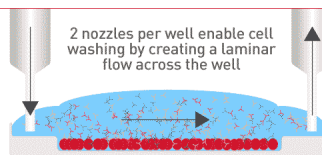


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Lymphoma B Cells Evade Apoptosis through the TNF Family Members BAFF/BLyS and APRIL¹

Bing He,* Amy Chadburn,* Erin Jou,* Elaine J. Schattner,† Daniel M. Knowles,* and Andrea Cerutti^{2*‡}

The mechanisms underlying the autonomous accumulation of malignant B cells remain elusive. We show in this study that non-Hodgkin's lymphoma (NHL) B cells express B cell-activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), two powerful B cell-activating molecules usually expressed by myeloid cells. In addition, NHL B cells express BAFF receptor, which binds BAFF, as well as transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation Ag (BCMA), which bind both BAFF and APRIL. Neutralization of endogenous BAFF and APRIL by soluble TACI and BCMA decoy receptors attenuates the survival of NHL B cells, decreases activation of the prosurvival transcription factor NF- κ B, down-regulates the antiapoptotic proteins Bcl-2 and Bcl-x_L, and up-regulates the proapoptotic protein Bax. Conversely, exposure of NHL B cells to recombinant or myeloid cell-derived BAFF and APRIL attenuates apoptosis, increases NF- κ B activation, up-regulates Bcl-2 and Bcl-x_L, and down-regulates Bax. In some NHLs, exogenous BAFF and APRIL up-regulate c-Myc, an inducer of cell proliferation; down-regulate p53, an inhibitor of cell proliferation; and increase Bcl-6, an inhibitor of B cell differentiation. By showing that nonmalignant B cells up-regulate BAFF and APRIL upon stimulation by T cell CD40 ligand, our findings indicate that NHL B cells deregulate an otherwise physiological autocrine survival pathway to evade apoptosis. Thus, neutralization of BAFF and APRIL by soluble TACI and BCMA decoy receptors could be useful to dampen the accumulation of malignant B cells in NHL patients. *The Journal of Immunology*, 2004, 172: 3268–3279.

B cell responses are highly dependent upon engagement of CD40 by CD40 ligand (CD40L),³ a TNF family member expressed by Ag-activated CD4⁺ T cells (1). By cooperating with B cell Ag receptor (BCR) and T cell cytokines, including IL-4, CD40L stimulates B cells to proliferate in the germinal center (GC) of secondary lymphoid follicles (2). BCR, CD40L, and cytokines would also play a key role in Ig V(D)J gene somatic hypermutation (SHM), class switch DNA recombination (CSR), and B cell clonal selection. SHM increases the Ab affinity for Ag by introducing point mutations within the V(D)J exon encoding the Ag-binding Ig V region (3), whereas CSR modulates

the Ab effector functions by substituting the H chain C region of IgM and IgD with that of IgG, IgA, or IgE (4). Finally, clonal selection eliminates GC B cells expressing low affinity and self-reactive Igs, thereby allowing B cells with high affinity for Ag to differentiate to memory B cells and Ab-secreting plasma cells (5).

GC B cells undergo programmed cell death or apoptosis unless positively selected by Ag, CD40L, or IL-4 (5). These stimuli rescue GC B cells from death signals transmitted through Fas, a CD40-inducible receptor that engages FasL on Ag-activated CD4⁺ T cells (6). Ultimately, B cell survival results from up-regulation of antiapoptotic and concomitant down-regulation of proapoptotic intracellular proteins. In general, the antiapoptotic proteins Bcl-2 and Bcl-x_L play a key role in the regulation of B cell survival (7). Another crucial prosurvival protein is NF- κ B, a transcription factor required for the up-regulation of Bcl-2 and Bcl-x_L (8, 9). The Bcl-2 pathway must be tightly regulated, because abnormal Bcl-2 expression favors the survival of autoreactive and aberrant B cells otherwise doomed to die. Consistent with this, Bcl-2 transgenic mice develop autoimmune disorders and B cell lymphomas (10, 11).

Lymphomas comprise the fifth most common cancer type in the U.S., with ~55,000 cases of non-Hodgkin's lymphoma (NHL) each year. NHLs include multiple clinical entities that derive from the transformation and clonal expansion of mature B cells at distinct stages of differentiation (12–14). Mantle cell lymphoma (MCL), chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), and marginal zone lymphoma (MZL) derive from mantle zone CD5⁺ naive B cells, CD5⁺ memory B cells, and marginal zone (MZ) CD5⁻ B cells, respectively (15–17). In contrast, Burkitt's lymphoma (BL) and follicular lymphoma (FL) originate from follicular GC B cells, including centroblasts (CBs) and centrocytes (CCs) (15). Finally, most, but not all, diffuse large cell B cell lymphoma (DLCL) tumors emerge from either GC B cells or activated B cells (18).

Departments of *Pathology and Laboratory Medicine and †Medicine, Weill Medical College, and ‡Immunology Graduate Program, Weill Graduate School of Medical Sciences, Cornell University, New York, NY 10021

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² Address correspondence and reprint requests to Dr. Andrea Cerutti, Weill Medical College, Cornell University, 1300 York Avenue, New York, NY 10021. E-mail address: acerutti@med.cornell.edu

³ Abbreviations used in this paper: CD40L, CD40 ligand; AID, activation-induced deaminase; AML, acute myeloid leukemia; APRIL, a proliferation-inducing ligand; BAFF, B cell-activating factor belonging to the TNF family; BAFF-R, BAFF receptor; BCMA, B cell maturation Ag; BCR, B cell Ag receptor; BL, Burkitt's lymphoma; CB, centroblast; CC, centrocyte; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; CSR, Ig class switch DNA recombination; DLCL, diffuse large cell lymphoma; FL, follicular lymphoma; GC, germinal center; IKK, I κ B kinase; Luc, luciferase reporter plasmid; mBAFF, membrane-bound BAFF; MCL, mantle cell lymphoma; MM, multiple myeloma; MZL, marginal zone lymphoma; NHL, non-Hodgkin's lymphoma; NIK, NF- κ B-inducing kinase; p, phospho; PARP, poly-ADP-ribose-polymerase; PB, peripheral blood; PI, propidium iodide; p-I κ B α , phosphorylated I κ B α ; SA, streptavidin; sBAFF, soluble BAFF; SHM, Ig V(D)J gene somatic hypermutation; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor; TRAF, TNFR-associated factor.

NHLs are characterized by diverse molecular abnormalities that promote the proliferation, enhance the survival, block the differentiation, or alter the genome integrity of malignant B cells (14). Nonrandom reciprocal translocations are frequent NHL-associated oncogenic events that originate from aberrant targeting of non-Ig loci by the SHM and CSR machineries (19). The resulting illegitimate gene recombination alters the transcription of key growth-, survival-, or differentiation-associated proto-oncogenes, such as *MYC*, *BCL-2*, and *BCL-6*, by placing them under the control of powerful Ig enhancer/promoter DNA regions (11–13, 20–22). Nevertheless, NHL B cells often overexpress c-Myc, Bcl-2, Bcl-6, and other crucial regulatory proteins, including NF- κ B (23), in the absence of translocations. Although aberrant SHM of promoter regions 5' of key genes plays an important role (24), the mechanisms underlying the overexpression of growth and survival proteins in tumoral B cells remain unclear.

NHL patients display increased circulating levels of B cell-activating factor of the TNF family (BAFF; also known as BLyS) (25), a molecule that promotes peripheral B cell survival as well as CD40-independent CSR and Ab production (26–29). BAFF is produced by myeloid cells (28, 30) and binds to transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), B cell maturation Ag (BCMA), and BAFF receptor (BAFF-R; also known as BR3), three receptors preferentially expressed by B cells (31, 32). Like CD40 (1), TACI, BCMA, and BAFF-R recruit TNF receptor-associated factors (TRAFs) (33–35) and elicit nuclear translocation of NF- κ B-Rel proteins by activating NF- κ B-inducing kinase (NIK) and I κ B kinase (IKK) (36–38). Once in the nucleus, BAFF-induced NF- κ B would activate key survival-associated genes, including Bcl-2 (39). Interestingly, TACI and BCMA also bind a proliferation-inducing ligand (APRIL), a BAFF-related molecule produced by myeloid cells that induces B cell proliferation, CSR, and plasma cell survival (28, 40, 41).

The following considerations prompted us to hypothesize that BAFF and APRIL play important roles in NHL. First, like Bcl-2 transgenic mice (10), BAFF-transgenic mice develop follicular and MZ B cell hyperplasia as well as autoimmunity (31). Second, the 13q32–34 locus encompassing the *BAFF* gene is often amplified in NHLs (42), and soluble BAFF (sBAFF) is increased in patients with NHL (25) and Sjogren's syndrome (43), an autoimmune disorder associated with MZL. Third, BAFF and APRIL have been recently shown to enhance the survival and proliferation of several tumors, including CLL and multiple myeloma (MM) (44–47). We show in this study that autocrine and paracrine BAFF and APRIL promote the accumulation of malignant B cells from different types of NHL by attenuating apoptosis.

