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# Context-Specific BAFF-R Signaling by the NF-κB and PI3K Pathways

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## SUMMARY

**BAFF is a soluble factor required for B cell maturation and survival. BAFF-R signals via the noncanonical NF-κB pathway regulated by the TRAF3/NIK/IKK1 axis. We show that deletion of *Ikk1* during early B cell development causes a partial impairment in B cell maturation and BAFF-dependent survival, but inactivation of *Ikk1* in mature B cells does not affect survival. We further show that BAFF-R employs CD19 to promote survival via phosphatidylinositol 3-kinase (PI3K), and that coinactivation of *Cd19* and *Ikk1* causes a profound block in B cell maturation at the transitional stage. Consistent with a role for PI3K in BAFF-R function, inactivation of PTEN mediates a partial rescue of B cell maturation and function in *Baff*$^{-/-}$ animals. Elevated PI3K signaling also circumvents BAFF-dependent survival in a spontaneous B cell lymphoma model. These findings indicate that the combined activities of PI3K and IKK1 drive peripheral B cell differentiation and survival in a context-dependent manner.**

## INTRODUCTION

BAFF is the most critical soluble factor for peripheral B cell maturation and survival, and dysregulated BAFF expression is associated with lupus-like autoimmunity and B cell non-Hodgkin (B-NHL)-like lymphoma (Mackay et al., 2010; Rickert et al., 2011). BAFF-R expression is induced on newly formed B cells that are poised to egress from the bone marrow and enter the spleen, and is further upregulated as transitional B cells mature to become follicular or marginal zone (MZ) B cells (Hsu et al., 2002; Meyer-Bahlburg et al., 2008; Stadanlick et al., 2008). Consistent with the pattern of BAFF-R expression, BAFF or BAFF-R deficiency imposes a block at the transitional T1-T2 maturation step due to failed survival, while follicular and MZ B cells are reduced by >90% and do not recover with age (Miller and Hayes, 1991; Schiemann et al., 2001; Thompson et al., 2001). Provision of a survival signal in the form of forced Bcl-2 expression rescues the transitional B cell block, leading to the generation of follicular B cells; however, MZ B cell formation remains impaired, indicating that BAFF-R engagement also imparts essential differentiation signals (Rahman and Manser, 2004; Sasaki et al., 2004; Tardivel et al., 2004).

In early work distinguishing the canonical (IKK2/Nemo-dependent) from the noncanonical (IKK1-dependent) NF-κB pathways, it was observed that BAFF-R engagement efficiently induced the cleavage of p100 (encoded by *NF-κB2*) into p52, allowing it to pair with RelB to drive gene expression (Claudio et al., 2002; Kayagaki et al., 2002; Senftleben et al., 2001). Cleavage of p100 is enabled by IKK1-dependent phosphorylation, which requires upstream activation by NIK (Senftleben et al., 2001; Xiao et al., 2001). In unstimulated B cells, cytosolic TRAF3 is bound to NIK and mediates its continual ubiquitination and degradation (Vallabhapurapu et al., 2008; Zarnegar et al., 2008b). BAFF-R engagement relieves this suppression by redirecting the ubiquitin-mediated degradation machinery to target TRAF3, allowing for newly formed NIK to persist (Chan et al., 2010). Consistently, gene-targeted mice lacking TRAF3 in B cells (Gardam et al., 2008; Xie et al., 2007), or mice expressing a mutated NIK molecule that cannot interact with TRAF3 (Sasaki et al., 2008), have been found to exhibit BAFF-independent B cell accumulation. The canonical NF-κB pathway has been shown to prime the noncanonical pathway by driving the expression of *NF-κB2* (Dejardin et al., 2002). In this regard, studies have shown that the B cell receptor (BCR) induces p100 to facilitate BAFF-R signaling (Stadanlick et al., 2008). In addition, BAFF-R has some intrinsic capacity to activate canonical NF-κB signaling (Hildebrand et al., 2010). While inhibition of RelB by

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p100 is relieved by cleavage of p100 into p52, p100 has been shown to aggregate and act as an inhibitor of p50:p65 (Basak et al., 2007). Moreover, NIK was recently shown to be destabilized by IKK1 phosphorylation (Razani et al., 2010). Thus, there are both positive and negative feedback mechanisms regulating the NF-κB pathways in B cells.

The majority of studies of BAFF-R signaling have focused on signaling via the TRAF/IKK/NF-κB pathway. However, the phosphatidylinositol (PtdIns) 3-kinase (PI3K) pathway has also been implicated in BAFF-R function (Baracho et al., 2011). The class IA PI3Ks consist of three catalytic isoforms (p110α, p110β, and p110δ) that form heterodimers with adaptor subunits (p85α, p55α, p50α, p85β, and p55γ) that regulate the location and enzymatic activity of the PI3K heterodimer. PtdIns(3,4,5)$P_3$ is also the primary substrate for the phosphoinositide 3-phosphatase PTEN, which directly antagonizes PI3K activity. Activation of downstream pathways is initiated by the recruitment of effector molecules such as PDK1, Akt, Btk, and PLCγ2, which bear pleckstrin homology (PH) domains that directly bind PtdIns(3,4,5)$P_3$ (Baracho et al., 2011). p110δ-deficient B cells exhibit impaired BAFF-induced survival (Henley et al., 2008), and combined inactivation of p110α/δ results in failed B cell generation or accumulation (Ramadani et al., 2010). Using Akt phosphorylation as a surrogate readout, investigators have observed that BAFF induces PI3K activity with both rapid and delayed kinetics (Otipoby et al., 2008; Patke et al., 2006). Thus, there is experimental evidence supporting a role for the PI3K pathway in BAFF-R function, but it is unclear whether this is a primary or ancillary role relative to the noncanonical NF-κB signaling pathway.

Here, we report the surprising finding that acute mature B cell survival is unaffected by the inducible loss of *Ikk1*, whereas early deletion of *Ikk1* results in an incomplete block in B cell maturation and BAFF responsiveness. We also provide evidence that CD19-dependent activation of the PI3K pathway is an important contributor to BAFF-mediated B cell survival. Thus, PI3K activity is pivotal for both BCR and BAFF-R signaling, underscoring its significance as a therapeutic target in autoimmune disease and B cell malignancy.

## RESULTS

### BAFF-Mediated Mature B Cell Survival Is IKK1 Independent

Although both NF-κB and PI3K pathways are activated downstream of BAFF-R engagement by BAFF, and loss of either *Baff* or *Baff-r* expression results in a block at the transitional stage of B cell maturation, it is unclear whether mature B cells still require IKK1 and/or PI3K for maintenance and survival. To address this issue, we generated a mouse strain in which IKK1 expression can be inducibly ablated in mature B cells by intercrossing mice containing a *loxP*-flanked *Ikk1* allele (*Ikk1*$^{L}$) (Liu et al., 2008) with the recently described *hCD20Tam*$^{Cre}$ strain (Khalil et al., 2012) bearing a *loxP*-regulated enhanced yellow fluorescent protein (EYFP) reporter cassette (Srinivas et al., 2001). Following administration of tamoxifen, Cre recombinase is rapidly activated with concomitant expression of EYFP and deletion of *Ikk1* in B cells expressing Cre. Strikingly, we found that deletion of *Ikk1* in mature B cells did not result in depletion of mature B cells 1 or 2 weeks following induction of Cre with tamoxifen (Figure 1A). Flow-cytometric analysis showed that in *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre}$ mice, on average, over 70% of cells were YFP$^+$ (and thus deleted *Ikk1*; Figure 1B). Separation of CD21$^{int}$CD23$^{hi}$ follicular cells and CD21$^{hi}$CD23$^{int/low}$ MZ B cells 7 days after tamoxifen injection showed that both subsets of YFP$^+$ B cells persisted equally well in the spleens of *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre}$ mice (Figure 1B).

Consistent with our in vivo observations, in vitro survival assays showed that B cells isolated from *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre}$ mice survived as well as control B cells in media alone or with BAFF stimulation (Figure 1C). By immunoblotting whole-cell lysates from sorted YFP$^+$ and YFP$^-$ B cells, we confirmed that the survival of *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre}$ B cells was not due to residual expression of IKK1 protein (Figure 1D). Interestingly, we also found that p52 was present in similar amounts in YFP$^+$ and YFP$^-$ B cells from *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre}$ mice, and could be generated de novo upon BAFF stimulation (Figure 1D).

Since the *hCD20Tam*$^{Cre}$ inducible system does not account for the contribution of p100 cleavage that occurred before tamoxifen-induced *Ikk1* inactivation, we intercrossed *Ikk1*$^{L/L}$ mice with *Cd19*$^{Cre}$ mice to eliminate IKK1 prior to the onset of BAFF-R expression. *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice exhibited a 40%–50% reduction in mature B cells (Figure 2A), but B cell development was not blocked at the T1-T2 maturation stage as observed in mice lacking BAFF/BAFF-R (Figures 2A and 2B) (Sasaki et al., 2004) or mice reconstituted with *Ikk1*$^{-/-}$ fetal liver cells (Kaisho et al., 2001). Bromodeoxyuridine (BrdU)-labeling experiments revealed that phenotypically mature splenic B cells in *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice exhibited a more rapid turnover, whereas mature recirculating B cells analyzed from the bone marrow of *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ and control mice had similar rates of turnover (Figure 2C). *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ B cells responded to BAFF, albeit less effectively than control B cells (Figure 2D). At the biochemical level, splenic B cells from *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice showed efficient ablation of IKK1 and impaired, but not absent, cleavage of p100 (Figure 2E). Moreover, p100 cleavage reached completion following in vitro BAFF stimulation of *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ B cells (Figure 2E). Altogether, these findings indicate that the loss of IKK1 imposes a bottleneck at the transitional B cell stage, but B cells that successfully traverse this stage become long-lived, mature, recirculating B cells that do not strictly require IKK1 for tonic BAFF-R signaling. Moreover, the results of the in vitro stimulation assays raise the possibility that another ser/thr kinase can partially compensate for the loss of IKK1 in the processing of p100 to generate p52.

