PI3 Kinase Signals BCR-Dependent Mature B Cell Survival

Lakshmi Srinivasan,[1] Yoshiteru Sasaki,[1,5] Dinis Pedro Calado,[1] Baochun Zhang,[1] Ji Hye Paik,[2] Ronald A. DePinho,[2] Jeffrey L. Kutok,[3] John F. Kearney,[4] Kevin L. Otipoby,[1] and Klaus Rajewsky[1,*]

[1]Program of Cellular and Molecular Medicine, Children's Hospital, and Immune Disease Institute, Harvard Medical School, Boston, MA 02115, USA
[2]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115, USA
[3]Department of Pathology, Brigham and Women's Hospital, Boston, MA 02115, USA
[4]Department of Microbiology, The University of Alabama at Birmingham, 35294, USA
[5]Present address: Riken Center for Developmental Biology, Kobe, Hyogo, 650-0047, Japan
*Correspondence: rajewsky@idi.harvard.edu
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SUMMARY

Previous work has shown that mature B cells depend upon survival signals delivered to the cells by their antigen receptor (BCR). To identify the molecular nature of this survival signal, we have developed a genetic approach in which ablation of the BCR is combined with the activation of specific, BCR dependent signaling cascades in mature B cells in vivo. Using this system, we provide evidence that the survival of BCR deficient mature B cells can be rescued by a single signaling pathway downstream of the BCR, namely PI3K signaling, with the FOXO1 transcription factor playing a central role.

INTRODUCTION

Mature B cells in the peripheral immune system depend on two central survival determinants, the B cell antigen receptor (BCR) and the receptor for the tumor necrosis factor (TNF) family cytokine, BAFF (Lam et al., 1997; Ng et al., 2005). Whereas the BCR controls the response of the cells to cognate antigen, the BAFF-R senses BAFF produced by neighboring cells in the B cell habitat and is thus critically involved in controlling B cell numbers. In BAFF or BAFF-R deficient mice, mature B cells scarcely develop, and BAFF depletion in adult animals leads to rapid apoptosis of these cells (Batten et al., 2000; Thompson et al., 2000; Yan et al., 2001). In the case of the BCR, using systems of induced and stage-specific gene targeting, Lam et al. (1997) and Kraus et al. (2004) demonstrated that mature B cells undergo apoptosis upon in vivo BCR ablation or mutation of one of its signaling units, the Igα polypeptide chain, and disappear from the body with a half-life of 3-6 days. This indicated that the BCR on resting, mature B cells transmits "tonic" survival signals into the cells, either upon interaction with ligands in the environment or spontaneously. The notion of "tonic" BCR signals keeping mature B cell alive is supported by additional, more indirect evidence (Bannish et al., 2001; Grande et al., 2007; Stadanlick et al., 2008).

Which pathways downstream of the BCR and BAFF-R signal B cell survival? Upon BAFF engagement, the BAFF-R signals mainly through the alternative NF-κB pathway, and there is evidence that BAFF-R-mediated activation of the latter is critical for mature B cell survival, although additional factors may be involved (Mecklenbrauker et al., 2004; Sasaki et al., 2006). However, in the case of the BCR, which can activate multiple signaling cascades, the nature of the survival signal has remained unresolved. One recent hypothesis is based on evidence that BCR engagement can activate the canonical NF-κB signaling pathway and mature B cells require canonical NF-κB signals for their in vivo maintenance (Cariappa et al., 2000; Pasparakis et al., 2002). This led Stadanlick et al. (2008) to propose a model in which mature B cell survival is based on an NF-κB-mediated crosstalk between BCR and BAFF-R. In this model, BCR-mediated canonical NF-κB signals enhance BAFF-R signaling by transcriptional upregulation of the genes encoding BAFF-R on the one hand and p100, a critical component of the alternative NF-κB pathway, on the other (Bonizzi and Karin, 2004). However, while this mechanism may well play a role upon B cell activation, its role in B cell maintenance remained speculative and is not easily compatible with other experimental evidence (Ruland et al., 2001; Xue et al., 2003).

