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J Immunol 2010; 184:5527-5536; Prepublished online 16 April 2010;
doi: 10.4049/jimmunol.1000146
http://www.jimmunol.org/content/184/10/5527

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/04/16/jimmunol.1000146.DC1

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Requirement for BAFF and APRIL during B Cell Development in GALT

Venkata A. Yeramilli and Katherine L. Knight

The effects of B cell-activating factor belonging to the TNF family (BAFF) on B cell maturation and survival in the mouse are relatively well understood. In contrast, little is known about the role of BAFF in B cell development in other mammals, such as rabbits, that use GALT to develop and maintain the B cell compartment. We examined the expression and requirement of BAFF and a proliferation-inducing ligand (APRIL) during peripheral B cell development in young rabbits. By neutralizing BAFF and APRIL in neonates with a soluble decoy receptor, transmembrane activator calcium modulator and cyclophilin ligand interactor-Fe, we found a marked reduction in the number of peripheral B cells, but found no change in the bone marrow (BM) compartment. In the appendix, the size and number of proliferating B cell follicles were greatly reduced, demonstrating that although BAFF/APRIL is dispensable for B cell development in BM, it is required for B cell development in GALT. We found that all rabbit B cells expressed BAFF receptor 3, but did not bind rBAFF, suggesting that the BAFF-binding receptors (BBRs) are bound by endogenous soluble BAFF. Further, we found that B cells themselves express BAFF, suggesting that the soluble BAFF bound to BBRs may be endogenously produced and stimulate B cells in an autocrine fashion. Additionally, we propose that this chronic occupancy of BBRs on B cells may provide a tonic and/or survival signal for the maintenance of peripheral B cells in adults after B lymphopoiesis is arrested in BM. The Journal of Immunology, 2010, 184: 5527–5536.

B cell-activating factor belonging to the TNF family (BAFF), discovered almost a decade ago, has emerged as an important regulator of B cell homeostasis and survival. BAFF binds three receptors: BAFF-R/BAFF receptor 3 (BR3), transmembrane activator calcium modulator and cyclophilin ligand interactor (TACI), and B cell maturation Ag (BCMA) (1–4). A closely related cytokine, a proliferation inducing ligand (APRIL), binds to two of these receptors: BCMA and TACI (5–7). Different species use these cytokines and their receptors to develop and maintain their B cell compartment through unique and conserved mechanisms.

Mice deficient in BAFF or its receptor BR3 exhibit a block in B cell development beyond the transitional T1 stage and lack marginal zone (MZ) and conventional B2 B cells, showing that BAFF is required for peripheral B cell maturation and survival (2, 8, 9). In contrast, mice deficient in TACI have increased numbers of B cells and serum Ig, suggesting that this BAFF/APRIL receptor has a negative regulatory role in controlling the size of the B cell pool (10). B cell maturation appears normal in both APRIL- and BCMA-deficient mice; however, class switching to IgA and survival of long-lived bone marrow (BM) plasma cells are impaired in April−/− and BCMA−/− mice, respectively (11–13). These findings demonstrate that BAFF and APRIL have distinct roles in B cell biology.

In contrast to TACI−/− mice, humans that lack TACI (patients with common variable immunodeficiency) have normal numbers of circulating B cells, but have decreased Ig levels (14, 15), suggesting that in humans, TACI plays a role in Ig production. Similar to BR3-deficient mice, patients with common variable immunodeficiency that lack BR3 also develop severe B cell lymphopenia due to the arrest of B cell development at the transitional B cell stage (16). Even though these phenotypes do not manifest until adulthood, these findings show that human B cells rely on BAFF signals for their maturation and survival.

Since the discovery of BAFF in humans and mice, BAFF has been cloned from chickens, ducks, quails, pigs, and rabbits, indicating that BAFF is evolutionarily conserved (17–21). However, the receptors for BAFF do not appear to be conserved. In chickens, no evidence for homologs of BCMA and APRIL were found, leaving birds with only BAFF and two receptors, BR3 and TACI, to control their B cell compartment (22). In young chicks, B cells develop in the bursa of Fabricius, which is a major component of GALT. In vivo neutralization of BAFF with a decoy receptor severely impaired B cell development in chickens, demonstrating that BAFF is required for development of immature B cells (22, 23). Similar to chickens, other animals, such as rabbits, sheep, cattle, and pigs, use GALT to develop their B cells (24–28). Although the molecular events that govern Ig gene rearrangement and somatic diversification have been investigated in some of these species, little is known about the mechanism by which B cell survival and homeostasis is maintained.

