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Cutting Edge: The Dependence of Plasma Cells and Independence of Memory B Cells on BAFF and APRIL

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Memory B (BMEM)3 cells and bone marrow (BM) plasma cells (PCs) (BM-PCs) are quiescent and long-lived with the ability to survive in the absence of overt Ag from years to decades (1). Members of the TNF family of ligands, specifically BAFF (B cell-activating factor of the TNF family) and APRIL (a proliferation-inducing ligand), have been implicated in B cell subset survival but their precise roles in supporting post-GC B cell differentiation and survival are still unresolved (2). Both of these ligands bind the receptors TACI (transmembrane activator and calcium modulator ligand interactor) and BCMA (B cell maturation Ag), with BAFF additionally and exclusively binding the BAFF receptor (BAFF-R). While APRIL can also bind to heparin sulfate proteoglycans (3, 4). As the mediators that govern the longevity of BMEM cells are currently unknown and given the indispensable role of BAFF in B lineage survival, we undertook a detailed analysis of the role of BAFF and APRIL in BMEM cell survival. We have shown BCMA to be essential for the persistence of long-lived BM-PCs (5). However, whether BAFF and/or APRIL sustains long-lived BM-PC survival remains unresolved. The studies presented herein definitively establish the in vivo roles of BAFF and APRIL in the survival of BMEM cells and BM-PCs.

In this study, we find that BAFF and APRIL do not play a role in supporting the survival and function of BMEM cells in vivo. Furthermore, we show that either BAFF or APRIL is sufficient to support PC survival but BM-PCs cannot survive in the absence of both of these ligands. These data provide important new insights into the roles of BAFF and APRIL on long-lived humoral immunity.

Materials and Methods

Mice, immunizations, and reagents

This study was approved by the Institutional Animal Care and Use Committee of Dartmouth College (Lebanon, NH). BALB/c mice were purchased from the National Cancer Institute (Bethesda, MD) and APRIL knockout (ko) mice were previously described (6). For immunizations, 10 μg of PE (Cyanotech) or 100 μg of (4-hydroxy-3-nitrophenyl)acetyl (NP) plus keyhole limpet hemocyanin (KLH) (NP28-KLH; BioSearch Technologies) adsorbed to alum (Pierce) was injected i.p. in a volume of 200 μl. For secondary challenge, 10 μg of PE in PBS in a volume of 200 μl was injected i.p.

The following Abs and staining reagents were used: IgG1 (clone A85-1), IgG2a/b (clone R2-40), CD138 (clone 281-2), streptavidin-PerCP, and rat IgG1b anti-mouse isotype control (clone KLH/G2b-1-2) from BD Pharmin- gen; CD38 (clone 90), B220 (clone B6), CD23 (clone B3B4), and IgD (clone 11-26c) from eBioscience; peanut agglutinin from Vector Laboratories; and TACI (clone 166010), and BAFF-R (clone 204406) from R&D Systems. TACI Ab was labeled with Alexa Fluor 488 (Molecular Probes).

Abbreviations used in this paper: BMEM cell, memory B cell; APRIL, a proliferation inducing ligand; BAFF, B cell activating factor of the TNF family; BMEM, B cell maturation Ag; BM, bone marrow; BM-PC, bone marrow plasma cell; GC, germinal center; het, heterozygous; KLH, keyhole limpet hemocyanin; mFc4, mutated Fc γ region; NF, naive follicular (B cell); NP, (4-hydroxy-3-nitrophenyl)acetyl; PC, plasma cell; Spl-PC, splenic plasma cell; TACI, transmembrane activator and calcium modulator ligand interactor.

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Generation of fusion proteins

An expression construct for the soluble mouse TACI-Ig fusion protein was generated at ZymoGenetics by fusing DNA sequences encoding the pre-pro signal sequence derived from human tissue plasminogen activator, the extracellular domain of mouse TACI (aa 2-82), and a mutated Fc region (mFc4) from the C57BL/6 mouse IgG2c H chain. To create mFc4, the amino acid substitution L235E was introduced in the C57BL/6 IgG2c Fc to reduce binding to FcγRI and FcγRII, and the substitutions E318A, K320A, and K322A were introduced to reduce complement fixation (7, 8). Soluble mABFR-Ig fusion protein using amino acid residues 2-76 from the extracellular domain of BAFF-R and soluble mFc4 lacking a receptor protein fusion was generated in a similar fashion. Mice were injected i.p. with 100 μg of fusion or control protein three times a week in 200 μl of PBS.

