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Critical Role of B Cell Lymphoma 10 in BAFF-Regulated NF-κB Activation and Survival of Anergic B Cells

Mei Yu,*‡ Yuhong Chen,† Yinghong He,*‡ Andrew Podd,‡,‡ Guoping Fu,† Jacqueline A. Wright,§ Eden Kleiman,§ Wasif N. Khan,§ Renren Wen,† and Demin Wang*‡,‡

Anergy is a key physiological mechanism for restraining self-reactive B cells. A marked portion of peripheral B cells are anergic B cells that largely depend on BAFF for survival. BAFF activates the canonical and noncanonical NF-κB pathways, both of which are required for B cell survival. In this study we report that deficiency of the adaptor protein B cell lymphoma 10 (Bcl10) impaired the ability of BAFF to support B cell survival in vitro, and it specifically increased apoptosis in anergic B cells in vivo, dramatically reducing anergic B cells in mice. Bcl10-dependent survival of self-reactive anergic B cells was confirmed in the Ig hen egg lysozyme/soluble hen egg lysozyme double-transgenic mouse model of B cell anergy. Furthermore, we found that BAFF stimulation induced Bcl10 association with IκB kinase β, a key component of the canonical NF-κB pathway. Consistently, Bcl10-deficient B cells were impaired in BAFF-induced IκBα phosphorylation and formation of nuclear p50/c-Rel complexes. Bcl10-deficient B cells also displayed reduced expression of NF-κB2/p100, severely reducing BAFF-induced nuclear accumulation of noncanonical p52/RelB complexes. Consequently, Bcl10-deficient B cells failed to express Bcl-xL, a BAFF-induced NF-κB target gene. Taken together, these data demonstrate that Bcl10 controls BAFF-induced canonical NF-κB activation directly and noncanonical NF-κB activation indirectly. The BAFF-R/Bcl10/NF-κB signaling axis plays a critical role in peripheral B cell tolerance by regulating the survival of self-reactive anergic B cells.

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Abbreviations used in this article: Bcl10, B cell lymphoma 10; CARMA1, caspase recruitment domain membrane-associated guanylate kinase protein 1; CBM, CARMA1/Bcl10/MALT1; FO, follicular; HEL, hen egg lysozyme; IKK, IκB kinase; MALT1, MALT lymphoma translocation protein 1; PKC, protein kinase C; PLC, phospholipase C; sHEL, soluble hen egg lysozyme.

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complex-dependent degradation of TNFR-associated factor 3, preventing NF-κB–inducing kinase degradation and resulting in the subsequent activation of the noncanonical NF-κB pathway (37, 38). NF-κB–inducing kinase activates IkB kinase (IKKα), and activated IKKα phosphorylates p100, leading to processing of NF-κB2 from a p100 precursor to a p52 product. Then p52 dimerizes with RelB to form the p52/RelB active heterodimer that translocates to the nucleus and regulates gene expression (35, 36). Additionally, BAFF-R can activate NF-κB through the canonical pathway that involves the activation of the IKK complex consisting of IKKα, IKKβ, and NEMO (39, 40). Activated IKK complex induces the phosphorylation and subsequent degradation of IkBs. Removal of IkBs results in the nuclear translocation of p50-containing NF-κB heterodimers and subsequent initiation of gene transcription. Despite progress in this area of research, the mechanisms by which BAFF-R mediates the activation of NF-κB in both the canonical and noncanonical pathways are not fully understood.

B cell lymphoma 10 (Bcl10) is an adaptor protein characterized by an N-terminal caspase recruitment domain and a C-terminal Ser/Thr-rich region (41–45). Bcl10 is essential for BCR-mediated NF-κB activation (46, 47). Bcl10, along with caspase recruitment domain family member-associated guanylate kinase protein 1 (CARMA1) and membrane-associated guanylate kinase protein 1 (MALT1), forms a three-component complex that couples BCR-induced protein kinase C (PKC) activity to IKK complex activation (48–51). Bcl10 deficiency impairs BCR-mediated NF-κB activation (47). Additionally, Bcl10 plays a critical role in TCR-, FceR-, and TLR4-induced NF-κB activation, and its deficiency impairs NF-κB activation by these receptors (46). Although Bcl10 plays a critical role in NF-κB activation by multiple receptors, its role in BAFF-R–mediated NF-κB activation is not known.

