMV-shuttle vector in frame with rabbit Fcy. Following homologous recombination and integration of Apx-CRD40-Ig, Apx-CRD2-Ig into the Ad genome, we selected recombinant clones (Invitrogen) and Ad constructs were transfected into QBI-293A cells (Qigene, Carlsbad, CA). Viral particles were purified on cesium chloride gradients, titered (Qigene), and stored at −80°C. Recombinant viral particles (1010 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 CD24high cells that were either CD21low/CD21+ or IgMlow/IgM+. All flow cytometry data were acquired with FACScanto or FACScantoII or FACSAria (BD Biosciences), gated on live lymphocyte-sized cells on the basis of forward and side scatter, and analyzed using FlowJo software (Tree Star, Ashland, OR). All FACS plots using fluorescent reagents are depicted on a logarithmic 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 mm3) 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 I) and the following indirect reagents: Cy2- or Cy3-conjugated streptavidin and Cy2- or Dylight 549-conjugated goat (Fab) anti-mouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Rabbit IggAFF (20) was biotinylated using NHS-LC biotin (Pierce Biochemicals, Rockford, IL).

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

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&K 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

Identified by CD24high cells and thus serves as a unique marker to identify transitional B cells. To determine whether these transitional B cells expressed similar levels on both T1 and T2 B cells (Fig. 1B), and thus, serves 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 CD24highCD38highCD1020high (23), we analyzed the CD24high cells for these markers and found that the T1 B cells were CD10highCD38high, whereas the T2 B cells were CD10highCD38high (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 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

NA-CTGAGGGGACCTGGGAGGCGG-3’ and ASr Cd21Bam, 5’- TGATCCGACCCAGCCTGG-3’ and reverse, 5’-TGGATCCCCGATCCTG-3’). First-round PCR was performed using the following primers: forward, 5’-CTCTGGCACAGGAGGC-3’ and reverse, 5’-AGTGGGAGAGAGAGA-3’. Aliquots (2 μl) of first-round PCR products were used as template for the second-round PCR using primers, as follows: forward, 5’-CAGTACCAGGGGCGG-3’ and reverse, 5’-GAGTGGGACCCAGCCTGG-3’.

One of the problems with these transitional B cells is that they are homogenous and cannot express a large number of immunoglobulin isotypes. Therefore, we conducted an analysis of the CD24high B cells in the spleen (CD24low and CD21+), which we henceforth refer to as T1 and T2 B cells, respectively, and a CD24+CD21+ subset, designated mature (M) B cells (Fig. 1A, upper). T1 B cells were IgMlow CD62Llow, 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 CD24highCD38highCD1020high (23), we analyzed the CD24high cells for these markers and found that the T1 B cells were CD10highCD38high, whereas the T2 B cells were CD10highCD38high (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 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 CD24high cells in the peripheral blood (PB), and these had a lower expression of CD21 compared with mature B cells (Fig. 1B). These cells were IgMlowCD62Llow (Fig. 1B), suggesting that PB contains only a T1-like population of transitional B cells. We observed a similar T1-like CD24highCD21low subset in GALT (Apx, 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 BAFF in vivo. Newborns were injected with a soluble decoy receptor (TACI-Ig) and we found a dramatic decrease in splenic T2 BAFF in vivo. Newborns were injected with a soluble decoy receptor (TACI-Ig) and we found a dramatic decrease in splenic T2 BAFF in vivo. Newborns were injected with a soluble decoy receptor (TACI-Ig) and we found a dramatic decrease in splenic T2 BAFF in vivo.

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 unmaturated 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 germinal center (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+ 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+ 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 (CD24high CD21lowIgMlowCD62Llow) in the BM of young rabbits (Fig. 4A) and a CD24lowCD21low population that we thought might include proB and preB cells (arrow in Fig. 4A). To test whether proB and preB cells are CD24+, 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+. 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 IgMlow cells in spleen, Apx, and PB, which we previously described as putative transitional B cells (20). In this study, we show that these BAFF-binding cells are CD24+ B cells (Fig. 2B) and that all T1 B cells in the spleen and Apx are Ki-67+ (Fig. 2C), indicating that they are proliferating.