### Sustained PtdIns(3,4,5)$P_3$ Signaling Restores B Cell Development in *Baff*$^{-/-}$ Mice

Since IKK1-dependent signaling events cannot solely account for BAFF-R function, we sought to identify additional pathways that may complement IKK1 activity. Several reports have shown that BAFF-R can engage the PI3K pathway (Henley et al., 2008; Otipoby et al., 2008; Patke et al., 2006; Woodland et al., 2008). We confirmed these findings, showing that BAFF induced rapid activation of Akt (Figure S1A). Addition of the p110δ-specific inhibitor IC87114 blocked Akt activation and impaired

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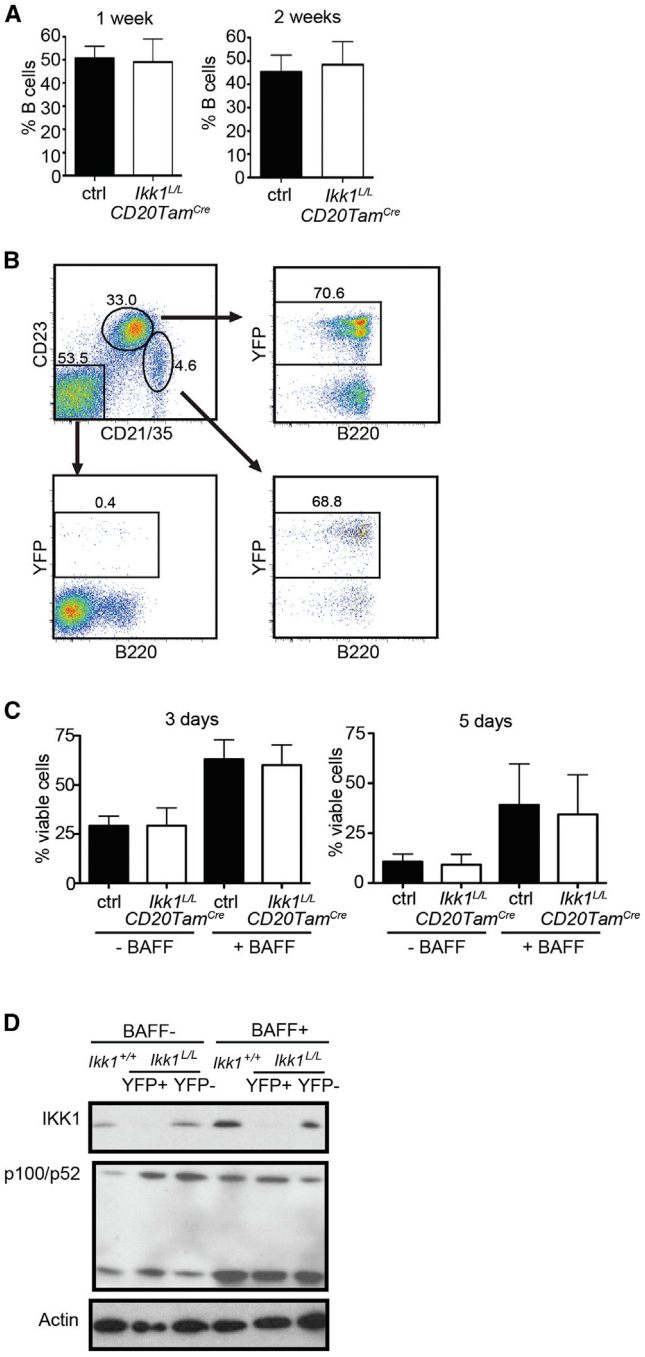


**Figure 1. IKK1-Deficient Mature B Cells Show Normal In Vivo Survival and BAFF-Mediated Survival In Vitro**

(A) *Ikk1* deletion was induced in mature B cells by tamoxifen injection of *Ikk1*$^{L/L}$ *hCD20Tam*$^{Cre+}$ mice on three consecutive days. *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre-}$ or *Ikk1*$^{+/+}$ *hCD20Tam*$^{Cre+}$ mice were used as controls (ctrl). Mice were sacrificed 1 week or 2 weeks after the last tamoxifen injection and the percentage of B cells in the spleen was determined by flow cytometry. Graphs show means + SD from three independent experiments.

(B) The percentage of YFP$^+$ B cells 7 days after tamoxifen injection was comparable between CD21$^{int}$CD23$^{hi}$ follicular B cells and CD21$^{hi}$CD23$^{int/low}$ MZ B cells. YFP expression was not detected in non-B cells (B220$^-$). Data shown are representative of two experiments.

(C) To study BAFF-mediated survival in vitro, mice were sacrificed after the last tamoxifen injection, and B cells were purified and stimulated with 10 ng/ml BAFF. The percentage of viable B cells 3 days or 5 days after culture was determined by flow cytometry. Graphs show mean + SD from three independent experiments.

(D) Splenic B cells from tamoxifen-treated *Ikk1*$^{L/L}$*hCD20Tam*$^{Cre+}$ mice were stimulated overnight with 25 ng/ml BAFF or incubated in medium alone. p100 cleavage and p52 generation were visualized by western blotting. Absence of IKK1 in Cre$^+$ cells (YFP$^+$) was confirmed by western blot analysis. Actin was used as loading control. Data shown are representative of two experiments.

BAFF-dependent B cell survival (Figure S1B). To address the physiologic significance of BAFF-dependent PI3K activity, we bred *Pten*$^{L/L}$*Cd19*$^{Cre}$ mice onto the BAFF-deficient background (*Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$). In *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells, the absence of PTEN results in sustained activation of the PI3K pathway due to impaired hydrolysis of the PI3K lipid product PI(3,4,5)$P_3$. Consistent with previous reports (Anzelon et al., 2003; Suzuki et al., 2003), B cell-specific deletion of *Pten* resulted in a skewing toward the MZ B cell fate (Figures 3A and 3B). In contrast, *Baff*$^{-/-}$ mice exhibited a dramatic reduction in all peripheral B cell subsets, owing to a block at the transitional stage of maturation (Figures 3A and 3B). Strikingly, in BAFF-deficient mice lacking expression of *Pten*, we observed a significant recovery in B cell maturation with no apparent bias toward the MZ B cell subset (Figures 3A and 3B). In this regard, the size of the CD21/35$^{hi}$CD1d$^+$ and CD9$^+$ B cell subsets was comparable in wild-type and *Pten*$^{L/L}$ *Baff*$^{-/-}$*Cd19*$^{Cre}$ mice (data not shown). BCR signaling promotes *Baff-r* expression (Rowland et al., 2010; Smith and Cancro, 2003) and in turn, BAFF signaling upregulates surface expression of CD21/35 and CD23 on B cells (Gorelik et al., 2004). Here, we found that constitutive activation of the PI3K pathway restored CD21/35, but not CD23, expression in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ splenic B cells (Figure 3C; data not shown). These data indicate that downstream of BAFF-R signaling, PI3K supports CD21/35 surface expression (Figure 3C), while CD23 expression is upregulated by BAFF-R signaling in a PI3K-independent manner or is downregulated by elevated PI3K signaling. Consistent with flow-cytometric analyses, histological staining of spleen sections from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice confirmed that *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice did not have an expansion of MZ B cells as was observed in *Pten*$^{L/L}$*Baff*$^{+/+}$ *Cd19*$^{Cre}$ mice, and that the overall splenic architecture in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice was similar to that in wild-type controls (Figure S2A).

## Antigen-Specific Immune Responses and Germinal Center Formation Are Intact in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ Mice

Despite the paucity of mature B cells in mice lacking expression of BAFF or BAFF-R, small germinal centers (GCs) are formed and some immunoglobulin G (IgG) is produced (Miller and Hayes, 1991; Rahman et al., 2003; Vora et al., 2003). However, the GC response is transient, with impaired proliferation and an associated failure to form mature follicular dendritic cell networks (Rahman and Manser, 2004; Rahman et al., 2003; Vora et al.,