Our current studies find that Bcl10 directly controls BAFF-mediated canonical NF-κB activation and induces the expression of NF-κB2/p100, thus indirectly regulating BAFF-mediated noncanonical NF-κB activation. This BAFF-R/Bcl10/NF-κB signaling axis specifically supports the survival of self-reactive anergic B cells in vivo. Impairment of this axis by Bcl10 deficiency alters the peripheral tolerance mechanism for self-reactive B cells, switching from anergy to deletion.

**Materials and Methods**

**Mice**

Bcl10-deficient mice have been previously described (47). Heterozygous Bcl10-deficient mice were bred to generate wild-type control and Bcl10-deficient mice. Ig hen egg lysozyme (IgHEL) transgenic mice (C57BL/6 MD4) and soluble HEL (sHEL) transgenic mice (C57BL/6 ML5) were obtained from The Jackson Laboratory. These transgenic mice were bred with ML4 and ML5 mice to generate wild-type Ig HEL, Bcl10-deficient Ig HEL, wild-type IgHEL, HEL, and Bcl10-deficient IgHEL, HEL mice. Mice used for the experiment were generally 2–4 mo old except where specifically indicated. All mouse procedures were approved by the Institutional Animal Care and Use Committee.

**Flow cytometry**

Single-cell suspensions from the spleen of the experimental and control mice were treated with Gey’s solution to lyse RBCs and resuspended in PBS with 2% FBS. The cells were then stained with a combination of fluorescence-conjugated Abs. PE-Cy7-conjugated anti-CD19 (2D1-21, 0193), allophycocyanin-conjugated anti-IgM (17-5790), FITC-conjugated anti-BAFF-R (11-5943), Cy-Chrome-conjugated anti-B220 (15-0452), PE-conjugated anti-B220 (12-0452), biotin-conjugated anti-AAA4.1 (CA93; 13-5892), PE-conjugated anti-CD23 (12-0232), and PE-Cy7-conjugated streptavidin (25-4317) were purchased from eBioscience. PE-conjugated anti-β2 (IgG2a) and FITC-conjugated anti-IgM (11-0402) were purchased from SouthernBiotech. Allophycocyanin-conjugated streptavidin (554067) was purchased from BD Pharmingen. Stained cells were analyzed on a BD LSR II cytometer with BD FACSDiva software.

**B cell isolation**

The splenic CD93+/– immature (also known as AA4.1+) and CD93– (AA4.1–) mature B cells were isolated from wild-type and Bcl10-deficient mice as previously described (52). Briefly, splenic B cells were isolated by negative selection using anti-CD4-, anti-CD8-, and anti-CD11b-coated MACS microbeads (Miltenyi Biotec) and then stained with biotin-conjugated anti-CD3 (eBioscience). Immature B cells (CD93+) were isolated using streptavidin-conjugated microbeads with the flow through constituting the mature B cells (CD93–). For NF-κB2/p100 mRNA expression studies, mature B cells were also isolated from CD93-depleted splenocytes by positive selection using biotin-conjugated anti-CD23 (eBioscience) Abs and streptavidin-conjugated microbeads. Purity of CD93– and CD93+ CD23+ B cells was ~90 and 95%, respectively, as determined by anti-B220 Ab staining followed by flow cytometry.

**Immunoprecipitation and Western blotting analysis**

Splenic AA4.1+ mature B cells (1 x 10^6) from wild-type and Bcl10-deficient mice were stimulated with BAFF (100 ng/ml) at 37°C for the indicated times and then lysed in lysis buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 3 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin). Cell lysates were subjected to Western blotting analysis with the indicated Abs. For detecting IkB phosphorylation, splenic mature B cells were pretreated with MG132 for 30 min before BAFF stimulation. For communoprecipitation, wild-type AA4.1+ mature B cells (2 x 10^6) were stimulated with BAFF (200 ng/ml) or anti-IgM (10 μg/ml) for the indicated times. Cell lysates were precleared with Sepharose beads and subsequently incubated with anti-Bcl10 Ab-conjugated Sepharose beads (sc-5273 AC; Santa Cruz Biotechnology) at 4°C overnight. After washing five times with cold lysis buffer, bound proteins were eluted in 2X Laemmli buffer at 95°C for 5 min and subjected to 10% SDS-PAGE, followed by Western blotting analysis with the indicated Abs.

