

Novel Function of TNF Cytokines in Regulating Bone Marrow B Cell Survival

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Two newly identified tumor necrosis factor (TNF) family cytokines, B cell activation factor from the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), have recently been shown to enhance the maturation and survival of peripheral B cells. However, whether BAFF and APRIL are expressed in the bone marrow (BM) microenvironment and if these two cytokines modulate early B cell development remain unclear. In the present study, we have detected the abundant expression of BAFF and APRIL transcripts in BM non-lymphoid cells. Low levels of BAFF and APRIL mRNA are also found in developing B cells. Furthermore, we have determined the expression patterns of BAFF receptors during B lymphopoiesis. In cultures, both recombinant BAFF and APRIL significantly promote the survival of precursor B cells whereas only BAFF can suppress apoptosis of immature B cells. These findings suggest that BAFF and APRIL, in addition to their well established role in regulating peripheral B cell growth, can modulate the survival of developing B cells in the BM. *Cellular & Molecular Immunology*. 2004;1(6):447-453.

Key Words: B cell, apoptosis, cytokine

Introduction

B cell development in mouse bone marrow (BM) is a complex process that depends critically upon control of cell production by proliferation and cell loss by apoptosis, leading to the generation of a functional B cell repertoire. Developing B cells progress through a series of differentiation stages defined by Ig gene rearrangements, cell surface determinants and growth factor requirements. During ontogeny, progenitor B cells undergo IgH chain gene rearrangement and further differentiate into precursor B cells that synthesize cytoplasmic μ H chains and transiently express the pre-B cell receptor on the membrane (1-3). Following the rearrangement of IgL chain genes, immature B lymphocytes are formed with the cell surface expression of IgM (4, 5). Self-reactive B cells are eliminated when they encounter membrane-bound self-antigens. After extensive selection against those immature B cells with autoreactivity, newly-generated functional B cells then

leave the BM and migrate *via* the blood stream to the peripheral lymphoid organs, where they finally become mature B lymphocytes (6). While proliferative factors that modulate B cell production have been extensively studied, much less is known of the factors that regulate B cell selection during development (2, 7-9). Our previous studies have shown that precursor B cells in the BM are prone to apoptotic selection at two differentiation stages, associated with IgH chain gene rearrangement and B cell receptor expression, respectively (10). These findings indicate that selection by apoptosis is a primary mechanism for eliminating aberrant or autoreactive B cells. Intrinsic and extrinsic signals that can trigger or inhibit lymphocyte apoptosis have been recently identified, but current understanding of lymphocyte selection and its implication in autoimmune development is still limited (11-15). Further studies on novel factors that can modulate B cell maturation and apoptosis will provide deeper insight into the mechanisms of lymphocyte development and selection.

The tumor necrosis factor (TNF) cytokine superfamily plays a crucial role in immune regulation by modulating lymphocyte proliferation and apoptosis, which is implicated in maintaining immune homeostasis and self-tolerance (16). B cell activation factor from the TNF family (BAFF, also known as B lymphocyte stimulator, THANK, TALL-1 and zTNF4) is a newly discovered TNF family cytokine that has been found to promote survival and

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Abbreviations: BM, bone marrow; SP, spleen; THY, thymus; TNF, tumor necrosis factor; BCMA, B cell maturation antigen; BAFF, B cell activation factor; TACI, transmembrane activator and CAML interactor; APRIL, a proliferation-inducing ligand; BR-3, BAFF receptor-3.

expansion of mature B cells (17). BAFF binds to three distinct TNF receptors named BCMA (B cell maturation antigen), TACI (transmembrane activator and CAML interactor) and BR-3 (BAFF receptor-3) on mature B cells in the periphery and exhibits a strong function for B cell activation and survival both *in vitro* and *in vivo* (18, 19). Moreover, TACI and BCMA also bind to APRIL (a proliferation-inducing ligand), a TNF cytokine that shares high sequence homology with BAFF (20). Both membrane-bound and soluble BAFF can induce proliferation of anti-IgM-stimulated peripheral B lymphocytes, and *in vivo* administration of recombinant BAFF protein to mice enhances B cell production and polyclonal hypergammaglobulinemia (21). Like BAFF, APRIL is also released as a biologically active protein and has been found to enhance the proliferation of peripheral B and T cells in cultures (22). Available data indicate that BAFF is produced by myeloid cells such as macrophages, neutrophils and dendritic cells. Although several lines of evidence reveal that BAFF can promote maturation and survival of newly formed immature B cells from BM, it remains unclear whether BAFF and/or APRIL are expressed in the BM and if these cytokines can modulate the survival and growth of developing B cells (23-25).