In an attempt to directly assess the molecular nature of the BCR-mediated survival signal for mature B cells, we have developed a system, in which conditional ablation of the BCR in mature B cells in vivo is combined with the conditional activation of candidate signaling cascades in the same cells. Using this approach, we find that mature B cells losing their BCR are fully rescued by activation of the PI3K signaling pathway, but not that of canonical NF-κB. Thus, PI3K signaling, which has recently emerged as a critical determinant of B cell development (Aiba et al., 2008; Deane and Fruman, 2004; Herzog et al., 2008; Omori et al., 2006; Verkoczy et al., 2007) also provides a critical survival signal for mature B cells.

RESULTS

Experimental Design

For targeted deletion of the BCR in mature B cells, we used the *CD21-cre* transgene, expressed when B cells differentiate from
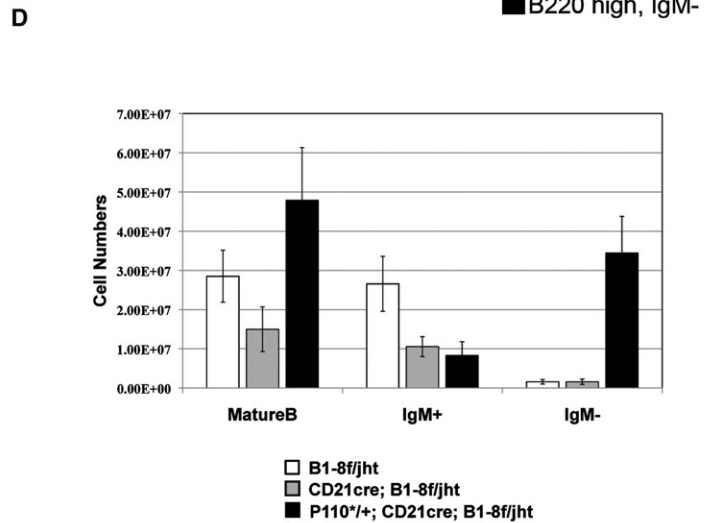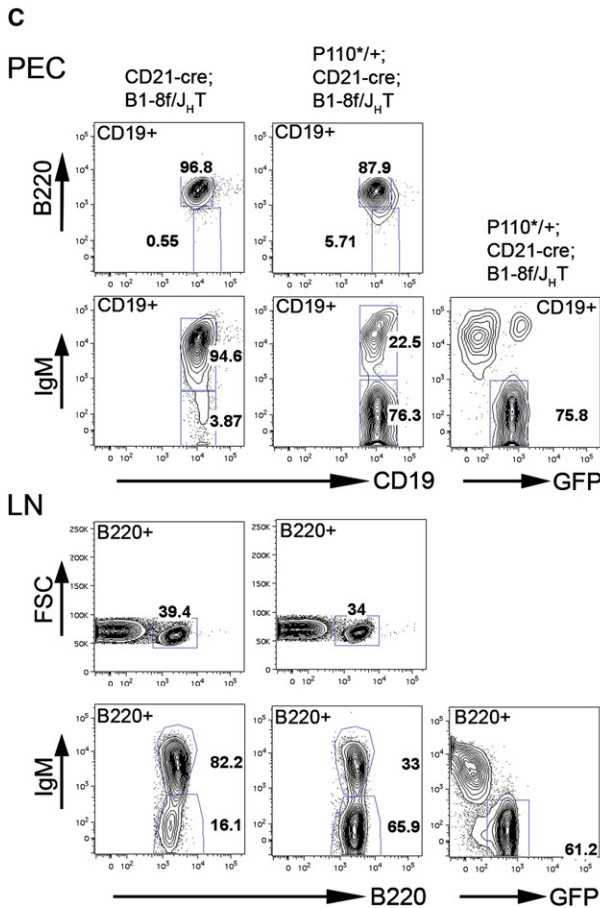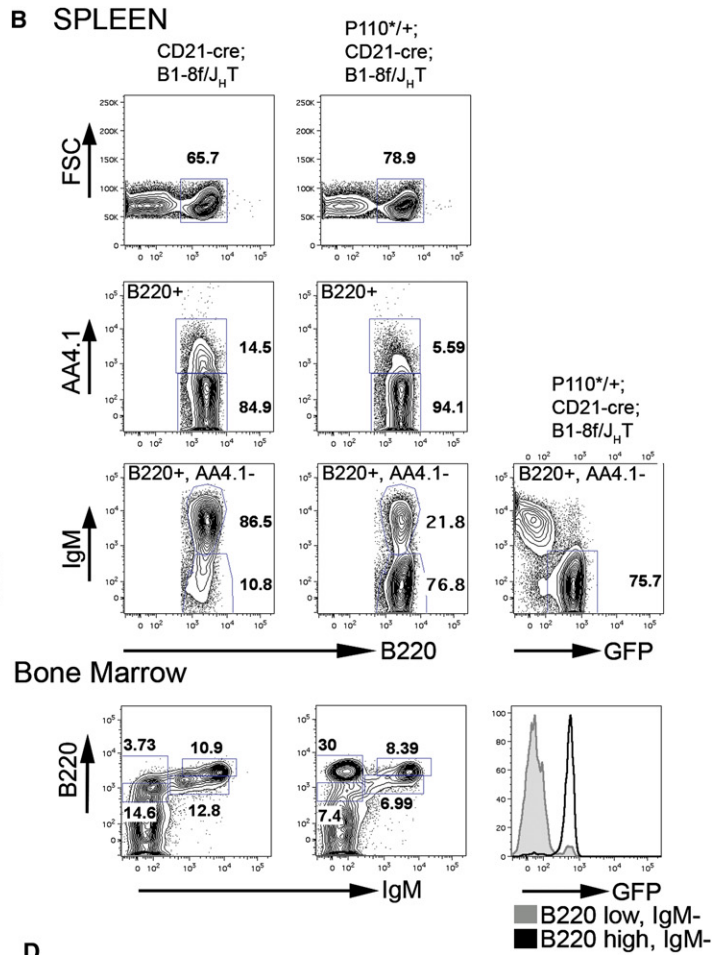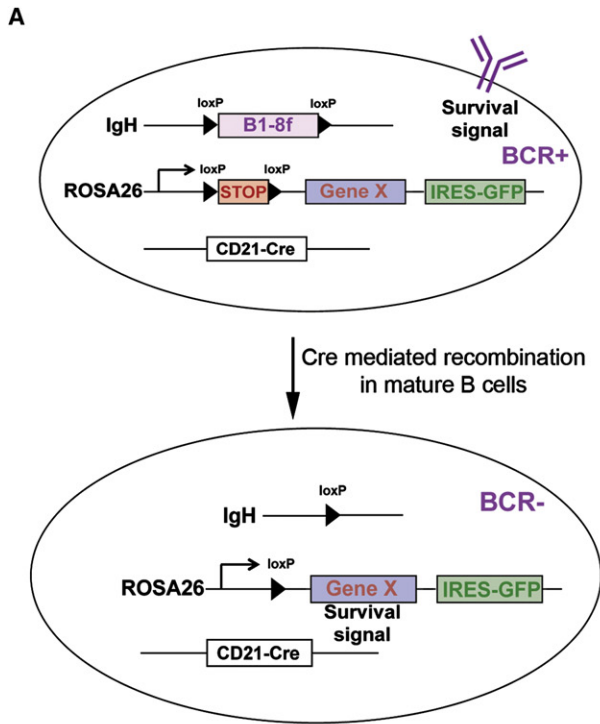