**Preparation of cytoplasmic and nuclear extracts**

Splenic CD93– mature B cells (1 x 10^6) from wild-type and Bcl10-deficient mice were stimulated with BAFF (200 ng/ml) for 3 or 16 h and then lysed in the lysis buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 20% glycerol, 1% NP-40). Cell lysates were incubated with 30 μg/ml leupeptin, followed by incubation on ice for 15 min. The mixtures were then centrifuged at 16,000 g for 15 min. The supernatants were collected as the cytoplasmic extracts. The pellets were washed with buffer A (20 mM HEPES pH 7.9, 10 mM KCl, 0.2 mM EDTA, 1 mM DTT, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin). After incubation for 15 min, Nonidet P-40 was added to a final concentration of 0.5%. The mixtures were vortexed for 10 s and spun at 16,000 x g for 30 s. The supernatants were collected as the cytoplasmic extracts. The pellets were washed with buffer A once and resuspended in 50 μl buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 2 mM EDTA, 1 mM DTT, 3 μg/ml aprotinin, 2 μg/ml pepstatin, and 1 μg/ml leupeptin), followed by incubation on ice for 15 min. The mixtures were spun at 16,000 x g for 5 min and the supernatants were collected as the nuclear extracts.

**Quantitative RT-PCR**

Splenic CD93+ CD23+ mature or CD93– immature B cells from wild-type and Bcl10-deficient mice were stimulated with BAFF (100 ng/ml) for 4 or 16 h. RNA was extracted from the cells using an RNeasy Mini kit (Qiagen).
and quantified. Equal amounts of mRNA were used to generate cDNA using a QuantiTect reverse transcription kit (Qiagen). Real-time PCR was performed with NF-κB/B2/p100 primers (Mm00479807), other FAM-labeled probes, and TaqMan Universal Master mix using a StepOne real-time PCR system (Applied Biosystems). The relative p100 mRNA fold induction was calculated relative to 18S rRNA.

Propidium iodide staining assay

Splenic AA4.1+ mature or AA4.1- immature B cells from wild-type or Bcl10-deficient mice were cultured at a density of 2 × 10^5 cells/ml with or without BAFF (100 ng/ml) for the indicated times, followed by propidium iodide staining and FACS analysis.

BrDU incorporation assay

The in vivo BrdU labeling assay was performed as described (47). In brief, mice were injected i.p. with 1 mg BrdU (Sigma-Aldrich) in 0.2 ml PBS at 12-h intervals for 4 d. Splenocytes from BrdU-treated mice were stained with anti-B220. Cells were then fixed and stained with anti-BrdU according to the manufacturer's instructions of an FITC BrdU flow kit (559619; BD Pharmingen). The degree of BrdU positivity in the gated B cells was analyzed by FACS.

Results

Bcl10 deficiency severely impairs BAFF-mediated B cell survival

All peripheral B cells require signals from both the BCR and BAFF-R for their survival (20–25). Self-reactive anergic B cells display even greater dependence on BAFF-R signaling for their survival (25). In present study, we examined the role of Bcl10 in BAFF-mediated B cell survival. Splenic CD93+ B cells, which contain T1, T2, and An1 anergic subpopulations, and CD93- B cells, which are mature follicular (FO) and marginal zone B cells, were isolated from wild-type and Bcl10-deficient mice. The cells were cultured in the absence or presence of BAFF, and cell viability was determined at various time points. In the absence of BAFF, both wild-type and Bcl10-deficient CD93+ and CD93- B cells underwent apoptosis over time, although more mutant cells than corresponding wild-type cells died after the initial time point (Fig. 1). As expected, addition of BAFF markedly rescued both wild-type CD93+ and CD93- B cells from apoptosis (Fig. 1). Importantly, CD93+ B cells from Bcl10-deficient mice displayed no survival response to BAFF (Fig. 1, right). Although BAFF increased viability of Bcl10-deficient CD93+ B cells, this was markedly reduced compared with wild-type CD93+ B cells (Fig. 1, left). Thus, BAFF-mediated immature B cell survival is entirely Bcl10-dependent, whereas mature B cells only partially depend on Bcl10 for their survival.