In young rabbits, B cells develop in the appendix, which is considered a mammalian homolog of the avian bursa (29). During development, the appendix is seeded by immature B cells that migrate from the BM. In the BM, these B cells undergo a proliferative expansion, form organized follicles, and somatically diversify their Ig genes. These diversified B cells then enter the circulation and serve as the preimmune Ab repertoire (30–32). Using a germ-free (GF)
appendix model, we previously demonstrated that colonization of the appendix with commensal bacteria is required for the proliferative expansion of B cells and diversification of Ig genes (33). Further, upon surgical removal of the organized GALT in neonatal rabbits, the percent of B cells in the periphery is significantly reduced (34, 35). These studies demonstrate the importance of GALT in the development and maintenance of the B cell compartment. Further, B lymphopoiesis in the BM is dramatically reduced as the rabbit ages (36, 37), and it is unclear how the peripheral B cell pool is maintained when there is little influx of newly made B cells from the BM. In this study, we investigated the role of BAFF during the early phase of B cell development in GALT and characterized the expression of BAFF-binding receptors in adult rabbits.

**Materials and Methods**

**Recombinant adenovirus**

Adenoviral constructs expressing TACI-Fc (extracellular portion of human TACI fused to human Fc) and mouse Fc (kindly provided by Dr. Tong Zhou, University of Alabama at Birmingham, Birmingham, AL) were transfected into QBI-293A cells (Qbiogene, Carlsbad, CA). Viral particles were isolated after multiple freeze-thaw cycles using cesium chloride gradients and titered using the 50% TCID method (Qbiogene) and stored at −80°C. Recombinant viral particles (10^10 in 0.3 ml PBS) were injected i.p. into newborn rabbit pups, and the rabbits were sacrificed 7–10 d later. All animal studies were reviewed and approved by the institutional animal care and use committee at Loyola University Chicago, Maywood, IL.

**Flow cytometry and immunohistochemistry**

For flow cytometry, Abs and indirect reagents were as follows: anti-IgM (clone 367; BD Biosciences, San Jose, CA), anti-CD4 (clone Ken-4; BD Biosciences), anti-rabbit L chain (KLK stock), anti-CD79a (clone HM47; BD Biosciences), anti-CD9 (clone MM2; Antigenix America, Huntington Station, NY), anti-CD43 (clone L11/43; Antigenix America), anti-CD14 (clone K4; Antigenix America), anti-CD11b (clone 198; Antigenix America), anti-MHC class II (MHC-II; clone 2C4; BD Biosciences), anti-CD44 (clone W4/86; Chemicon International, Temecula, CA), goat anti-human BAFF (Antigenix America) (38), goat anti-human BR3 (R&D Systems, Minneapolis, MN) (38), FITC goat Fab anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and streptavidin-PE/APC.

**FIGURE 1.** Immunohistochemistry and in situ hybridization to detect BAFF and APRIL in GALT. A, Immunofluorescent staining for IgM and BAFF in appendix sections from CV (4 wk old), GF (4 wk old), and GF appendix rabbit in which the appendix was colonized with *B. subtilis* and *B. fragilis* (7 wk old). Original magnification ×100. B, In situ hybridization to detect APRIL (left panel) and BAFF (right panel) transcripts in appendix (4-wk-old CV). Original magnification ×200 (left panel), ×100 (right panel). C, In situ hybridization to detect BAFF transcripts in a neonatal appendix (1-wk-old CV). Original magnification ×50. D, dome; F, follicle; FAE, follicle-associated epithelium; VE, villus epithelium.