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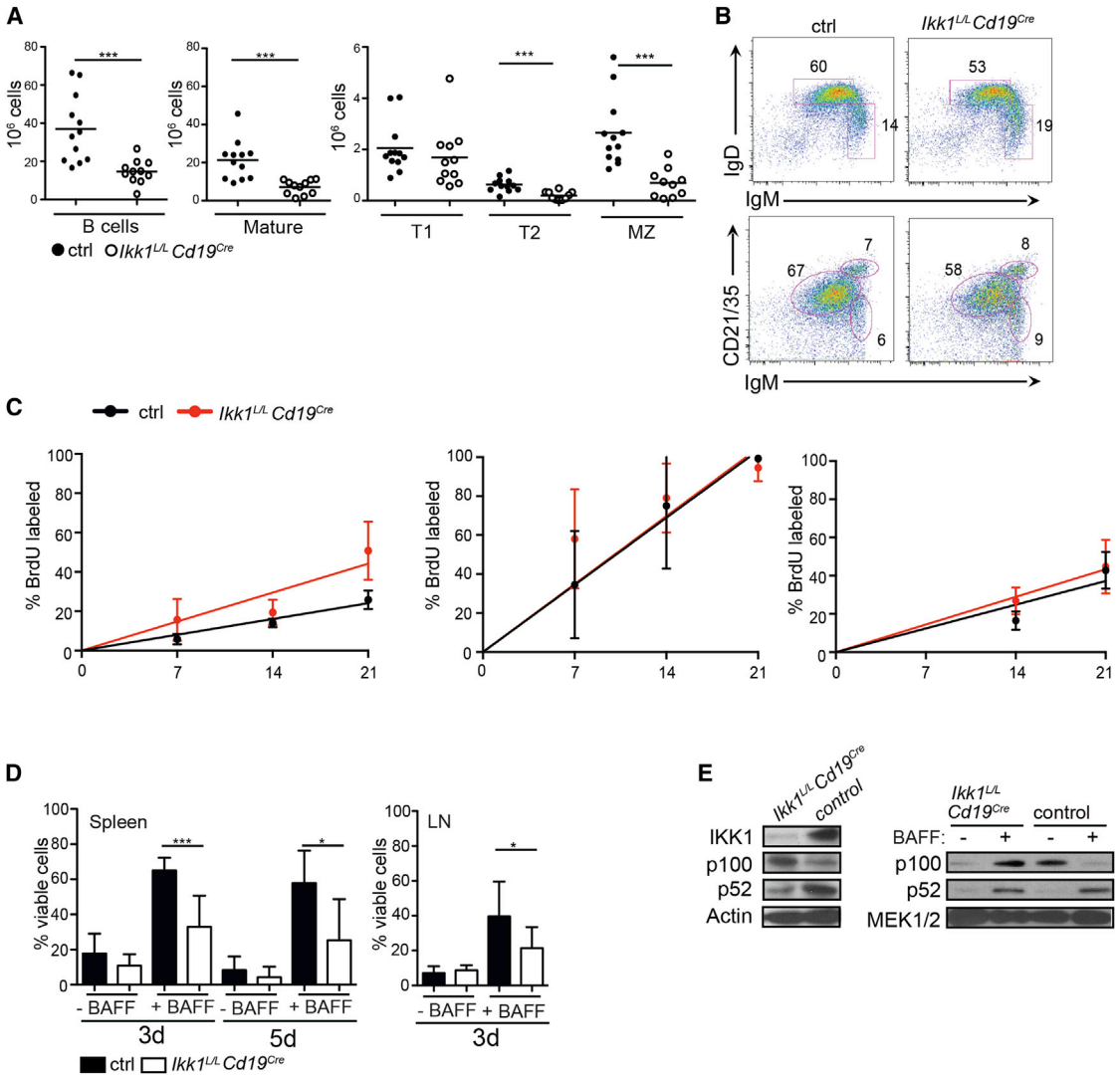


**Figure 2. IKK1 Deletion Early in B Cell Development Results in an Incomplete Block in B Cell Maturation**

(A) Graphs show the total cell numbers of B cells (left panel) and B cell subsets (middle and right panels) in spleens obtained from *Ikk1*$^{L/L}$*CD19*$^{Cre+}$ and control mice. *Ikk1*$^{L/L}$*CD19*$^{Cre-}$ or *Ikk1*$^{+/+}$ *CD19*$^{Cre+}$ mice were used as controls (ctrl). B cell subsets were identified by cell surface markers: B220$^{+}$, total B cells; B220$^{+}$CD21$^{int}$IgM$^{low}$, mature B cells; B220$^{+}$CD21$^{low}$IgM$^{hi}$, T1 B cells; B220$^{+}$CD21$^{hi}$CD23$^{hi}$IgM$^{hi}$, T2 B cells; B220$^{+}$CD21$^{hi}$CD23$^{int/low}$, MZ B cells.

(B) B cell maturation in the spleen was analyzed by flow cytometry. Plots are representative of >11 mice analyzed.

(C) Mice were continuously provided BrdU in the drinking water and euthanized after 7, 14, or 21 days of treatment. Cells were harvested from the spleen and the bone marrow and stained with a BrdU antibody and for surface markers as follows: (left) splenic follicular (B220$^{+}$, IgM$^{+}$, CD23$^{hi}$, and CD21$^{lo}$) B cells; (center) bone marrow B cell progenitors (B220$^{+}$, IgM$^{-}$, and IgD$^{-}$); (right) recirculating mature B cells (B220$^{+}$, IgD$^{+}$, and IgM$^{lo}$) in the bone marrow. Four experimental and CD19Cre control mice (10–15 weeks old) were used per time point and rates of turnover were calculated by linear regression analysis. Error bars represent SD.

(D) B cells from spleens enriched for mature B cells (CD23$^{+}$CD43$^{-}$) or from lymph nodes (LNs; B220$^{+}$CD43$^{-}$) were stimulated with 10 ng/ml BAFF and the percentage of viable cells was determined by flow cytometry after 3 days and/or 5 days in culture. The graph summarizes seven samples for each genotype and time point. Error bars represent SD.

(E) Left panel: protein lysates from freshly isolated splenic B cells were assayed for p100 cleavage by western blotting. Right panel: p100 processing to p52 in LN B cells stimulated overnight with 25 ng/ml BAFF versus unstimulated cells.

2003). As in the case of MZ B cell formation, ectopic expression of Bcl-2 does not rescue the GC response in *Baff*$^{-/-}$ mice, resulting in the accumulation of B cells bearing an immature phenotype and disrupted follicular architecture (Rahman and Manser, 2004). Thus, BAFF signaling is critical for the survival of transitional and mature recirculating B cells, and for promoting MZ and GC B cell differentiation.

Although sustained PtdIns(3,4,5)$P_3$ signaling in *Baff*$^{-/-}$ mice lacking *Pten* allowed for B cell development beyond the transitional stage, we sought to determine whether the mature B cells found in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice were functional. To this end, we immunized *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice and control animals with nitrophenol-keyhole limpet hemocyanin (NP-KLH) in alum and measured the relative levels of NP-specific serum

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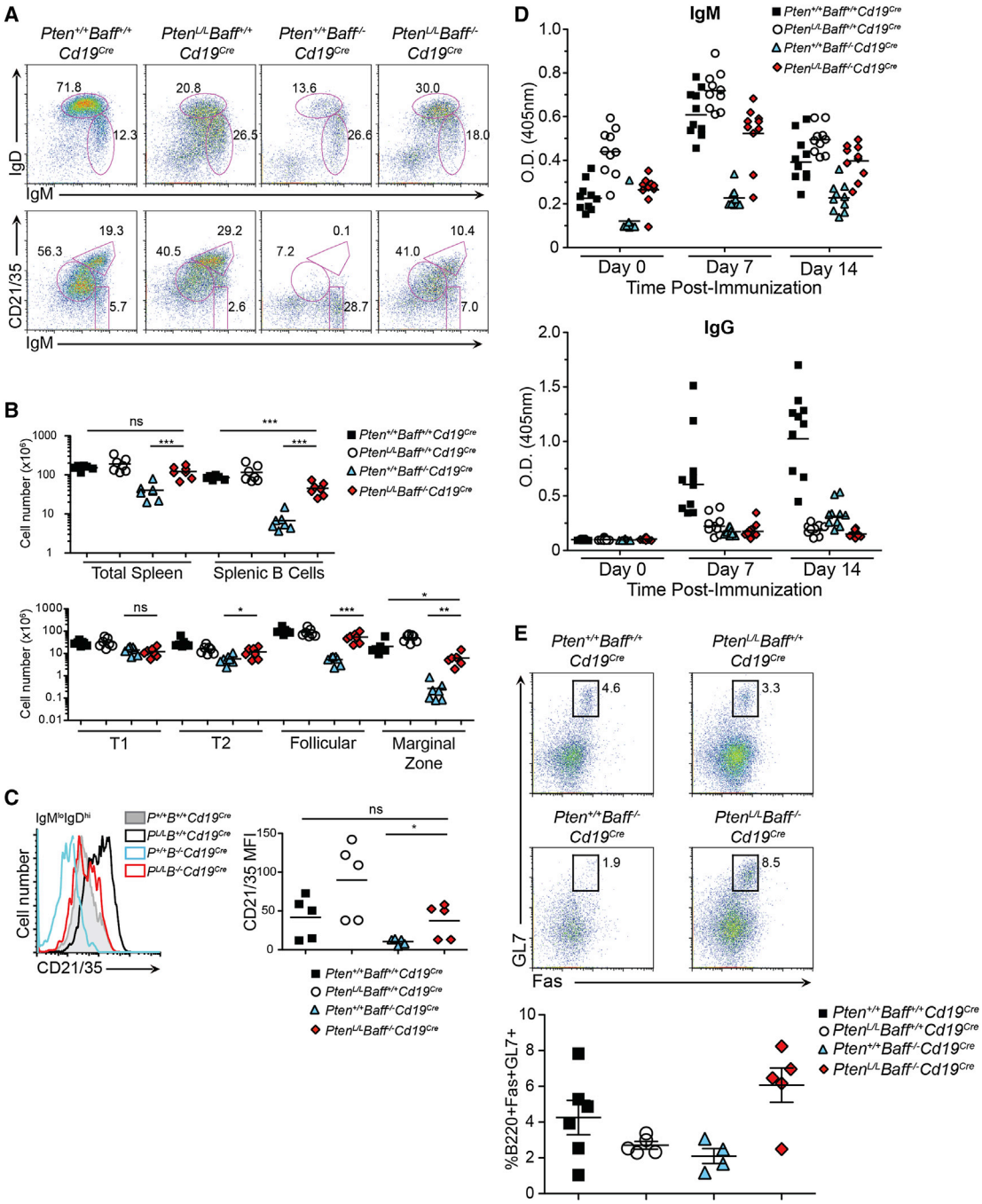


**Figure 3. Constitutively Active PI3K Restores B Cell Development in *Baff*$^{-/-}$ Mice**

(A) Flow cytometry of B220+ splenic cells from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice. Data are representative of eight mice per group.