transitional to mature cells (Kraus et al., 2004), in combination with the *B1-8f* allele, in which the B1-8 VDJ gene segment, sitting in its physiological position in the *IgH* locus, is flanked by loxP sites (Lam et al., 1997). We complemented this system by a third genetic element, which would rescue the BCR deficient cells by concomitant, Cre-mediated activation of a survival signal mimicking the one normally provided by the BCR. Following a strategy which we had previously developed (Sasaki et al., 2006), we generated a series of *ROSA26* alleles harboring cDNAs of constitutively active signaling molecules, preceded by a *loxP* flanked STOP cassette and marked by a GFP gene under the control of an internal ribosomal entry site downstream of the inserted cDNA. In combination with *CD21-cre* and *B1-8f*, cDNA and GFP expression would coincide with BCR deletion in mature B cells, allowing us to determine whether a signaling pathway is activated which rescues BCR^{neg} mature B cells (Figure 1A).

Constitutively Active PI3K Rescues BCR-Negative B Cells

One of the constitutively active signaling molecules that we conditionally expressed from the *ROSA26* locus was MP110*, a constitutively active form of P110α, the catalytic subunit of PI3K (Klippel et al., 1996). For simplicity, we will refer to this allele, which is described in Figure S1 (available online), as *P110** throughout the paper.