**FIGURE 2.** BAFF expression in B cells. A, Quantitative PCR for BAFF transcripts from 55D1 B cells, BM CD11b+ myeloid cells, PB CD14+ monocytes, and IgM+ appendix (Apx), spleen (Spl), and PB B cells. Data are represented as fold changes of BAFF mRNA relative to BM CD11b+ myeloid cells. Error bars = SEM derived from triplicate PCR reactions. B, Western blot of lysates from 55D1 cells, BM CD11b+ myeloid cells, and IgM+ Apx, Spl, and PB B cells probed with anti-BAFF. C, Flow cytometric analysis of IgM+ cells from appendix, spleen, PBL, and B cell lines, 55D1 and PBL-1, stained with biotinylated TACI-Fc (open histograms) and human IgG as isotype control (shaded histograms). D, Flow cytometric analysis of CHO cells transfected with FL BAFF and stained with biotinylated TACI-Fc (open histogram) or biotinylated human IgG as isotype control (shaded histogram). Data are representative of two (quantitative PCR), four (Western blot), and three (flow cytometry) experiments. Mock represents CHO cells transfected with empty vector.
(BD Biosciences). BAFF and TACI-Fc were biotinylated using NHS-LC biotin (Pierce, Rockford, IL). All flow cytometry data were collected on an FACS Canto (BD Biosciences) and gated on lymphocyte-sized cells on the basis of forward and side scatter, then analyzed using FlowJo software (Tree Star, Ashland, OR). For immunohistochemistry, acetone-fixed cryosections (7 μm) were blocked with goat serum and stained with the following Abs: anti-IgM, anti-CD4, anti-Ki-67 (clone B56; BD Biosciences), anti-CD9, goat anti-human BAFF, anti-CD11c (clone 3/22; R&D Systems), anti-rabbit macrophage Ab (clone RAM11; Dako, Carpinteria, CA) and biotinylated BAFF. Cy2- or Cy3-conjugated streptavidin and Cy2-conjugated goat (Fab) anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used as secondary reagents. Slides were viewed under a Leica DM IRB microscope (Leica Microsystems, Bannockburn, IL) and images captured using MagnaFire 2.1C digital camera system (Optronics, Goleta, CA); grayscale images were edited using ImageJ software (National Institutes of Health, Bethesda, MD). The GF appendix and Bacillus subtilis- and Bacteroides fragilis-colonized appendix tissues used for immunohistochemistry were obtained from rabbits previously described (33).

Quantitative real-time PCR
Total RNA was isolated from 5SD1 B cells, FACS-sorted IgM⁺ B cells, BM CD11b⁺ cells (gated on side scatter-high cells), and peripheral blood (PB) CD14⁺ monocytes using the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized (starting with 100 ng RNA for each sample) using the Superscript III First-Strand Synthesis kit (Invitrogen, Carlsbad, CA). BAFF and β-actin (endogenous control) were PCR-amplified with primers: rbBAFF forward 5′-tgattgcag acagtgcacacagca-3′ and rbBAFF reverse 5′-agg-taccctgcttgctgacact-3′; and rb-actin forward 5′-agttggtgtaaacaagctgtcaggt-3′ and rb-actin reverse 5′-agcagctacattctgtcaggtt-3′, respectively, on an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). The relative levels of BAFF mRNA normalized against β-actin were analyzed using the 2⁻ΔΔT method (39).

Cloning and expression of BAFF and APRIL
Soluble BAFF (sBAFF) (aa 134–284) was PCR-amplified from rabbit appendix cDNA (OS RbBAFF 5′-aagcttgtaggtgtaggtggaag-3′ and AS RbBAFF 5′-ctcgagcaacaacttcagtgcacc-3′) and cloned into pET-24b vector (Novagen, Madison, WI). sBAFF was expressed in Escherichia coli BL21 (DE3) cells (Invitrogen) and purified from the insoluble fraction using an Ni²⁺-NTA agarose column (Qiagen). BAFF was dialyzed against a refolding buffer (0.05 M glycine, 0.03 M NaOH, 0.4 M L-arginine, 1 mM DTT [pH 10]) for 24 h at 4°C, followed by dialysis in PBS at 4°C (all chemicals purchased from Sigma-Aldrich, St. Louis, MO). Full-length (FL) BAFF was PCR-amplified from appendix cDNA (OS FLRbBAFF 5′-ctcgaggccgccaccatggatgactccacg-3′ and AS FLRbBAFF 5′-aagcttcaacaacttcagtgcaccgaagaa-3′) and cloned into pEGFP-N1 vector (Clontech Laboratories, Palo Alto, CA), such that BAFF was in-frame with enhanced GFP. Chinese hamster ovary (CHO) cells were transfected with BAFF-GFP fusion construct and cultured in the presence of 50 μM CMK (Calbiochem, San Diego, CA) for 3 d to minimize shedding of BAFF. Only GFP-positive cells were analyzed by flow cytometry for binding to biotinylated TACI-Ig. Soluble APRIL (sAPRIL) (aa 105–250) was PCR-amplified from appendix cDNA using primers: OS