Materials and Methods

Nonmalignant cells

Normal B cells were isolated from the peripheral blood (PB) and tonsils of healthy subjects. PB CD19⁺ or IgD⁺ B cells were purified through a magnetic cell-sorting device (Miltenyi Biotec, Auburn, CA) after incubating PBMCs with biotin-conjugated Abs to CD19 (BD PharMingen, San Diego, CA) or IgD (Southern Biotechnology Associates, Birmingham, AL) and streptavidin (SA)-conjugated MicroBeads (Miltenyi Biotec). A similar strategy was used to segregate tonsillar IgD⁺CD38⁻, IgD⁺CD38⁺, IgD⁻CD38⁺, IgD⁻CD38⁻, CD38⁺CD77⁺, and CD38⁺CD77⁻ B cells. Briefly, tonsillar B cells were enriched by SRBC rosetting and magnetically sorted into CD38⁺ and CD38⁻ fractions with a PE-conjugated mAb to CD38 (BD PharMingen) and anti-PE-MicroBeads. Then, CD38⁺ B cells were treated with MultiSort Release Reagent (Miltenyi Biotec) and divided into two aliquots. One aliquot was separated into IgD⁺CD38⁺ and IgD⁻CD38⁺ fractions using a FITC-conjugated mAb to IgD and anti-FITC-MicroBeads. Another aliquot was separated into CD38⁺CD77⁺ and CD38⁺CD77⁻ fractions using a FITC-conjugated mAb to CD77 (BD PharMingen) and anti-FITC-MicroBeads. CD38⁻ B cells were separated

into IgD⁺CD38⁻ and IgD⁻CD38⁻ fractions using a FITC-conjugated mAb to IgD and anti-FITC MicroBeads. Finally, a biotinylated mAb to CD14 (BD PharMingen) and SA-conjugated MicroBeads were used to sort monocytes from PBMCs. Macrophages obtained by incubating monocytes with 100 U/ml IFN- γ (Sigma-Aldrich, St. Louis, MO) for 4 days were cocultured with B cells at a 1:5 ratio.

Malignant cells

NHL subtypes were diagnosed according to the criteria established by the World Health Organization (48), and specimens were obtained after obtaining informed consent. Mononuclear cells from PB or lymph node specimens of patients with CLL, MCL, FL, BL, or DLCL were enriched in CD19⁺ B cells by magnetically removing T, NK, and myeloid cells with biotinylated mAbs to CD3, CD11c, CD14, and CD16 (BD PharMingen) and SA-conjugated MicroBeads. After this procedure, >90% of CD19⁺ B cells were clonal and expressed either Ig κ or Ig λ (BD PharMingen). Cells were used immediately or were frozen in DMSO. BL2, BL16, BL30, BL74, Ramos, Bjab, HBL-1, and HBL-3 BL cell lines and the acute myeloid leukemia (AML) HL-60 cell line were obtained from American Type Culture Collection (Manassas, VA) and R. Dalla-Favera (Columbia University, New York, NY).

Cell cultures and reagents

Cells were cultured in complete RPMI 1640 medium supplemented with 10% FCS unless otherwise specified. Recombinant BAFF (Research Diagnostics, Flanders, NJ), APRIL MegaLigand (Alexis Biochemicals, San Diego, CA), CD40L (Immunex, Seattle, WA), and IL-4 (Schering-Plough, Kenilworth, NJ) were used at 0.1 μ g/ml, 0.1 μ g/ml, 0.5 μ g/ml, and 500 U/ml, respectively. MOPC-21 control Ig (Sigma-Aldrich), TACI-Ig (Alexis Biochemicals), BCMA-Ig (Cell Sciences, Norwood, MA), and CD40-Ig (Ansell, Bayport, MN) were used at 30 μ g/ml. BCR (or surface Ig) was engaged with 2 μ g/ml Immunobead reagent (Irvine Scientific, Camarillo, CA), which binds both H and L chains of human Igs. Cycloheximide (Sigma-Aldrich) was used at 100 μ g/ml.

Flow cytometry

IgD, CD14, CD38, and CD77 were labeled with FITC- or PE-conjugated Abs. Membrane-bound BAFF (mBAFF) was labeled with a mouse Buffy-2 Ab to BAFF (Alexis Biochemicals) and a PE-conjugated anti-mouse Ab (BD PharMingen). Biotinylated IgD was labeled with SA-PerCP and used in triple-fluorescence studies together with an FITC-conjugated mAb to CD38 or CD77 and a PE-labeled Ab to BAFF. Surface BAFF-binding activity was measured with a human BAFF protein fused to murine CD8 (Ansell) and a PE-conjugated Ab to mouse CD8 (BD PharMingen). Cells were acquired using a FACSCalibur analyzer and mean fluorescence intensity was corrected for isotype control Ab staining.

Fluorescence microscopy

Cells were cytospun, fixed, and washed as previously described (28). Nuclei were visualized with 4',6-diamidino-2'-phenylindole dihydrochloride (Roche, Indianapolis, IN). Rabbit IgG to BAFF (Upstate Biotechnology, Lake Placid, NY) and goat IgG to APRIL (Alexis Biochemicals) were labeled with Cy3- and fluorescein-conjugated secondary Abs (Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. Slides were analyzed and photographed using a fluorescence microscope (Zeiss Axio-plan 2; Atto Instruments, Rockville, MD).

Confocal microscopy

Mouse Ab to BAFF (Alexis Biochemicals) was labeled with a Cy5-conjugated (blue) anti-mouse Ab (Jackson ImmunoResearch Laboratories). Goat Abs to TACI and BCMA (Santa Cruz Biotechnology, Santa Cruz, CA) were labeled with an Alexa 456 (red)-conjugated anti-goat Ab (Molecular Probes, Eugene, OR). Rabbit Abs to TRAF2 and TRAF6 (Santa Cruz Biotechnology) were labeled with an Alexa 488 (green)-conjugated anti-rabbit Ab (Molecular Probes). Primary and secondary Abs were used at 1/200 and 1/1000 dilutions, respectively. Coverslips were applied with Slow Fade reagent (Molecular Probes). Cells were visualized using an LSM510 laser-scanning microscope (Carl Zeiss, Jena, Germany).

Proliferation and apoptosis assays

To measure DNA synthesis, 10^4 cells/200 μ l were seeded in 96-well plates and pulsed with 1 μ Ci of [3 H]TdR on day 2 of culture. After 18 h, cells were harvested to measure [3 H]TdR uptake. Alternatively, 5×10^4 cells were incubated in a final volume of 100 μ l. Formazan release was measured after 2 days through the Celltiter 96 AQ assay following the manufacturer's instructions (Promega). Cell viability was evaluated by seeding 5×10^6 cells in six-well plates at a final volume of 5 ml. After 2 or 4 days, viable cells were counted with a trypan blue exclusion test. Cell cycle analysis was performed on 3×10^6 cells fixed in 70% ethanol at 4°C overnight and incubated in 1 ml of PBS containing 50 μ g/ml propidium iodide (PI) and 10 U/ml RNase A (Sigma-Aldrich) for 30 min at room temperature in the dark. Apoptosis was assayed using an Annexin V^{FITC} apoptosis detection kit (Oncogene Research Products, San Diego, CA). Cells stained with PI and annexin V were analyzed by flow cytometry.

RT-PCRs

Activation-induced cytidine deaminase (AID; 382 bp), APRIL (417 bp), BAFF (398 bp), CD40L (450 bp), β -actin (593 bp), TACI (323 bp), BCMA (326 bp), and BAFF-R (260 bp) were RT-PCR-amplified for 25 cycles (28, 49). BAFF transcripts were quantified through real-time PCR as previously reported (45). Briefly, cDNA synthesis and RT-PCR were performed in a single 50- μ l reaction containing 25 μ l of OneStep RT-PCR Master Mix with AmpliTaq Gold DNA polymerase, 1.25 μ l of 40 \times MultiScribe reverse transcriptase, 125 mM double fluorescently labeled probe for BAFF (5'-CCACCAGCTCCAGGAGAAGGCAACTC-3') or cyclophilin B (5'-AGCATCTACGGTGAGCGCTTCCCC-3'), 300 nM forward and reverse primers for BAFF (5'-ACCCGCGGACTGAAAATCT-3' and 5'-CACGCTTATTCTGCTGTCTGA-3', respectively) and cyclophilin B (5'-GGA GATGGCACAGGAGGAAA-3' and 5'-CGTAGTGCTTCAGTTGAAGT TCTCA-3', respectively), and 5 μ g of RNA. BAFF was measured with an ABI PRISM 7000 sequence detection system (PE Applied Biosystems, Foster City, CA) and normalized for cyclophilin B.

Plasmids and luciferase reporter assays

The -839/+232 genomic DNA segment encompassing the BAFF promoter was cloned into a promoterless pGL3-Basic luciferase (Luc) reporter vector as previously reported (49). Cells (20×10^6 /ml) were transfected with 40 μ l of DNA-TE solution containing 20 μ g of BAFF-luciferase (BAFF-Luc) or a minimal NF- κ B-driven reporter vector encompassing two NF- κ B-binding κ B sites (κ B-Luc) and 200 ng of pRL-TK control vector (Promega). In some experiments cells were cotransfected with 10 μ g/ml empty pcDNA3 or I κ B α -pcDNA3 expression vector. Electroporation was performed at 625 V/cm (BL16) or 525 V/cm (Bjab) and 950 μ F using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells ($1 \times 10^6 \times$ ml) were cultured for 48 h. Firefly and *Renilla* activities were measured with the Dual-Luciferase Assay System (Promega) to assess promoter activity and transfection efficiency, respectively.