(B) Absolute numbers of splenocytes and splenic B220$^{+}$ B cells (top panel), and splenic B cell subsets (bottom panel). Data are from five experiments with seven mice per group; small horizontal lines indicate mean.

(C) Expression of CD21/35 on B220$^{+}$-gated IgM$^{lo}$IgD$^{hi}$ splenic B cells from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice. MFI, mean fluorescence intensity.

(D) ELISA of NP-specific IgM (top) or IgG (bottom) in the sera of *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice prior to immunization (day 0), and 7 or 14 days postimmunization with 100 μg NP-KLH in alum.

(E) Flow-cytometric analysis of splenic GC B cells (B220$^{+}$ gated) from immunized mice (top). The graph summarizes the percentage of B220$^{+}$GL7$^{+}$Fas$^{+}$ B cells 14 days postimmunization (bottom). Error bars represent SEM.

See also Figure S2.

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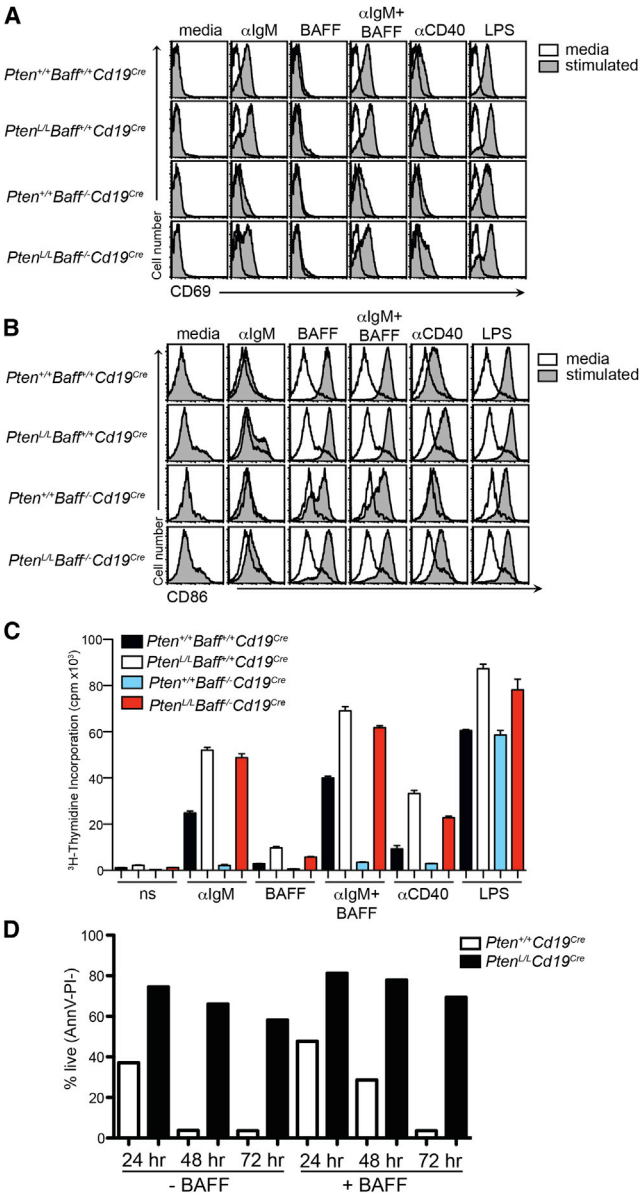


**Figure 4. Upregulation of Activation Markers and Proliferation Are Restored in *Baff*$^{-/-}$ B Cells Lacking *Pten***

(A) Flow-cytometric analysis of CD69 expression on *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells following stimulation with the indicated mitogens.

(B) As in (A), expression of CD86.

(C) Purified splenic B cells from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice were stimulated as indicated. Proliferation was determined at 48 hr by $^{3}$H-thymidine incorporation. All assays were conducted in triplicate and SDs are shown as error bars. Data are representative of three independent experiments, with two mice per group per experiment.

(D) *Pten*$^{+/+}$*Cd19*$^{Cre}$ or *Pten*$^{L/L}$*Cd19*$^{Cre}$ mature LN B cells were left untreated or were cultured in the presence of BAFF, and cell viability was assessed by Annexin V (AnnV) and propidium iodide (PI) staining. The graph shows the percentage of live (AnnV$^{-}$PI$^{-}$) cells at each time point. Data are representative of three experiments with five mice per group total.

See also Figure S1.

IgM and IgG antibody at 7 and 14 days postimmunization. *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice produced elevated levels of NP IgM antibody at 7 and 14 days postimmunization as compared with *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice, and their responses were statistically indistinguishable from those of normal *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ controls (Figure 3D, top). Consistent with previously published studies (Anzelon et al., 2003; Suzuki et al., 2003), *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ mice displayed a significant reduction in NP IgG antibodies, likely due to the fact that sustained and elevated PtdIns(3,4,5)$P_3$ signaling inhibits class switch recombination by terminating Foxo1-dependent *Aicda* transcription (Dengler et al., 2008; Omori et al., 2006) (Figure 3D, bottom). Correspondingly, in spite of robust antigen-specific IgM production, the *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice showed a virtual absence of NP-specific IgG and resembled *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ mice in this respect (Figure 3D).

To confirm that the absence of NP-specific IgG was not due to defective GC formation in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice, we assessed the presence of GCs in immunized control mice and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice. Flow-cytometric analysis of splenocytes from immunized mice showed that unlike *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice, *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice produced abundant B220$^{+}$PNA$^{+}$GL7$^{+}$ GC B cells (Figure 3E). In fact, the *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice harbored a greater percentage of GC B cells than their normal or PTEN-deficient counterparts. In addition, staining of spleen sections with B220 and PNA showed robust GCs in immunized *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice, consistent with flow-cytometric data (Figure S2B). Thus, antigen-driven B cell responses are recovered in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice, whereas repression of class switch recombination remains a dominant effect of *Pten* inactivation.

## PTEN-Deficient B Cells from *Baff*$^{-/-}$ Mice Are Responsive to Extracellular Stimuli and BCR Engagement

Given the robust in vivo responses of *Pten*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ B cells following immunization, we next sought to determine whether *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells display the activation and proliferative properties of mature B cells responding to specific stimuli. To this end, purified splenic *Pten*$^{+/+}$*Baff*$^{+/+}$*CD19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*CD19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*CD19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ B cells were cultured in the presence of BAFF, anti-igM F(ab')$_2$ (with or without BAFF), agonistic CD40 antibody, or lipopolysaccharide. Consistent with previous reports (Anzelon et al., 2003; Suzuki et al., 2003), expression of the activation markers CD69 and CD86 was augmented on PTEN-deficient B cells (Figures 4A and 4B). In contrast, expression of CD69 and CD86 was significantly reduced or absent on B cells from BAFF-deficient animals following treatment with various stimuli (Figures 4A and 4B). Notably, constitutive activation of the PI3K pathway by the loss of PTEN expression in BAFF-deficient B cells restored B cell responsiveness and induction of CD69 and CD86 expression under all conditions examined. In this regard, *Pten*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ B cells resembled control B cells (Figures 4A and 4B). Consistent with these data, *Pten*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ B cells also proliferated robustly following stimulation with numerous mitogenic stimuli and were comparable to *Pten*$^{L/L}$*Baff*$^{+/+}$*CD19*$^{Cre}$ B cells (Figure 4C). Since inhibition of

PI3K impairs BAFF-R signaling (Figures S1A and S1B), we also confirmed that sustained activation of the PI3K pathway in PTEN-deficient B cells promotes BAFF-induced survival (Figure 4D). Thus, the competence of B cells from *Pten*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ mice to respond productively to BCR engagement and costimulation supports the strong antibody responses in vivo.

## PI3K-Driven B Lymphomagenesis Is Unperturbed in the Absence of BAFF

We recently reported a model of spontaneous B cell lymphoma in mice harboring B cell-specific deletion of genes encoding PTEN and SHIP phosphatases (Miletic et al., 2010). This model demonstrated not only enhanced survival of *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ lymphoma cells in the presence of BAFF but also a proliferative response to BAFF. Moreover, *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ lymphoma B cells continued to expand upon adoptive transfer into sublethally irradiated *Baff*$^{-/-}$ recipients. Here, we sought to determine whether BAFF is required for B lymphoma initiation as well as progression in *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ mice. To this end, we crossed *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ mice onto the *Baff*$^{-/-}$ background (*Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$). B cell development in *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ mice was comparable to that observed in BAFF-expressing *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ mice, with B cell numbers similar to those found in wild-type controls (Figures 5A and 5B). Strikingly, *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ mice developed lethal lymphoma with onset and penetrance similar to those observed in BAFF-sufficient *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ mice (Figure 5C). Moreover, the lymphoma cells that expanded in *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*CD19*$^{Cre}$ mice were phenotypically similar (B220$^{lo}$CD5$^{+}$CD11b$^{+}$) to lymphoma B cells from *Pten*$^{L/L}$*Ship*$^{L/L}$*CD19*$^{Cre}$ mice (Figure 5D). Collectively, these data indicate that BAFF is not required for B lymphomagenesis when PI3K signaling is highly dysregulated.