Bcl10 deficiency causes drastic reduction in anergic B cells

Prior studies have shown that the survival function of BAFF is particularly important for self-reactive anergic B cells (22–25). Based on our finding that Bcl10-deficient B cells survived poorly in response to BAFF, we examined the effects of loss of Bcl10 on the survival of anergic B cell population. In the spleen, B220+CD93+ cells are largely mature FO B cells (B220+CD93+CD23-IgMlo), whereas B220+CD93- cells contain T1 (B220+CD93+CD23-IgMlo), T2 (B220+CD93+CD23-IgMhi), and T3-type An1 anergic (B220+CD93+CD23-IgMlo) populations (19). Previous studies have shown that FO B cells were reduced in the spleens derived from Bcl10-deficient relative to wild-type mice (47). In this study we found that T1 B cells were slightly increased and T2 B cells were markedly increased in the spleens derived from Bcl10-deficient mice relative to those from wild-type animals (Fig. 2A, 2B). In contrast, the percentage and number of splenic An1 anergic B cells (B220+CD93+CD23-IgMlo) was drastically reduced in Bcl10-deficient relative to wild-type mice (Fig. 2A–C). Thus, Bcl10 deficiency results in a drastic reduction in self-reactive anergic B cells.

Bcl10 deficiency specifically increases anergic B cell apoptosis in vivo

Next, we examined the rate of apoptosis in mutant An1 anergic B cells. We stained splenocytes from wild-type or Bcl10-deficient mice with anti-B220, anti-CD93, anti-IgM, and anti-CD23 and then analyzed them for apoptosis by TUNEL assay. Bcl10-deficient An1 anergic B cells had an increased rate of apoptosis relative to that of the corresponding wild-type B cells (Fig. 2D). In contrast, Bcl10-deficient T1, T2, and FO B cells had similarly low rates of apoptosis relative to those of the corresponding wild-type B cell subpopulations, as previously reported (Fig. 2D) (47). These data demonstrate that Bcl10 deficiency specifically impairs the survival of An1 anergic B cells in vivo.

Reduced Bcl10-deficient anergic B cells in IgHEL sHEL mouse model

To further investigate a role of Bcl10 in BAFF-mediated survival of An1 anergic B cells, Bcl10-deficient mice were crossed with Ig HEL transgenic mice, which bear rearranged H and Ig L chain genes encoding a BCR that specifically recognizes HEL (16). An1 B cells have an increased rate of apoptosis when cultured in the presence of HEL (19). In the absence of self-Ag sHEL, the spleens from Bcl10-deficient Ig HEL transgenic mice displayed a slight reduction of total splenic B cells compared with wild-type controls (Fig. 3A, 3B). FACS analysis of splenocytes with B220, CD93, IgM, and CD23 staining showed that the spleens from both wild-type and Bcl10-deficient Ig HEL transgenic mice had a large population of FO mature B cells, a small population of T1 B cells, a moderate population of T2 B cells, and no An1 anergic B cells (Fig. 3C). As expected, chronic exposure of wild-type Ig HEL B cells to sHEL induced self-reactive transgenic B cells into An1 anergic B cells (Fig. 3C, second row) and reduced the population of splenic B cells in wild-type Ig HEL sHEL relative to wild-type Ig HEL transgenic mice (Fig. 3A, 3B). In contrast, in the absence of Bcl10, sHEL drastically reduced splenic B cells, especially An1 anergic B cells, in the spleens of Bcl10-deficient relative to wild-type Ig HEL sHEL mice (Fig. 3). These results confirm that Bcl10 deficiency results in a drastic reduction of An1 anergic B cells in the well-defined Ig HEL sHEL transgenic model of B cell anergy.

The severe reduction of the An1 anergic B cells in Bcl10-deficient Ig HEL sHEL mice led us to examine whether the reduction is due to...
increased apoptosis of the mutant cells. TUNEL assay demonstrated that the apoptosis rates of splenic B cells were markedly increased in Bcl10-deficient relative to wild-type IgHELsHEL double-transgenic mice (Fig. 4A, 4B). Of note, the apoptosis rates of splenic B cells from both wild-type and mutant IgHEL single-transgenic mice were equally low (Fig. 4A, 4B).