![Flow cytometric identification of transitional B cell subsets.](image)

**FIGURE 1.** Flow cytometric identification of transitional B cell subsets. A, Staining of splenic B cells for T1 (CD24highCD21low) (dashed), T2 (CD24highCD21low) (gray), and mature (M) (CD24low/CD21+) (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+ 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.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>T1</th>
<th>T2</th>
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<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>Saccular 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>

*The number in parentheses indicates the number of rabbits analyzed. ND, not detectable; T1, CD24/CD21low/CD62Llow/CD10high/CD38low; T2, CD24high/CD21CD62L/CD10/CD38high.
FIGURE 2. Functional analysis of transitional B cells. A. Flow cytometric analysis of Apx and spleen cells from TACI-Ig–treated and control (Ig)-treated rabbits stained with anti-CD21 and anti-CD24 mAb. Flow cytometric analysis of Apx and spleen cells from conventional rabbits stained with B, anti-CD24 and soluble rBAFF and C. upper, anti-CD24 and anti-I-L chain and lower, anti-Ki67 (open histograms) of T1 cells (from upper diagram). Shaded histogram = isotype control. D. Flow cytometric analysis of sorted splenic T1 (CD24<sub>high</sub>CD21<sub>low</sub>) (upper) and mature B cells (CD24<sub>low</sub>CD21<sup>+</sup>) (lower) stained with annexin V and propidium iodide (PI) after 12–15 h in culture with anti-Ig (10 μg/ml) [goat F(ab′)] anti-rabbit IgG (H + L); Jackson ImmunoResearch Laboratories]. E. Somatic diversification of V<sub>H</sub> regions of PCR-amplified VDJ genes from splenic (Spl) and Apx T1 B cells. The horizontal scale bar represents average number of nucleotide changes/V<sub>H</sub> gene (excluding D and J regions); each dot represents one V<sub>H</sub> gene sequence. Sequences obtained from three adult rabbits are shown. Data in B–D are representative of two to three independent experiments. Data in A are representative of two control and three TACI-Ig–treated rabbits.

(Fig. 4B). In addition to early B cell progenitors, the CD24<sub>low</sub>CD21<sup>+</sup> population might include other lineages such as CD24<sup>+</sup> stromal cells and common lymphoid progenitors (27). In contrast to young rabbits, we found few, if any, CD24<sub>high</sub> T1 B cells in the BM of adult rabbits (Fig. 4C). This finding is consistent with the absence of B lineage precursors (proB and preB) in the BM of adult rabbits (19).

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 localize transitional B cells in GALT during this process, we examined the Apx from an IgH transgenic (Tg) rabbit. These IgH Tg rabbits are deficient in B cells early in ontogeny, but IgM<sup>B</sup> B cells accumulate gradually first in the Apx and SR and later in PP, MLN, blood, and spleen (30). The delayed temporal appearance of B cells in these rabbits offered an opportunity to study the early stages of peripheral B cell development. We examined one IgH Tg rabbit that lacked IgM<sup>B</sup> B cells in the periphery (PB and spleen), but had a few B cells in GALT (Fig. 5A). We found that these IgM<sup>B</sup> B cells in the Apx were CD21<sub>low</sub> (Fig. 3C, right), and CD20<sup>+</sup> (Fig. 3C, lower), suggestive of a transitional B cell phenotype. The CD20<sup>+</sup> B cells were scattered, predominantly in the domes and villi of underdeveloped B cell follicles located between large T cell areas (Fig. 3C, lower). Similarly, we identified transitional B cells in the dome and villous regions of Apx from conventional neonatal rabbits (3 and 6 d of age) (Fig. 3D). Similar to neonates, we found CD20<sup>+</sup> cells located in the villous regions of the Apx of a 4-wk-old rabbit (data not shown). We conclude that during development, the transitional B cells migrate to the domes and villi of the Apx prior to differentiating into follicular B cells. The unique and close proximity of GALT transitional B cells to the intestinal lumen suggests to us that these B cells may interact with commensal bacteria or bacterial-derived products that promote B cell activation and maturation in GALT.