## Augmented PI3K Signaling by BAFF-R Does Not Affect the Noncanonical NF-κB Pathway and Promotes Mcl-1 Function

To determine whether there is biochemical crosstalk or synergy between the PI3K and NF-κB pathways downstream of BAFF-R, we examined p100 expression and p52 generation in *Pten*$^{+/+}$*Cd19*$^{Cre}$ and *Pten*$^{L/L}$*Cd19*$^{Cre}$ B cells. Freshly isolated B cells from both mouse lines exhibited similar amounts of p100 and the p52 cleavage product, indicating similar in vivo responses to endogenous BAFF (Figure 6A). Accordingly, exposure to BAFF in vitro resulted in efficient conversion of p100 to p52 in control and in PTEN-deficient B cells (Figure 6A). Induction of p100 by BCR stimulation was also similar in control and PTEN-deficient B cells, indicating that canonical NF-κB signaling was not augmented by heightened activation of the PI3K pathway.

BAFF has been characterized chiefly as a prosurvival factor. The targets of BAFF-dependent survival have yet to be identified, but we focused on the prosurvival Bcl-2 family member Mcl-1, which is regulated primarily in a posttranslational manner that requires PI3K signaling and has previously been implicated in BAFF-R signaling (Maurer et al., 2006; Woodland et al., 2008). Mcl-1 is phosphorylated by GSK-3β, leading to degradation of Mcl-1. The loss of Mcl-1 is countered by Akt-mediated phosphorylation and subsequent inactivation of GSK-3β (Maurer et al., 2006). To examine this regulatory cascade, we measured levels of pAkt (Ser473), pGSK-3β (Ser9), and Mcl-1 in freshly isolated splenic B cells from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice. Both *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells showed elevated levels of phosphorylated Akt as well as phosphorylation of GSK-3β on inhibitory serine 9, the site that is phosphorylated by Akt, as compared with control *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ or *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells (Figure 6B). Consistent with these results, we also found elevated levels of Mcl-1 in both *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ B cells (Figure 6B). It is possible that some of these differences reflect the altered distribution of B cell subsets between these strains (Figures 3A and 3B). Nevertheless, *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice displayed reduced total splenic B cell numbers but a similar subset distribution compared with normal *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ control mice (Figures 3A and 3B).

Mcl-1 promotes cell survival through the direct binding and sequestration of the proapoptotic BH3 family member Bim (Maurer et al., 2006). Correspondingly, we found an elevated amount of Bim associated with Mcl-1 in *Pten*-deficient B cells as compared with control B cells (Figure 6C). Together, these data suggest that activation of PI3K downstream of BAFF-R may promote B cell survival in part via maintenance of Mcl-1 expression and sequestration of Bim by Mcl-1.

## BAFF-R Signaling Employs both the IKK1 and CD19/PI3K Pathways

Although it is known that PI3K is activated in B cells downstream of BAFF-R, how PI3K is recruited to BAFF-R remains unclear. Unlike noncanonical NF-κB signaling, which has been shown to be dependent upon TRAF3 for activation downstream of BAFF-R (Rickert et al., 2011), we found that Akt activation was not affected in a positive or negative manner in mice lacking TRAF3 in B cells (*Traf3*$^{L/L}$*Cd19*$^{Cre}$; Figure 7A). Thus, while TRAF3 ablation permits BAFF-independent B cell survival (Gardam et al., 2008; Xie et al., 2007), this effect is apparently not due to augmented PI3K signaling.

Downstream of the BCR, PI3K p110δ can act on membrane substrates via p85α-mediated recruitment to the transmembrane adaptor CD19 as well as to the cytosolic adaptor BCAP (Baracho et al., 2011; So and Fruman, 2012). To determine whether CD19 may also act as a coreceptor for BAFF-R signaling, we treated *Cd19*$^{+/+}$ and *Cd19*$^{-/-}$ (aka *Cd19*$^{Cre/Cre}$) splenic and lymph node (data not shown) B cells with BAFF and examined them for phosphorylation of CD19 on the p85-binding sites Y513 and Akt S473. We found that BAFF-R binding induced robust phosphorylation of CD19 (Y513) and that expression of CD19 augmented Akt activation (Figure 7B). Impaired BAFF-R signaling correlated with reduced survival of *Cd19*$^{-/-}$ B cells cultured in the presence of BAFF (Figure 7C). Together, these results indicate that CD19 is a critical component of BAFF-R signaling that may recruit PI3K to BAFF-R in a manner analogous to its role in BCR signaling.

In agreement with earlier findings, *Cd19*$^{-/-}$ mice displayed a modest reduction in mature B cells and a near absence of MZ

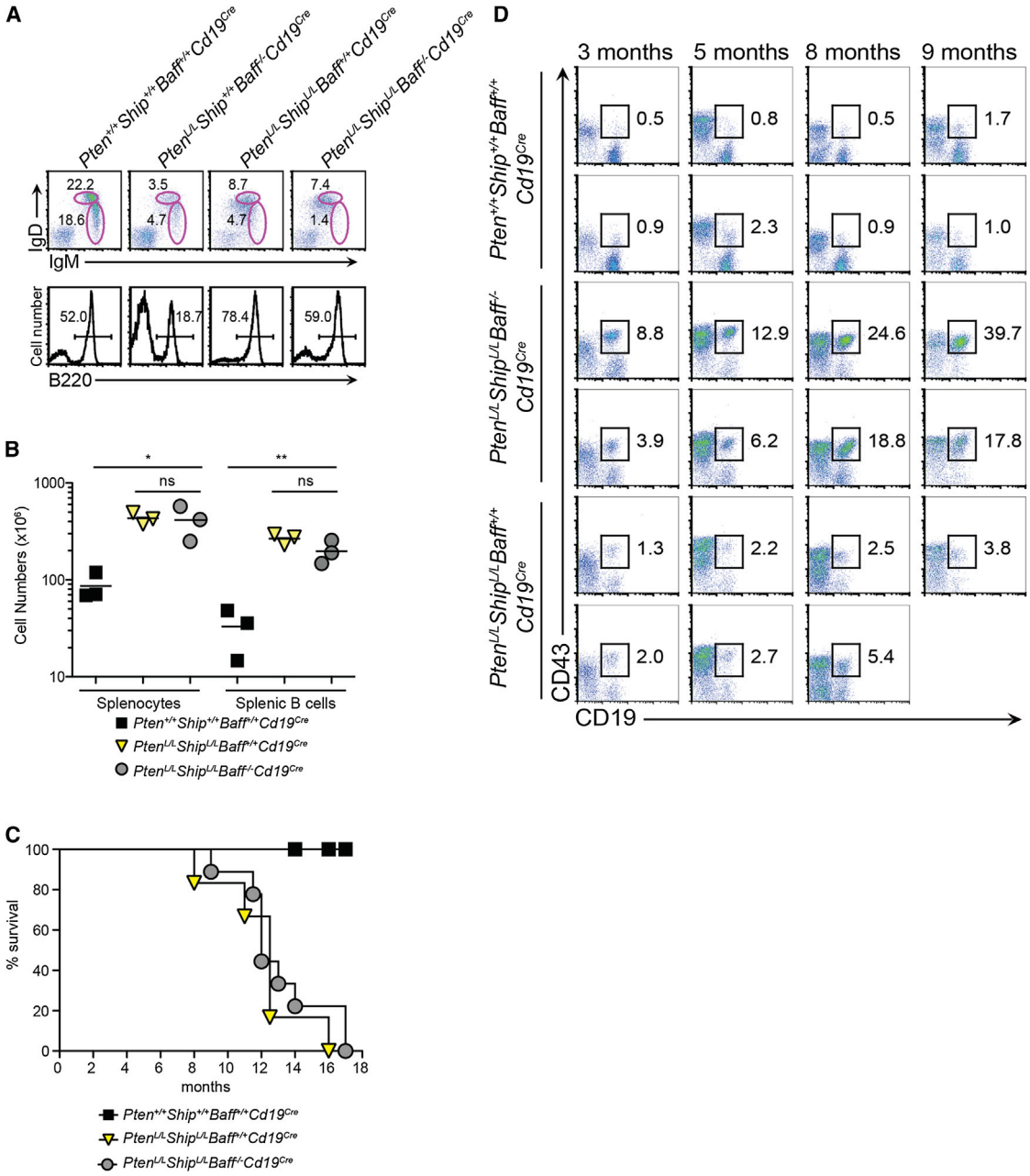


**Figure 5. Lymphoma Development in *Pten*$^{L/L}$*Ship*$^{L/L}$*Cd19*$^{Cre}$ Mice Occurs in a BAFF-Independent Manner**

(A) Flow-cytometric analysis of B220$^{+}$-gated splenic cells from *Pten*$^{+/+}$*Ship*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, Pten+/+Ship+/+*Baff*$^{-/-}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice. Data are representative of two independent experiments with at least two mice per group.

(B) Absolute numbers of splenocytes and splenic B cells (n = 3 mice per group; small horizontal lines indicate mean).

(C) Kaplan-Meier survival curve of Pten$^{+/+}$Ship$^{+/+}$Baff$^{+/+}$Cd19$^{Cre}$ (n = 7), Pten$^{L/L}$Ship$^{L/L}$Baff$^{+/+}$Cd19$^{Cre}$ (n = 6), and Pten$^{L/L}$Ship$^{L/L}$Baff$^{-/-}$Cd19$^{Cre}$ (n = 9) mice.