Cell preparation

To enrich splenocytes for B_{MEM} cells or for PCs before cell sorting or phenotyping, splenocytes were incubated with anti-IgG1-biotin (clone 11-26c; e Bioscience) and anti-IgM-biotin (clone DS-1; BD Pharmingen), with these cells removed using the EasySep biotin selection kit for mouse cells (Stem Cell Technologies). Before sorting B_{MEM} cells, non-B cells were removed using the EasySep mouse B cell enrichment kit (Stem Cell Technologies). CD11c positive selection was performed using an EasySep mouse CD11c positive selection kit (Stem Cell Technologies). FACS was performed on a BD FACScan flow cytometer. Cell sorts were performed on a BD FACSaria with 10,000–100,000 cells sorted and postsort analysis indicating purities exceeding 98% (Flow Cytometry Facility at Dartmouth Medical School).

ELISPOT/ELISA analysis

Single cell suspensions from BM and spleen were counted and cells were apportioned to PE or NP_{20}-BSA-coated Multiscan 96-well plates (Millipore), with PCs detected by HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 polyclonal Abs (Southern Biotechnology Associates). For analysis of Ag-specific IgG1 serum Ab levels, ELISA plates were coated with either NP_{30}-BSA or NP_{20}-BSA and the Ab levels were detected with IgG1-HRP as previously described (9).

Real-time PCR analysis

Real-time PCR was performed as previously described (5). Primers for the control gene mouse GAPDH were 5′-GTTCTGATGATGTGGTGGGA-3′ (forward) and 5′-CGGAGATGATGACCTTTTG-3′ (reverse). BCMA primers were 5′-ATCTTCTGTTGGGCTGACCTT-3′ (forward) and 5′-CCTTGAGGCTGTGCCTTTGACG-3′ (reverse).

Results

B_{MEM} cells express TACI but not BAFF-R or BCMA

Following immunization with PE, Ag-specific B cells can be enumerated by their ability to bind PE (9, 10). We identified GC B cells as B_{220}^+ PE^+ CD38^- PNA^- (where PNA is peanut agglutinin) cells, with these cells detectable by day 7 postimmunization and disappearing by day 30 (Fig. 1A). We identified B_{MEM} cells as B_{220}^+ PE^+ CD38^- IgD^- cells that arose by day 21 and were detectable for the lifetime of the mouse (Fig. 1A) (10). Mature naive follicular (NF) B cells were defined as B_{220}^- CD23^- CD38^- IgD^- (Fig. 1A).

The expression of TACI and BAFF-R on NF B cells, day 7 PE^- GC B cells, and day 60 PE^- B_{MEM} B cells was determined by flow cytometry (Fig. 1B). B_{MEM} cells expressed TACI but not BAFF-R (Fig. 1B). NF B cells expressed both BAFF-R and TACI, and GC B cells expressed only BAFF-R (Fig. 1B). The specificity of the Ab staining was confirmed by adding 100 μg of either soluble BAFFR-Ig or TACI-Ig during the staining step of NF B cells, and this eradicated anti-BAFF-R or anti-TACI mAb staining, respectively (data not shown).

The mRNA expression levels of BCMA within NF B cells, day 7 PE^+ GC B cells, and day 60 PE^+ B_{MEM} B cells were measured because receptor detection by flow cytometry was unreliable. We and others have previously reported increased mRNA expression of BCMA by PCs (5, 11). As such, splenic PCs (Spl-PCs) and BM-PCs were used as positive controls for BCMA mRNA expression, with Spl-CD11c^- dendritic cells used as a negative control. No BCMA expression was detectable in highly purified (> 99%), electronically sorted NF B cells, day 7 PE^- GC B cells, or day 60 PE^- B_{MEM} B cells, indicating that this receptor may be exclusively expressed by PCs (Fig. 1C). Taken together, these data show that NF B cells express both BAFF-R and TACI, GC B cells express only BAFF-R, and B_{MEM} cells express only TACI.

BAFF and APRIL do not impact B_{MEM} cell survival or function in vivo

Given that BAFF and/or APRIL are critical for the survival of multiple B-2 lineage B cell subsets by signaling through either BAFF-R or BCMA and that B_{MEM} cells express only TACI, we sought to determine the requirement of these ligands for B_{MEM} cell survival. To address this, PE-immunized mice were treated with a fully murine BAFFR-Ig, which binds and neutralizes BAFF, or murine TACI-Ig, which binds and neutralizes both

![Image](https://www.jimmunol.org/)

