

BAFF regulates B cell survival by downregulating the BH3-only family member Bim via the ERK pathway

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The B cell activating factor belonging to the tumor necrosis factor family (BAFF) is required for B cell survival and maturation. The mechanisms by which BAFF mediates B cell survival are less understood. We found that BAFF and a proliferation-inducing ligand (APRIL), which are related, block B cell antigen receptor (BCR)-induced apoptosis upstream of mitochondrial damage, which is consistent with a role for Bcl-2 family proteins. BCR ligation strongly increased expression of the proapoptotic Bcl-2 homology 3-only Bcl-2 protein Bim in both WEHI-231 and splenic B cells, and increases in Bim were reversed by BAFF or APRIL. Small interfering RNA vector-mediated suppression of Bim blocked BCR-induced apoptosis. BAFF also induced Bim phosphorylation and inhibited BCR-induced association of Bim with Bcl-2. BAFF induced delayed but sustained stimulation of extracellular signal-regulated kinase (ERK) and its activators, mitogen-activated protein kinase/ERK activating kinase (MEK) and c-Raf, and MEK inhibitors promoted accumulation and dephosphorylation of Bim. These results suggest that BAFF inhibits BCR-induced death by down-regulating Bim via sustained ERK activation, demonstrating that BAFF directly regulates Bim function. Although transitional immature type 1 (T1) B cell numbers are normal in Bim^{-/-} mice, T2 and follicular mature B cells are elevated and marginal zone B cells are reduced. Our results suggest that mature B cell homeostasis is maintained by BAFF-mediated regulation of Bim.

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Abbreviations used: APRIL, a proliferation-inducing ligand; BAFF, B cell activating factor belonging to the TNF family; BCMA, B cell maturation antigen; BCR, B cell antigen receptor; BH3, Bcl-2 homology 3; $\Delta\psi_m$, mitochondrial membrane potential; DUSP, dual-specificity phosphatase; ERK, extracellular signal-regulated kinase; FO, follicular; FOP, FO precursor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK activating kinase; MKP, MAPK phosphatase; MZ, marginal zone; MZP, MZ precursor; PARP, poly(ADP-ribose)polymerase; siRNA, small interfering RNA; T1, transitional immature type 1; TACI, transmembrane activator and cyclophilin ligand interactor.

Apoptosis plays an essential role in the development and maintenance of cellular homeostasis of the mammalian immune system. The survival and death of hematopoietic cells, including B lymphocytes, is finely tuned; excessive apoptosis may lead to immunodeficiencies, whereas too little cell death may cause autoimmunity and cancers (1, 2). During B lymphocyte development, progression from the splenic immature transitional immature type 1 (T1) B cell to the T2 stage is critical for establishing long-lived mature B cells (3). Thus, at the bone marrow immature stage and the splenic T1 stage, B cells expressing either nonfunctional or autoreactive B cell antigen receptors (BCRs) are deleted by neglect and negative selection, respectively (4). Components of both the BCR signaling pathway (e.g., Ig α and Syk) and the B cell activating factor belonging to the TNF family

(BAFF; BLyS/TALL-1/THANK/zTNF4) are required for T1 to T2 B cell progression (5–8).

BAFF is both a B cell survival and maturation factor (7–9). It binds three TNF family receptors: BAFF-R (BR3), B cell maturation antigen (BCMA), and transmembrane activator and cyclophilin ligand interactor (TACI) (7, 8). A second, highly related homologue, a proliferation-inducing ligand (APRIL), also binds TACI and BCMA but not BAFF-R (7, 8). The mechanisms by which BAFF regulates B cell survival are not well-defined. BAFF blocks nuclear translocation of protein kinase C δ (PKC δ), and BAFF-mediated B cell survival is impaired in PKC δ KO mice (10). Like other antiapoptotic TNF homologues such as CD40L and receptor activator of NF- κ B ligand, BAFF promotes NF- κ B activation (11–14). BAFF activates NF- κ B via two distinct mechanisms: (a) I κ B degradation and subsequent nuclear translocation of active NF- κ B dimers and (b) NF- κ B-inducing kinase-mediated processing of p100 precursors to active p52 subunits. Mice deficient in both NF- κ B1 and

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NF- κ B2, similar to BAFF KO animals (7, 8), have a defect in progression of B cells from the T1 to T2 B cell stage. Consistent with a role for NF- κ B signaling in BAFF-mediated B cell survival, BAFF enhances mRNA levels of three NF- κ B-regulated antiapoptotic Bcl-2 family members, Bcl-2, Bcl-x_L, and A1/Bfl-1 (11, 12, 14, 15).

The Bcl-2 homology 3 (BH3)-only subgroup of the Bcl-2 family includes Bid, Bad, Bik, Bim, Bmf, Hrk/DP5, Noxa, and Puma. These proteins share only the BH3 domain with other Bcl-2 family members and are proapoptotic (2, 16, 17). BH3-only proteins serve as sentinels for specific apoptotic stimuli. They initiate programmed cell death via interaction with and blockade of prosurvival Bcl-2 family members (18). In particular, the BH3-only protein Bim is critical for apoptosis of hematopoietic cells, including B and T lymphocytes, macrophages, and granulocytes (19). Indeed, experiments using Bim KO mice indicate that Bim is required for negative selection of T lymphocytes and is up-regulated by TCR ligation (20). Similarly, BCR-induced apoptosis is strongly reduced in immature and mature B cells from Bim KO mice, and the deletion of autoreactive B cells is also inhibited (21). Furthermore, autoantigen-stimulated B cells from Ig/HEL double transgenic mice express elevated levels of Bim mRNA and protein (22).

The proapoptotic activity of Bim can be regulated at both the transcriptional and posttranslational levels. In response to growth factor withdrawal and concomitant blockade of the phosphatidylinositol 3-kinase-Akt pathway, Bim mRNA levels are up-regulated by activation of the fork-

head-like transcription factor FoxO3A/FKHRL-1 (23). At least two mechanisms exist for the posttranslational control of Bim: phosphorylation and ubiquitination. Phosphorylation of Bim is mediated by either extracellular signal-regulated kinase (ERK) or c-Jun NH₂-terminal protein kinase promoting changes in its proapoptotic activity (24–30). ERK-induced phosphorylation of Bim promotes its ubiquitination and subsequent degradation via the proteasome (26, 27, 30).

The B cell phenotype of BAFF transgenic mice is remarkably similar to that observed in mice lacking Bim. Both lines of mice have enlarged spleens, with elevated numbers of mature B cells and autoantibody-secreting plasma cells. Older mice also develop systemic lupus erythematosus-like autoimmune kidney disease (19, 31). Based on these similarities, we hypothesized that BAFF may promote B cell survival by down-regulating Bim. Using the immature WEHI-231 B cell line, which is a model for the deletion of autoreactive B cells (32, 33), and splenic B cells, we found that BCR signaling up-regulates Bim, and that BAFF blocks BCR-induced increases in Bim. BAFF signaling induced phosphorylation of Bim via a mitogen-activated protein kinase (MAPK)/ERK activating kinase (MEK)-dependent pathway and also promoted sustained ERK activation. Our results suggest that BAFF inhibits BCR-induced cell death by down-regulating Bim and that BAFF promotes Bim down-regulation via sustained activation of ERK. Based on the phenotype of Bim^{-/-} mice, we propose that BAFF regulates Bim at the transitional B cell stage to control B cell homeostasis.