Immunoblots

Membrane proteins were purified from 15×10^6 cells with a ProteoPrep Membrane Extraction Kit (Sigma-Aldrich). Total proteins were obtained by treating 20×10^6 cells with a standard lysis buffer. Proteins (20 μ g) were fractionated onto a 10% SDS-PAGE and transferred to nylon membranes. After blocking, membranes were probed with Abs to BAFF (Upstate Biotechnology), APRIL, TACI, BCMA, I κ B α , Bcl-2, Bcl-x₁, Bcl-6, c-Myc, p53, actin (Santa Cruz Biotechnology), p38, phospho (Thr¹⁸⁰/Tyr¹⁸²)-p38, phospho (Ser³²/Ser³⁶)-I κ B α (Cell Signaling Technology, Beverly, MA), and poly ADP-ribose polymerase (PARP; BD PharMingen). Membranes were then washed and incubated with an appropriate secondary Ab (Santa Cruz Biotechnology). Proteins were detected with an ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

Immunoprecipitations

Cells (20×10^6) were lysed on ice in 1% Nonidet P-40 lysis buffer containing protease inhibitors for 30 min. Cell lysates (500 μ g) were centrifuged for 15 min at 14,000 \times g and precleared with protein G- or A-Sepharose beads (Santa Cruz Biotechnology) for 2 h. Cleared lysates were first incubated with 5 μ g of a goat Ab to an irrelevant Ag, TACI, or BCMA (Santa Cruz Biotechnology) overnight at 4°C and then incubated with 2 μ l of protein G- or A-Sepharose beads for 1 h at 4°C. Beads were washed three times, resuspended in sample buffer, and boiled. After centrifugation, the immunoprecipitated material was immunoblotted with Abs to different proteins, including CD80 (BD PharMingen). To detect sBAFF, fluids from 1×10^6 /ml cell cultures were concentrated with centrifugal filter devices (Millipore, Bedford, MA), incubated with a mAb to BAFF (Upstate Bio-

technology), immunoprecipitated with protein A-Sepharose beads, and probed with an appropriate secondary Ab.

EMSA

Nuclear proteins were extracted from 5×10^6 cells as previously reported (28, 50). A double-stranded oligonucleotide probe encompassing a consensus κ B site from the I κ B gene promoter was end-labeled with [γ - 32 P]ATP by T4 kinase and used at \sim 30,000 cpm in each EMSA reaction. Reaction samples were prepared as previously described (28, 50), incubated at room temperature for 15 min, and electrophoresed through a 6% nondenaturing polyacrylamide gel. The composition of DNA-bound nuclear NF- κ B-Rel complexes was determined by incubating reaction mixtures with 1 μ l of Ab to p65, c-Rel, Rel-B, p50, or p52 (Santa Cruz Biotechnology) for 15 min at room temperature before adding the radiolabeled probe (28, 50).

Results

TACI, BCMA, BAFF-R, BAFF, and APRIL expression in B cells and myeloid cells from healthy subjects

In healthy subjects, PB B lymphocytes include IgD⁺CD38⁻ naive and IgD⁻CD38⁻ memory B cells, whereas B lymphocytes from secondary lymphoid organs encompass IgD⁺CD38⁻ naive, IgD⁺CD38⁺ founder GC, IgD⁻CD38⁺ GC, and IgD⁻CD38⁻ memory B cells. GC B cells can be further distinguished into CD77⁺ CBs and CD77⁻ CCs. Normal PB CD19⁺ B cells as well as tonsillar naive, founder GC, and memory B cell fractions expressed surface BAFF-binding activity, but no or low mBAFF (Fig. 1A and Table I). Similar B cells contained TACI, BCMA, and BAFF-R transcripts, but lacked BAFF and APRIL transcripts as well as transcripts for AID (Fig. 1B and Table I), a GC B cell-specific enzyme that plays a key role in CSR and SHM (3). In contrast, total GC B cells as well as purified CBs and CCs expressed BAFF, APRIL, and AID in addition to TACI, BCMA, and BAFF-R transcripts. All mature B cell subsets lacked transcripts for CD40L and (not shown) CD68, which are expressed by T cells and macrophages, respectively. Unlike B cells, PB or tonsillar CD14⁺ myeloid cells expressed mBAFF, but not BAFF-binding activity (Fig. 1A). Consistent with this, CD14⁺ myeloid cells expressed BAFF and APRIL, but not TACI, BCMA, or BAFF-R transcripts (Fig. 1B). Similar myeloid cells lacked AID and CD40L transcripts. Thus, all normal B cell subsets express TACI, BCMA, and BAFF-R, whereas BAFF and APRIL are confined to normal GC B cells and myeloid cells.

TACI, BCMA, BAFF-R, BAFF, and APRIL expression in B cells and myeloid cells from NHL patients

The expression of TACI, BCMA, BAFF-R, BAFF, and APRIL in NHL remains unclear. Unlike normal B cells, fresh malignant CD19⁺ B cells from CLL/SLL, FL, BL, or DLCL expressed surface BAFF-binding activity as well as mBAFF (Fig. 2A and Table I). In general, DLCL B cells expressed more mBAFF than CLL/SLL, FL, or BL B cells (Table I). CD14⁺ myeloid cells from all NHL patients lacked BAFF-binding activity, but expressed more mBAFF than CD14⁺ myeloid cells from healthy subjects. Malignant CLL/SLL, MCL, BL, FCL, and DLCL B cells also expressed TACI, BCMA, and BAFF-R as well as BAFF, APRIL, and AID transcripts. Finally, malignant B cells from some CLL/SLL and DLCL cases contained CD40L transcripts, although less than BAFF and APRIL transcripts. Thus, NHL B cells express TACI, BCMA, and BAFF-R and are exposed to both autocrine and paracrine BAFF and APRIL.

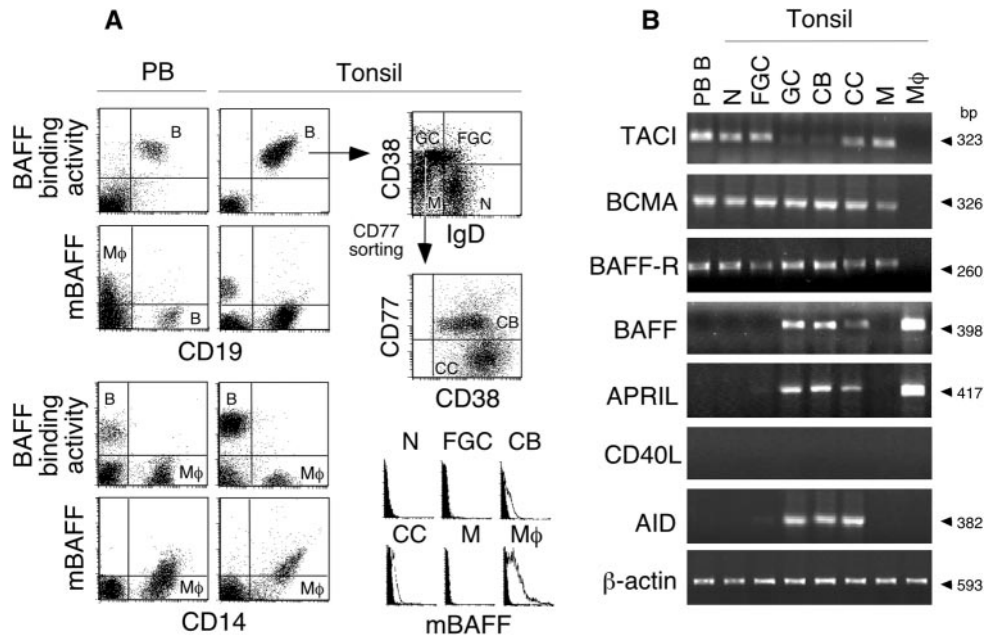


FIGURE 1. TACI, BCMA, BAFF-R, BAFF, and APRIL surface proteins and transcripts in normal B cells. *A, Left*, BAFF-binding activity, mBAFF, CD19, and CD14 on PB and tonsillar mononuclear cells. B and Mφ in the cytograms indicate B cells and CD14⁺ monocytes/macrophages. *Right*, IgD⁺CD38⁻ naive B cells (N), IgD⁺CD38⁺ founder GC B cells (FGC), IgD⁻CD38⁺ GC B cells (GC), and IgD⁻CD38⁻ memory B cells (M) were isolated after tonsillar B cell staining for CD38 and IgD. Purified CD38⁺ B cells were further fractionated into CD38⁺CD77⁺ CBs and CD38⁺CD77⁻ CCs. mBAFF was analyzed on all these B cell subsets and Mφ. *B*, TACI, BCMA, BAFF-R, BAFF, APRIL, CD40L, AID, and β-actin transcripts in PB CD19⁺ B cells and tonsillar N, FGC, GC, M, and Mφ. The data in *A* and *B* represent one of four similar experiments.

TACI, BCMA, BAFF, and APRIL expression in lymphoma and myeloid cell lines

BAFF and APRIL expression was further evaluated in NHL B cell lines. Total lysates from normal PB IgM⁺ (IgD⁺) B cells contained TACI and BCMA, but lacked BAFF and APRIL proteins (Fig. 3A). In contrast, total lysates from IgM⁺ BL B cell lines contained BAFF and APRIL in addition to TACI and BCMA proteins. As expected, a control AML cell line contained BAFF and APRIL, but not TACI and BCMA, proteins. Purified membrane proteins from normal IgM⁺ B cells included BCMA, but not BAFF (Fig. 3B). In contrast, purified membrane proteins from

IgM⁺ BL B cell lines included BCMA, BAFF, as well as a 17-kDa protein corresponding to sBAFF, a BAFF cleavage byproduct that retains the ability to bind and activate membrane-anchored receptors, including BCMA (26). Although including BAFF, purified membrane proteins from a control AML cell line lacked BCMA as well as sBAFF. Unlike normal IgM⁺ B cells, IgM⁺ BL B cells and control AML cells released sBAFF in the culture fluids (Fig. 3B). Finally, BAFF and APRIL proteins could be visualized in the cytoplasm of IgM⁺ BL B cells and control AML cells, but not in that of normal IgM⁺ B cells (Fig. 3C). Thus, NHL B cell lines contain more BAFF and APRIL, express more mBAFF, and release more sBAFF than normal B cells.