In the present study, we have characterized the expression patterns of BAFF and APRIL and their receptors during normal B cell development in the BM. Our results from semi-quantitative RT-PCR analyses have demonstrated that BM B cells express both BAFF and APRIL transcripts and that the expression of receptors for BAFF and APRIL is developmentally regulated among B cells at various differentiation stages. Furthermore, both recombinant BAFF and APRIL markedly enhance the survival of precursor B cells whereas only BAFF can suppress apoptosis of immature B cells. These results provide new insight in understanding B cell growth and apoptosis during normal development.

Materials and Methods

Mice

Male Balb/c mice at 12-16 weeks of age were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in a pathogen-free animal facility at the University of Hong Kong. All experimental procedures were approved by the University Committee on the Use of Live Animals in Teaching and Research.

Preparation of cell suspensions

BM cells were collected by flushing femoral shafts with cold MEM supplemented with 10% heat-inactivated FCS (Life Technologies, Grand Island, NY). Cell suspensions from the thymus (THY) and spleen (SP) of normal mice were prepared by mechanical disruption of the tissue through a cell strainer. Red blood cells were lysed by incubating cells in 1 ml ACK buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) for 3 min on ice. After the lysis of erythrocytes, the number of nucleated cells was determined using a hemacytometer. Aliquots of cell samples were either assayed immediately or incubated at 37°C in a humidified atmosphere with 5% CO₂, as

described (10).

Immunostaining and flow cytometry

For phenotypic analysis, single-cell suspensions were stained with the following mAbs: FITC-B220 (RA3-6B2) and phycoerythrin-conjugated IgM (R6-60.2) purchased from PharMingen (San Diego, CA). Cells were incubated with combinations of labeled antibodies in PBS containing 2% FCS. Immunostained cells were analyzed with an EPICS-Altra flow cytometer (Beckman Coulter). A minimum of 10,000 events per sample were collected from various phenotypically defined cell subpopulations. Cell debris and clumps were excluded by setting a gate on forward scatter *vs.* side scatter, as described (10). BM precursor B cells (B220⁺IgM⁻) and immature IgM⁺ B cells from normal mice were purified by cell sorting. When reanalyzed after sorting, sorted cell fractions were routinely > 96% pure.

Cell culture

Purified BM B220⁺IgM⁻ precursor B cells and IgM⁺ immature B cells were cultured for 5 days in RPMI 1640 medium supplemented with 2% heat-inactivated FCS unless otherwise specified. Recombinant BAFF, APRIL and BCMA-Fc (Alexis Co, Switzerland) were used at 200 ng/ml. Total cell numbers were monitored daily and cell viability was determined by trypan blue staining. Cultures were performed in triplicate, and results are presented as the mean ± SD of each triplicate.

Apoptotic assay

Freshly prepared splenic cell suspensions were cultured in serum-free RPMI 1640 medium with or without recombinant BAFF, APRIL and BCMA-Fc. After various time intervals of culturing, cell samples were phenotypically labeled and resuspended in the binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl₂). Two microliters of FITC-Annexin V (PharMingen) were added and incubated for 15 min in the dark at room temperature before flow cytometric analysis was performed, as described (10).

Cell cycle analysis

Phenotypically labeled and ethanol-fixed cell samples from the BM were suspended in 50 µg/ml RNase (Boehringer Mannheim Co., Mannheim, Germany) and 50 µg/ml propidium iodide (Sigma) in PBS and kept on ice in the dark until flow cytometric analysis, as described (10).