**FIGURE 1.** A. The expression profiles of NF B cells, GC B cells, and B_{MEM} cells for TACI, BAFF-R, and BCMA. BALB/c mice were primed with 10 μg of PE/alum i.p. and then analyzed for PE^- B cells (7 days) and 60 days (d60) later with corresponding naive BALB/c controls. PNA, peanut agglutinin. B. The expression of BAFF-R and TACI on NF B cells, day 7 PE^- GC B cells, and enriched day 60 PE^- B_{MEM} cells was detected using anti-BAFF-R and anti-TACI Abs (light lines) in comparison to isotype controls (filled dark lines), with the mean fluorescence intensity shown in the upper right corner of each plot. C. The mRNA expression levels of BCMA relative to GAPDH (rel exp gapdh) in sorted NF B cells, day 7 PE^- GC B cells, day 60 PE^- B_{MEM} cells, Spl-PCs, BM-PCs, and CD11c^- DCs were analyzed by real-time PCR. Spl-PCs and BM-PCs were harvested from BALB/c mice primed with NP_{20}-KLH/alum on day 0 and rechallenged on day 21, with B_{220}^- CD138^- cells sorted 6–7 days later. A and B are each representative of three independent experiments, and data C are pooled expression values from two independent sorts and real-time analyses.
BAFF and APRIL (12). As a control, the murine Fc portion of the fusion proteins, designated mFc4, was used. After BAFFR-Ig or TACI-Ig treatment for 2 wk, no decrease in the total number of PE$^+$ B$_{MEM}$ cells per spleen was observed compared with mFc4-treated immune mice (Fig. 2, A and D). The percentages of both PE$^+$ B$_{MEM}$ cells and total B220$^+$CD38$^+$IgG$^+$IgD$^+$ cells increased after BAFFR-Ig or TACI-Ig treatment (Fig. 2, A and B), likely due to the loss of the BAFF-dependent NF B cell subset (Fig. 2C) in which a 4-fold reduction was observed. When BAFFR-Ig or TACI-Ig treatment was extended to 3 wk, similar results were obtained (data not shown). TACI-Ig treatment induced a rapid decline of NF B cells by day 5 and reached baseline levels by day 11, with these levels continuing through day 15. In contrast, TACI-Ig had no effect on the number of PE$^+$ B$_{MEM}$ cells throughout this time course (Fig. 2, E and F).

To test the possible impact of BAFF/APRIL blockade on the recall response of B$_{MEM}$ cells upon secondary Ag encounter, PE-immunized mice treated with fusion proteins or mFc4 were recalled with soluble PE and the number of PE$^+$ IgG$^+$ PCs in both the spleen and BM were analyzed 5 days later. After recall, we observed no difference in the number of PE$^+$ IgG$^+$ PCs present in the spleen or BM (Fig. 2G). Together, these data suggest that BAFF and APRIL are not required for B$_{MEM}$ cell survival or function in vivo.

**Redundant roles of BAFF and APRIL in supporting long-lived BM-PC survival**

To investigate whether BAFF, APRIL, or both support PC survival, mice previously immunized against PE were treated for 3 wk with TACI-Ig, BAFFR-Ig, or mFc4 and the numbers of long-lived Spl-PCs and BM-PCs were examined thereafter. TACI-Ig treatment, as previously published, reduced the numbers of PCs in both the spleen and BM (data not shown) (5). However, blockade of BAFF alone with BAFFR-Ig had no impact on Spl-PC or BM-PC survival (data not shown). These findings together implicate a role for APRIL or APRIL together with BAFF in sustaining PC survival.

To directly test the requirement of APRIL in BM-PC survival as well as its role in affinity maturation, immune mice genetically deficient in APRIL (APRIL ko) were tested alongside heterozygous (APRIL het) littermate controls (6). Both APRIL ko and APRIL het were immunized with NP$_x$KLH (Fig. 3). NP$_x$-specific, long-lived BM-PCs were enumerated in immunized APRIL het and APRIL ko mice after 3 wk of fusion protein treatment (Fig. 3). APRIL ko mice produced numbers of BM-PCs indistinguishable from those observed in the APRIL het

The total numbers of NF B cells per spleen and PE$^+$ B$_{MEM}$ cells per spleen from each treatment group are shown in C and D, respectively. A kinetic analysis of the numbers of NF B cells or PE$^+$ B$_{MEM}$ cells over 15 days of either mFc4 or TACI-Ig treatment is shown in E and F, respectively. NF and PE$^+$ B$_{MEM}$ cells per spleen were quantified on the indicated days after the beginning of treatment, with values averaged from four mice (E and F). To examine the impact of fusion protein treatment on B$_{MEM}$ cell function, mice immunized and treated with fusion proteins as in A were recalled at the end of treatment with 10$^4$ PE in PBS i.p., with PE$^+$ IgG$^+$ PCs from the spleen and BM enumerated by ELISPOT 5 days later (G). ASCs, Ab-secreting cells. A representative experiment shown of three performed for A–D and G and two performed for E and F. Results of Student’s t test are shown above each graph as comparisons between treatment groups; ns, no significance; ***, p < 0.0001.
controls, establishing that APRIL was dispensable for BM-PC survival in vivo (Fig. 3). Additionally, APRIL ko mice generated similar frequencies of high-affinity NP-specific IgG1, indicating that APRIL does not play an essential role in affinity maturation (data not shown). Treatment of either APRIL ko or het mice with TACI-Ig significantly reduced the numbers of BM-PCs (Fig. 3). By contrast, treatment of APRIL ko mice with BAFFR-Ig-depleted BM-PCs, while BAFFR-Ig treatment had no effect on PC numbers in APRIL het mice (Fig. 3). These data suggest that BAFF or APRIL can support BM-PC survival and that antagonism of one ligand alone does not lead to loss of BM-PC, whereas blockade of both BAFF and APRIL reduces BM-PCs in vivo.