As compound mutant mice expressing *P110** in mature B cells died soon after birth, possibly due to the expression of *CD21-cre*, and consequently P110*, in the forebrain (Schmidt-Supprian and Rajewsky, 2007), we studied the requirement of PI3K activity for the survival of BCR^{neg} B cells in chimeric mice, generated by transfer of fetal liver cells of the compound mutants into syngeneic RAG2/common γ chain deficient recipients, which are lymphocyte deficient (Cao et al., 1995). Lymphocyte development was reconstituted in the chimeric mice, and no lethality was observed in recipients of either *CD21-cre;B1-8f/J$_H$T* or *P110**/+;CD21-cre;B1-8f/J$_H$T* fetal liver cells. *J$_H$T* is a null allele of the *IgH* locus (Gu et al., 1993), introduced into the system to limit IgH expression to the *B1-8f* allele.

In accord with our previous work (Kraus et al., 2004), IgM^{neg} B cells were detectable in spleens from *CD21-cre; B1-8f/J$_H$T* fetal liver chimeric mice, but did not accumulate beyond around 10% of total mature B cells in steady state (Figure 1B). In striking contrast, 77% of splenic mature B cells (B220+, AA4.1-) from *P110**/+; CD21-cre; B1-8f/J$_H$T* chimeras were IgM^{neg}. Essentially all and only those cells expressed GFP, indicating that Cre-mediated recombination occurred at the *IgH* and the *ROSA26* locus with equal efficiency, and

consistent with a requirement of P110* expression for the survival of these cells. The increased percentage of mature (B220+, AA4.1-) B cells in the chimeras carrying the P110* allele is consistent with the counter selection of such cells when they loose their BCR in the absence of P110* expression (Kraus et al., 2004).

Rescue of BCR^{neg} mature B cells by P110* was also seen in other lymphoid organs. Thus, a large fraction of mature (B220 high) B cells in the bone marrow of *P110**/+; CD21-cre; B1-8f/J$_H$T* chimeras were IgM^{neg} and expressed GFP, whereas in the absence of the P110* allele all B220 high cells were IgM^{pos} (Figure 1B, bottom row). Large numbers of GFP expressing IgM^{neg} B cells also accumulated in the peritoneal cavity and lymph nodes of compound mutant chimeras carrying the *P110** allele (Figure 1C).

Numerically, the rescue of BCR^{neg} mature B cells by P110* was extremely effective. *P110**/+; CD21-cre; B1-8f/J$_H$T* chimeras had more than twice the number of splenic B cells of their P110*-negative counterparts, with almost all of the increase being due to the accumulation of IgM^{neg} cells (Figure 1D). In comparison to mice without ongoing BCR ablation in mature B cells (*B1-8f/JHT*), the P110* rescue of IgM^{neg} B cells generated a somewhat expanded peripheral B cell compartment (Figure 1D), partly due to a moderate expansion of the Marginal Zone (MZ) B cell subset (Figure S2).

P110*-Rescued BCR-Negative B Cells Resemble Normal Mature B Cells

Histologically, a normal organization of BCR^{neg} B cells into follicles and adjacent MZ was seen in the spleen, with a majority of BCR^{neg}, B220+ cells intermingled with a minority of BCR^{pos}, B220+ cells that had not undergone Cre-mediated recombination (Figures 2A and 2B). When analyzed by flow cytometry, mutant and control cells were identical in forward scatter analysis and expressed similar levels of the B cell markers B220, CD19 and CD21 (Figure 2C). CD23 was about 2-fold downregulated on the cells compared to controls, a phenomenon that will be further discussed below. Significantly, no upregulation of the activation markers CD95, CD69 and MHC II was seen in the rescued cells, suggesting that they were in a resting state like normal, mature B cells (Figure 3A). Accordingly, 84% of these cells did not incorporate bromodeoxyuridine (BrdU) into their DNA over a 20 day feeding period, similar to the 80% unlabelled cells in the controls (Figure 3B,C; Forster and Rajewsky, 1990). In contrast, 50% of the IgM^{neg} B cells in compound mutants lacking the P110* allele incorporated BrdU, indicative of their rapid turnover (Kraus et al., 2004). An unexpected finding was the low degree of BrdU incorporation

Figure 1. Constitutively Active PI3K Rescues BCR-Negative B Cells

(A) Schematic representation of the experimental strategy.