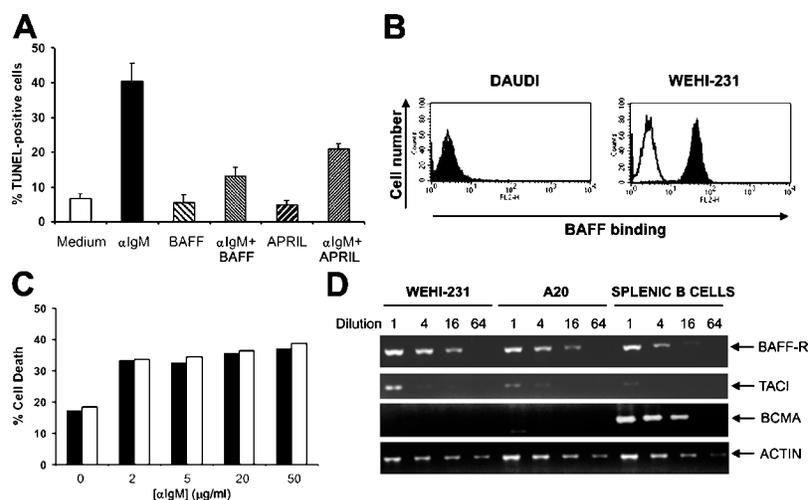


Figure 1. BAFF and APRIL block BCR-induced apoptosis in WEHI-231 B Cells. (A) WEHI-231 cells were treated for 48 h with anti-IgM in the presence or absence of BAFF or APRIL, and apoptotic cells were quantified by FACS using TdT-mediated dUTP nick-end labeling. Results show the means \pm SD from three independent experiments. (B) Binding of BAFF to Daudi (left) and WEHI-231 (right) B cell lines was detected by FACS using biotinylated BAFF and streptavidin-PE. Biotinylated BAFF + streptavidin-PE (shaded) and streptavidin-PE only (bold line) traces are shown. Results shown are from one of three experiments. (C) Daudi cells were treated for

48 h with anti-IgM in the presence (open bar) or absence (shaded bar) of BAFF or etoposide (as a positive control), and cell death was quantified by FACS using Mitotracker Red CMXRos. Results shown are from one of two experiments. (D) BAFF-R, TACI, BCMA, and β -actin (as a loading control) were amplified by RT-PCR using gene-specific primers from fourfold serial dilutions of WEHI-231, A20, and splenic B cell cDNA (Table S1, available at <http://www.jem.org/cgi/content/full/jem.20051283/DC1>). PCR products were resolved on NuSieve 3:1 agarose.

RESULTS

BAFF and APRIL inhibit BCR-induced cell death in WEHI-231 B cells

WEHI-231 B cells, like T1 B cells, undergo programmed cell death in response to BCR engagement (33–35). We initially tested the effect of BAFF and APRIL on BCR-induced cell death in WEHI-231 B cells. BCR engagement with goat anti-mouse IgM F(ab')₂ fragments induced apoptosis, and treatment with either BAFF or APRIL inhibited BCR-induced cell death (Fig. 1 A). Thus, WEHI-231 B cells express functional receptors for BAFF and APRIL, and BAFF and APRIL block BCR-induced cell death. Using a biotinylated derivative of BAFF, we found that WEHI-231 B cells have high relative levels of BAFF binding (Fig. 1 B). In contrast, the Burkitt's lymphoma line Daudi did not bind BAFF, and, as expected, BAFF did not block BCR-induced cell death in Daudi B cells (Fig. 1 C). RT-PCR analysis revealed that WEHI-231 B cells expressed similar levels of BAFF-R and moderately higher TACI levels compared with A20 and splenic B cells, but unlike splenic B cells, which expressed BCMA, neither WEHI-231 nor A20 cells expressed BCMA mRNA (Fig. 1 D). As APRIL binds only to TACI and BCMA (7, 8), these results suggest that APRIL-induced blockade of BCR-mediated cell death in WEHI-231 B cells is most likely mediated through TACI.

BAFF and APRIL inhibit BCR-induced apoptosis upstream of mitochondrial inner membrane depolarization and caspase-9 cleavage

To define how BAFF and APRIL block BCR-induced cell death, we next examined the effect of BAFF and APRIL on various components of the BCR-induced cell death pathway, including the effector caspase substrate poly(ADP-ribose) polymerase (PARP), caspase-3, and caspase-9 (36, 37). Anti-IgM antibodies induced cleavage of full-length PARP to its apoptotic fragment within 24 h, which was further increased at 48 h (Fig. 2 A). BAFF or APRIL strongly reduced anti-IgM-mediated PARP cleavage (Fig. 2 A). BCR cross-linking also resulted in increases in cleaved caspase-3 and processing of pro-caspase-9 (Fig. 2 A), which is required for activation of caspase-3-like effector caspases in WEHI-231 B cells (38). BAFF or APRIL blocked BCR-induced accumulation of both cleaved caspase-3 and cleavage of pro-caspase-9 (p47) to its large active 35-kD fragment (p35; Fig. 2 A). Thus, BAFF and APRIL block BCR-induced cell death upstream of BCR-induced pro-caspase-9 and -3 processing.

Because mitochondrial perturbations including the release of cytochrome C and inner membrane depolarization are upstream of caspase-9 and -3 activation (39) and are required for pro-caspase-9 processing during BCR-induced apoptosis (38), we tested whether BAFF could block BCR-induced depolarization of the mitochondrial inner membrane. A decrease in mitochondrial membrane potential ($\Delta\psi_m$) was detected 24 h after BCR ligation, decreasing further by 48 h (Fig. 2 B). This loss of mitochondrial inner membrane potential after BCR ligation was abrogated by BAFF in a dose-dependent manner (Fig. 2 C). Thus, BAFF

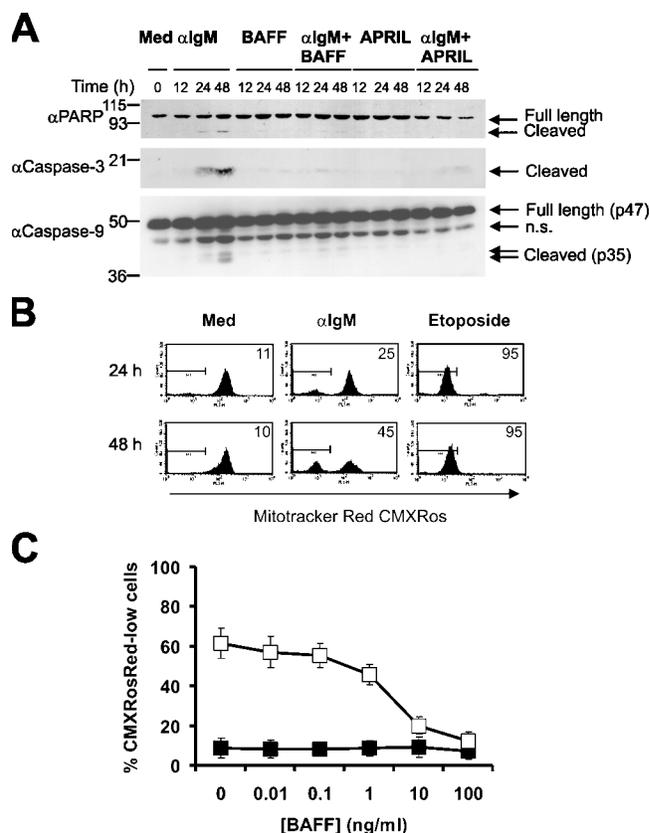


Figure 2. BAFF and APRIL block BCR-induced cell death upstream of caspase-3 and -9 activation and mitochondrial inner membrane depolarization. (A) WEHI-231 cells were treated for the indicated times with anti-IgM antibodies in the presence or absence of BAFF or APRIL. Immunoblots were probed with anti-PARP, anti-cleaved caspase-3, or anti-caspase-9. n.s., nonspecific. (B) WEHI-231 cells were treated for 24 or 48 h with either medium, anti-IgM, or etoposide (as a positive control). (top right) Percentages of Mitotracker Red CMXRos-low cells are shown. (C) WEHI-231 cells were stimulated for 48 h with the indicated concentrations of BAFF in the presence (\square) or absence (\blacksquare) of anti-IgM. $\Delta\psi_m$ was determined by FACS using Mitotracker Red CMXRos. Results shown are the means \pm SD from three experiments.

and APRIL block the BCR-induced cell death pathway upstream of mitochondrial damage.