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**FIGURE 3.** Analysis of the B cell compartment in TACI-Fc–treated and control (Fc) rabbits. A, Immunofluorescent staining for IgM and Ki-67 in the appendix and for IgM and CD4 in spleen (original magnification ×100). B, Average number of Ki-67⁺ B cell follicles per appendix section; a total of four sections from each of the three TACI-Fc– or Fc-treated rabbits were examined. Error bars = SEM. C, Flow cytometry of IgM and CD4 stained cells in appendix, spleen, and PBL. D, Flow cytometry of BM stained with Abs to surface L chain, cytoplasmic CD79a, cytoplasmic IgM, CD43, and MHC-II. Data are representative of three control rabbits and five TACI-Fc–treated rabbits.
RbsAPRIL, 5'-agatgcagctccacaccgaa-3' and ASRbAPRIL, 5'-tcacagt-cagttccacaccc-3'. The underlined nucleotides in the reverse primer indicate a stop codon. The sAPRIL PCR product was cloned into pDISPLAY vector (Invitrogen) and transfected into 293T cells. Transfected cells were cultured in serum-free media, and the supernatant containing sAPRIL was harvested after 4 to 5 d and used in B cell-stimulation assays. The nucleotide sequence of all constructs was confirmed by nucleotide sequence analysis.

**Western blot**

55D1 B cells, FACS-sorted IgM⁺ B cells, and BM CD11b⁺ cells (10⁶) were lysed in buffer containing 4% SDS, 3% DTT, 40% glycerol, and 0.065 M Tris (pH 6.8) and electrophoresed on 12% SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P Transfer Membrane (Millipore, Bedford, MD) and probed with a cross-reactive goat anti-human BAFF Ab (Antigenix America) (38), followed by biotinylated rabbit Fab anti-goat IgG (Rockland, Gilbertsville, PA), streptavidin-HRP (Jackson ImmunoResearch Laboratories), and SuperSignal substrate (Pierce). To demonstrate equal loading of lysates, blots were stripped and reprobed with anti-β-actin Ab (clone AC-15; Sigma-Aldrich).

**In situ hybridization**

Paraformaldehyde-fixed (4%) sections (10–12 μm) were hybridized with 10–30 ng/ml digoxigenin-labeled BAFF or APRIL riboprobes at 70°C for 20 h in a humidified chamber. Hybridized sections were stringently washed and incubated with HRP-F(ab')-anti-digoxigenin (Dako), followed by biotinyl tyramide (Dako) and alkaline phosphatase-F(ab')-anti-biotin (Dako). Sections were developed with BCIP/NBT (Vector Laboratories, Burlingame, CA) and examined with the Leica DM IRB microscope as described above (Leica Microsystems). BAFF and APRIL riboprobes were prepared as described (40). GenBank accession numbers (www.ncbi.nlm.nih.gov/) for nucleotide sequences used to prepare riboprobes were: for BAFF, ABP01347 (nt 417–873) and for APRIL, EF494239 (nt 1–753).

**In vitro stimulation of B cells**

For costimulatory experiments, 10⁵ splenocytes and appendix cells (in 100 μl) were cultured with anti-Ig (10 μg/ml) [goat (F(ab')²) anti-rabbit IgG (H +L); Jackson ImmunoResearch Laboratories] and varying concentrations of soluble BAFF or supernatant containing sAPRIL. Proliferation was assessed by [³H]thymidine incorporation (1 μCi/well) (Amersham Biosciences, Piscataway, NJ) incorporation using a liquid scintillation counter (PerkinElmer, Waltham, MA). As a positive control for proliferation, splenocytes and appendix cells were cultured with irradiated murine CD40L-transfected CHO cells in a 100:1 ratio, respectively, and pulsed with [³H]thymidine. For survival assays, appendix B cells were depleted of CD4⁺ T cells using anti-CD4 and anti-mouse Ig-coated magnetic beads (MACS; Miltenyi Biotec, Auburn, CA). Enriched B cells (10⁶/ml) were cultured in either media alone or BAFF (3 μg/ml) or heat-inactivated BAFF (95°C for 30 min). Live cells in culture were enumerated after 48 and 96 h. To assess BAFF binding to in vitro-activated B cells, mononuclear cells from PB, spleen, and appendix were cultured in the presence of anti-Ig (10 μg/ml) and CD40L-expressing CHO cells as described above. After 3 d in culture, live B cells were analyzed by flow cytometry for BAFF binding.