(B) Representative FACS analysis of spleen and bone marrow lymphocytes. Numbers represent percentages of cells within the lymphocyte gate or of cells in the gate indicated in the FACS plot. Expression of P110* leads to accumulation of BCR^{neg} mature B cells (B220+, AA4.1-) in the spleen that are GFP positive. The histogram plot in the bone marrow section compares GFP expression in the progenitor gate (B220 low, IgM^{neg}) and the IgM^{neg} mature B cell gate (B220 high, IgM^{neg}) from *P110**/+; CD21-cre; B1-8f/J$_H$T* mice.

(C) FACS analysis of lymphocytes from peritoneal cavity (PEC, top two rows) and lymph nodes (LN, bottom two rows).

(D) Comparison of B cell numbers from the spleen of *B1-8f/J$_H$T* (white bars), *CD21-cre; B1-8f/J$_H$T* mice (gray bars) and *P110**/+; CD21-cre; B1-8f/J$_H$T* mice (filled bars), mean and SD of a minimum of 6 mice per genotype.

A

C57Bl/6

100X

P110*/+; CD21-cre;
B1-8f/J$_H$T

100X

B

B1-8f/J$_H$T

P110*/+; CD21-cre;
B1-8f/J$_H$T

B220   MOMA   RS3-1,IgMa

C

% of Max

CD21-cre;
B1-8f/J$_H$T

P110*/+;
CD21-cre;
B1-8f/J$_H$T

FSC   B220   CD19   CD23   CD21
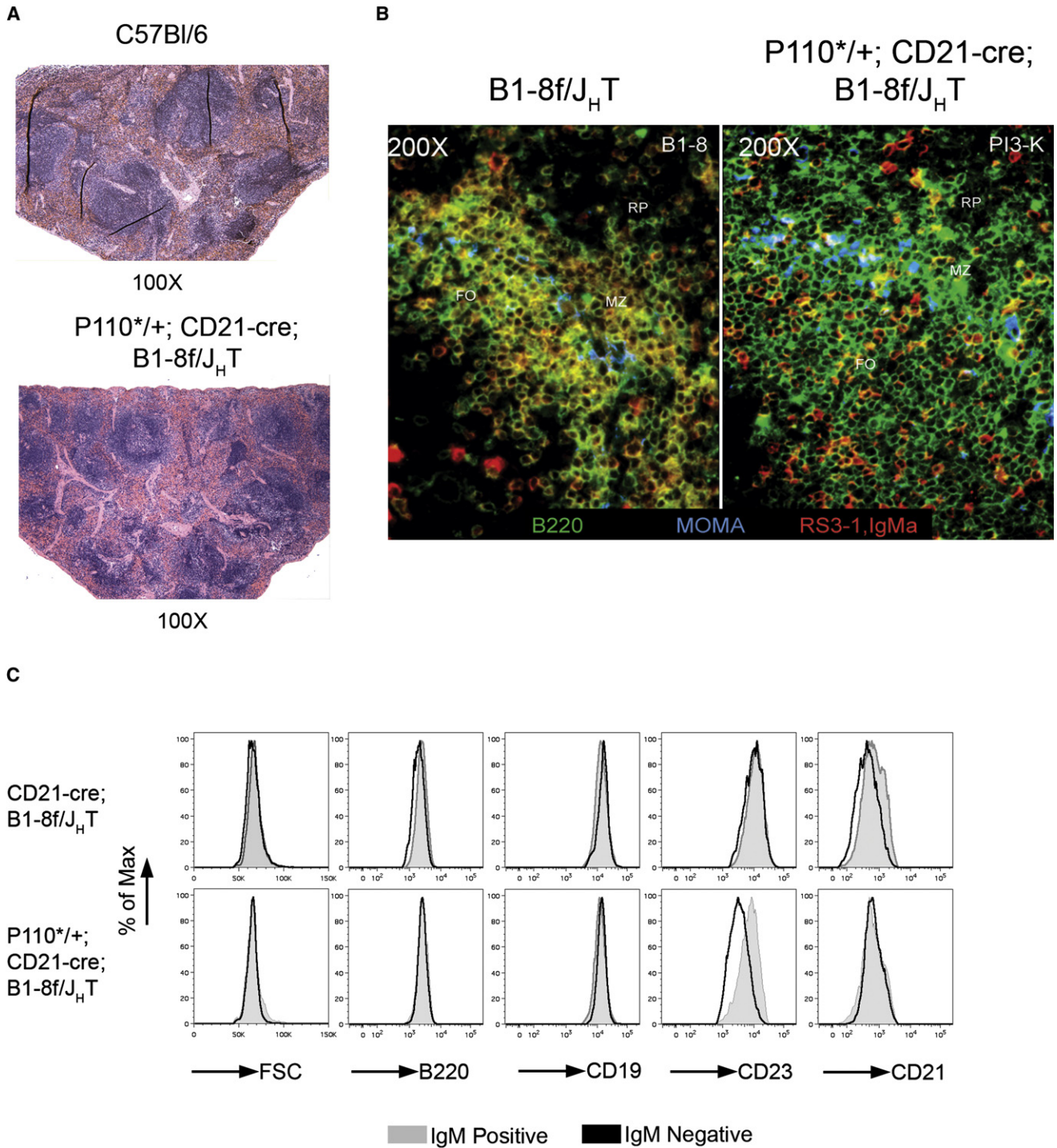
IgM Positive   IgM Negative