BAFF and APRIL down-regulate BCR-induced expression of Bim

The fact that BAFF blocks BCR-induced cell death upstream of mitochondrial damage suggested that BAFF may regulate apoptosis by controlling the activity of one or more Bcl-2 family proteins. The phenotypes of B cells from BAFF transgenic and Bim KO mice are strikingly similar, with elevated numbers of mature B cells and plasma cells (19, 31). Hence, we tested whether BAFF and BCR cross-linking reciprocally regulated Bim levels in WEHI-231 B cells. Similar to other cells, including primary mouse B cells (21, 22), WEHI-231 B cells expressed much higher levels of BimEL than BimL or BimS (Fig. 3 A). BCR cross-linking induced a

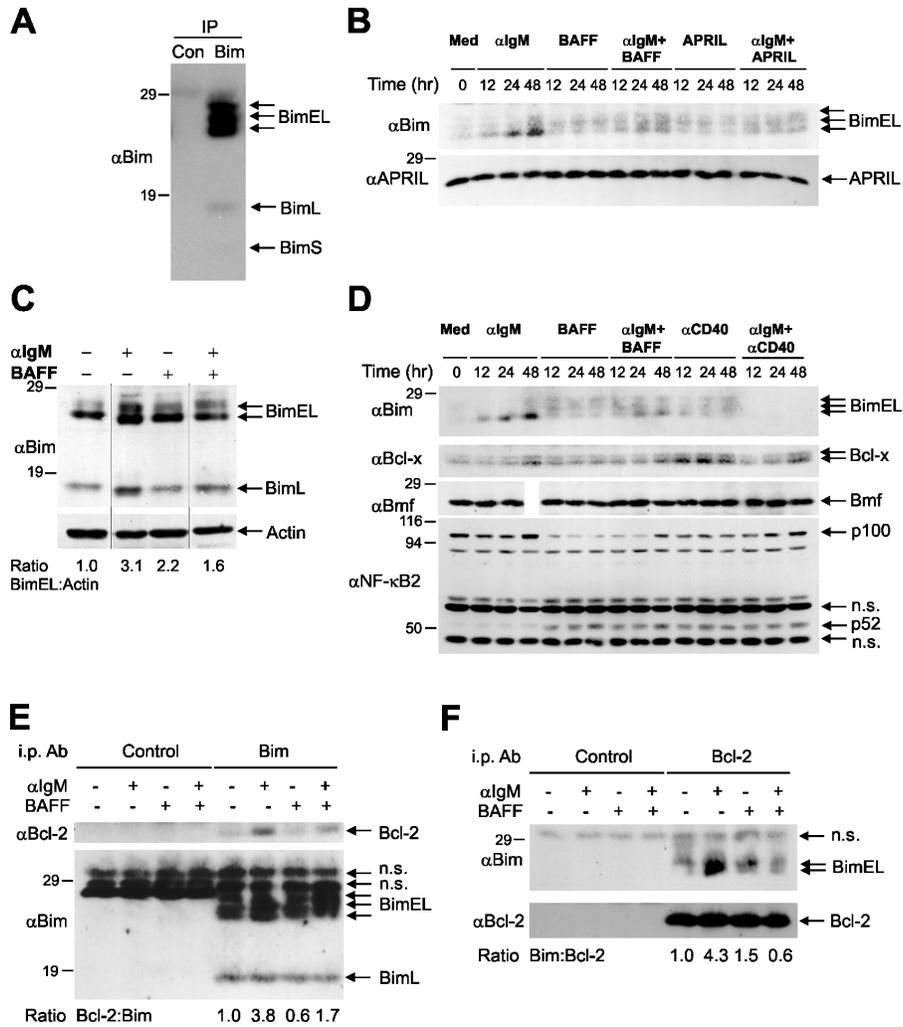


Figure 3. BAFF- and APRIL-induced down-regulation of Bim correlates with a blockade of BCR-induced apoptosis. (A) NP-40 lysates from WEHI-231 cells were immunoprecipitated with anti-Bim mAb or IgG_{2a} isotype control. Immune complexes were probed with anti-Bim serum. Positions of Bim isoforms including multiple BimEL species are shown by arrows. (B) WEHI-231 cells were incubated for 0–48 h with anti-IgM, BAFF, or APRIL (as in Fig. 2 A). Western blots were probed with anti-Bim or anti-APRIL serum (loading control). (C) Purified mouse splenic B cells were incubated with anti-IgM and/or BAFF for 24 h. Immunoblots were probed with anti-Bim or anti-actin serum (loading control). The ratio of BimEL to actin

was assessed by scanning densitometry. Dividing lines separate images from different parts of the same gel. (D) WEHI-231 cells were treated for the indicated times with anti-IgM, BAFF, or anti-CD40. Immunoblots were probed with anti-Bim, anti-Bcl-x_L, anti-Bmf, or anti-NF-κB2 serum. Non-specific (n.s.) proteins serve as loading controls. (E and F) WEHI-231 cells were incubated with anti-IgM antibodies and/or BAFF as indicated. Bim (E) or Bcl-2 (F) were immunoprecipitated from NP-40 lysates, which were probed with anti-Bcl-2 or anti-Bim, stripped, and re-probed with anti-Bim or anti-Bcl-2, respectively. Nonspecific (n.s.) bands are indicated. The ratio of Bcl-2 to Bim was quantified by scanning densitometry.

dramatic increase in the levels of BimEL detectable at 24 h and maximal at 48 h (Fig. 3 B). BAFF or APRIL markedly reduced BCR-mediated increases in BimEL protein levels (Fig. 3 B), and this correlated with blockade of BCR-induced cell death (Fig. 1 A). We then tested whether BAFF also down-regulated Bim expression in mouse splenic B cells. BCR ligation increased the levels of Bim protein (both BimL and BimEL isoforms) threefold in splenic B cells (Fig. 3 C). Similar to our results in WEHI-231 B cells, BAFF markedly reduced BCR-induced Bim expression in splenic B cells to levels seen in unstimulated B cells (Fig. 3 C).

Because BAFF and APRIL blocked BCR-induced expression of Bim, we tested whether engagement of CD40, another TNF receptor whose ligation blocks BCR-induced cell death (32), also reduced BCR-induced Bim expression. CD40 ligation also blocked BCR-mediated increases in BimEL protein (Fig. 3 D). In contrast, neither BAFF nor CD40 ligation regulated expression of Bmf, another BH3-only Bcl-2 family member. We also examined whether BAFF regulated expression of two key prosurvival Bcl-2 family members, Bcl-x_L or Bcl-2, in these cells. Although CD40 ligation increased the levels of Bcl-x_L

protein, BAFF did not up-regulate Bcl- x_L or Bcl-2 expression (Fig. 3 D and see Fig. 5 C). However, BAFF did regulate the association of Bim with Bcl-2. In the absence of BCR cross-linking, little Bim associated with Bcl-2 (Fig. 3, E and F). However, BCR cross-linking promoted a strong association of Bcl-2 with Bim (Fig. 3, E and F). Importantly, Bim-Bcl-2 interactions were considerably reduced in B cells treated with both anti-IgM and BAFF (Fig. 3, E and F).