**Results**

**BAFF and APRIL expression in GALT**

Our previous studies using a GF appendix model showed that commensal bacteria are required to stimulate B cell proliferation and Ig diversification in GALT (33). Intestinal microbiota may contribute to these processes by regulating the expression and secretion of stimulatory cytokines, such as BAFF. To test this possibility, we examined BAFF expression in GF and conventional (CV) appendixes by immunohistochemistry and found that although BAFF was readily detected in the B cell follicle areas of CV appendixes, little to no BAFF was detected in the GF appendixes (Fig. 1A, top and middle panels). Following introduction of two commensal organisms, *B. subtilis* and *B. fragilis* (33), BAFF was detected in the B cell follicle areas (Fig. 1A, bottom panel). These data demonstrate that intestinal microbiota is required for BAFF expression and localization in GALT. By in situ hybridization, APRIL transcripts were detected near the follicle-associated epithelium and villous epithelium (Fig. 1B, left panel). In contrast, BAFF transcripts were detected around the B cell follicles (Fig. 1B, right panel), where proliferating B cells reside (Supplemental Fig. 1A). We considered that, because BAFF is known to be expressed by macrophages and dendritic cells (DCs) (1, 41), the BAFF in B cell follicle areas could be derived from resident macrophages and DCs. However, by immunohistochemistry, macrophages and DCs were detected predominantly in the domes (Supplemental Fig. 1B), whereas most of the BAFF transcripts were detected in the B cell follicles and some in the domes (Fig. 1C). These data suggest that the BAFF in B cell follicles is not produced by DCs and macrophages, but instead is produced by B cells.

**Expression of BAFF by B cells**

To directly test if B cells produce BAFF, we analyzed 55D1 B cells and FACS-sorted IgM⁺ B cells from appendix, spleen, and PB and

![FIGURE 4. In vitro stimulation of GALT B cells with rBAFF and rAPRIL. Splenocytes and appendix cells cultured for 72 h with anti-Ig (10 μg/ml) and increasing concentrations of sBAFF (A) or supernatant containing sAPRIL (B) (5–40%). Proliferation was assessed by [³H]thymidine incorporation. C, Splenocytes and appendix B cells cocultured with and without CD40L-expressing CHO cells in a 100:1 ratio, respectively. Proliferation was assessed by [³H]thymidine incorporation. D, CD4⁺-depleted appendix B cells were cultured in either medium alone or in media supplemented with sBAFF or denatured sBAFF. Live cells in culture were enumerated after 48 and 96 h. Error bars = SEM derived from triplicate wells. Data are representative of three independent experiments.](http://www.jimmunol.org/figs/5530/fig4.jpg)
performed real-time PCR and Western blot analyses. Because myeloid cells express BAFF (41), we included CD11b⁺ myeloid cells from the BM and CD14⁺ monocytes from PB as positive controls. By both assays, we found that IgM⁺ B cells from appendix, spleen, and PB expressed BAFF (Fig. 2A, 2B). To determine if BAFF expression can be detected on the surface of B cells, we stained primary B cells and two B cell lines, 55D1 and PBL-1 (42, 43), with biotinylated TACI-Fc. Some of the B cells bound TACI-Fc (Fig. 2C), albeit weakly, indicating that they express BAFF. As a positive control, CHO cells transfected with FL rabbit BAFF readily bound TACI-Fc (Fig. 2D). We also confirmed that the binding of TACI-Fc to B cells is not due to endogenous sBAFF bound to surface receptors (data not shown). Together, these results show that rabbit B cells express BAFF. Recent reports demonstrated BAFF transcript and protein expression in mouse and human B cells; however, membrane expression of BAFF was undetectable on most freshly isolated human peripheral B cells, presumably due to rapid shedding of BAFF (44, 45). In mice, only B1 B cells displayed low levels of membrane BAFF (46). Thus, in rabbits, the BAFF-expressing B cells may represent B1-like subsets.

**Requirement of BAFF/APRIL for GALT development**

To determine if BAFF and/or APRIL are required for B cell expansion in GALT, we used a soluble decoy receptor approach to neutralize these cytokines in vivo. We injected newborn rabbits with a recombinant adenovirus expressing TACI-Fc, and 7–10 d later, the appendix was analyzed by immunohistochemistry for the presence of Ki-67⁺–proliferating B cells (Fig. 3A). We found little to no Ki-67 expression in the appendix, and the B cell follicles were markedly reduced in size compared with age-matched controls injected with an adenovirus expressing only Fc. By quantifying the total number of follicles with proliferating B cells in appendix of TACI-Fc and Fc-treated rabbits, we found ~80% reduction in Ki-67⁺ follicles in the TACI-Fc–treated rabbits (Fig. 3B). Consistent with these immunohistochemical results, by flow cytometry, we found the percentage of IgM⁺ B cells was greatly reduced in TACI-Fc–treated rabbits as compared with Fc controls (Fig. 3C). Similar to the appendix, B cells in the spleen and PBLs were markedly reduced (Fig. 3A, 3C). In BM, we did not find any perturbation in the B cell compartment of TACI-Fc–treated rabbits (Fig. 3D). The frequency of precursor B cell populations, pro- and pre-B cells, as well as APCs (MHC-II⁺) and other hematopoietic (CD43⁺) precursors, were similar between rabbits injected with TACI-Fc and control. Taken together, these experiments indicate that neutralization of BAFF/APRIL by TACI-Fc blocked B cell development in GALT and peripheral tissues while leaving the BM B cell compartment intact.