Figure 2. P110*-Rescued IgM^{neg} B Cells Are Arranged in Follicles in the Spleen and Are Similar to IgM^{pos} B Cells

(A) Hematoxylin and Eosin staining of sections of spleen from a wild-type C57Bl/6 (B6) and *P110*/+; CD21-cre; B1-8f/J$_H$T* (PI3K, Fetal liver chimera) mice.

(B) Immunofluorescent staining of sections of spleen showing part of a single follicle from *B1-8f/J$_H$T* and *P110*/+; CD21-cre; B1-8f/J$_H$T* mice. B220, marking all B cells is stained in green, IgM is in red and MOMA1, marking metallophilic macrophages is in blue.

(C) Comparison of cell size (FSC), and surface expression of B cell markers B220, CD19, CD23 and CD21 between IgM^{pos} (gray) and IgM^{neg} (black) follicular B cells from spleen of *CD21-cre; B1-8f/J$_H$T* (top row) and *P110*/+; CD21-cre; B1-8f/J$_H$T* (bottom row) mice.
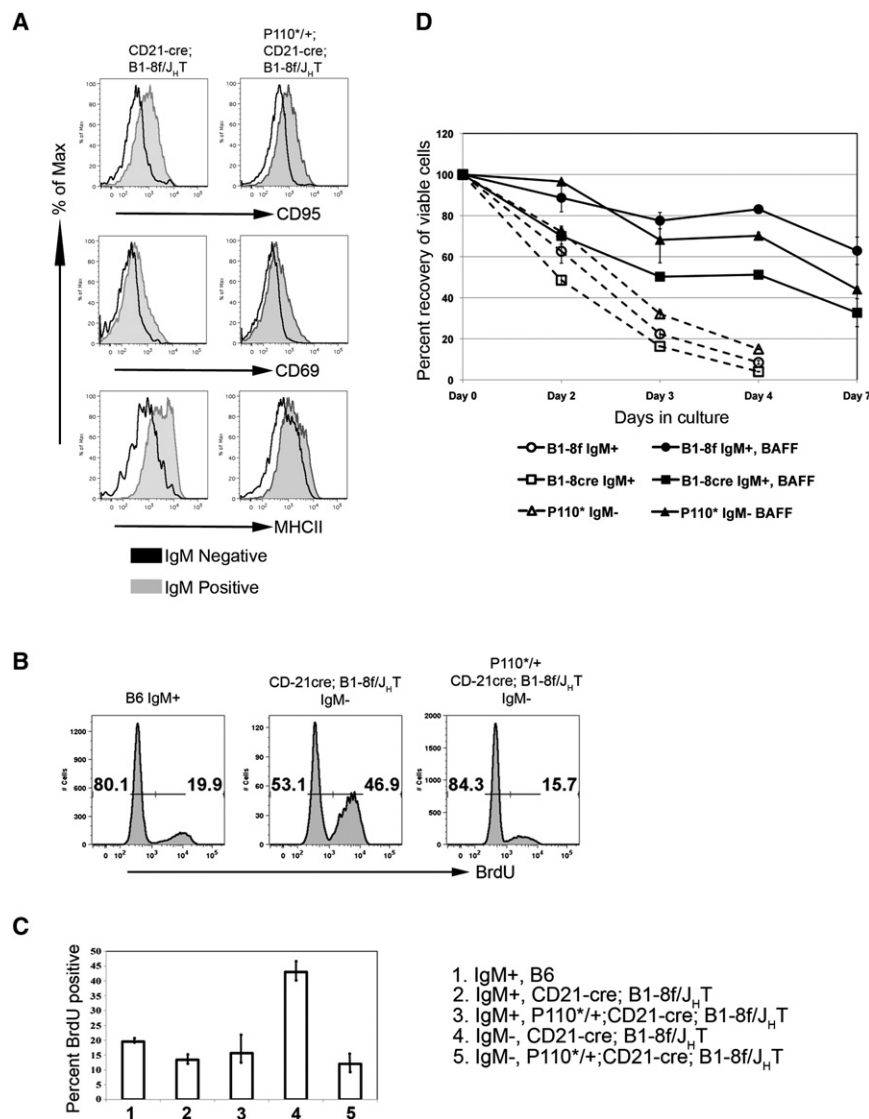
Figure 3. P110*-Rescued IgM^{neg} B Cells Are Resting B Cells Dependent on BAFF for Survival