Down-regulation of Bim blocks BCR-induced cell death

To test whether down-regulating Bim expression inhibits BCR-induced apoptosis in WEHI-231 B cells, endogenous Bim levels were knocked down by stable overexpression of Bim small interfering RNA (siRNA). BimEL levels were strongly reduced in B cells overexpressing Bim488siRNA, in contrast to B cells expressing a vector control or another Bim siRNA, Bim13siRNA (Fig. 4 A). Importantly, BCR-induced cell death was also decreased by 50–75% at a range of doses of anti-IgM antibodies in the Bim488siRNA-expressing B cells compared with control or Bim13siRNA-expressing cells (Fig. 4 B). These results show that down-regulating endogenous Bim levels strongly reduces BCR-mediated apoptosis in WEHI-231 B cells, similar to immature Bim KO splenic B cells (21). Together, our results suggest BAFF inhibits BCR-induced cell death by down-regulating Bim.

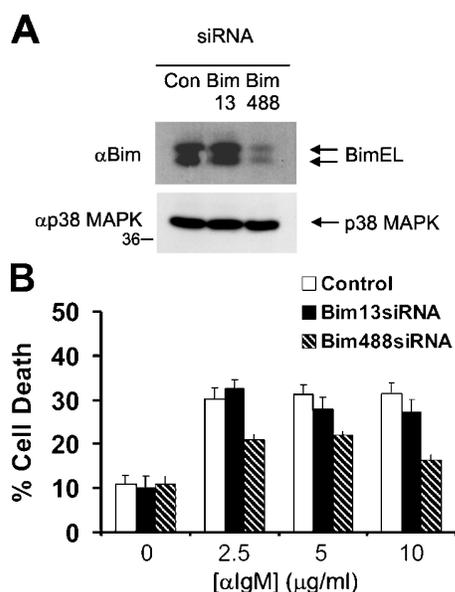


Figure 4. Down-regulation of Bim inhibits BCR-induced apoptosis in WEHI-231 B cells. (A) Immunoblots were prepared as in Fig. 2 and probed with anti-Bim or anti-p38 MAPK (loading control). BimEL species migrating with different mobilities are indicated by arrows. (B) Stable WEHI-231 cell lines expressing either Bim-specific siRNAs or a control vector were treated for 48 h with the indicated doses of anti-IgM, and $\Delta\psi_m$ was determined by FACS using Mitotracker Red CMXRos. Results shown are the means \pm SD from three independent experiments.

BAFF promotes Bim phosphorylation via sustained activation of a MEK–ERK–dependent pathway

In addition to lowering BimEL levels, BAFF signaling generated BimEL species with reduced mobility on SDS-PAGE (Fig. 3), suggesting that BimEL may be posttranslationally modified in B cells. Indeed, BimEL can be posttranslationally regulated via phosphorylation and/or ubiquitination in other cells (24–30). Thus, we tested whether the slower migrating forms of BimEL in WEHI-231 B cells treated with BAFF were caused by phosphorylation of BimEL. We compared the mobility of BimEL from BAFF-, anti-IgM-, and anti-CD40-stimulated B cells in the presence or absence of λ protein phosphatase, which dephosphorylates serine, threonine, and tyrosine residues (40). Treatment with λ protein phosphatase increased the mobility of BimEL in BAFF- and anti-CD40-treated B cells (Fig. 5 A). In contrast, the mobility of BimEL from WEHI-231 B cells stimulated with anti-IgM antibodies for 24 h was unaltered by λ protein phosphatase (Fig. 5 A).

Previous studies have shown that prosurvival signals, including the TNF family ligands receptor activator of NF- κ B ligand and nerve growth factor, induce phosphorylation of BimEL via the MEK–ERK pathway (24, 30). To test whether the MEK–ERK pathway was required for BAFF-induced BimEL phosphorylation in B cells, WEHI-231 cells were pretreated with U0126, a specific MEK inhibitor (41), before stimulation by BAFF. U0126 increased both the mobility and levels of BimEL from BAFF-treated B cells under conditions in which ERK phosphorylation was markedly inhibited (Fig. 5 B), suggesting that BAFF-induced BimEL phosphorylation via the MEK–ERK pathway down-regulates BimEL expression. In contrast, a specific inhibitor of p38 MAPK did not change the levels of BimEL from BAFF-treated B cells even though a moderate increase in electrophoretic mobility was observed (Fig. 5 B).

Because these data suggested that BAFF regulates Bim expression via a MEK-dependent pathway, we next tested whether BCR and BAFF signaling differ in their regulation of ERK activation using a phospho-specific ERK antibody that detects only the phosphorylated, activated forms of ERK. Although BCR cross-linking induced rapid activation of ERK1 and -2 within 30 min, this ERK activation subsequently declined below basal levels within 16 h and remained low (Fig. 5, C and D). In contrast, BAFF signaling promoted a delayed but sustained phosphorylation of ERK1 and -2 that was only evident 4 h after BAFF stimulation (Fig. 5, C and D). Importantly, in the presence of BAFF, BCR-induced ERK1 and -2 activation was sustained for up to 24 h (Fig. 5, C and D). BAFF may induce sustained ERK activation by stimulating kinases upstream of ERK or, alternatively, by inhibiting protein phosphatases such as the dual-specificity phosphatases (DUSPs) of MAPK phosphatase (MKP), which dephosphorylate ERK (42). To investigate these possibilities, we compared the effects of anti-IgM antibodies and BAFF on the phosphorylation of two upstream kinases: MEK and one of its activators, the MAPKKK mem-