Table I. BAFF and APRIL in normal and malignant B cells

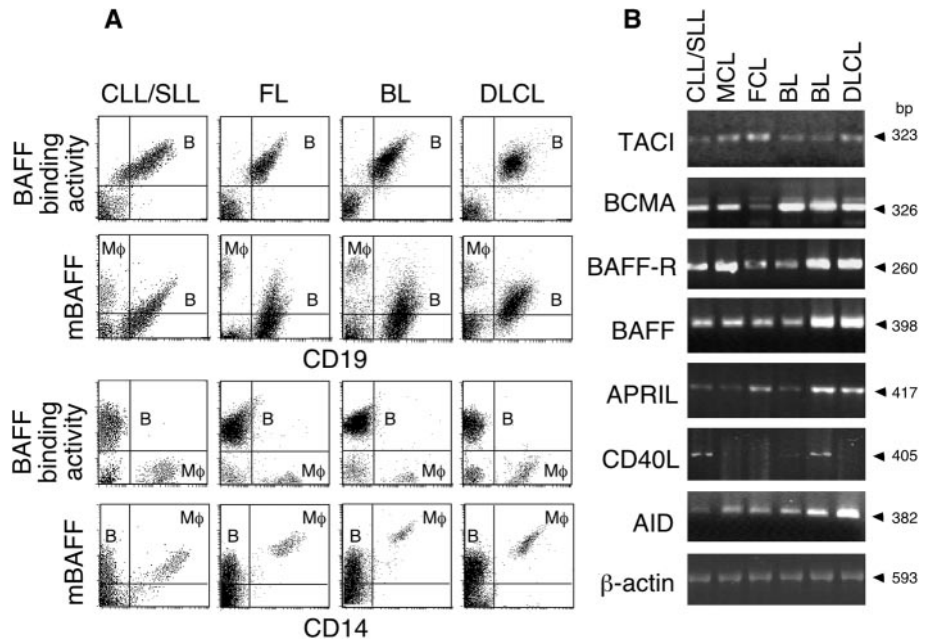
	BAFF		APRIL
	Transcript ^a	Protein ^b	Transcript ^a
Normal PB B Cells			
CD19 ⁺ (n = 4)	-	- ^c	-
Normal tonsillar B cells			
IgD ⁺ CD38 ⁻ (n = 4)	-	-	-
IgD ⁺ CD38 ⁺ (n = 4)	-	-	-
IgD ⁻ CD38 ⁺ (n = 4)	+ (4/4)	-/+ (4/4)	+ (4/4)
IgD ⁻ CD38 ⁻ (n = 4)	-	-	-
Malignant B cells			
CLL/SLL (n = 4)	+ (4/4)	+ (3/4)	+ (4/4)
MCL (n = 4)	+ (4/4)	ND	+ (4/4)
FL (n = 4)	+ (4/4)	+ (3/4)	+ (4/4)
BL (n = 4)	+ (4/4)	++ (4/4)	+ (4/4)
DLCL (n = 4)	+ (4/4)	+++ (4/4)	+ (4/4)
Malignant B cell lines			
BL (n = 8)	+ (8/8)	-/+ (8/8)	+ (8/8)

^a BAFF and APRIL were RT-PCR-amplified for 25 cycles.

^b mBAFF was determined after B cell treatment with sodium citrate buffer (pH 4.5) to remove receptor-bound BAFF.

^c -, -/+, +, ++, and +++ correspond to the following mean fluorescence intensities: <2, 2-5, 6-10, 11-20, and >20, respectively.

FIGURE 2. TACI, BCMA, BAFF-R, BAFF, and APRIL surface proteins and transcripts in NHL B cells. **A**, BAFF-binding activity, mBAFF, CD19, and CD14 on PB or lymph nodal mononuclear cells from one representative CLL/SLL, MCL, FCL, BL, and DLCL case of four. **B** and **Mφ** in the cytograms indicate B cells and CD14⁺ monocytes/macrophages. **B**, TACI, BCMA, BAFF-R, BAFF, APRIL, CD40L, AID, and β -actin transcripts in PB or lymph node B cells and myeloid cells from one representative CLL/SLL, FL, BL, or DLCL case of four.



Autocrine and paracrine BAFF and APRIL enhance NHL B cell accumulation by attenuating apoptosis
 The following experiments were performed to assess whether BAFF and APRIL enhance NHL B cell survival. The number of

viable cells spontaneously declined upon incubation with FCL, DLCL, and BL B lymphocytes with medium alone for 2 days (Fig. 4A). This decline was not affected by adding control Ig or CD40-Ig, a soluble decoy receptor that blocks the binding of CD40 on B

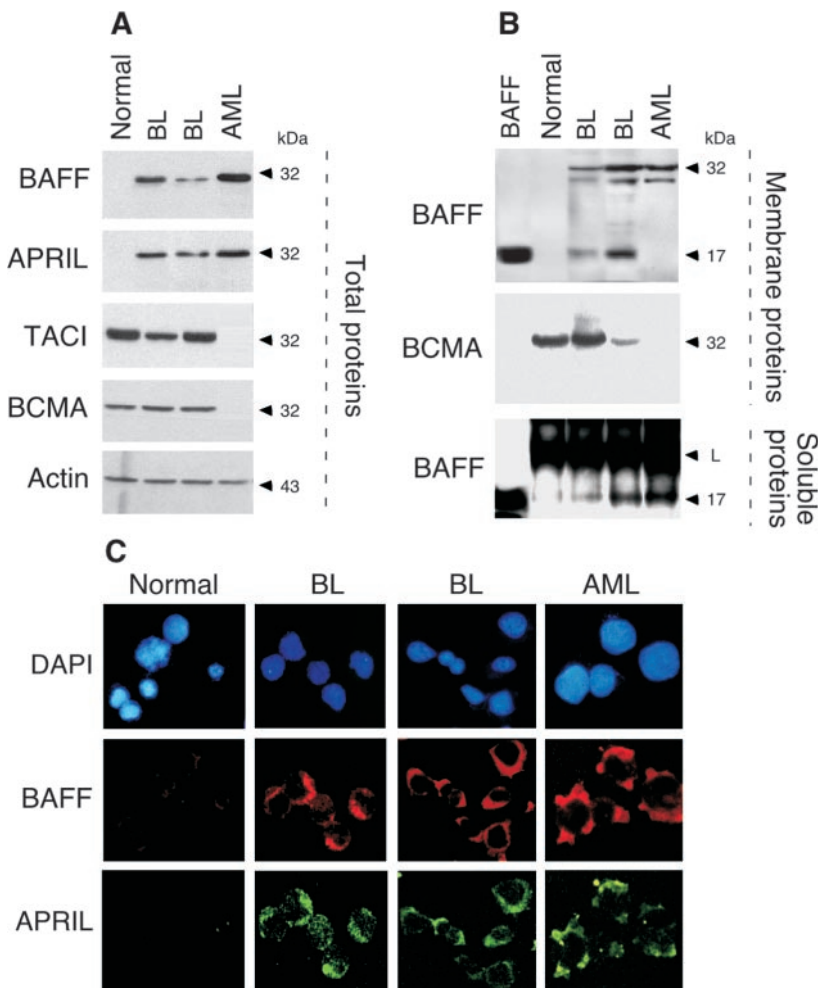


FIGURE 3. TACI, BCMA, BAFF, and APRIL proteins in NHL B cell lines. **A**, BAFF, APRIL, TACI, BCMA, and actin total proteins from normal PB IgM⁺ B cells, IgM⁺ BL16 and Ramos BL cell lines, and HL60 AML cells. **B**, BAFF and BCMA membrane proteins from normal PB IgM⁺ B cells, IgM⁺ BL16 and Ramos BL cell lines, and HL60 AML cells. The bottom panel shows sBAFF immunoprecipitated from the culture fluids of cells incubated for 6 days with medium alone. L, L chain of the immunoblotting anti-BAFF Ab. Recombinant sBAFF (5 ng/ml; first lane) was used as a control. **C**, BAFF (red) and APRIL (green) proteins in nonmalignant PB IgM⁺ B cells and malignant Ramos and BL16 BL B cells or HL60 AML cells. 4',6-Diamidino-2'-phenylindole dihydrochloride (blue) was used to counterstain cell nuclei. The data in A–C represent one of three similar experiments.

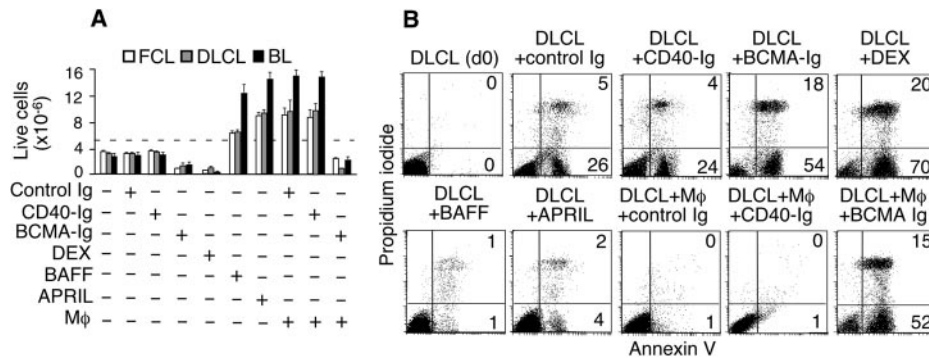


FIGURE 4. Autocrine and paracrine BAFF and APRIL increase NHL B cell survival. *A*, Viable FCL, DLCL, and BL B cells on day 0 and after incubation for 2 days with control Ig, CD40-Ig, BCMA-Ig, dexamethasone (DEX), BAFF, APRIL, and/or macrophages (Mφ). The dotted line indicates the initial cell input. Bars indicate the SD of triplicate determinations. *B*, DLCL B cells were cultured as described in *A* for 2 days and then stained with PI and annexin V. Numbers inside cytograms indicate the proportion of early annexin V⁺PI⁻ (bottom right number) and late annexin V⁺PI⁺ (top right number) apoptotic cells. The data in *A* and *B* represent one of three similar experiments.

cells by CD40L. In contrast, the number of viable NHL B cells was dramatically decreased by BCMA-Ig, a soluble decoy receptor that prevents the binding of BAFF and APRIL to B cell-anchored TACI, BCMA, and BAFF-R. A similar decrease was induced by dexamethasone, an NF-κB-inhibiting steroid often used to treat NHLs. Conversely, soluble BAFF, soluble APRIL, or macrophages increased the viability of NHL B cells. The prosurvival activity of macrophages was dependent upon BAFF and APRIL, but not CD40L, because it was inhibited by BCMA-Ig, but not CD40-Ig.