Semi-quantitative RT-PCR analysis

Total RNA was isolated from BM, THY, SP, peripheral blood mononuclear cells (PBMCs) prepared from Ficoll-Paque centrifugation as well as cell sorting-purified BM B cell fractions with Trizol reagent (Invitrogen, Carlsbad, CA). Samples were processed according to the manufacturer's protocol. RNA was precipitated by adding isopropanol. RNA was pelleted at 12,000 g for 10 min at 4°C, and washed with 70% ethanol. After drying in the vacuum concentrator (5301; Eppendorf, Germany) at 30°C, resuspended in diethylpyrocarbonate (DEPC)-treated water, and the quality of RNA isolation was confirmed by 1.5% agarose gel in TRIS acetate EDTA buffer. cDNA was synthesized with random primers and M-MLV-RT as described by the manufacturer (Invitrogen). PCRs were

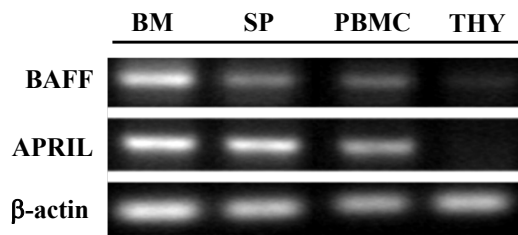


Figure 1. Expression of BAFF/APRIL in the immune system analyzed by semi-quantitative RT-PCR analysis. RNA was extracted from various lymphoid organs as well as PBMCs. Both BAFF and APRIL messengers were readily detected in BM, SP and PBMCs whereas only minimal amount of BAFF but not APRIL were expressed in THY. Data are representative of two independent experiments.

made semi-quantitative by varying the number of amplification cycles and performing dilutional analysis so that there was a linear relationship between the amount of cDNA used and the intensity of the PCR product. BAFF, APRIL, BCMA, BR-3, TACI, Bcl-2, Bcl-xL and β-actin sequences were amplified by PCR using the following gene-specific forward and reverse primers (Invitrogen): BAFF (347 bp), 5'-TGT TGT CCA GCA GTT TCA C-3', 5'-CTG CAG ACA GTC TTG AAT GA-3'; APRIL (700 bp), 5'-AGC CTC ATC TCC AGG CCA GAT-3', 5'-AAA CCC CAG GAA TGT TCC ATG CG-3'; TACI (263 bp), 5'-GCG CAC CTG TAG AGA CTT C-3', 5'-GCC TCA ATC CTG GAC CAT G-3'; BR-3 (605 bp), 5'-GCC CAG ACT CGG AAC TGT CCC A-3', 5'-GCC CAG TAG AGA TCC CTG GGT TCC-3'; BCMA (304 bp), 5'-GGC GCA ACA GTG TTT CCA CA-3', 5'-CTC GGT GTC GGC CTT GTC CA-3'; Bcl-2, 5'-TCG CTA CCG TCG TGA CTT C-3', 5'-AAA CAG AGG TCG CAT GCT G-3'; Bcl-xL, 5'-TAA GTG AGC AGG TGT TTT GGA C-3', 5'-GGG AGG TGA GAG GTG AGT GG-3'; β-actin (166 bp), 5'-GCA TTG CTG ACA GGA TGC-3', 5'-CCT GCT TGC TGA TCC ACA TC-3'. PCR reactions were performed using 0.5 mM of each deoxynucleotide triphosphate, and 0.5 μM sense and anti-sense primers with 1 U Taq polymerase (Promega) under the following conditions: BAFF, 55°C annealing temperature, 35 cycles; APRIL, 57°C annealing temperature, 38 cycles; TACI, 50°C annealing temperature, 35 cycles; BR-3, 55°C annealing temperature, 35 cycles; BCMA, 55°C annealing temperature, 35 cycles; β-actin, 50°C annealing temperature, 25 cycles. All reactions were performed using a thermal cycler for PCR (RoboCycler Gradient 96, Stratagene). The RT-PCR products were separated on 1.5% agarose gel in 1 × TAE buffer, and visualized by 1 μM ethidium bromide. Subsequently, the identity of RT-PCR products was confirmed by sequencing. All experiments were repeated at least three times. Controls were performed by applying the whole PCR master mix without cDNA. Base pair (bp) length was determined by using a DNA marker (Invitrogen).

Statistical analysis

Student's *t* test was performed where appropriate. Data are presented as the mean ± SEM. *p* values of < 0.05 were considered statistically significant.