We also examined whether impaired cell proliferation contributes to the drastic reduction of anergic B cells in mutant IgHELsHEL mice. In vivo BrdU labeling assay demonstrated that the BrdU labeling rates of splenic B cells were reduced in Bcl10-deficient relative to wild-type IgHEL single-transgenic mice (Fig. 4C, 4D). However, the in vivo BrdU labeling rates of splenic B cells were comparable between Bcl10-deficient and wild-type IgHELsHEL double-transgenic mice (Fig. 4C, 4D). Of note, splenic B cells in Bcl10-sufficient and Bcl10-deficient IgHELsHEL double-transgenic mice were mainly anergic (Fig. 3C). Compared to wild-type double-transgenic mice, mutant double-transgenic mice had a dramatic reduction of these anergic cells (Fig. 3A, 3C). Thus, Bcl10 deficiency mainly impairs the in vivo survival of anergic B cells in IgHELsHEL transgenic mice. Overall, these data demonstrate that in the absence of self-Ag, Bcl10-deficient peripheral B cells can usually survive in vivo, whereas in the presence of self-Ag, self-reactive Bcl10-deficient B cells undergo apoptosis instead of anergy.

**Bcl10 deficiency impairs BAFF-induced canonical NF-κB activation**

Owing to a critical role for BAFF in anergic B cell survival, Bcl10-dependent mechanisms that contribute to BAFF-R signaling were investigated. Bcl10 deficiency could reduce the expression of BAFF-R on B cells, resulting in an impairment of the ability of BAFF to support B cell survival. To examine this possibility, we compared BAFF-R expression on wild-type and Bcl10-deficient splenic B cells at different maturation stages. Based on expression of IgD and IgM, splenic B cells can be divided into IgMhiIgD− (T1), IgMloIgDhi (T2), and IgMloIgDlo (FO and An1) B cells (53). FACS analysis demonstrated that subpopulations of Bcl10-deficient B cells expressed comparable levels of BAFF-R relative to corresponding subsets of wild-type B cells (Fig. 5A). Thus, Bcl10 deficiency has no apparent effect on the expression of BAFF-R.

The other possible explanation for the impaired ability of BAFF to support the survival of Bcl10-deficient B cells is that Bcl10 is required for BAFF-R signaling. NF-κB activation by BAFF-R is essential for B cell survival (22, 30). BAFF-R can induce canonical NF-κB activation through IKKb-dependent degradation of IκB (39, 40). To study the potential role of Bcl10 in BAFF-induced canonical NF-κB activation, we first examined whether Bcl10 interacts with IKKb upon BAFF stimulation. Three independent coimmunoprecipitation analyses demonstrated that BAFF stimulation induced the association of Bcl10 with IKKb in wild-type splenic B cells, similar to anti-IgM-induced BCR engagement (Fig. 5B). In contrast, BAFF-induced association of Bcl10 with IKKb was absent in Bcl10-deficient splenic B cells (Fig. 5B, right). Of note, Bcl10 constitutively associated with MALT1 without stimulation, as previously described (54) (Fig. 5B, left). Thus, Bcl10 signaling complex recruits IKKb upon BAFF stimulation.

Next, we examined whether Bcl10 is involved in BAFF-induced canonical NF-κB activation by assessing IKKb-dependent IκB phosphorylation at Ser32/36. We compared IκB phosphorylation in wild-type and Bcl10-deficient splenic mature B cells following BAFF stimulation. BAFF-induced IκB phosphorylation was markedly reduced in Bcl10-deficient relative to wild-type cells (Fig. 5C). Thus, Bcl10 deficiency impairs BAFF-induced IKKb activation.

Furthermore, we investigated whether Bcl10 is required for BAFF-induced activation of the canonical NF-κB by gel mobility shift assays. As previously reported (39, 40), canonical NF-κB activation by BAFF stimulation occurred at an early time point (Fig. 5D) and involved the formation of p50/p100-Rel heterodimer (Fig. 5E) in wild-type mature B cells. However, this canonical NF-κB activation by BAFF was severely impaired in Bcl10-deficient mature B cells (Fig. 5D, 5E). Mutant B cells only displayed basal levels of transcriptionally inactive p50/p50 homodimers, which were not elevated by BAFF stimulation (Fig. 5E). Taken together, these data demonstrate that Bcl10 is critical for BAFF-induced canonical NF-κB activation in B cells.