**Stimulation of appendix B cells by BAFF and APRIL**

Inhibition of B cell development in GALT by the BAFF/APRIL decoy receptor suggested to us that appendix B cells may be stimulated to proliferate by these cytokines. To test if BAFF and APRIL costimulate GALT B cells, we cultured appendix cells with anti-Ig, sBAFF, and sAPRIL and measured proliferation by thymidine incorporation. Although splenocytes exhibited a robust dose-dependent proliferative response to both rBAFF and rAPRIL, appendix B cells showed only a modest 2-fold increase in thymidine incorporation (Fig. 4A, 4B). As a positive control for proliferation,
appendix B cells readily responded to CD40L stimulation (Fig. 4C). To investigate whether BAFF provides a survival signal to GALT B cells, we cultured appendix B cells with BAFF and found ~2- to 4-fold more viable B cells in rBAFF-containing cultures as compared with media alone, indicating that BAFF promotes survival of appendix B cells (Fig. 4D). We conclude that neither BAFF nor APRIL provide a strong costimulatory (proliferative) signal to appendix B cells. Appendix B cells also did not proliferate in response to anti-Ig stimulation alone, indicating that B cells in appendix are functionally different from those in spleen.

**Occupancy of BAFF-binding receptors on primary B cells**

To determine the expression pattern of BAFF-binding receptors (BBRs) on GALT and peripheral B cells, we stained B cells with biotinylated rBAFF and were surprised to find that most B cells from appendix and PB did not bind rBAFF (Fig. 5A). This was unexpected because studies with primary human and murine B cells showed rBAFF binding to almost all B cells (1, 2, 47, 48). We ruled out the possibility that the lack of binding was due to defective rBAFF reagent by testing it on B cell lines and found that rBAFF bound to five out of six B cell lines of rabbit, human, and mouse origin (Fig. 5B). We considered that the lack of binding of rBAFF to primary B cells could be due to the lack of BR3; however, by flow cytometry, B cells from appendix, PB, and spleen expressed BR3 (Fig. 6A). To test if the lack of rBAFF binding was due to occupied receptors, we used an anti-BAFF Ab that primarily detects surface-bound BAFF (44) and found that all B cells reacted with this Ab (Fig. 6B), suggesting that the BBRs have endogenous BAFF already bound and therefore are unavailable to bind rBAFF. However, primary B cells that were activated in vitro with anti-Ig and CD40L did bind rBAFF (Fig. 6C), suggesting that upon activation, B cells either lose surface-bound BAFF and/or synthesize new BBRs that can bind rBAFF. We conclude that in rabbits, most primary B cells do not bind rBAFF because the BBRs are already occupied.

**Binding of rBAFF to PB monocytes and splenic MZ B cells**

As shown in Fig. 5A, we observed some cells in PB and spleen that bound rBAFF. Surprisingly, most of these rBAFF-binding cells were MHC-II⁺, CD11b⁺, and CD14⁺ (Fig. 7B), indicating that BAFF binds PB monocytes. Ritter et al. (49) showed that CD44 could be used as a marker to distinguish between myeloid and lymphoid cells, and we first used anti-CD44 mAb to determine if CD44 could distinguish rabbit myeloid and lymphoid cells. We identified CD44⁺ and CD44⁻ subsets of MHC-II⁺ cells in PB (Fig. 7C, upper panel) and showed that the CD44⁺ subset comprised of mostly CD14⁺ and CD9⁻ myeloid cells (Fig. 7C, lower middle and right panels) and the CD44⁻ subset comprised of L chain⁺ B cells (Fig. 7C, lower left panel). By using CD44 as a pan marker to distinguish rabbit myeloid and lymphoid cells (Fig. 7D), we confirmed that rBAFF binds strongly to CD44⁺ myeloid cells and only weakly to CD44⁻ B cells.

In the spleen, most of the BAFF-binding cells were B cells (Fig. 5A). Because in vitro-activated B cells bound rBAFF (Fig. 6C), and MZ B cells are likely activated and express high levels of TACI (50), we tested if the subset of splenic B cells that bound rBAFF were MZ B cells. Because CD9 is expressed on some MZ B cells in mice (51), we used anti-rabbit CD9 mAb to identify MZ B cells in rabbit spleen. By immunohistochemistry, we found BAFF binding and CD9 expression around the margins of B cell follicles (Fig. 8A), suggesting that BAFF may be binding to MZ B cells. By flow cytometry, using anti-CD9 mAb, rBAFF, and anti-L chain, we found that a subset of CD9⁺ B cells bound rBAFF (Fig. 8B). Taken together, we conclude that rBAFF binds PB monocytes and to a subset of CD9⁺ splenic MZ B cells.