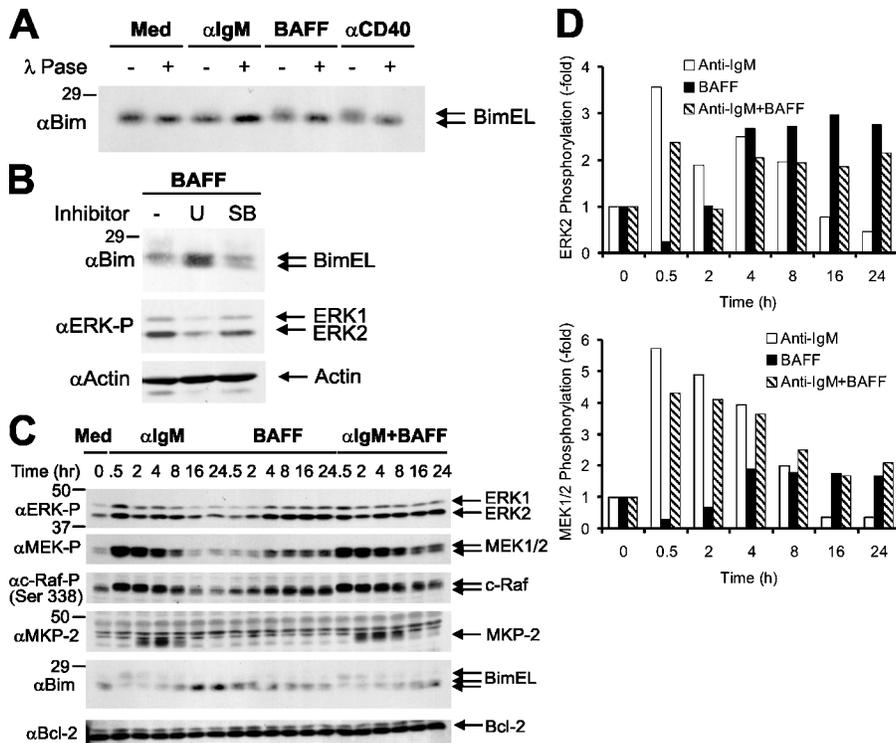


Figure 5. BAFF induces Bim phosphorylation via sustained activation of the MEK-ERK pathway. (A) WEHI-231 cells were treated for 24 h with medium, anti-IgM, BAFF, or anti-CD40. Cell lysates were incubated with or without λ protein phosphatase. Immunoblots were probed with anti-Bim serum. (B) WEHI-231 cells were pretreated for 2 h with DMSO vehicle, 5 μ M MEK inhibitor U0126 (U), or 5 μ M p38 MAPK inhibitor SB203580 (SB) before incubation with BAFF for 24 h. Blots were probed

with anti-Bim, anti-phospho-ERK, or anti-actin (loading control). (C and D) WEHI-231 cells were treated for 0–24 h with anti-IgM in the presence or absence of BAFF. Immunoblots were probed with either anti-phospho-ERK, anti-phospho-MEK, anti-phospho-c-Raf (Ser 338), anti-MKP2, anti-Bim, or anti-Bcl-2 serum. In (D), immunoblots for ERK2 (top) or MEK1/2 (bottom) were quantified by scanning densitometry to measure the fold change in each phosphorylated protein.

ber c-Raf, whose phosphorylation on Ser 338 is required for c-Raf activation in response to many stimuli (43). In addition, we monitored expression of two MKPs, MKP-2/DUSP4 and MKP-3/DUSP6, which contain ERK-binding FXFP motifs (42). Similar to our results with ERK, BCR cross-linking induced strong but transient MEK and c-Raf phosphorylation that declined below basal levels after 16–24 h, whereas BAFF induced delayed but sustained increases in both MEK and c-Raf phosphorylation (Fig. 5, C and D). BCR ligation also strongly up-regulated MKP-2 levels, whereas MKP-3 levels were not substantially affected by BCR cross-linking (Fig. 5 C and not depicted). Interestingly, the initial BCR-mediated increases in MKP-2 protein correlated with down-regulation of BCR-mediated ERK activation, which was consistent with the idea that MKP-2 may be a critical negative feedback regulator of BCR-induced ERK activation in B cells. In contrast, BAFF signaling did not induce MKP-2 expression, which is consistent with its ability to promote sustained ERK activation (Fig. 5 C). Consistent with the distinct patterns of BCR- and BAFF-induced ERK activation, BCR cross-linking also promoted rapid but transient phosphorylation of Bim that was maximal after 30 min, followed by its sustained accumulation at later

times (Fig. 5 C). Importantly, BAFF markedly blocked BCR-induced Bim accumulation (Fig. 5 C). In summary, these results suggest that sustained ERK activation by BAFF correlates with its ability to promote down-regulation of Bim and strongly suggest that BAFF promotes Bim down-regulation via sustained activation of ERK. Furthermore, the data suggest that BAFF induces ERK activation by activating upstream kinases such as c-Raf rather than blockade of BCR-mediated expression of MAPK phosphatases such as MKP2/DUSP4.

Effect of individual BAFF receptors on BCR-induced cell death and Bim down-regulation

To examine the effects of individual BAFF receptors in regulating BCR-induced apoptosis and Bim expression in WEHI-231 B cells, we developed a chimeric BAFF receptor system. We created constructs where the extracellular and transmembrane regions of each BAFF receptor were substituted with the extracellular and transmembrane portions of mouse CD8 α (Fig. 6 A). WEHI-231 B cells were retrovirally transduced with CD8 α -control, -BAFF-R, -TACI, and -BCMA, and stable clonal cell lines were established. Each chimeric BAFF receptor was expressed at simi-

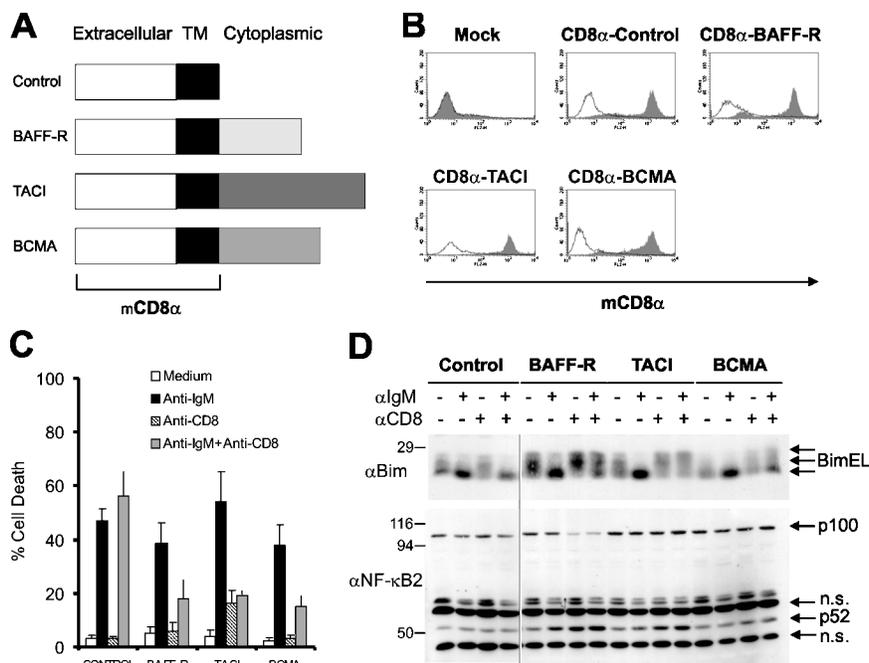


Figure 6. Blockade of BCR-induced apoptosis by chimeric BAFF receptors correlates with changes in Bim mobility and expression. (A) Chimeric BAFF receptors were generated by in-frame fusion of the extracellular and transmembrane domains of mouse CD8 α to the cytoplasmic regions of BAFF-R, TACI, and BCMA. (B) Surface levels of chimeric BAFF receptors on clonal WEHI-231 cell lines were quantified by FACS using biotinylated anti-CD8 α mAb. Isotype control (open trace) and CD8 α

lar levels on the clonal cell lines (Fig. 6 B). We incubated each chimeric CD8 α –BAFF receptor–expressing cell line with anti-IgM, anti-CD8 α , or a combination of both antibodies for 48 h and quantified cell death. Cross-linking either chimeric CD8 α –BAFF-R, –TACI, or –BCMA but not a control CD8 α receptor lacking a cytoplasmic domain substantially inhibited BCR-induced apoptosis (Fig. 6 C). Thus, engagement of each BAFF receptor—BAFF-R, TACI, or BCMA—blocks BCR-induced cell death in WEHI-231 B cells.