**Bcl10 deficiency indirectly impairs BAFF-induced noncanonical NF-κB activation**

Previous studies have shown that BCR-mediated canonical NF-κB activity contributes to the basal as well as induced expression of NF-κB2/p100, which is a critical substrate in BAFF-mediated noncanonical RelB/p52 activation and B cell survival (55, 56).
Bcl10 deficiency disrupts BCR-mediated NF-κB activation (46, 47). Our Western blot analysis of the expression of the NF-κB family members revealed that the basal level of NF-κB2/p100, but not other NF-κB members, was reduced in Bcl10-deficient relative to wild-type splenic mature B cells (Fig. 6A). However, the NF-κB2/p100 protein was efficiently processed into p52 in the presence of BAFF in both wild-type and Bcl10-deficient mature B cells, as indicated by reduction in NF-κB2/p100 protein (Fig. 6B). Consistent with reduced NF-κB2/p100 protein in the Bcl10-deficient B cells, BAFF-induced NF-κB2/p100 mRNA expression was also reduced in Bcl10-deficient relative to wild-type immature and mature B cells (Fig. 6C). Thus, lack of Bcl10 reduces basal and BAFF-induced expression of NF-κB2/p100 in B cells. This limits the p100 substrate necessary for the optimal activation of noncanonical NF-κB in Bcl10-deficient B cells.

It is well established that constant BAFF exposure induces noncanonical NF-κB activation through a relatively well-defined pathway that requires processing of NF-κB2/p100 into p52. The p52 then dimerizes with RelB to form p52/RelB heterodimer (35, 36). Our results showed that induction of NF-κB DNA binding activity by constant BAFF exposure was severely reduced in Bcl10-deficient relative to wild-type B cells (Fig. 6D).

These NF-κB DNA–binding complexes in wild-type B cells were supershifted strongly by anti-p50 and slightly by anti–c-Rel or anti-RelB, whereas the supershift with anti-RelA/p65 or anti-p52 was least prominent (Fig. 6E). However, anti-RelA/p65 and anti-p52 reduced intensity of BAFF-induced NF-κB bands (Fig. 6E). These results suggest that in addition to the expected p52/RelB heterodimer (35, 36), p50/RelA and p50/c-Rel heterodimers were also formed in response to BAFF.
In contrast, the NF-κB DNA–binding complexes in Bcl10-deficient B cells were supershifted or reduced in intensities by all Abs used except anti-p52 (Fig. 6E). These results are consistent with reduced canonical NF-κB activation (p50/RelA and p50/c-Rel heterodimers) in Bcl10-deficient B cells. Furthermore, a severe reduction in the formation of p52/RelB heterodimer was in agreement with reduced basal p100 protein levels combined with an inability to transcriptionally upregulate p100. Nonetheless, the limiting amounts of NF-κB2/p100 available in Bcl10-deficient B cells were efficiently processed into p52 and subsequently translocated into nucleus (Fig. 6B). Taken together, these data demonstrate that Bcl10 deficiency severely impairs the formation of NF-κB complexes.

**FIGURE 5.** Bcl10 deficiency impairs BAFF-induced canonical NF-κB activation. (A) Expression of BAFF-R. Splenocytes from wild-type and Bcl10-deficient mice were stained with Abs to CD19, IgM, IgD, and BAFF-R. Expression levels of BAFF-R on total splenic (CD19+), T1 (IgMhiIgDhi), T2 (IgMhiIgDlo), and FO (IgMhiIgDhi) B cells were measured by FACS analysis. (B–E) Splenic mature B cells (CD93+) were isolated from wild-type and Bcl10-deficient mice. (B) BAFF-induced association of Bcl10 with IKKβ. Wild-type or Bcl10-deficient mature B cells were stimulated with BAFF or anti-IgM for the indicated times. Cell lysates were immunoprecipitated with anti-Bcl10-conjugated Sepharose beads, followed by Western blotting analysis with the indicated Abs. (C) BAFF-induced IkBo phosphorylation. Cells were pretreated with MG132 and subsequently stimulated with BAFF for the indicated times. Cell lysates were subjected to direct Western blotting analysis with anti–phospho-IκBα or anti-actin Abs. (D and E) BAFF-induced canonical activation of NF-κB. Cells were stimulated with (+) or without (−) BAFF for 3 h and total cell lysates were subjected to NF-κB gel mobility shift (D) and Ab supershift (E) analysis. Arrows indicate the position of NF-κB complexes (D) and p50/p50 homodimers (E). Data are representative of three (A, C–E) or two (B) independent experiments.