**Binding of rBAFF to a subset of IgMlo B cells**

Although most freshly isolated B cells from adult rabbit PB and appendix did not bind rBAFF (Fig. 5A), in neonates, we found a subset of IgMlo B cells from appendix and spleen that did bind rBAFF (Fig. 9A, left panel). In the appendix follicles, these rBAFF⁺ cells were located in the basolateral region (Fig. 9B), where we find Ki-67⁺–proliferating B cells (Fig. 3A), suggesting that these subsets are cycling in vivo. Further, in TACI-Fc–treated rabbits, over 30% of the B cells in appendix were IgM⁺ and bound rBAFF, whereas only ~10% of the B cells in the control rabbits bound rBAFF (Fig. 9A). This accumulation of rBAFF⁺ IgMlo B cells in TACI-Fc–treated rabbits suggests that this subset depends on BAFF for maturation and represents transitional-like B cells that arise early in development. In support of this idea, we found that most B cells in the PB of a newborn rabbit (2 d old) were IgMlo, and many of these bound rBAFF (Fig. 9C).

**Discussion**

Mammals develop and maintain their B cell compartment through unique and conserved mechanisms. In both humans and mice, growing evidence suggests that BAFF plays an important role in peripheral B cell selection at the transitional stage and survival postmaturation (52). However, in other mammals, such as rabbit, pig, and sheep, in which GALT is required for B cell development, there are no studies describing the role of BAFF in B cell maturation and survival. In this study, we examined the role of rabbit BAFF and APRIL during peripheral B cell development.

In young rabbits, immature B cells from the BM migrate to GALT, where in the presence of commensal microbiota, they form organized follicles, undergo proliferative expansion, and somatically diversify.
their Ig genes (30–32). Dramatic reduction in the size and number of these proliferating B cell follicles in the appendix by administration of TACI-Fc showed that BAFF/APRIL is required for this early phase of B cell development in GALT. In the appendix, BAFF may provide a maturation and proliferative signal to the incoming immature B cells, as described for transitional (T2) B cells in the murine spleen (53), and provide a survival signal to B cells after the proliferative expansion (54). Results from our in vitro analysis of appendix B cells, in which BAFF provides a survival signal to appendix B cells, supports this second possibility. Additionally, BAFF may also aid in the recruitment of immature B cells from the BM to GALT. Badr et al. (55) recently reported a novel role for human BAFF in B cell chemotaxis by showing that BAFF increased the chemotactic response of primary human B cells to a few chemokines, including CXCL13. Interestingly, abundant expression of BAFF (this paper and Ref. 38) and CXCL13 (40) is found in the developing rabbit appendix, suggesting that BAFF may similarly enhance the chemotactic response of incoming B cells to CXCL13 produced by stromal cells/follicular DCs.

**FIGURE 7.** BAFF-binding to PB monocytes. Flow cytometric staining of PBL with rBAFF and anti–MHC-II (A); anti-CD11b; and anti-CD14 (B). C, Top panels, Flow cytometric analysis of MHC-II+ cells for CD44 expression. CD44hi and CD44lo subsets indicated by vertical dashed line. Bottom panels, Analysis of CD44lo cells for L chain expression (left panel) and CD44hi cells for CD14 and CD9 expression (middle and right panels). D, Flow cytometric staining of CD44lo and CD44hi subsets of MHC-II+ cells with rBAFF. Shaded histogram indicates isotype control.
In mice, blockade of BAFF results in loss of mature B cells and an arrest in B cell maturation at the transitional T1 stage (9). Further, BAFF appears to be dispensable for B cell development in the BM. In rabbits, neutralization of BAFF resulted in a similar loss of peripheral B cells without affecting the BM B cell compartment, indicating that BAFF plays a crucial role in peripheral B cell development. These results also suggested to us that the residual B cells in TACI-Fc–treated rabbits may either reflect B cell subpopulations similar to murine T1 and T2 B cells and/or a few mature B cell subsets that are not dependent on BAFF for their development. Although TACI-Fc can bind and therefore neutralize both BAFF and APRIL, the phenotypes observed in TACI-Fc–treated rabbits appear similar to BAFF-deficient mice, suggesting that the lack of BAFF rather than APRIL is likely responsible for the observed B cell deficiency.