Additional experiments were performed to assess whether BAFF and APRIL attenuate apoptosis. DLCL B cells included early and late apoptotic elements upon incubation with control Ig or CD40-Ig for 2 days (Fig. 4*B*). Early apoptotic cells expressed surface phosphatidylserine, an annexin V-binding phospholipid usually confined to the inner portion of the membrane, whereas late apoptotic DLCL B cells expressed surface phosphatidylserine and captured PI, a DNA-binding compound that penetrates severely damaged cells. Unlike control Ig or CD40-Ig, BCMA-Ig increased early and late apoptotic DLCL B cells. A similar increase was induced by dexamethasone, whereas BAFF or APRIL decreased apoptotic DLCL B cells. Macrophages had a similar antiapoptotic effect, which was reversed by BCMA-Ig, but not by CD40-Ig. Similar results were obtained with fresh FL and BL B cells (not shown).

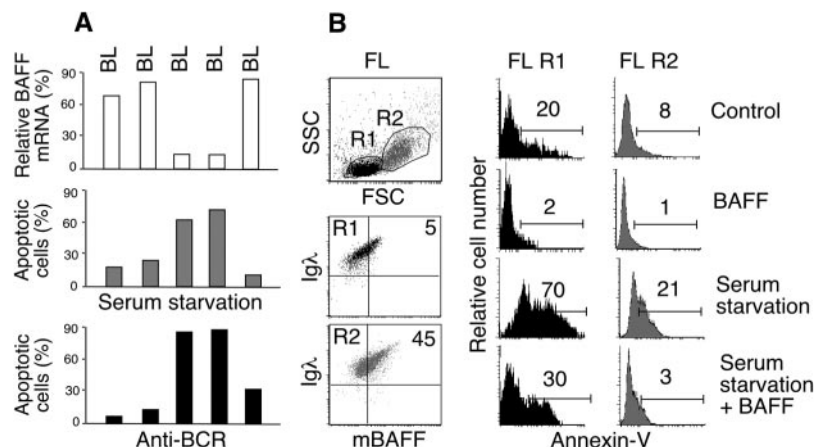
The prosurvival activity of autocrine BAFF was further investigated in IgM⁺ BL B cell lines. Some BL lines contained an amount of BAFF transcripts comparable to that detected in the

control AML cell line, HL60. Other BL cell lines contained lesser amounts of BAFF transcripts (Fig. 5*A*). BL B cell lines expressing more BAFF transcripts were more resistant to serum starvation- or BCR-induced apoptosis than BL B cell lines expressing less BAFF transcripts. A positive correlation between BAFF expression and NHL B cell resistance to apoptosis was also found in fresh NHL B cells. Large FCL B cells expressing more mBAFF were more resistant to serum starvation-induced apoptosis than clonally related small FCL B cells expressing less mBAFF (Fig. 5*B*). Exogenous BAFF partially rescued both mBAFF^{high} and mBAFF^{low} FCL B cells from apoptosis. Collectively, our results indicate that BAFF and APRIL attenuate NHL B cell apoptosis through both autocrine and paracrine pathways.

Autocrine and paracrine BAFF and APRIL shift the Bcl-2 family ratio to favor NHL B cell survival

Bcl-2 and Bcl-x_L are NF-κB-inducible Bcl-2 family members that enhance cell survival by interfering with the release of cytochrome *c* from mitochondria (7–9). Cytochrome *c* initiates apoptosis by activating caspases, a family of intracellular proteases that degrade intracellular proteins such as PARP (7). Unlike Bcl-2 and Bcl-x_L, the Bcl-2 family member Bax enhances apoptosis by facilitating cytochrome *c* release (7). Compared with DLCL B cells incubated with control Ig, freshly isolated DLCL B cells incubated for 2 days with BCMA-Ig or TACI-Ig increased spontaneous PARP cleavage, down-regulated Bcl-2 and Bcl-x_L, and up-regulated Bax (Fig. 6*A*). Conversely, DLCL B cells attenuated spontaneous PARP cleavage, up-regulated Bcl-2 and Bcl-x_L, and down-regulated Bax

FIGURE 5. Autocrine BAFF increases NHL B cell resistance to apoptosis. *A*, Top, Relative BAFF mRNA levels in BL16, BL74, Ramos, Bjab, and HBL-3 BL lines. The AML HL60 cell line was assigned an expression value of 100. Middle and bottom, Percentage of annexin V⁺ cells from BL lines incubated for 2 days with serum-depleted medium (serum starvation) or anti-BCR. *B*, mBAFF expression and annexin V-binding activity on small (R1) Igλ⁺ and large (R2) Igλ⁺ FL B cells incubated for 2 days with regular medium (control), BAFF, serum-depleted medium, or serum-depleted medium plus BAFF. Numbers indicate the percentage of positive cells. The data in *A* and *B* represent one of three similar experiments.



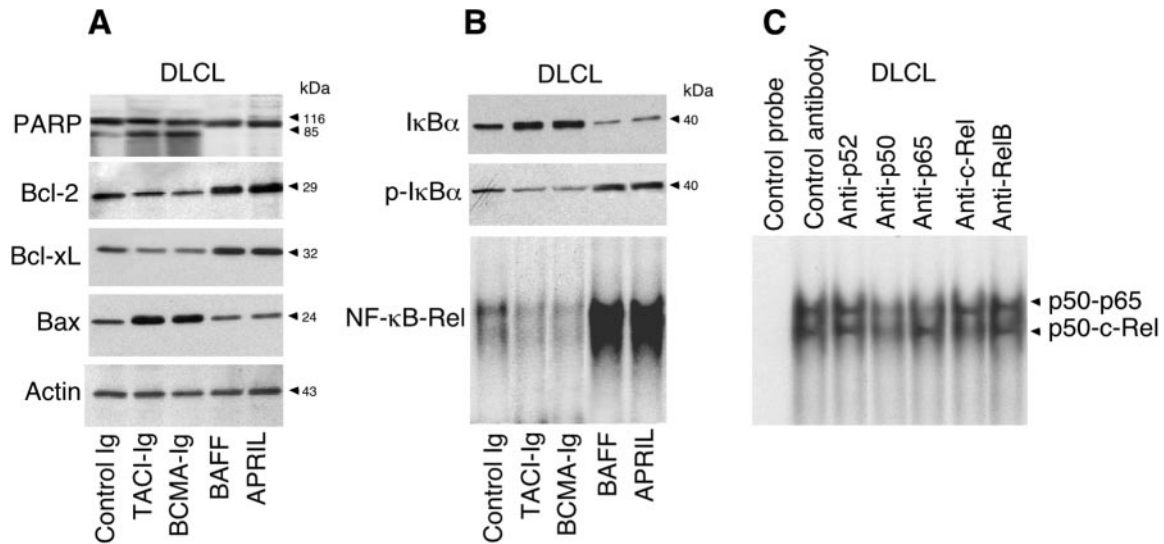


FIGURE 6. Autocrine and paracrine BAFF and APRIL up-regulate antiapoptotic, but down-regulate proapoptotic, Bcl-2 family members to favor NHL B cell survival. *A*, PARP, Bcl-2, Bcl-x_L, Bax, and actin proteins in DLCL B cells incubated with control Ig, TACI-Ig, BCMA-Ig, BAFF, or APRIL for 2 days. *B*, IκBα and p-IκBα proteins in DLCL B cultured as described in *A*. Nuclear NF-κB-Rel binding to DNA was assayed after 6 h. *C*, Composition of NF-κB-Rel protein-DNA complexes in DLCL B cells incubated for 6 h with BAFF. The data in *A–C* represent one of three similar experiments.

upon exposure to BAFF or APRIL. Similar results were obtained with fresh FL and BL B cells (not shown) as well as with BL B cell lines, including BL16 (Fig. 7*A*). Thus, both autocrine and paracrine BAFF and APRIL shift the Bcl-2 family ratio to favor NHL B cell survival.

Autocrine and paracrine BAFF and APRIL activate NF-κB in NHL B cells

In resting B cells cytoplasmic p50, p65 (RelA), and c-Rel NF-κB-Rel proteins occur as inactive dimers bound to IκBα (51, 52). After stimulation, B cells phosphorylate IκBα through an IKK complex that includes two catalytic subunits, α and β, as well as a regulatory subunit, γ. In this classical pathway, degradation of phosphorylated IκBα (p-IκBα) is followed by translocation of NF-κB-Rel dimers from the cytoplasm to the nucleus (52). In addition

to inducing proliferation, differentiation, and survival genes, such as *Bcl-2* and *Bcl-x_L* (8, 9), NF-κB turns on the *IκBα* gene. This negative feedback limits further NF-κB activation. Unstimulated DLCL B cells incubated with a control Ab constitutively expressed p-IκBα and contained DNA-binding nuclear complexes corresponding to p50-c-Rel and p50-p65 (Fig. 6, *B* and *C*). In the presence of TACI-Ig or BCMA-Ig, DLCL B cells up-regulated IκBα, but down-regulated p-IκBα and NF-κB-Rel binding to DNA. Conversely, DLCL B cells incubated with BAFF or APRIL down-regulated IκBα, but up-regulated p-IκBα and NF-κB-Rel binding to DNA.