(D) Expansion of B220$^{-/low}$CD19$^{+}$ lymphoma B cells in peripheral blood of *Pten*$^{+/+}$*Ship*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice as determined by flow cytometry at the indicated time points. Data shown are from two representative animals for each group. The *Pten*$^{L/L}$*Ship*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ animal shown in the bottom row died before 9 months of age.

B cells (Figure 7D). However, unlike *Baff*$^{-/-}$ mice, the T2 population was unaffected (Figures 3A, 3B, and 7D, top). Thus, to determine whether BAFF-R may differentially utilize the IKK1 and CD19/PI3K pathways in transitional, mature, and MZ B cell subsets, we generated mice lacking both CD19 and IKK1 in B cells (*Ikk1*$^{L/L}$*Cd19*$^{Cre/Cre}$). Strikingly, these mice exhibited a strong block in peripheral B cell maturation that was comparable to that observed in *Baff*$^{-/-}$ mice (Figures 3A, 3B, and 7D, bottom). Indeed, *Ikk1*$^{L/L}$*Cd19*$^{Cre/Cre}$ B cells were nonresponsive to BAFF stimulation in vitro (Figure 7C). These findings suggest that the

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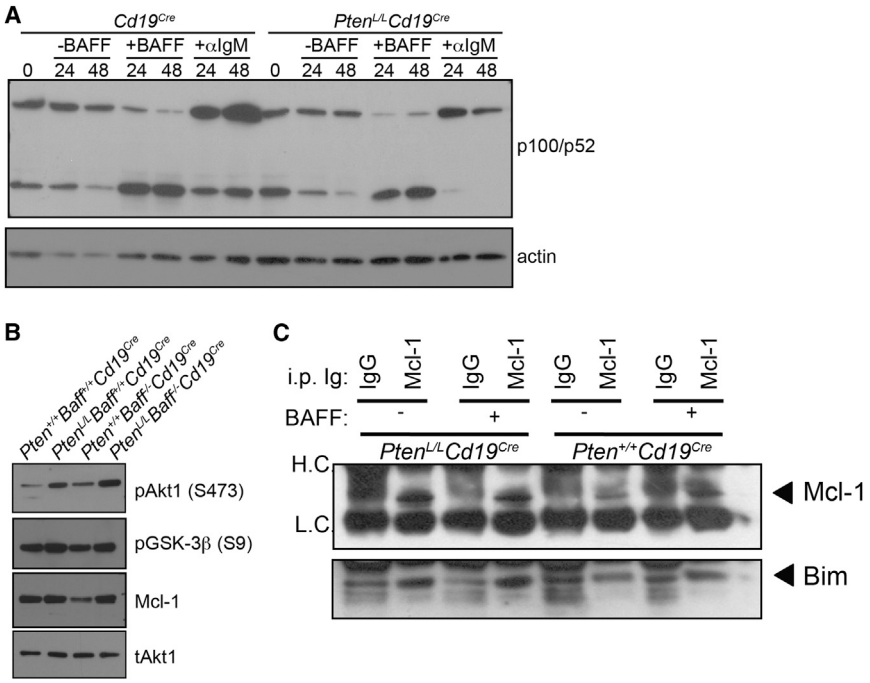


**Figure 6. Augmented PI3K Signaling by BAFF-R Does Not Affect the Noncanonical NF-κB Pathway and Promotes Mcl-1 Function**

(A) *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ and *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$ B cells were left untreated or were cultured in the presence of BAFF or anti-igM F(ab′)$_2$ fragments. Activation of noncanonical NF-κB was determined by western blotting with antibodies against p100/p52. Membranes were stripped and reprobed for actin as a loading control.

(B) Western blots of protein lysates from freshly isolated *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, or *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ splenic B cells probed with pAkt1 (S473), GSK-3β (S9), Mcl-1, or Akt1 antibodies.

(C) *Pten*$^{+/+}$*Cd19*$^{Cre}$ and *Pten*$^{L/L}$*Cd19*$^{Cre}$ B cells were left untreated or were treated with BAFF. Lysates were generated and Mcl-1 was immunoprecipitated. Immunoprecipitates were resolved by SDS-PAGE and membranes were probed with antibodies against Bim and Mcl-1.

IKK1 and CD19/PI3K pathways act in parallel to mediate BAFF-R signaling in newly formed B cells.

## DISCUSSION

It has been shown in numerous studies that BAFF depletion causes the rapid loss of transitional, mature, and GC B cells. BAFF-R signaling via the noncanonical NF-κB pathway is thought to occur similarly in these B cell subsets. Early studies showed that fetal-liver-derived B cells from *Ikk1*$^{-/-}$ mice presented a block at the late transitional (T2) B cell stage (Kaisho et al., 2001), but we found that *Ikk1* inactivation in early B cells resulted in only a partial block at the T2 stage. This apparent discrepancy might be explained by the recent discovery of a role for IKK1 in early B cell generation, and perhaps a greater dependence on IKK1 activity for fetal- versus bone-marrow-derived B cells (Balkhi et al., 2012). BrdU-labeling studies revealed that splenic B cells bearing a mature phenotype exhibited a higher turnover in *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice, suggesting that they were relatively short-lived. However, turnover of mature recirculating B cells in the bone marrow of *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice was unaffected by the loss of IKK1, consistent with results obtained from the inducible loss of IKK1 using the *hCD20Tam*$^{Cre}$ system. One possible explanation for these findings is that inhibitory p100 accumulates in transitional B cells and descendant mature B cells in the spleens of *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice, predisposing them to apoptosis and failed entry into the long-lived mature recirculating B cell pool.

The lack of a role for IKK1 in mature B cell survival is consistent with our previous observation of intact B cell maturation and survival in knockin mice expressing a mutant IKK1 molecule that cannot be phosphorylated by NIK (IKK$^{AA}$) (Mills et al., 2007). In contrast, the IKK$^{AA}$ mice exhibit a complete block in GC B cell differentiation (Mills et al., 2007), suggesting that BAFF-R/NIK/IKK1 signaling may be important for priming the survival and differentiation pathways that are set in place after antigen encounter. Expression of a constitutively active form of IKK2 or disruption of NIK degradation also allows for BAFF-independent B cell maturation (Sasaki et al., 2006, 2008). Elevated NIK activity has been shown to activate the canonical NF-κB pathway as well as the noncanonical pathway (Zarnegar et al., 2008a). Thus, the B cell phenotypes observed in mice expressing constitutively active IKK2 or NIK may have similar biochemical underpinnings in abnormally augmenting canonical NF-κB-dependent gene transcription.

Given that inactivation of the noncanonical NF-κB pathway is insufficient to explain the biologic effects of BAFF depletion on mature B cells, we focused on the PI3K pathway, which has previously been implicated in BAFF-R function (Baracho et al., 2011). The PI3K pathway serves multiple functions in cell growth, proliferation, survival, and differentiation. Correspondingly, BAFF stimulation also primes B cells for cell-cycle entry and protein synthesis (Huang et al., 2004; Patke et al., 2006). These effector pathways likely account, in part, for the observed defects in the MZ and GC B cell compartments in mice bearing defects in PI3K/Akt signaling (Calamito et al., 2010; Clayton et al., 2002; Zhang et al., 2012).

The majority of studies of PI3K function in B cells have focused on BCR-induced PI3K activity, including the recruitment of CD19 as a coreceptor. In this regard, inactivation of CD19 or p110δ yields similar defects in the generation of MZ, B-1, and GC B cells (Clayton et al., 2002; Engel et al., 1995; Okkenhaug et al., 2002; Rickert et al., 1995); however, dual ablation of p110α/δ leads to a nearly complete block in B cell development at the pro-B cell stage (Ramadani et al., 2010). Here, we show that CD19 contributes to BAFF-mediated survival, consistent with BAFF-induced CD19 phosphorylation and Akt activation. Intriguingly, this finding suggests that BAFF-R employs signaling