**FIGURE 6.** Bcl10-deficient splenic B cells express reduced levels of p100, indirectly affecting BAFF-induced noncanonical NF-κB activation. (A) Expression of NF-κB/Rel family members. Protein levels of NF-κB/Rel family members in total cellular extracts from splenic mature B cells were determined by direct Western blotting analysis with the indicated Abs. Densitometric analysis of protein bands was performed and normalized to actin loading control. Levels of each NF-κB protein in Bcl10-deficient and wild-type B cells were compared by giving wild-type an arbitrary value of 1. The bar graph shows the relative p100 protein levels calculated from three independent experiments. p < 0.01. (B) BAFF-induced NF-κB2/p100 processing and nuclear translocation. Following 16 h culture of splenic mature B cells with or without BAFF, cytoplasmic and nuclear extracts were prepared and subjected to Western blotting with the indicated Abs. GAPDH was used as cytoplasmic protein loading control and YY1 as nuclear protein loading control. (C) BAFF-induced NF-κB2/p100 mRNA expression. Splenic immature (CD93+) and mature (CD93+ CD23+) B cells were either stimulated with BAFF or left nonstimulated for 16 h. NF-κB2/p100 mRNA expression was determined by quantitative real-time PCR. Relative fold induction in response to BAFF was normalized to 18S rRNA and calibrated to nonstimulated sample for the corresponding genotype. (D and E) BAFF-induced noncanonical activation of NF-κB. Splenic mature B cells were constantly stimulated with BAFF for 16 h and total cell lysates were subjected to NF-κB gel mobility shift (D) and Ab supershift (E) analysis. Arrows indicate the position of NF-κB complexes. (F) BAFF-induced ERK and Akt activation. Splenic mature B cells were stimulated with BAFF for the indicated times and cell lysates were subjected to Western blotting analysis with the indicated Abs. Data are representative of three (A, B, D, E) or two (F) independent experiments, or data are representative of at least three experiments that used one animal from each genotype per experiment (C).
of not only p50/RelA and p50/c-Rel complexes, typically activated by the canonical NF-κB pathway, but also p52/RelB complexes, typically activated by the noncanonical NF-κB pathway. The impairment of noncanonical NF-κB activation in Bcl10-deficient B cells is likely due to the reduction of NF-κB2/p100, which is in part regulated by the canonical NF-κB pathway.

In addition to the NF-κB pathway, BAFF stimulation promotes B cell survival by downregulating the proapoptotic molecule Bim via ERK activation as well as by activating the prosurvival kinase Akt (57, 58). To address the role that Bcl10 plays in these pathways, we examined whether Bcl10 is involved in BAFF-induced activation of ERK and Akt. Splenic mature B cells were isolated from wild-type and Bcl10-deficient mice and then stimulated with BAFF. The activation of ERK was evaluated by immunoblotting with Abs that detect phosphorylation of Thr202/Tyr204 within ERK1 and Thr185/Tyr187 within ERK2. BAFF-induced activation of ERK was comparable in Bcl10-deficient and wild-type B cells (Fig. 6F). Additionally, the activation of Akt was evaluated by immunoblotting with Abs that detect phosphorylation of Ser473 within Akt. BAFF-induced activation of Akt was biphatic. The initial activation, which occurred 5–10 min poststimulation, was reduced to basal level 30 min later, followed by a second phase of activation at 24 h (Fig. 6F). This biphatic activation of Akt by BAFF was not affected in Bcl10-deficient mature B cells (Fig. 6F). Thus, Bcl10 is not required for BAFF-induced activation of ERK and Akt.

**Bcl10 deficiency impairs BAFF-dependent NF-κB target gene expression**

The activation of the NF-κB pathway by BAFF stimulation ultimately upregulates the expression of antiapoptotic genes, such as Bcl-xL (22, 30). To confirm that Bcl10 plays an important role in BAFF-mediated survival of B cells through the NF-κB pathway, we examined BAFF-induced upregulation of the prosurvival NF-κB target gene, Bcl-xL, in Bcl10-deficient B cells. Following BAFF stimulation, the level of Bcl-xL proteins was markedly upregulated in wild-type B cells (Fig. 7). In contrast, the upregulation of Bcl-xL proteins was abolished in Bcl10-deficient B cells (Fig. 7). Of note, the amount of Bcl-2 proteins remained unaltered before and after BAFF stimulation in both wild-type and Bcl10-deficient B cells (Fig. 7). Therefore, Bcl10 is required for BAFF-induced expression of Bcl-xL, a prosurvival gene of the Bcl-2 family that is regulated by NF-κB.