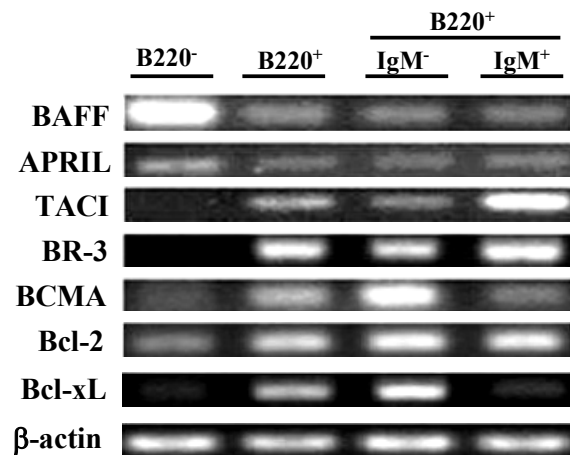


Figure 2. Transcript levels of BAFF receptors among developing B cells in the BM. Cell-sorting purified B220⁺ B cells, B220⁻ non-B cells and B cell subsets (B220⁺IgM⁻ precursors and IgM⁺ immature B cells) were obtained from freshly prepared BM suspensions of normal mice. BAFF and APRIL transcripts were detectable in developing B cells whereas mRNA levels of BAFF receptor expression were developmentally regulated. Moreover, Bcl-2 family genes were differentially expressed in developing B cells in the BM. Data are representative of three independent experiments.

Results

Expression of BAFF and APRIL and their receptors during B cell development

To determine whether BAFF and APRIL are expressed in various lymphoid organs, we examined the transcript levels of these two genes by semi-quantitative RT-PCR. Both BAFF and APRIL mRNA levels were readily detected in the BM, SP and PBMCs from normal mice. In contrast, only a minimal amount of BAFF and no detectable APRIL were expressed in the thymus (Figure 1). Furthermore, levels of BAFF and APRIL mRNA were highly expressed in B220⁻ non-lymphoid cells but only traceable amount of transcripts were detected in B220⁺ B lineage cells in the BM (Figure 2). The PCR products for BAFF and APRIL were excised and sequenced to confirm their identity (data not shown). To establish the expression patterns of BAFF receptors among developing B cells, we purified B220⁺ IgM⁻ precursor B cells and IgM⁺ immature B cells from freshly prepared BM suspensions by cell-sorting. Among three receptors for BAFF, BR-3 mRNA expression was detected exclusively in B lineage cells, with a higher level expressed at the immature B cell stage. Interestingly, BCMA was highly expressed in precursor B cells and then significantly down-regulated among IgM⁺ immature B cells; in contrast, TACI expression was low in precursor B cells but markedly upregulated at the immature B cell stage. These findings suggest that expression of BAFF receptors is developmentally regulated during B lymphopoiesis in BM.

The mechanisms by which BAFF enhances peripheral B cell survival appears to be achieved through modulating expression of the Bcl-2 family molecules (26). To examine the expression patterns of Bcl-2 family genes during B cell

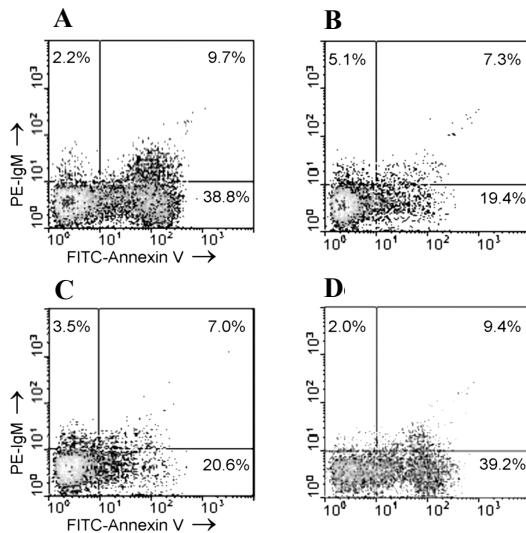


Figure 3. Effects of BAFF and APRIL on suppressing apoptosis of BM B cells. Freshly prepared BM cell suspensions were cultured for 16 h in serum-free RPMI medium alone (A) or with recombinant BAFF (B), APRIL (C) and BAFF plus BCMA-Fc (D), respectively. Flow cytometric analysis showed significantly reduced apoptotic incidence of IgM⁺ immature B cells when cultured with BAFF and APRIL. Moreover, the protective effect of BAFF on B cell apoptosis was specifically blocked by treatment with BCMA-Fc. Data are representative of two independent experiments.

development, we detected that Bcl-xL transcripts were highly expressed in B220⁺IgM⁻ precursor B cells and significantly down-regulated in immature B cells. However, levels of Bcl-2 mRNA remained similar between precursors and immature B cells, consistent with previous findings that Bcl-xL was involved in regulating the survival of early precursor B cells (27).