As BAFF and APRIL strongly down-regulated BCR-induced Bim expression (Fig. 3 B), we next examined the effect of engaging each BAFF receptor on the blockade of BCR-induced Bim expression by treating each WEHI-231–CD8 α –BAFF receptor B cell line with anti-IgM antibodies in the presence or absence of anti-CD8 α for 48 h followed by immunoblotting of Bim. Ligation of chimeric CD8 α –BAFF-R and, in particular, CD8 α –TACI resulted in both decreases in electrophoretic mobility and reduced Bim expression (Fig. 6 D). As a positive control, processing of NF- κ B2 from its p100 precursor into the active p52 subunit was also monitored. Ligation of chimeric BAFF-R, but not TACI or BCMA, induced strong NF- κ B2 p100 processing (Fig. 6 D), which was consistent with a previous study (13). Ligation of chimeric TACI also moderately increased p52 levels, but in the apparent absence of p100 processing.

cell surface (shaded trace) expression levels are shown. (C and D) Clonal WEHI-231 cell lines expressing chimeric BAFF receptors were incubated for 48 h with the indicated stimuli. Cell death was quantified by trypan blue staining. Results shown are the means \pm SD from at least three independent clonal lines. Bim and NF- κ B2 were detected by immunoblotting of cell lysates as described in Fig. 3. A dividing line separates images from different parts of the same gel. n.s., nonspecific.

Bim KO mice have elevated numbers of splenic T2 and follicular (FO) mature B cells but reduced marginal zone (MZ) B cells

Bim KO mice have increases in splenic B cell numbers (19); however, it is unclear whether this represents a global dysregulation of all splenic B cell populations or defects in specific B cell subsets. To address this issue, we analyzed the numbers of splenic T1, T2, FO, and MZ B cell populations in Bim KO mice. The numbers of total splenic B cells were elevated two- to threefold in Bim^{-/-} compared with WT mice (unpublished data) (19). These differences were reflected in the 2.5- and 4-fold increases in FO and T2–FO precursor (FOP) splenic B cell numbers and a 1.5-fold increase in T2–MZ precursor (MZIP) B cells, respectively (Fig. 7, A and B). Remarkably, Bim KO mice had similar numbers of T1 B cells and twofold fewer MZ B cells in the spleen compared with WT mice (Fig. 7 A), suggesting that regulation of Bim by BAFF or other TNF family ligands is particularly important at the T2 and FO B cell stages, whereas other BH3-only Bcl-2 family members may control homeostasis of T1 and MZ B cell populations. We next tested whether Bim was required for spontaneous- and BCR-induced cell death in different splenic B cell subsets using B cells sorted based on their expression of CD21, CD23, and CD24. Spontaneous apoptosis of immature T2, mature FO, and MZ B cells was strongly reduced in Bim KO mice compared with their WT counter-

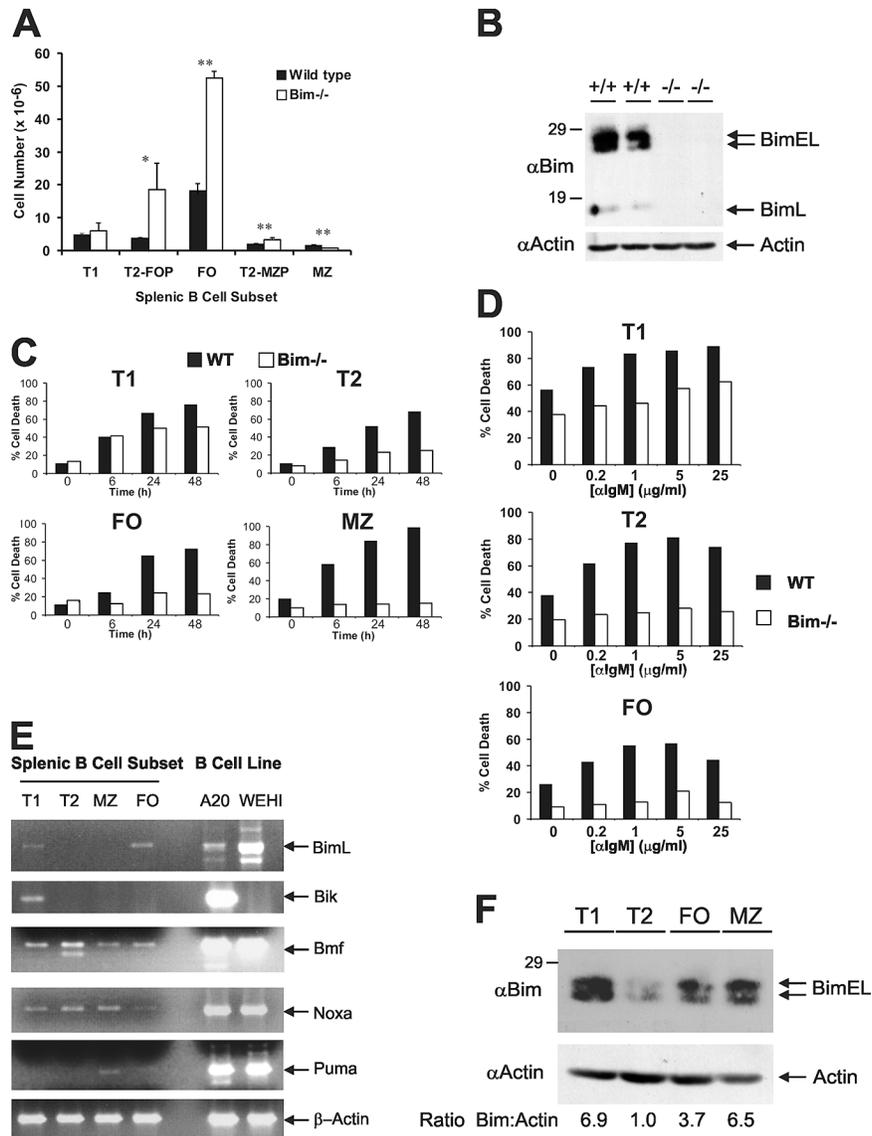


Figure 7. Splenic B cell subsets are dysregulated in Bim KO mice and differ in their expression of BH3-only Bcl-2 family members.

(A) Splenic B cells were isolated from WT and Bim^{-/-} mice, stained with αIgD-FITC, αCD21-PE, and αIgM-PerCP, and the numbers of T1, T2-FOP, FO, T2-MZP, and MZ B cells were determined. Data shown are the means ± SEM from three mice per phenotype. MZ B cell numbers in WT and Bim KO mice were $1.7 \pm 0.2 \times 10^6$ and $0.73 \pm 0.09 \times 10^6$, respectively. $P < 0.05$ (*) and $P < 0.02$ (**) were determined using an unpaired *t* test. (B) Cell lysates of isolated splenic B cells from WT and Bim^{-/-} mice were probed with anti-Bim or anti-actin (loading control). (C) T1, T2, FO, and MZ splenic B cell subsets were purified by FACS cell sorting using

αCD21-FITC, αCD23-APC, and αCD24-PE. Spontaneous cell death was quantified at the indicated times by FACS using Mitotracker Red CMXRos. (D) T1, T2, and FO splenic B cell subsets were isolated as described in C and treated for 18 h with the indicated doses of anti-IgM, and cell death was analyzed as described in C. (E) T1, T2, FO, and MZ splenic B cell subsets were purified as described in C. Bim, Bik, Bmf, Noxa, Puma, and β-actin (loading control) were amplified by RT-PCR (Table S1), and PCR products were resolved as in Fig. 1D. (F) Splenic B cell subsets were isolated by FACS cell sorting as described above. Immunoblots were probed with anti-Bim or anti-actin (loading control).

parts (Fig. 7 C), whereas spontaneous cell death of T1 B cells was only moderately reduced after 24–48 h. BCR-induced apoptosis was also strongly blocked in T2 and FO splenic B cell populations from Bim KO compared with WT mice, whereas BCR-induced cell death was observed in T1 B cells from Bim KO mice, albeit at reduced levels compared with WT mice (Fig. 7 D).