In mice, transitional B cells are IgM\textsuperscript{hi} BR3\textsuperscript{hi} and accumulate upon BAFF blockade (56). Recently, Meyer-Bahlburg et al. (53) identified a subset of transitional B cells that are cycling in vivo. Using rBAFF as a tool, we unexpectedly identified an IgM\textsuperscript{lo} subset (IgM\textsuperscript{lo} rBAFF\textsuperscript{+}) in neonates and in the appendix; these cells were localized in the basolateral region of the B cell follicle, where we find Ki-67\textsuperscript{+} cells, suggesting that these cells are cycling. Further, these IgM\textsuperscript{lo} cells accumulated in TACI-Fc–treated rabbits, suggesting that the IgM\textsuperscript{lo} rBAFF\textsuperscript{+} cells represent transitional-like B cells. Experiments to further characterize these putative transitional-like B cells are in progress. We performed the BAFF neutralization experiments initially in neonates to identify the signals required for the proliferative expansion of B cells in GALT. However, based on the results of this study, it will be important to neutralize BAFF in adult rabbits and determine if transitional-like B cells can be identified in the periphery in the absence of ongoing lymphopoiesis in the BM.

Almost all human and murine B cells bind rBAFF and express BR3 as the dominant receptor (1, 2, 47, 48). However, in rabbits, we were surprised to find that most B cells from GALT and PB did not bind rBAFF. We showed that this lack of binding is not due to the absence of BR3, because almost all B cells expressed BR3. Rather, we find that this lack of binding is due to prior engagement of BBRs with endogenous BAFF. Carter et al. (57) first demonstrated this phenomenon of BBR occupancy on human B cells isolated from patients with systemic lupus erythematosus that have 2- to 10-fold elevated levels of serum BAFF (58). They found that the level of labeled rBAFF bound to freshly isolated B cells was significantly reduced in patients with systemic lupus erythematosus compared with healthy controls due to occupied receptors. Elevated levels of serum BAFF have also been reported in other autoimmune diseases in humans and mouse models (3, 59, 60). BAFF transgenic mice develop autoimmune symptoms, suggesting that too much BAFF signaling can interfere with peripheral B cell selection and allow autoreactive B cells to escape these checkpoints during B cell development (61). The saturated level of occupied BBRs on rabbit B cells raises the possibility that serum BAFF levels are elevated, and it will be important to measure this and also to determine if the sBAFF bound to B cells is derived from B cells themselves. Using the cross-reactive anti-BAFF Ab described in this study, we attempted to measure serum BAFF and were unable to detect BAFF by ELISA, presumably due to low sensitivity of the Ab.

B lymphopoiesis occurs in rabbit only early in ontogeny, and there appears to be little to no de novo production of B cells in the BM of adults (36, 37). This raises the question as to how the B cell...
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The occupation of BCRs on B cells provides a tonic/survival signal after B lymphopoiesis is arrested. Although constantly occupied BCRs may provide a positive survival signal, they can also negatively interfere with any ongoing peripheral B cell selection, as seen in BAFF-transgenic mice. These observations suggest that BAFF signaling in B cells is carefully regulated to maintain B cell homeostasis.

In summary, this is the first study that describes a functional role for BAFF during peripheral B cell development in a mammal that depends on GALT for generating the B cell repertoire. Our results demonstrate that rabbit BAFF plays an important role in peripheral B cell development. In addition, rabbit GALT and peripheral B cells express BAFF, and BBRs are occupied by endogenous BAFF. We suggest that this chronic occupancy may provide a tonic signal for the longevity and/or maintenance of B cells in adult rabbits after the arrest of lymphopoiesis.

Acknowledgments

We thank Dr. Dennis Lanning for performing the in situ hybridization experiments and Dr. Fareema Bilwani for performing the quantitative PCRs. We acknowledge the help of Patricia Simms in the FACS Core Facility at Loyola University Chicago (Maywood, IL). We also thank Dr. Chander Raman, University of Alabama at Birmingham, Birmingham, AL, for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

References


**Figure S1: Immunohistochemical analysis of appendix for B cells, macrophages and DCs.**

**A)** Immunofluorescent staining of appendix with anti-IgM and anti-Ki-67. **(B)** Immunofluorescent staining of neonatal appendix (1 wk of age) for B cells (anti-IgM) and dendritic cells (DCs) (anti-CD11c) (*top panel*) and macrophages (MΦ) (RAM11) (*bottom panel*). The dotted line indicates a representative follicle (F) and dome (D).