BAFF and APRIL enhance the survival of developing B cells

To directly assess whether BAFF can modulate the survival of developing B cells, we cultured freshly prepared BM cell suspensions in serum-free RPMI medium with or without recombinant BAFF (200 ng/ml) or APRIL (200 ng/ml) for 16 h, a sensitive short-term culture system that allows the accumulation of apoptotic cells without being phagocytosed by macrophages (10). As shown in Figure 3, BAFF significantly suppressed apoptosis of IgM⁺ immature B cells as compared to the control value ($48.2 \pm 6.8\%$ vs. $72.5 \pm 5.9\%$, $p < 0.01$). This protective effect of BAFF on B cell survival was specifically blocked by a soluble decoy receptor BCMA-Fc, suggesting that BAFF binding to its receptors is essential for its effect on B cell growth. Similar results were obtained among IgM⁺ B cells when cultured with APRIL. To determine if BAFF can differentially affect the survival of developing B cells at various stages of differentiation, we purified BM B220⁺IgM⁻ precursor B cells and IgM⁺ immature B cells by cell-sorting and cultured cell fractions for 5 days in 2% FCS-RPMI media with recombinant BAFF and APRIL, respectively. As shown in Figure 4, BAFF showed potent activity in enhancing the survival of both precursor B cells and immature B cells, whereas APRIL appeared to only prolong the survival of B220⁺IgM⁻ precursors but not IgM⁺ B cells.

BAFF and APRIL do not promote the maturation of precursor B cells

To determine whether BAFF-induced proliferation accounts for increased numbers of viable B cells in culture, we purified B220⁺IgM⁻ precursor B cells by cell-sorting and performed cell cycle analysis on cultured B cell samples by flow cytometry. Cell cycle distributions in B220⁺IgM⁻ precursor B cells did not show any obvious change after being cultured with BAFF and APRIL. No difference in the cycling population (S + G₂/M) was found between precursor B cells treated with or without BAFF ($12.4 \pm 2.5\%$ vs. $11.7 \pm 3.2\%$, $p > 0.05$). Similar results were observed among precursor B cells cultured with recombinant APRIL. As shown in Figure 5, B220⁺IgM⁻ precursor B cells cultured with media in the absence of IL-7 for 72 h maintained their pre-B cell phenotype. However, cultures of precursor B

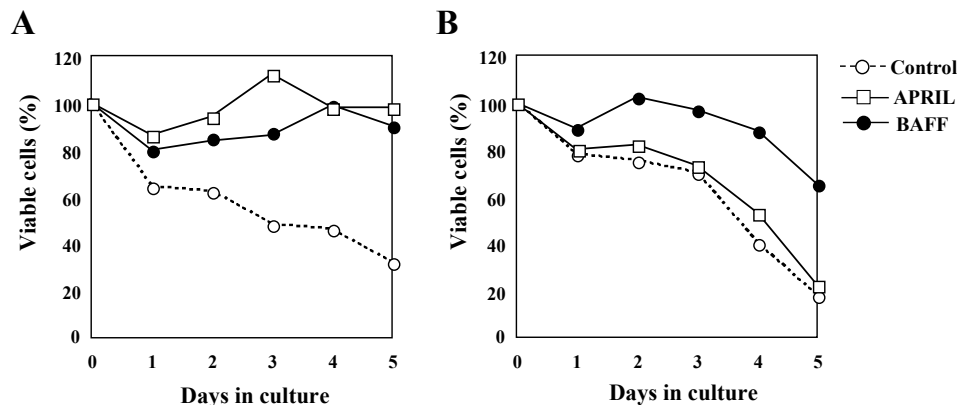


Figure 4. Enhanced survival of BM developing B cells by BAFF and APRIL in cultures. Sorting-purified BM B220⁺IgM⁻ precursors (A) and IgM⁺ immature B cells (B) were cultured with 2% FCS-RPMI for 5 days in the absence and presence of BAFF and APRIL, respectively. Viable cells were daily enumerated. Data represent the mean value of triplicate cultures, representative of two independent experiments.