Expression of Bim and other BH3-only Bcl-2 family members in mouse splenic B lymphocyte subsets

The results in Fig. 7 suggest that different subpopulations of splenic B cells may express distinct profiles of Bim and other BH3-only Bcl-2 members. In addition, a recent study shows that Bim is expressed at increasing levels during B cell development in the bone marrow (44). Thus, T1, T2, FO, and

MZ B cells were isolated by cell sorting, and the levels of BH3-only Bcl-2 family members were analyzed by RT-PCR. Bim mRNA was detected in T1 but not T2 B cells and was expressed at highest levels in FO B cells (Fig. 7 E). Strikingly, Bik mRNA was selectively expressed in T1 B cells but not detected in T2, FO, or MZ B cells (Fig. 7 E). Two other BH3-only Bcl-2 family members, Bmf and Noxa, were more ubiquitously expressed, with the highest mRNA levels observed in T2 B cells. In contrast, Puma mRNA appeared to be relatively restricted to MZ B cells (Fig. 7 E). As mRNA expression of Bcl-2 family members may not correlate with protein expression (45), we also examined Bim protein levels in splenic B cell subsets (Fig. 7 F). Bim protein was expressed in each splenic B cell subset, with T2 B cells expressing the lowest levels of Bim (Fig. 7 F).

DISCUSSION

Our results suggest that BAFF-mediated down-regulation of Bim is a key mechanism by which BAFF promotes B cell survival. The finding that BAFF affects B cell fate via its regulation of Bim is consistent with the striking similarity observed between BAFF-transgenic and Bim KO mice. Both have dysregulated B cell maturation leading to elevated numbers of mature B cells and autoantibody-secreting plasma cells (Fig. 7 A) (19, 31). Autoantigen-binding B cells appear to have increased dependence on BAFF for their survival compared with naive B lymphocytes (22). Thus, in a host with a diverse B cell repertoire with limiting quantities of BAFF, autoantigen-binding B cells, which receive too little BAFF to be protected from BCR-induced apoptosis, are competitively eliminated. Importantly, the levels of Bim protein were higher in autoantigen-binding B cells compared with naive B cells, suggesting that BAFF acts, at least in part, by blocking the function of Bim (22). Our finding that BAFF down-regulates expression of Bim protein and also promotes its phosphorylation thus provides clear evidence that BAFF directly regulates Bim and antagonizes its function (Figs. 3 and 5), underscoring that Bim is an important target of BAFF-induced B cell survival.

BAFF appears to inhibit BCR-induced cell death by promoting down-regulation of the proapoptotic BH3-only family protein Bim rather than up-regulating antiapoptotic Bcl-2 family members (Figs. 3 and 5). Indeed, BAFF did not reproducibly change the levels of three key prosurvival Bcl-2 subfamily proteins, Bcl-x_L, Bcl-2, or Mcl-1 (Figs. 3 and 5 and not depicted). A similar lack of effect of BAFF on Bcl-2, Bcl-x_L, Mcl-1, or A1 at the mRNA and protein level has also been observed in purified B cells (22, 46, 47). In contrast, ligation of CD40, another antiapoptotic TNF receptor, both down-regulated Bim and up-regulated Bcl-x_L (Fig. 3 D). It is possible that BAFF's inability to up-regulate Bcl-x_L reflects its preferential activation of the type 2 (p52-dependent) as opposed to the type 1 (p50-dependent) NF-κB pathway (47). However, another report showed that BAFF up-regulates Bcl-x_L mRNA and protein via a p50 (NF-κB1)-dependent pathway in mature mouse B cells (14). Al-

though an explanation for these differences remains unclear, the coordinate activation of both type 1 and 2 NF-κB pathways plays an important role in BAFF-mediated B cell survival (12–14).

Bim is a critical regulator of hematopoietic cell homeostasis including both B and T lymphocytes (19–21, 44, 48). In particular, studies using immunoglobulin- and TCR-transgenic mice or self-antigen mouse models indicate Bim is required for the deletion of autoreactive B and T cells (20, 21, 48). Thus, our results showing that siRNA-mediated knockdown of Bim strongly reduces BCR-induced apoptosis are consistent with these studies and another report using a human B cell line (49).

Intriguingly, analysis of the splenic B cell subsets in Bim KO mice revealed increases in specific subsets as opposed to a general increase in all B cell populations (Fig. 7 A). The numbers of T2-FOP and FO B cells were increased 4- and 2.5-fold, respectively, suggesting that Bim is a key negative regulator of homeostasis in these B cell subsets. In contrast, Bim KO and WT mice had similar numbers of T1 B cells (Fig. 7 A), suggesting that either Bim is not critical for the homeostasis of T1 B cells, even though Bim protein is expressed in T1 B cells (Fig. 7 F), or that other BH3-only proteins function in concert with Bim to control homeostasis. Consistent with these ideas, T1 B cells from Bim KO mice remained sensitive to both spontaneous- and BCR-induced cell death, albeit at reduced levels compared with WT mice (Fig. 7, C and D). A good candidate is Bik, which may regulate BCR-induced cell death (50). Not only do T1 B cells express Bik mRNA (Fig. 7 E), but T1 B cells from BAFF-R mutant mice express more Bik compared with WT mice (51). Although no obvious defects in total splenic B cell numbers were apparent in Bik KO mice (52), a detailed analysis of transitional and MZ B cells was not performed. The fact that T2 and FO B cells are elevated in Bim^{-/-} mice suggests that BAFF-mediated control of Bim may be particularly important for the homeostatic control of these B cell populations. Our result that Bim is expressed at higher levels in T1 compared with T2 B cells suggests that BAFF may function at the T2 stage to down-regulate Bim (Fig. 7 F), which is consistent with studies showing that BAFF induces survival of T2 B cells (7–9). Surprisingly, splenic MZ B cell numbers were reduced in Bim^{-/-} mice (Fig. 7 A). Similar decreases in MZ B cell numbers were also observed in Eμ-Bcl-2 transgenic mice (53). These mice also had enhanced BCR-induced calcium responses consistent with a model in which strong BCR signals favor FO versus MZ B cell development (54). Thus, the absence of Bim may lead to more free Bcl-2, such as in Bcl-2 transgenic mice, providing an explanation for the reduced MZ B cell numbers. Collectively, our results suggest that in addition to its role in promoting cell death, Bim may also regulate B cell lineage decisions, a possibility we are now investigating.

BAFF-induced blockade of BCR-induced cell death and down-regulation of Bim correlated with sustained activation of the MEK-ERK signaling pathway (Figs. 3, 5). Ligation of

CD40 also induced a similar sustained pattern of ERK activation in WEHI-231 B cells (55). Indeed, pharmacological blockade of BAFF-mediated ERK activation using the MEK inhibitor U0126, strongly elevated BimEL protein levels (Fig. 5 B), suggesting that the MEK-ERK pathway is required for BAFF-induced BimEL down-regulation. Previous studies have also shown that activation of the ERK pathway in response to serum survival factors, PMA, IL-3, and M-CSF, induces Bim phosphorylation, ubiquitination, and degradation via the 26S proteasome (26, 27, 29, 30). In addition, phosphorylation of Bim may reduce its ability to directly interact with the proapoptotic multidomain Bcl-2 family protein Bax, thereby preventing oligomerization of Bax and subsequent cell death (29). We observed that BAFF blocked BCR-induced interactions between Bim and Bcl-2 (Fig. 3, E and F). In future studies, it will be important to address whether BAFF-induced phosphorylation of Bim perturbs its interaction with pro-survival Bcl-2 family members such as Bcl-2, Bcl-x_L, and Mcl-1. The apoptotic activity of another BH3-only protein, Bad, is regulated by a similar mechanism, in which Akt-mediated phosphorylation of Bad promotes its association with 14-3-3 scaffold proteins and sequesters Bad from Bcl-x_L (56, 57).