**Discussion**

Previous studies have demonstrated that the survival of all peripheral B cells as well as maintenance of anergic B cells depends on signals from both the BCR and BAFF-R (20–24). Our findings demonstrate that Bcl10 was essential for anergic B cell survival. This Bcl10 function was apparent in the diverse repertoire as well as Ig HEL transgenic model. The Ig HEL single transgene did not appear to affect T1 and T2 B cells and only slightly reduced mature FO B cells in Bcl10-deficient relative to wild-type control mice, indicating that Bcl10 function was not critical for the survival of peripheral B cells in the absence of self-Ag. In Ig HEL single transgenic model, soluble self-Ag, sHEL, drove self-reactive B cells into anergy in both wild-type and mutant mice. Anergic B cells that depend more heavily on BAFF underwent more apoptosis in the absence of Bcl10. Thus, the BAFF-R/Bcl10/NF-κB signaling axis contributes to the establishment of peripheral B cell tolerance by maintaining anergic B cells and preventing these B cells from self-Ag-induced elimination.

BAFF can induce canonical and noncanonical NF-κB activation pathways, both of which are required for B cell survival (35, 39). Whereas BAFF-R–induced activation of the noncanonical NF-κB pathway is relatively well studied, the membrane-proximal signaling cascades upstream of the canonical pathway remain unclear. Our current studies demonstrate that Bcl10 directly participated in BAFF-induced activation of canonical NF-κB via mechanisms involving direct association of Bcl10 with IKKβ. This results in the activation of the IKK complex consisting of IKKα, IKKβ, and NEMO. Our observation that noncanonical NF-κB activation by BAFF was impaired in Bcl10-deficient mice suggests a role for Bcl10 in the regulation of this NF-κB pathway as well. A direct role for Bcl10 in activation of the noncanonical pathway was ruled out by our data showing that Bcl10 deficiency did not affect NF-κB2/p100 to p52 processing. Instead, this defect was found to be an indirect effect of Bcl10 deficiency on the expression of NF-κB2/p100. Thus, our results suggest that reduced availability of NF-κB2/p100 limited the extent of noncanonical NF-κB activation in Bcl10-deficient B cells.

It is well known that Bcl10 forms a ternary complex with CARMA1 and MALT1 (CBM) to couple PKC activity to IKK complex activation during BCR-induced canonical NF-κB activation (48–51). Our data demonstrating a requirement for Bcl10 in the activation of the canonical pathway suggest that the CBM complex is required for BAFF-induced activation of the IKK complex. This hypothesis is supported by our finding that Bcl10 is constitutively associated with MALT1, suggesting that Bcl10/MALT1 partner with CARMA1 to form the CBM complex to activate the IKK complex during BAFF-R signaling. This possibility remains to be investigated, and the role of MALT1 in the activation of the canonical NF-κB pathway remains unknown. However,
a recent study has demonstrated that MALT1 regulates BAFF-induced noncanonical NF-κB activation in B cells. MALT1 deficiency impairs BAFF-induced phosphorylation and processing of NF-κB2/p100, as well as ReIB nuclear translocation, suggesting a direct role for MALT1 in the noncanonical NF-κB activation (60). Additionally, the role of CARMA1 in BAFF-induced canonical NF-κB activation is also unknown. Our findings suggest that the Bcl10-containing CBM complex mediates the canonical NF-κB pathway. Our results lay the foundation for further studies to determine the contribution of CARMA1 and MALT1 to BAFF-induced canonical NF-κB activation.

The membrane-proximal steps of the BCR signaling cascade that lead to the formation of the CBM complex have been well studied. Upon BCR ligation, protein tyrosine kinases Lyn, Syk, and Btk are sequentially activated, and B cell linker protein and CD19 are lead to the formation of the CBM complex have been well studied. Additionally, the role of CARMA1 in BAFF-induced canonical NF-κB activation. Our results lay the foundation for further studies to determine the contribution of CARMA1 and MALT1 to BAFF-induced canonical NF-κB activation.

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Disclosures

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References

48. Thome, M. 2004. CARMA1, BCL-10 and MALT1 in lymphocyte development