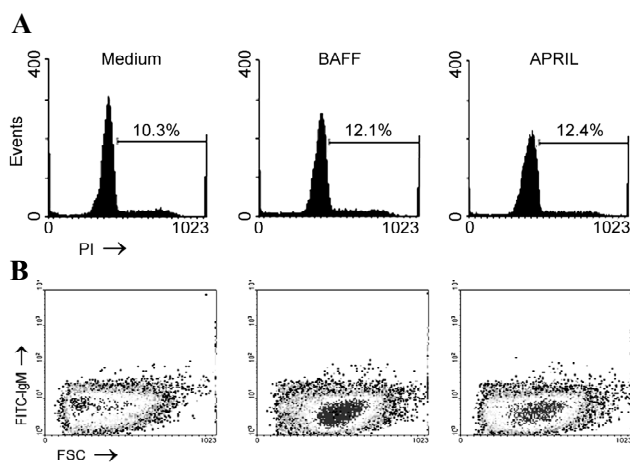


Figure 5. BAFF and APRIL do not enhance maturation of precursor B cells. Sorting-purified BM B220⁺IgM⁻ precursors were cultured with medium (2% FCS-RPMI), BAFF and APRIL for 3 days and analyzed by flow cytometry. (A) Cultured cells were performed with nuclear staining (propidium iodide) for cell cycle analysis. The frequencies of cycling B cells were indicated in the representative flow cytometric histograms. (B) Viable cells in the cultures were analyzed for the surface expression of IgM. Data are representative of three independent experiments.

cells with either BAFF or APRIL for 72 h did not result in increased frequency of IgM⁺ immature B cells, suggesting that BAFF and APRIL do not enhance the differentiation process of precursor B cells. When cultured with recombinant IL-7, 55 ± 12.5% of B cells became IgM⁺ immature B lymphocytes. These results indicate that BAFF and APRIL may function merely as survival factors for developing B cells in BM.

Discussion

In the present study, we have determined the expression patterns of BAFF and APRIL and their receptors during B lymphopoiesis in BM. Our studies *in vitro* demonstrated that both recombinant BAFF and APRIL significantly enhance the survival of precursor B cells while only BAFF can increase longevity at the immature B cell stage, indicating a previously unrecognized role for BAFF and APRIL in regulating early B cell development in normal mouse BM.

B cell development in BM is a very selective process that requires finely tuned differentiation and survival signals to maintain a delicate balance between cell production by proliferation and cell loss by apoptosis (7, 11). Our recent studies support the notion that apoptosis is an important mechanism in modulating normal B lymphopoiesis (28). TNF family cytokines play a crucial role in the development of the immune system and the maintenance of lymphocyte homeostasis, and BAFF in particular has been shown to enhance the survival of newly generated immature B cells from BM as well as splenic B cells at the transitional stage (23). In the current study, we have revealed that BAFF and APRIL transcripts are abundantly expressed by BM “B220⁺” non-lymphoid cells.

Moreover, receptors for BAFF and APRIL are differentially expressed at various stages of B cell differentiation, suggesting a mechanism by which BAFF and/or APRIL may modulate early B cell development in the BM. Our findings indicate that these two TNF cytokines derived from non-lymphoid cells and BM stromal microenvironment may constitute important niches for B cell survival. Current studies have clearly demonstrated that BAFF is predominantly expressed in myeloid lineage cells such as macrophages, dendritic cells and granulocyte colony stimulating factor-treated neutrophils. Interestingly, we have detected low levels of both BAFF and APRIL mRNA expression in BM developing B cells while the transcripts of these two genes are not detectable in splenic B lymphocytes (L Lu et al., unpublished). It remains to be determined whether constitutive expression of BAFF transcripts in developing B cells has any functional implication in modulate cell survival in an autocrine fashion. Further studies are being performed to confirm the expression of BAFF and APRIL at the protein level.