Similar results were obtained with fresh FL and BL B cells (not shown) and with BL cell lines, including BL16 (Fig. 7*B*). BL16 B cells down-regulated IκBα as early as 10 min after stimulation with BAFF or APRIL (Fig. 7*C*). By 6 h, stimulated BL16 B cells

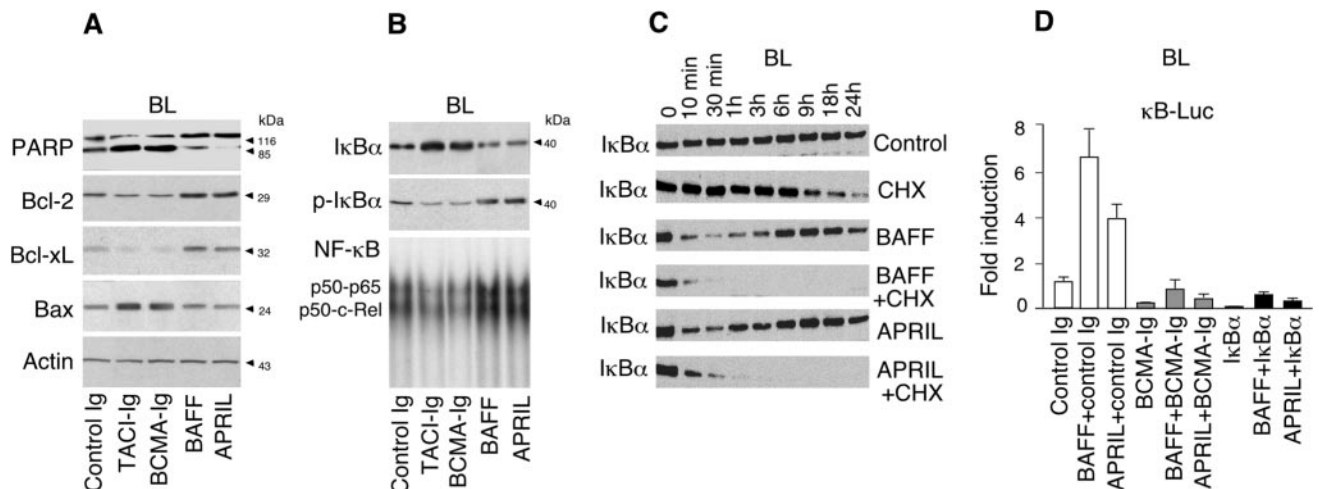


FIGURE 7. Autocrine and paracrine BAFF and APRIL activate NF-κB in NHL B cells. *A*, PARP, Bcl-2, Bcl-x_L, Bax, actin, IκBα, and p-IκBα proteins in BL16 B cells incubated with control Ig, TACI-Ig, BCMA-Ig, BAFF, or APRIL for 2 days. *B*, Nuclear NF-κB-Rel binding to DNA in BL16 cells cultured as described in *A* for 6 h. *C*, IκBα protein in BL16 cells incubated with medium alone (control), cycloheximide (CHX), BAFF, APRIL, BAFF plus CHX, or APRIL plus CHX for the indicated times. *D*, BL16 B cells cotransfected with κB₂-LUC and empty pcDNA3 or IκBα-pcDNA3 were incubated in the presence or the absence of control Ig, BCMA-Ig, BAFF, and/or APRIL. Luciferase activity was measured after 2 days and expressed as fold induction. Bars indicate the SD of triplicate determinations. Data in *A–D* represent one of three similar experiments.

re-up-regulated $\text{I}\kappa\text{B}\alpha$ unless exposed to the protein synthesis inhibitor cycloheximide. Additional experiments verified whether NF- κB induced by BAFF and APRIL turns on gene transcription. In the presence of BAFF or APRIL, BL16 B cells activated a luciferase reporter plasmid containing an NF- κB -dependent minimal promoter with two κB sites (κB -Luc; Fig. 7D). BCMA-Ig, but not control Ig, inhibited the activation of κB -Luc by BAFF or APRIL, and this inhibition was comparable to that induced by overexpressing $\text{I}\kappa\text{B}\alpha$. Thus, BAFF and APRIL activate NF- κB in NHL B cells through both autocrine and paracrine pathways.

TACI and BCMA constitutively signal in NHL B cells

The following experiments were performed to verify whether TACI and BCMA constitutively signal in NHL B cells. TACI and BCMA colocalized with BAFF, TRAF-2, and TRAF-6 in discrete areas of unstimulated BL16 B cells (Fig. 8A). Consistent with this, TACI and BCMA immunoprecipitated with BAFF, APRIL, TRAF-2, TRAF-6, $\text{I}\kappa\text{B}\alpha$, and p- $\text{I}\kappa\text{B}\alpha$ in BL16 B cells (Fig. 8B). In similar B cells, TACI immunoprecipitated with BCMA, and BCMA with TACI. In contrast, TACI and BCMA did not coimmunoprecipitate with CD80, a costimulatory molecule expressed by BL16 B cells. Furthermore, a control mAb did not immunoprecipitate TACI and BCMA in BL cells. Conversely, anti-TACI and anti-BCMA mAbs did not immunoprecipitate TACI and BCMA in control AML cells. Exposure of BL16 cells to BAFF up-regulated the amount of TRAF2 coimmunoprecipitating with TACI and that of TRAF6 coimmunoprecipitating with BCMA. In similar BL16 cells, BAFF up-regulated the coimmunoprecipitation of p- $\text{I}\kappa\text{B}\alpha$, but down-regulated that of $\text{I}\kappa\text{B}\alpha$ with TACI and BCMA. These findings suggest that autocrine BAFF and APRIL recruit a TACI-BCMA-TRAF2-TRAF6 complex that continuously activates NF- κB .

APRIL cooperates with BAFF to enhance NHL B cell proliferation

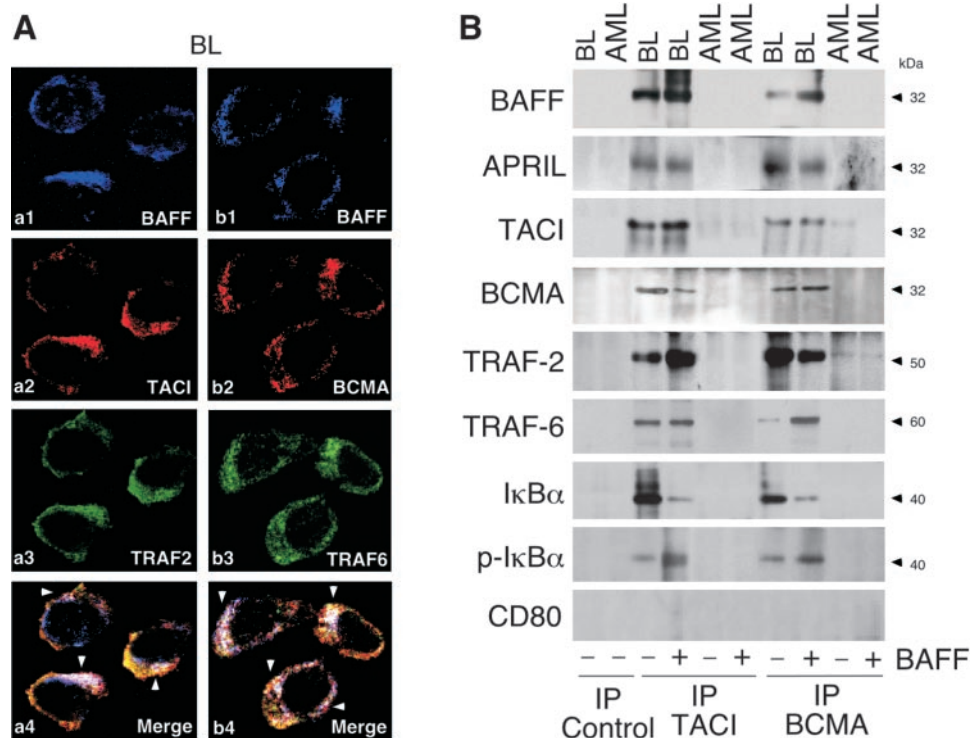
Additional experiments were performed to verify whether BAFF and APRIL modulate NHL B cell proliferation. Fresh FL, DLCL,

and BL B cells up-regulated the synthesis of DNA upon exposure to APRIL, but not BAFF (Fig. 9A). Similarly, BL B cells increased the release of formazan, a metabolite produced by actively proliferating cells, upon exposure to APRIL, whereas BAFF or BCR engagement did not affect and inhibited formazan release, respectively (Fig. 9B). Notably, NHL B cells exposed to both BAFF and APRIL released more formazan than NHL B cells exposed to BAFF or APRIL alone. Unlike BAFF, APRIL increased the proportion of actively cycling BL B cells in the S and G₂/M phases of the cell cycle (Fig. 9C). Similar BL B cells down-regulated p53 (Fig. 9D), an inhibitor of cell proliferation (53), and up-regulated c-Myc, an inducer of cell proliferation (54), after exposure to BAFF or APRIL. Furthermore, BAFF and, to a larger extent, APRIL up-regulated the expression of p-p38, a mitogen-activated protein kinase that facilitates c-Myc-induced cell proliferation (55) as well as that of Bcl-6, a proto-oncogene that inhibits GC B cell differentiation and favors lymphomagenesis (20, 22, 56, 57). Thus, BAFF and APRIL deliver distinct signals that cooperatively stimulate NHL B cell growth.