Discussion

The findings of our studies demonstrate that: 1) murine B\textsubscript{MEM} cells express TACI but not BCMA or BAFF-R; 2) the survival and function of B\textsubscript{MEM} cells are BAFF- and APRIL-independent; and 3) either BAFF or APRIL can support the survival of long-term BM-PCs.

Our finding that murine B\textsubscript{MEM} cells express TACI and little BAFF-R or BCMA (Fig. 1, B and C) is in contrast to results from studies analyzing human CD27\textsuperscript{+} B\textsubscript{MEM} cells, which reportedly express high levels of BAFF-R along with detectable levels of BCMA protein (13, 14). However, there is general agreement that B\textsubscript{MEM} cells in both human and mice express high levels of TACI protein (Fig. 1B) (13, 14). Our data indicating that NF B cells express both BAFF-R and TACI while GC B cells express only BAFF-R (Fig. 1, B and C) are in agreement with previous human studies, although other investigators also report the expression of BCMA by GC B cells, which we did not observe (13, 14). Together, these data suggest there may be species-specific differences in distribution of BAFF-R and BCMA expression across the mature B cell subsets.

The ability of BAFF to provide survival signals for immature B cells, mature NF B cells, and BM-PCs has been extensively reported (2, 5). BAFF has also been found to enhance CD27\textsuperscript{+} human B\textsubscript{MEM} cell survival in vitro (11). However, in our studies the neutralization of both BAFF and APRIL did not affect the survival or recall capacity of B\textsubscript{MEM} cells in vivo (Fig. 2). This suggests that B\textsubscript{MEM} cells are the first identified B-2 lineage subset that survives independently of BAFF and APRIL.

We previously reported that soluble BAFF or APRIL supports BM-PC survival in vitro. Furthermore, we demonstrated that the treatment of mice with either TACI-Ig treatment or genetic deletion of BCMA eliminated BM-PCs in vivo (5). In the present study, we have shown that BAFF and APRIL are redundant in their ability to support BM-PC survival, because elimination of both (through either TACI-Ig treatment or genetic ablation of APRIL accompanied by BAFFR-Ig treatment) was needed to impair BM-PCs survival in vivo (Fig. 3). These findings complement and help explain the enhanced efficacy observed when TACI-Ig is used in the treatment of a murine model of systemic lupus erythematosus in comparison to BAFFR-Ig treatment (15). Our data showing that BAFFR-Ig does not deplete BM-PCs in vivo additionally validates our previous findings that PCs do not survive through BAFF-R signaling (Fig. 3). A recent report by Ingold et al. observed that blockade of both BAFF and APRIL but not blockade of BAFF alone prevented the appearance of newly formed BM-PCs shortly after immunization (4). Our data taken together with this report suggest that either BAFF or APRIL is required for both newly formed and long-lived BM-PC survival (4).

The therapeutic ramifications of these data are significant. CD20-targeted B cell depletion therapy in humans has been met with resounding success in the treatment of autoimmune and malignant B cell disorders. However, this course of treatment eliminates CD27\textsuperscript{+} B cells because these cells express CD20 and does not directly target BM-PCs, as these cells express little CD20. Because dual BAFF/APRIL blockade depletes BM-PCs in mice, the ability to effectively treat patients with malignant or autoimmune BM-PCs is of great interest. Multiple myeloma is an incurable B cell malignancy characterized by clonal accumulation of malignant plasma cells in the bone marrow, and it has recently been reported that BAFF and APRIL blockade ablates multiple myeloma cells in vivo more effectively than BAFF blockade alone (16). Finally, the knowledge that BAFF blockade depletes numerous B cell subsets except B\textsubscript{MEM} cells indicates a potential advantage of therapeutically depleting patients of specific B cell subsets while leaving their immunological history intact. Indeed, even after BAFF or APRIL blockade, a patient’s B\textsubscript{MEM} cell compartment might still persist and allow recall responses against previously encountered pathogens.

In conclusion, we have examined the roles of BAFF and APRIL in long-lived humoral memory. We found that BAFF and APRIL are not required for B\textsubscript{MEM} cell survival or function. However, the presence of either BAFF and/or APRIL alone is required to support the survival of BM-PCs, exhibiting redundancy in function between these two TNF ligands.

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Disclosures

S. R. D. is an employee of Zymo Genetics, Inc. and holds stock options in the company.
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