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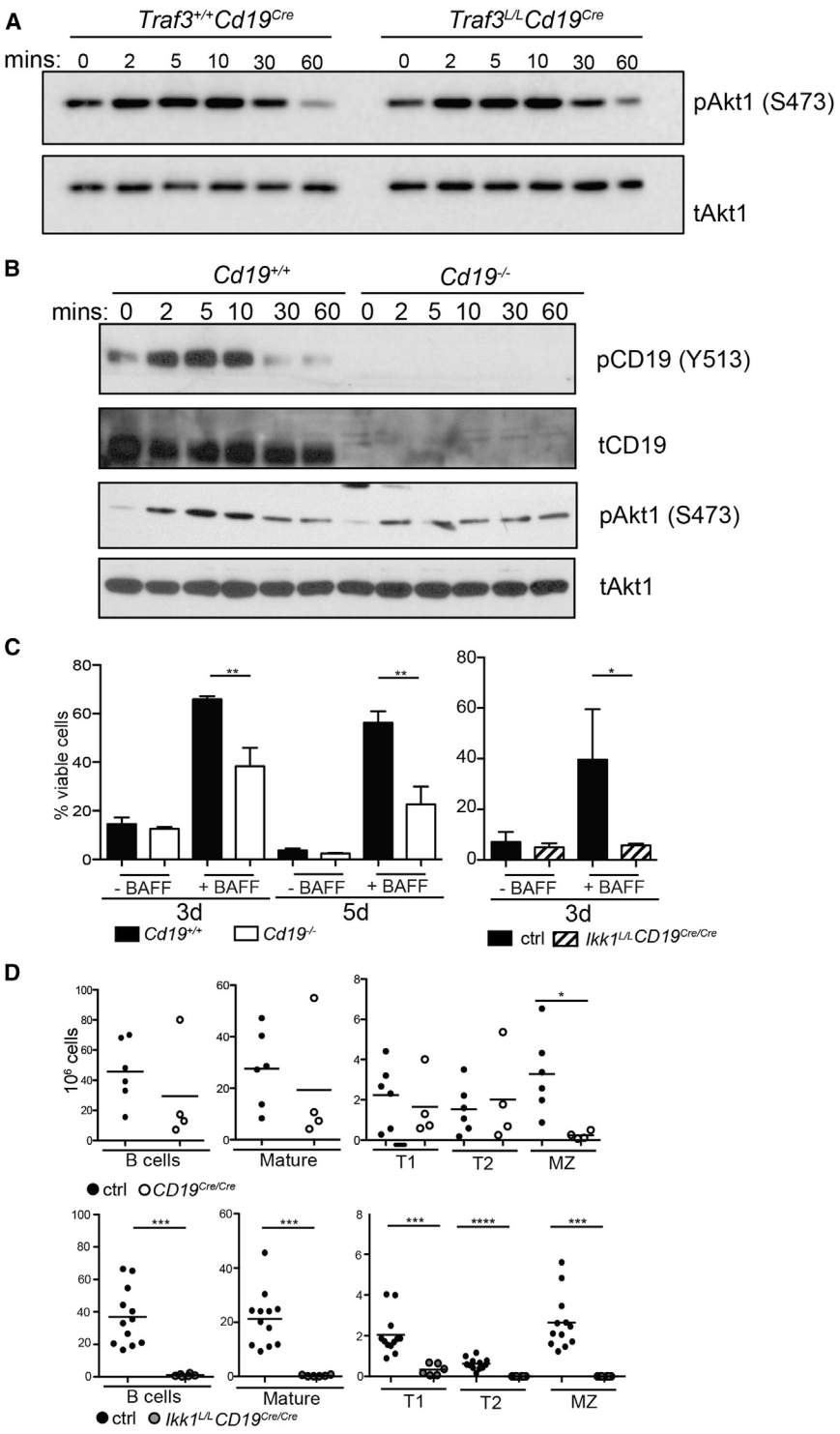


**Figure 7. BAFF-Induced Signaling Is Attenuated in B Cells Lacking Expression of CD19**

(A) Western blots of protein lysates from *Traf3*$^{+/+}$*Cd19*$^{Cre}$ or *Traf3*$^{L/L}$*Cd19*$^{Cre}$ splenic B cells treated for the indicated time points with BAFF were probed with pAkt1 (S473) or tAkt1 antibodies.

(B) Western blots of protein lysates from *Cd19*$^{+/+}$ or *Cd19*$^{-/-}$ splenic B cells treated for the indicated time points with 25 ng/ml BAFF were probed with pCD19 (Y513), CD19, pAkt1 (S473), or tAkt1 antibodies.

(C) *Cd19*$^{+/+}$ or *Cd19*$^{-/-}$ LN B cells were left untreated or were cultured in the presence of 25 ng/ml BAFF and the percentage of viable cells was determined by flow cytometry after 3 days and/or 5 days in culture (left panel). Graphs summarize data from three individual mice in technical triplicates per genotype. LN B cells from *Ikk1*$^{L/L}$*CD19*$^{Cre/Cre}$ (IKK1 and CD19 double-deficient) and control mice were treated with 10 ng/ml BAFF or were cultured in medium alone, and cell viability was assessed 3 days later (right panel). Graphs summarize results from three independent experiments with seven control samples and three *Ikk1*$^{L/L}$*CD19*$^{Cre/Cre}$ samples in total. These measurements were part of the experiments described in Figure 2C; therefore, results shown for *Ikk1*$^{L/L}$*CD19*$^{Cre/Cre}$ samples can be directly compared with the *Ikk1*$^{L/L}$*CD19*$^{Cre/+}$ samples shown in Figure 2C.

(D) Total splenic B cell numbers and cell numbers of the indicated B cell subsets from *CD19*$^{-/-}$ and control mice are shown in the top panel. B cell subsets were defined as in Figure 2A. Analysis of total cell numbers for *Ikk1*$^{L/L}$*CD19*$^{Cre/Cre}$ and control mice is shown in the bottom panel. These mice were analyzed in parallel with mice presented in Figure 2A; therefore, results shown for *Ikk1*$^{L/L}$*CD19*$^{Cre/Cre}$ mice can be directly compared with data from the *Ikk1*$^{L/L}$*CD19*$^{Cre/+}$ mice shown in Figure 2A. Error bars represent SD.

components associated with the BCR in a "coreceptor" capacity. This assertion is supported by the recent work of Tusche et al. (2009), who reported a role for Syk in BAFF-R signaling. Findings that BAFF activates Btk also support the possible linkage of the BCR and PtdIns(3,4,5)$P_3$ signaling downstream of BAFF-R (Shinners et al., 2007). Thus, previous studies showing that the BCR is required for continued B cell survival may have incorporated homeostatic signaling by BAFF-R (Lam et al., 1997).

To further evaluate the PI3K pathway in BAFF-R signaling, we performed gain-of-function studies by inactivating *Pten* in B cells. This alteration is similar to that achieved by expressing constitutively active PI3K (p110), in that PTEN loss leads to the sustained presence of PtdIns(3,4,5)$P_3$. We previously showed that PTEN loss leads to the preferential expansion of the MZ and B-1 B cell compartments, and complements CD19 deficiency (Anzelon et al., 2003). Here, we show that loss of PTEN supports B cell maturation and function in BAFF-deficient mice. Interestingly, the distribution of peripheral B cell subsets in *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice is more similar to that

observed in wild-type animals than to that found in *Pten*$^{L/L}$*Baff*$^{+/+}$ *Cd19*$^{Cre}$ mice, suggesting that PTEN loss does not mask residual B cell defects in *Baff*$^{-/-}$ mice. Moreover, unlike ectopic Bcl-2 expression (Rahman and Manser, 2004; Tardivel et al., 2004), the partial rescue of the BAFF defect is not confined to enhanced B cell survival, but also extends to B cell differentiation and antigen-dependent responses. That said, a full restoration of the mature recirculating B cell pool is not observed in *Pten*$^{L/L}$*Baff*$^{-/-}$ *Cd19*$^{Cre}$ mice, likely reflecting the importance of IKK1 activity at the transitional B cell stage. This hypothesis is further supported by the phenotype of *Cd19*$^{Cre/Cre}$*Ikk1*$^{L/L}$ double-deficient mice, underscoring a synergistic relationship between CD19/PI3K and IKK1 signaling.

BAFF induces the transcription of the prosurvival factors *A1*, *Bcl-x*$_L$, and *Pim2* (Enzler et al., 2006; Hatada et al., 2003; Hsu et al., 2002). Consistent with the role of BAFF in generating T2 B cells, early studies of *Bcl-x*$_L$$^{-/-}$ mice revealed a reduced percentage of IgM$^+$IgD$^-$ B cells (Motoyama et al., 1995). However, the B cells that overcome this bottleneck exhibit normal survival as mature recirculating cells (Motoyama et al., 1995), which may be similar to the phenotype we observed in *Ikk1*$^{L/L}$*Cd19*$^{Cre}$ mice. Although *Pim2*$^{-/-}$ and *NF-kB2*$^{-/-}$ B cells showed similar defects in BAFF-mediated survival in vitro (Enzler et al., 2006), inactivation of all three *Pim* genes resulted in only a subtle defect in peripheral B cells in younger mice (Mikkers et al., 2004). Induction of A1 transcription by BAFF is not strictly correlated with increased protein expression (Hatada et al., 2003). Moreover, A1 represents a quartet of highly similar genes, one of which (A1a) has been shown to be dispensable for BAFF-mediated survival, suggesting that A1 induction by BAFF may not be critical (Hatada et al., 2003).

Mcl-1 has been linked to BAFF signaling (Giltiay et al., 2010; Woodland et al., 2008), but it is not a transcriptional target of NF-κB. Mcl-1 protein is extremely labile and earlier studies have shown that it is essential for early B cell generation (Opferman et al., 2003). More recently, Vikstrom et al. (2010) demonstrated that Mcl-1 is essential for GC and, to a lesser extent, follicular B cell survival. By contrast, loss of Bcl-xL is inconsequential for GC B cell differentiation and survival (Vikstrom et al., 2010). We show that PTEN loss promotes Mcl-1 expression, likely due to inactivation of GSK-3 by Akt and resultant disruption of GSK-3-dependent Mcl-1 degradation (Maurer et al., 2006). Thus, our data suggest that Mcl-1 regulation is an important target of PI3K-mediated survival in mature B cells.

Inhibition of the PI3K pathway is of broad interest for applications in oncology, including the treatment of B cell malignancies. The first-in-class small-molecule inhibitor GS-1101, which is selective for p110δ, has met with considerable success in the clinic and is now entering phase 3 clinical trials for the treatment of B cell chronic lymphocytic leukemia. In addition, phase 2 trials are under way for the use of GS-1101 in the treatment of indolent B-NHL (follicular lymphoma, small lymphocytic lymphoma, lymphoplasmacytoid lymphoma, and MZ lymphoma). The efficacy of these inhibitors is largely attributed to the inhibition of BCR-mediated signaling. However, our findings suggest a reappraisal of the molecular basis of these BCR-targeting strategies to take into account the consequences of impaired BAFF-R signaling that may nonetheless be acting through the BCR complex. As such, BAFF-depletion regimens may be effective in combined therapies with small-molecule inhibitors targeting BCR signaling. Based upon the mouse lymphoma studies presented here, we would also predict that BAFF-depletion therapy would not be effective in lymphoma cases where PI3K signaling is elevated.