BAFF promoted delayed but sustained activation of the MEK-ERK pathway as assessed by increased phosphorylation of c-Raf, MEK, and ERK (Fig. 5, C and D). In contrast, BCR signaling induced rapid but transient ERK activation. Earlier experiments have shown that differences in the duration or kinetics of ERK activation may “encode” distinct biological outcomes such as cell proliferation and differentiation (58). The dual specificity MAPK phosphatase MKP-2/DUSP4 was strongly up-regulated in response to BCR but not BAFF signaling (Fig. 5 C). Importantly, sustained BCR signaling promotes MKP-2/DUSP4 up-regulation and subsequent inhibition of ERK activation, which correlates with accumulation of Bim (Fig. 5). In the future, it will be important to test whether MKP-2/DUSP4 is induced by activation of the MEK-ERK pathway and thereby functions as part of a negative feedback loop to down-regulate BCR-induced ERK activation and promote cell death. This possibility is supported by the observation that transcriptional induction of another MKP family member, PAC-1/DUSP2, which is also transcriptionally up-regulated by BCR ligation, requires MAPK activation (59).

MATERIALS AND METHODS

Reagents. Human BAFF and a biotinylated derivative were gifts from J. Gross (Zymogenetics, Seattle, WA). Human APRIL, mouse BAFF, and rabbit anti-Bmf serum were purchased from Axxora. Rabbit anti-Bim serum was obtained from StressGen Biotechnologies. Rabbit antisera specific for cleaved caspase-3, caspase-9, PARP, Bcl-x_L, phospho-ERK, and phospho-MEK were purchased from Cell Signaling Technology. Rabbit antisera specific for p38 MAPK, NF-κB2, MKP-2, and goat anti-actin serum were obtained from Santa Cruz Biotechnology, Inc. Rat anti-Bim (14A8) mAb was purchased from Kamiya Biomedical. Goat anti-mouse IgM F(ab')₂ fragments were purchased from Jackson ImmunoResearch Laboratories. Biotin-conjugated anti-CD8α, FITC-labeled anti-IgD, PerCP-conjugated anti-IgM,

FITC- and PE-conjugated rat anti-CD21, and PE-conjugated rat anti-CD24 mAbs, a BD IMagnet, and a hamster anti-Bim (3F11) mAb were obtained from BD Biosciences. APC-conjugated rat anti-CD23 was purchased from Caltag. G418 sulfate, puromycin, U0126, and SB203580 were from EMD Biosciences. λ protein phosphatase and mouse anti-phospho-c-Raf (Ser 338) serum were obtained from Upstate Biotechnology.

B cells and cell lines. Mouse WEHI-231 and A20 and human Daudi B cell lines were obtained from American Type Culture Collection and cultured as described previously (60). Mouse splenic B cells were isolated using a BD-IMagnet, and T1, T2, FO, and MZ B cells were sorted using mAbs to CD21, CD23, and CD24 as previously described (6, 34). mAbs to IgM, IgD, and CD21 were used to analyze splenic B cells as T1, T2-FOP, T2-MZP, FO, and MZ based on relative expression of CD21 (61). For cell stimulations, WEHI-231 B cells were treated with 10 μg/ml anti-IgM in the presence or absence of 100 ng/ml BAFF or 100 ng/ml APRIL unless otherwise described in the figure legends.

Mice. Bim^{-/-} (B6.129-Bcl2l1^{tm1.1Ast/J}) and C57BL/6J mice were obtained from the Jackson Laboratory. Mice were housed under specific pathogen-free conditions, and all experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee.

CD8α-BAFF receptor retroviral constructs and retroviral transduction of WEHI-231 cells. Extracellular and transmembrane regions of mouse CD8α were amplified using *Pfu Turbo* DNA polymerase from pcDNA3-CD8α-IgαCT and ligated into the *XhoI* and *BamHI* sites of pLXSN (CLONTECH Laboratories, Inc.). *Pfu Turbo*-amplified cytoplasmic portions of each BAFF receptor were then inserted into the *BamHI* site of pLXSN-mCD8α. Constructs were verified by automated DNA sequencing. Vectors were transiently transfected into the ecotropic packaging line PE501 by calcium phosphate precipitation. PE501 viral supernatants were used to transduce the amphotropic packaging line PA317 and stable cell lines obtained by selection in 0.8 mg/ml G418. Retroviral supernatants were used to transduce WEHI-231 cells and stable polyclonal cell lines obtained by selection in 1 mg/ml G418. Clonal WEHI-231-CD8α-BAFF receptor-expressing cell lines were obtained by limiting dilution in the presence of G418.

Stable Bim siRNA-expressing WEHI-231 B cell lines. Bim siRNAs were designed to regions of mouse Bim common to all major isoforms (nt 13-31 and 488-506) as described previously (29). Oligonucleotides were synthesized according to the manufacturer's guidelines (Oligoengine), annealed to create *BglIII* and *HindIII* sites, phosphorylated, and ligated into shrimp alkaline phosphatase-treated vector to generate pSuperRetroPuro-Bim-siRNA. Inserts were verified by automated DNA sequencing. HEK293T cells were cotransfected with pSuperRetroPuro-control or -BimsiRNA vectors, pVPack-GP, and pVPack-10A1 by calcium phosphate precipitation. Viral supernatants were used to transduce WEHI-231 B cells in the presence of 10 μg/ml polybrene and polyclonal cell lines obtained by selection in 1 μg/ml puromycin.

RT-PCR. RT-PCR was performed as previously described (62).

Western blotting and immunoprecipitation. Cell lysates were prepared by brief sonication on ice, and immunoblotting was performed as described previously (60). Immunoprecipitations were performed using NP-40 lysis buffer (10 mM Na₂HPO₄, 150 mM NaCl, 2 mM EDTA, 1% NP-40, pH 7.2) containing 1 mM Na₃VO₄, 50 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF and immune complexes captured using protein G-sepharose beads. Bands from immunoblots were quantified by scanning densitometry software (Image version 1.63; National Institutes of Health).

λ protein phosphatase treatment. Cell lysates were prepared using Ige-pal Lysis Buffer without inhibitors (10 mM triethanolamine, 150 mM

NaCl, 1 mM EDTA, 1% Igepal, pH 7.8). After a 10-fold dilution in λ phosphatase buffer, lysates were incubated with 150 U λ protein phosphatase for 15 min at 37°C before SDS-PAGE.

Cell death assays. Apoptotic cells were detected by TdT-mediated dUTP nick-end labeling with an In Situ Cell Death Detection Kit (Boehringer Mannheim) as previously described (63). $\Delta\psi_m$ was measured by FACS using 50 nM Mitotracker Red CMXRos (Invitrogen). Data were analyzed using Cellquest software.

Online supplemental material. Table S1 shows the sequences of primers used for RT-PCR analysis of BAFF receptors and BH3-only Bcl-2 family members. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20051283/DC1>.

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