During the developmental process, levels of BCMA transcripts are significantly higher in pre-B cells while TACI mRNA expression is upregulated upon B cell maturation in a gradual fashion. Further studies are required to address the differential roles of BAFF receptors in signaling B cell functions. Using a well-established sensitive culture system (10), we have shown that both recombinant BAFF and APRIL significantly promote the survival of B220⁺IgM⁻ precursor B cells whereas only BAFF suppresses apoptosis of IgM⁺ B cells. Our previous studies have demonstrated that IL-7, a stromal cell-derived cytokine, can enhance survival of precursor B cells *via* modulating Bcl-2 family proteins (29). Members of the Bcl-2 family proteins are key regulators of the intrinsic cell death pathway. Expression of the anti-apoptotic genes including Bcl-2, Bcl-xL, Mcl-1 and A1 can prevent lymphocyte death to a variety of apoptotic stimuli. Several studies have reported that the action of BR-3 signaling is to enhance proliferation and survival of peripheral B cells by upregulating anti-apoptotic factors such as Bcl-2, A1 and Bcl-xL (26, 30). Thus, it is plausible to speculate that BAFF and APRIL may regulate survival of developing B cells by directly modulating the expression of Bcl-2 family genes. It has been well established that IL-7 plays many fundamental roles during murine B cell development (31). Apart from its trophic effect on B cell survival, IL-7 can enhance proliferation at discrete stages of B cell differentiation (32). However, it remains to be studied whether there are any cooperative interactions between IL-7 receptor- and BAFF receptor-mediated signaling pathways. Interestingly, both BAFF and APRIL have shown no effect on promoting the differentiation and maturation of precursor B cells in culture. Nevertheless, our data suggest that BAFF acts merely as a survival factor for developing B cells. Recent gene-targeting studies have shown that B cell development is not impaired in the BM of either BAFF or APRIL deficient mice, implying a dispensable role for either BAFF or APRIL in early B cell development (33-35). Nevertheless, these findings can not rule out the possibility that these two cytokines may play a complementary role in modulating B lymphopoiesis. BAFF

transgenic mice have an elevated number of B lymphocytes in the periphery and secrete various autoantibodies while B cell compartment in the BM is not affected. It is still unclear whether enhanced emigration of newly formed B cells to the peripheral lymphoid organs accounts for seemingly unexpanded developing B cell populations in the BM. Furthermore, BAFF and/or APRIL might exert their trophic effects under non-physiological conditions, which is supported by recent findings that BAFF^{+/+} stromal cells are necessary and sufficient for complete reconstitution of the B cell compartment in lethally irradiated mice (36).

Dysregulated B cell apoptosis has been implicated in the breakdown of immune tolerance and development of autoimmunity. Emerging evidence shows that serum levels of BAFF are elevated in human autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis and Sjogren's syndrome (37-39), and recent gene-targeting studies have suggested a potent role for BAFF in systemic autoimmune diseases. Transgenic expression of BAFF in mice leads to enlargement of the spleen and lymph nodes and increased numbers of peripheral B cells (19). In particular, B lymphoplasia is often accompanied by autoantibody production and hyperglobulinemia in these transgenic mice. Administration of recombinant BAFF into mice results in an enlarged pool of peripheral B cells and enhanced humoral immune responses (21). Therefore, elevated levels of BAFF proteins in inflamed tissue could lead to several consequences such as enhanced B cell survival and increased B cell activation and antibody production, resulting in lymphocyte-mediated tissue damage. Available information clearly demonstrates that BAFF is a crucial factor controlling B cell maturation and survival with the potential to break immune tolerance when overexpressed *in vivo*, but the biological role of APRIL remains poorly defined. Thus, elucidating the regulatory function of BAFF and APRIL in B cell differentiation and maturation may contribute to a fuller understanding of the development of autoimmune diseases (6, 24, 40).

In summary, we have determined expression patterns of BAFF and APRIL and their receptors in developing B cells. Although the exact mechanisms still need to be uncovered, our *in vitro* studies have established the trophic effect of these two novel TNF cytokines in modulating the survival of developing B cells. Further characterization of BAFF-mediated B cell growth should shed new light on the molecular mechanisms involved in the generation of a functional B cell repertoire.

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