Role of complement in the proliferative expansion of Apx B cells

During the early stages of peripheral B cell development in rabbits, GALT serves as a site for B cell expansion and Ig diversification (12). We previously demonstrated that commensal bacteria in the intestinal lumen are required to stimulate B cell proliferation and Ig diversification in GALT (21, 31). Intestinal bacteria may contribute to these processes by regulating the expression and secretion of various bacterial- and host-derived stimulatory molecules. In a germfree Apx, we found no C3 deposition, whereas in conventional appendices, C3 was readily identified in the B cell follicles (Fig. 5A). These results indicate that C3 expression/localization in the Apx is regulated by commensal bacteria. Furthermore, by flow cytometry, we found luminal bacteria were coated with C3 and IgA, and the IgA<sup>B</sup> bacteria appeared to have a greater deposition of C3 on the surface compared with IgA<sup>B</sup> bacteria (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 CD21 is required for B cell proliferation 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.

Costimulatory molecules required for the proliferative expansion of B cells in GALT

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-
Ig) (32). 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 (CD24highIgMlow) (dashed), M (CD24+ IgM+) (black), and non-B cells (N) (IgM– CD24–) (black) for CD20. Shaded histogram = isotype control. B, Immunohistological staining of spleen (6-wk-old) section for CD23 (follicular B cells) and CD20 (transitional B cells). The dotted line represents a B cell follicle. C, Flow cytometric and immunohistological analyses of tissues from an IgH transgenic rabbit stained for IgM and CD21 (upper) and CD20 and IgM (lower), respectively. D, Staining for CD20 in Apx from a conventional 3- and 6-d-old rabbit. Original magnification ×100.

FIGURE 3. Tissue localization of CD20+ transitional B cells. A, Flow cytometric staining of Apx B cells from a neonatal rabbit (1 wk of age) for T1 (CD24highIgMlow) (dashed), M (CD24+ IgM+) (black), and non-B cells (N) (IgM– CD24–) (black) for CD20. Shaded histogram = isotype control. B, Immunohistological staining of spleen (6-wk-old) section for CD23 (follicular B cells) and CD20 (transitional B cells). The dotted line represents a B cell follicle. C, Flow cytometric and immunohistological analyses of tissues from an IgH transgenic rabbit stained for IgM and CD21 (upper) and CD20 and IgM (lower), respectively. D, Staining for CD20 in Apx from a conventional 3- and 6-d-old rabbit. Original magnification ×100.

FIGURE 4. Flow cytometric analysis of T1 B cells in BM. A, BM cells from a young rabbit (8 wk of age) stained for CD21 and CD24, with histograms of IgM and CD62L staining of cells in T1 (dashed) and M (black) cell gates. Shaded histogram = isotype control. B, BM cells from a young rabbit (8 wk of age) stained for IgM, MHC II (left), and CD24, with CD24 histograms of cells in proB and preB and B cell gates (center and right). Shaded histogram = isotype control. C, BM cells from adult rabbits aged 1.5–2.2 yr stained for CD24 and IgM. All plots are representative of staining obtained from at least three rabbits.
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 HSAhigh 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 CD24high immature B cells exit the BM early in ontogeny as transitional (T1) B cells (IgMlowCD21lowCD62Llow) and traffic to GALT (Apx), where they enter the domes 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 GALT 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 participate 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 lymphopoiesis. 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 lymphopoiesis 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 Th1 B cells are maintained by self-renewal and are responsible for maintaining the B cell compartment in the absence of detectable B lymphopoiesis. 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 lymphopoiesis in the BM decreases in the elderly.

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Disclosures

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