BCR and CD40L up-regulate autocrine BAFF and APRIL in normal B cells

B cells might up-regulate BAFF and APRIL upon activation by canonical GC differentiation-inducing stimuli, including CD40L. PB naive B cells from healthy subjects up-regulated BAFF and APRIL transcripts as early as 6 h after exposure to CD40L and as early as 24 h after BCR cross-linking (Fig. 10A). In addition, naive B cells up-regulated BAFF and APRIL proteins upon stimulation through BCR engagement, CD40L, and/or IL-4 for 4 days (Fig. 10B). Neutralization of autocrine BAFF and APRIL by TACI-Ig or BCMA-Ig, but not control Ig, attenuated the viability (Fig. 10C) and IgM-secreting activity (Fig. 8D) of naive B cells incubated with CD40L and IL-4 for 7 days. In a BL B cell line, the up-regulation of mBAFF by CD40L and IL-4 was associated with increased activation of BAFF-Luc, a luciferase reporter plasmid driven by the *BAFF* gene promoter (Fig. 8, E and F). NF- κB was

FIGURE 8. TACI and BCMA constitutively signal in NHL B cells. **A**, BL16 BL B cells were stained for BAFF (blue; *a1*), TACI (red; *a2*) and TRAF-2 (green; *a3*) or BAFF (blue; *b1*), BCMA (red; *b2*), and TRAF-6 (green; *b3*). *a4* and *b4* correspond to overlays. Arrows point to white-appearing areas of colocalization of blue-, red-, and green-stained molecules. **B**, Total lysates from BL16 BL B cells or AML HL60 cells incubated with medium only (control) or BAFF for 6 h were immunoprecipitated (IP) with a control Ab or an Ab to TACI or BCMA. IP proteins were immunoblotted for BAFF, APRIL, TACI, BCMA, TRAF-2, TRAF-6, $\text{I}\kappa\text{B}\alpha$, p- $\text{I}\kappa\text{B}\alpha$, or CD80. The data in **A** and **B** represent one of three similar experiments.



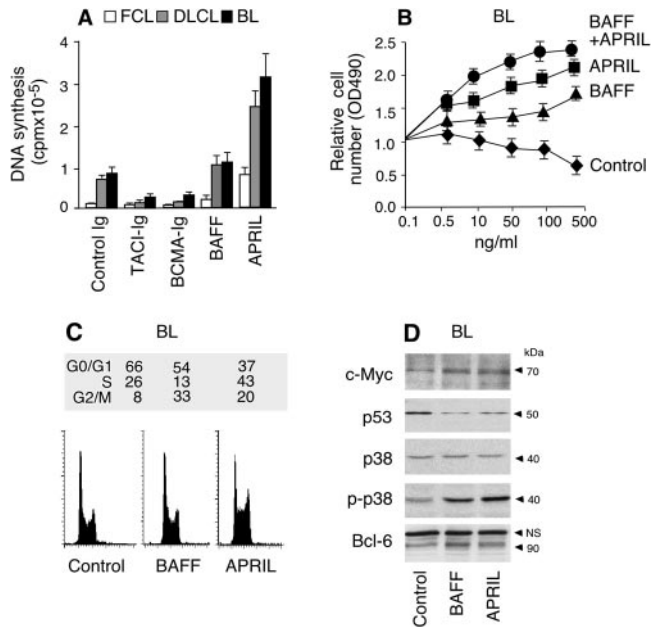


FIGURE 9. APRIL enhances the proliferation of NHL B cells. *A*, Fresh FCL, DLCL, and BL B cells were incubated with control Ig, BCMA-Ig, TACI-Ig, BAFF, and/or APRIL. DNA synthesis was evaluated after 4 days. Bars indicate the SD of triplicate determinations. *B*, Formazan release by fresh BL B cells incubated with anti-BCR (control), BAFF, and/or APRIL for 2 days. Bars indicate the SD of triplicate determinations. *C*, Fresh BL B cells were incubated with medium alone (control), BAFF, or APRIL for 4 days. Numbers indicate the proportion of cells in G₀-G₁, S, and G₂-M phases of the cell cycle. *D*, c-Myc, p53, p38, p-p38, and Bcl-6 proteins in BL B cells incubated for 2 days with medium alone (control), BAFF, or APRIL. The data in *A*–*D* represent one of three similar experiments.

crucial for the transcriptional activation of the *BAFF* gene promoter, because BL B cells overexpressing I κ B α failed to activate BAFF-Luc upon stimulation with CD40L and IL-4. Thus, normal B cells physiologically up-regulate active BAFF and APRIL upon BCR engagement or exposure to CD40L and IL-4.

Discussion

Engagement of BAFF-R by BAFF plays a key role in conservation of the normal mature B cell repertoire, including MZ, naive, GC, memory, and plasmacytoid B cells (29, 32, 41). By showing that CLL/SLL, MCL, FL, BL, and DLCL B cells express BAFF-R and attenuate apoptosis in response to BAFF, our findings suggest that NHL B cells share a key survival pathway with their normal B cell precursors. That BAFF exerts a critical prosurvival activity on different B cell neoplasias is further indicated by its ability to attenuate apoptosis in CLL and MM B cells (45–47). Intriguingly, BAFF enhances neoplastic B cell accumulation by cooperating with APRIL. This BAFF-related molecule would stimulate B cell survival through BCMA (41), which is expressed by NHL B cells as well as CLL and MM B cells (45–47). Alternatively, APRIL might drive pro-survival signals through an as yet unknown receptor.

By showing that macrophages attenuate the apoptosis of NHL B cells through a paracrine pathway that is inhibited by soluble BCMA and TACI decoy receptors, our findings indicate that BAFF and APRIL expressed on and released by bystander myeloid cells play a key role in malignant B cell survival. Intriguingly, macrophages from NHL patients express more mBAFF than macrophages from healthy subjects. This might stem from the expression and release of BAFF-inducing factors, including lympho-

toxin- α/β (58), by the progressively expanding malignant B cell clone. BAFF and APRIL would further stimulate malignant B cell survival through an autocrine pathway. Consistent with this, NHL B cells as well as CLL and MM B cells (45–47) express both BAFF and APRIL. Notably, BAFF is expressed on the surface of some, but not all, elements of a given NHL clone. These mBAFF⁺ cells would accumulate independently from external survival signals, whereas the remaining mBAFF⁻ cells probably require exogenous BAFF from myeloid cells to survive.

Our data raise the question of whether autocrine BAFF and APRIL expression is confined to malignant B cells or extends to normal B cells. We show that normal naive B cells rapidly up-regulate BAFF and APRIL upon exposure to GC differentiation-inducing stimuli, including CD40L. The up-regulation of BAFF by CD40L requires *trans*-activation of the *BAFF* gene promoter and may account for the ability of CD40L to turn on a noncanonical NF- κ B pathway in B cells that is usually induced by BAFF-R (37, 38, 59). By enhancing the survival of CD40-activated B cells, including GC CBs and CCs, autocrine BAFF and APRIL might optimize the Ab response to T cell-dependent Ags. This would explain why BAFF and APRIL are more abundant in GC-derived B cell tumors. Notably, our and previous (45–47) studies indicate that tumoral B cells express more APRIL and BAFF, namely mBAFF and sBAFF, than normal B cells. In addition, tumoral B cells constantly express BAFF and APRIL, whereas GC B cells down-regulate BAFF and APRIL upon differentiation to memory B cells. These observations imply that malignant B cells deregulate an otherwise physiological prosurvival autocrine pathway to gain growth advantage.

Neoplastic B cells would deregulate BAFF and APRIL through a number of mechanisms that probably take place after the initial transforming event. In most NHL B cells, SHM is actively ongoing (50, 60, 61) and might alter *BAFF* and *APRIL* gene transcription by aberrantly targeting 5' gene promoters (24). Abnormal BAFF expression might also stem from amplification of the 13q32–34 locus, an abnormality often detected in FL and DLCL (42). In DLCL and MZL, alterations of genes encoding the IKK-activating Bcl-10 and Malt proteins (14) might deregulate the nuclear translocation of NF- κ B, a key *trans*-activator of the *BAFF* gene promoter (49). Abnormal NF- κ B activation might also up-regulate BAFF in AIDS-associated and post-transplant NHLs (62). These B cell neoplasias often express latent membrane protein 1 and/or 2, two EBV-encoded NF- κ B-activating proteins that up-regulate BAFF and APRIL in B cells (49). Finally, chronic immune stimulation by self or foreign Ags might deregulate BAFF and APRIL expression in MZLs associated with Sjogren's syndrome and *Helicobacter pylori* infection (14, 31, 63).

In NHL B cells, autocrine and paracrine BAFF and APRIL attenuate apoptosis by shifting the balance of intracellular Bcl-2 family members toward survival. Consistent with this, BAFF up-regulates antiapoptotic Bcl-2 and Bcl-x_L, but down-regulates proapoptotic Bax. Conversely, TACI-Ig and BCMA-Ig down-regulate Bcl-2 and Bcl-x_L, but up-regulate Bax and, as shown by others (45), activate apoptosis-inducing caspases. Although unable to bind BAFF-R, APRIL rescues NHL B cells from apoptosis almost as efficiently as BAFF. This might reflect the activation of BCMA and/or an additional, as yet unknown, prosurvival receptor by APRIL (41). Intriguingly, BL B cell-bound BCMA is associated with TACI, suggesting that TACI, BCMA, and perhaps BAFF-R form promiscuous receptor complexes with abnormal signaling activity. BAFF and APRIL signaling could be further deregulated by chronic ligand-induced internalization of TACI, BCMA, and BAFF-R, which would continuously stimulate new TACI, BCMA,