## EXPERIMENTAL PROCEDURES

### Mice

*hCD20Tam*$^{Cre}$ animals (Khalil et al., 2012) were intercrossed with mice carrying the rosa26-flox-STOP-YFP allele (Srinivas et al., 2001), in which YFP is expressed upon Cre activation. *Ikk1*$^{L/L}$*CD20Tam*$^{Cre}$ and control animals were injected i.p. with 1 mg tamoxifen (Sigma-Aldrich) + 10% ethanol in olive oil on three subsequent days. *Pten*$^{L/L}$*Cd19*$^{Cre}$ mice (Anzelon et al., 2003) were crossed to *Baff*$^{-/-}$ (Schiemann et al., 2001) mice to generate a mouse line with B cell-specific deletion of *Pten* and absence of *Baff* expression in all tissues (*Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$). *Ikk1*$^{L/L}$ and *Cd19*$^{Cre}$ mouse lines were intercrossed to obtain IKK1-deficient mice (*Ikk1*$^{L/L}$*Cd19*$^{Cre}$) and IKK1 and CD19 double-deficient mice (*Ikk1*$^{L/L}$*Cd19*$^{Cre/Cre}$). All animals were maintained in the animal facility of the Sanford-Burnham Medical Research Institute (SBMRI). All protocols were approved by the Institutional Animal Care and Use Committee at SBMRI and were carried out in accordance with institutional guidelines and regulations.

### Histology

Spleens were embedded in Tissue-Tek O.C.T. (Sakura Finetek) and frozen at −80°C. Acetone-fixed sections were blocked for 1 hr with 1% BSA + 5% fetal bovine serum (FBS) in PBS and stained with a combination of various antibodies (Moma-1-bio, CD3-APC, B220-PE, B220-FITC, and PNA-FITC) for 2 hr at room temperature or overnight at 4°C, and streptavidin-Cy3 was added in a second staining step. Images were acquired on a Zeiss Axio ImagerM1 microscope (Zeiss).

### Flow Cytometry and Antibodies

Single-cell suspensions were prepared, counted, and stained with antibodies according to standard procedures. The following antibody clones were obtained from eBioscience: CD3 (145-2C11), IgM (II/41), IgD (11-26), CD19 (ID3), B220 (RA3-6B2), CD11b (M1/70), CD43 (S7), CD21 (4E3), CD23 (B3B4), CD4 (GK1.5), and CD8 (53-6.7). Biotinylated reagents were detected with streptavidin conjugated to a fluorescent marker (BD Biosciences). All data were collected on a FACSCanto flow cytometer (BD Biosciences).

### Immunizations and ELISA

Mice were immunized i.p. with 100 μg NP-KLH precipitated in alum (Imject; Pierce), and serum was collected 0, 7, and 14 days postimmunization. Costar EIA/RIA plates (Corning) were coated with 10 μg/ml NP$_{23}$-BSA (Biosearch Technologies) in PBS containing 0.05% sodium azide. Following blocking with 0.25% BSA in PBS, serial dilutions of the indicated serum samples were added. Alkaline phosphatase-labeled anti-mouse IgM or IgG antibody (Southern Biotech) and p-nitrophenylphosphate substrate (Sigma-Aldrich) were used for colorimetric detection at 405 nm using an ELx808 plate reader with KC4 software (BioTek Instruments).

### Cell Culture, Survival, and Proliferation Assays

B cell purification and in vitro stimulation were performed as previously described (Miletic et al., 2010). For survival assays, purified splenic or lymph node B cells were plated at a concentration of 1 × 10$^6$ cells/ml in 10% media. Survival was determined by flow cytometry analyzing the forward-scatter/side-scatter properties of the cells or by using the AnnV-FITC Apoptosis Detection Kit (BioVision) according to the manufacturer's instructions. For inhibition of PI3K p110δ, cells were pretreated with 10 mM IC87114 in DMSO (ICOS).

### Immunoblotting and Immunoprecipitations

Purified B cells were stimulated with 1 μg/ml anti-IgM F(ab′)$_2$ or with 25 ng/ml BAFF for the indicated time points, and then lysed on ice with RIPA buffer

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(PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 10 mM EDTA) supplemented with a protease inhibitor cocktail (Boehringer Mannheim), 10 mM sodium fluoride, and 1 mM $Na_3VO_4$, and phenylmethanesulfonylfluoride. Equal protein amounts were resolved on 10% Bis-Tris gels (Bio-Rad or Invitrogen) followed by western blotting for the indicated proteins. Antibodies raised against total IKK1, phospho-Akt (S473), total Akt, p100/p52, phospho-CD19 (Y513), total CD19, phospho-GSK-3β (S9), actin, and Bim were obtained from Cell Signaling Technology. Anti-Mcl-1 was purchased from Rockland Immunochemicals. Primary antibodies were detected using horseradish peroxidase-labeled donkey anti-rabbit (Jackson Immunoresearch) or anti-mouse antibodies (Amersham).

For coimmunoprecipitation, B cells were lysed in lysis buffer for 20 min on ice. Clarified lysates were incubated with 2 μg anti-Mcl-1 or control IgG antibodies overnight at 4°C. Protein A/G beads (GE Healthcare) were added for 1 hr at 4°C. Immunoprecipitates were washed as described previously (Maurer et al., 2006) and western blotting was performed as described above.

### BrdU Incorporation

Mice were provided 0.5 mg/ml BrdU (Sigma) + 2% sucrose in drinking water for up to 21 days. Bone marrow and splenic cells were isolated on days 7, 14, and 21, and stained with antibodies as indicated. After surface staining, the cells were fixed with BD Cytofix/Cytoperm (BD Biosciences) and permeabilized with permeabilization buffer (eBioscience), followed by permeabilization with 0.1% Triton X-100 (Sigma), a second fixation, and DNase (Sigma) treatment. The cells were then stained with an BrdU antibody (Invitrogen).

### Software and Statistical Analysis

Gimp (GNU Image Manipulation Program) and GraphPad Prism (GraphPad Software) were used for image editing and statistical evaluation, respectively. The significance of observed differences was evaluated by unpaired t test. The obtained p values are indicated as follows: ***$p < 0.001$, **$p < 0.005$, *$p < 0.05$.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.10.022.

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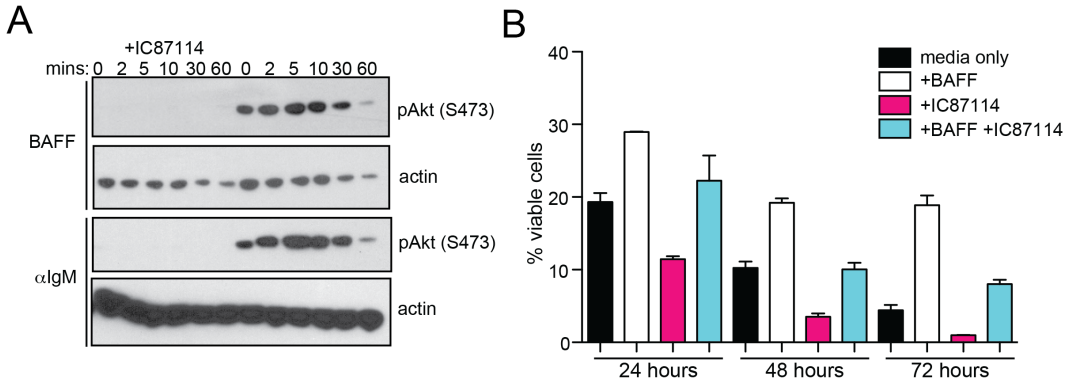
**Supplemental Figure 1. PI3K/Akt-dependent BAFF-R signaling.** (A) Western blots of protein lysates from freshly-isolated *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, or *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ splenic B cells probed with anti-pAkt1 (S473) antibodies or anti-actin antibodies as a loading control. (B) *Pten*$^{+/+}$*Cd19*$^{Cre}$ splenic B cells were cultured in medium or with 25 ng/mL BAFF in the presence or absence of IC87114, and cell viability was assessed by flow cytometry.

**Supplemental Figure 2. Splenic architecture in naïve and immunized mice.** (A) Fluorescent imaging of follicles from *Pten*$^{+/+}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{L/L}$*Baff*$^{+/+}$*Cd19*$^{Cre}$, *Pten*$^{+/+}$*Baff*$^{-/-}$*Cd19*$^{Cre}$, and *Pten*$^{L/L}$*Baff*$^{-/-}$*Cd19*$^{Cre}$ mice in frozen spleen sections stained with antibodies to CD3, Moma-1, and B220. Top row shows magnification with a 5x objective; bottom row shows 10x magnifications. Data are representative of n=5-6 mice per group. (B) Fluorescent imaging of splenic GC B cells 14 days post-immunization in frozen sections stained with antibody to peanut agglutinin (PNA) and B220. All data are representative of 3 independent experiments with a total of n=12-15 mice per group. Magnification shown is with a 10x objective.

# Figure S1, Related to Figure 4



# Figure S2, Related to Figure 3