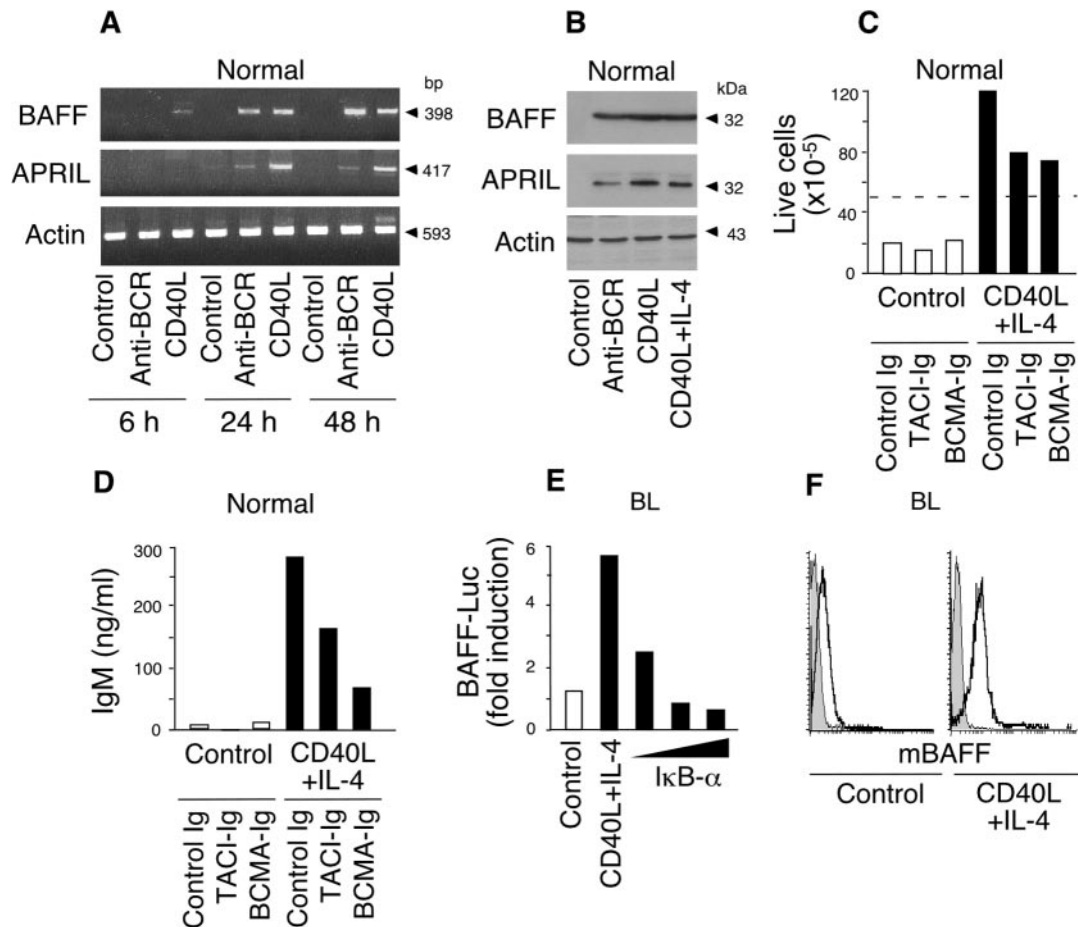


FIGURE 10. Nonmalignant B cells up-regulate BAFF and APRIL upon activation by BCR or CD40. *A* and *B*, BAFF, APRIL, and actin transcripts and total proteins in nonmalignant IgD⁺ B cells incubated with medium alone (control), anti-BCR, CD40L, and/or IL-4. *C* and *D*, Viability and IgM-secreting activity of nonmalignant IgD⁺ B cells after incubation with or without CD40L, IL-4, control Ig, TACI-Ig, and/or BCMA-Ig. The dotted line indicates initial cell input. *E*, Bjab BL B cells transfected with BAFF-Luc, empty pcDNA3, or 2, 10 or 20 μg of IκBα-pcDNA3 were incubated with medium alone (control; □) or CD40L plus IL-4 (■). Luciferase activity was measured after 2 days and is expressed as the fold induction. *F*, mBAFF (open profiles) on Bjab BL B cells incubated with medium alone (control) or CD40L plus IL-4 for 4 days. The shaded profiles depict cell staining with an isotype-matched control Ab. The data in *A–F* represent one of three similar experiments.

and BAFF-R synthesis. This might explain the diffuse cytoplasmic expression pattern of TACI and BCMA in NHL B cells.

NF-κB is crucial to up-regulate Bcl-2 and Bcl-x_L and attenuate B cell apoptosis (8, 9). In normal mouse B cells, BAFF-R typically turns on an alternative NF-κB pathway in which phosphorylation of cytoplasmic p100 (also known as NF-κB2) by TRAF-activated NIK and IKKα is followed by p100 ubiquitination and processing to p52, which then translocates to the nucleus in association with RelB (37–39). In similar B cells, BAFF-R as well as TACI and BCMA also activate a canonical NF-κB pathway (33–36), in which IκBα phosphorylation by a TRAF-activated IKKα-β-γ complex is followed by IκBα degradation and subsequent p50-c-Rel and p50-p65 nuclear translocation (51). Although unable to assess the role of BAFF-R, our experiments suggest that BAFF and APRIL produced by NHL B cells constitutively activate the canonical NF-κB pathway through BCMA and perhaps TACI. Consistent with this, BCMA and TACI on NHL B cells are constitutively associated with autocrine BAFF and APRIL as well as TRAF2, TRAF6, and p-IκBα. That autocrine BAFF and APRIL contribute to the deregulation of the canonical NF-κB pathway in NHL B cells is also shown by the ability of these malignant B cells to attenuate constitutive IκBα phosphorylation, IκBα degradation, and p50-c-Rel and p50-p65 nuclear translocation upon exposure to soluble TACI and BCMA decoy receptors. Conversely, NHL B

cells up-regulate IκBα phosphorylation, IκBα degradation, and p50-c-Rel and p50-p65 nuclear translocation upon exposure to exogenous BAFF or APRIL. Finally, preliminary data (not shown) suggest that NHL B cells activate the alternative NF-κB pathway upon exposure to BAFF, but not APRIL. Thus, it is likely that BAFF and APRIL cooperatively enhance NHL B cell survival by activating both classical and alternative NF-κB pathways through distinct receptors.

In addition to activating NF-κB (33), TACI limits the survival and expansion of activated B cells by triggering apoptosis (27, 64). The paradoxical ability of TACI to activate NF-κB and yet induce cell death is similar to that observed for other TNF receptor family members, which trigger either survival or death depending on the cell context (65). Thus, it is conceivable that TACI triggers apoptosis in normal B cells, but enhances survival in NHL B cells. The outcome of TACI signaling might also depend on the relative expression of BCMA and BAFF-R. Should this be the case, certain NHL B cells might become more sensitive to the prosurvival activity of BCMA and BAFF-R by down-regulating TACI.

We have recently shown that B cells exposed to BAFF and APRIL up-regulate AID (28). This enzyme is thought to initiate SHM and CSR by favoring the introduction of DNA breaks within the Ig V(D)J and switch regions (3). Because of its potential oncogenic activity (66), AID is only transiently expressed by activated B cells, including

GC B cells (3, 50). As shown by our data, B cell NHLs constitutively express AID (50, 67, 68) and actively undergo SHM and CSR in a variable fraction of the clone (50, 61). Ongoing SHM might select increasingly dangerous clonal variants by aberrantly targeting non-Ig loci (24), whereas ongoing CSR might favor the outgrowth of class-switched IgD⁻IgM⁻ elements with conserved responsiveness to environmental Ags (60, 69). In this regard, IgG-BCR is less sensitive than IgD-BCR and IgM-BCR to CD22, an inhibitory coreceptor that negatively modulates Ag-induced B cell growth by turning off the Ig α -Ig β signaling unit of the BCR complex (70). It is tempting to speculate that autocrine and paracrine BAFF and APRIL contribute to the deregulation of SHM and CSR by up-regulating AID and increasing the survival of AID-expressing NHL B cells.

By showing that soluble TACI and BCMA decoy receptors attenuate spontaneous NHL B cell DNA synthesis and that APRIL increases the proportion of NHL B cells entering the S and G₂-M phases of the cell cycle, our data suggest that APRIL stimulates malignant B cell growth through both autocrine and paracrine pathways. In BL B cells, APRIL-induced proliferation is associated with up-regulation of the growth-inducing proteins c-Myc and p-p38, down-regulation of the growth-inhibiting protein p53, and up-regulation of the GC B cell differentiation-inhibiting protein Bcl-6. Although inducing similar changes in c-Myc, p38, p53, and Bcl-6 expression, BAFF does not increase the proportion of NHL B cells in the S and G₂-M phases of the cell cycle. Yet, BAFF enhances the proliferation of NHL B cells exposed to APRIL. These observations and recent studies showing that BAFF augments the proliferation of MM B cells (47) suggest that BAFF and APRIL cooperatively stimulate the growth of tumoral B cells by delivering qualitatively distinct signals through different receptors.

Our finding that engagement of CD40 on tumoral B cells by CD40L up-regulates BAFF extends previous studies showing that certain NHLs express CD40L in addition to CD40 (71–73) and raises the possibility that multiple TNF-related ligands cooperatively enhance the accumulation of NHL B cells. This would be in line with a recent report showing that BAFF increases the proliferation and survival of normal B cells exposed to CD40L (39). In addition to attenuating apoptosis, BAFF and APRIL might increase the proliferation and limit the terminal differentiation of CD40L-induced NHL B cells by up-regulating Bcl-6. This transcriptional repressor retains actively proliferating B cells in the GC (56), and its down-regulation by CD40L (74) induces cell cycle arrest as well as maturation of both normal and malignant GC B cells to terminally differentiated plasma cells (57).

NHLs constitute a heterogeneous collection of diseases, and successful therapy will ultimately depend on detailed knowledge of the pathogenetic pathways involved in each lymphoma. Our findings outline a key survival pathway that is shared by a vast spectrum of B cell tumors. This implies that BAFF and APRIL inhibitors, such as soluble TACI and BCMA decoy receptors, could be used as adjuvants in the context of specific antilymphoma therapies. Given the ability of CD40L to cooperate with BAFF and APRIL, a combination of CD40L blockers and BAFF and APRIL inhibitors might be beneficial in selected NHL cases with increased CD40L expression.

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