(A) Comparison of surface expression of activation markers on IgM^{pos} (gray histograms) and IgM^{neg} (black histograms) mature follicular B cells (gated on B220+, AA4.1- and CD23 high) from the spleen of *CD21-cre; B1-8f/J$_H$T* (left column) and *P110*/+; CD21-cre; B1-8f/J$_H$T* (right column) mice.

(B) Representative FACS plots showing BrdU incorporation in B lymphocytes from spleen after 20 days of BrdU feeding of mice of indicated genotype. The numbers indicate the percentage of BrdU-positive or -negative cells within the mature B cell fraction (B220+, AA4.1-).

(C) Graphical representation of data in B. Also shown is incorporation of Brdu in the IgM^{pos} mature B cells from *CD21-cre; B1-8f/J$_H$T* and *P110*/+; CD21-cre; B1-8f/J$_H$T* fetal liver chimeric mice.

(D) P110*-rescued IgM^{neg} B cells are dependent on BAFF for survival in vitro. IgM^{pos} and IgM^{neg} mature follicular B cells from *B1-8f/J$_H$T* (B1-8f), *CD21-cre; B1-8f/J$_H$T* (B1-8cre) and *P110*/+; CD21-cre; B1-8f/J$_H$T* (P110*) mice were cultured in vitro in the presence (filled markers) or absence (open markers) of BAFF. Data are plotted as percent recovery of viable cells.

Failure of Canonical NF-κB Signaling and Constitutive MEK1 and Rac1 to Efficiently Rescue BCR-Negative B Cells

The efficient rescue of BCR^{neg} B cells by P110* contrasted with our failure to rescue such cells by activation of the canonical NF-κB pathway. We used for this purpose the conditional *IKK2ca* allele, from which constitutively active IκB kinase 2 (IKK2) is expressed, activating the canonical NF-κB pathway and rescuing B cells from BAFF dependency

in the population of IgM^{pos} B cells in the *CD21-cre; B1-8f/J$_H$T* mutant chimeras (Figure 3C). Apparently, many of these cells escaped Cre-mediated recombination by an unknown mechanism.

As the P110*-rescued BCR^{neg} cells are well controlled in terms of overall cell numbers, one would predict that they retain the dependence of normal mature B cells on the survival factor BAFF. On the other hand, BAFF:BAFF-R induced activation of the alternative NF-kB pathway leads to the phosphorylation of Akt, which is a main downstream target of PI3K (Patke et al., 2006; Otipoby et al., 2008). We therefore assayed the BAFF dependence of P110*-rescued BCR^{neg} B cells in cell culture experiments (Batten et al., 2000; Gross et al., 2001) and found that the survival of P110*-rescued and control cells was equally dependent on BAFF (Figure 3D).

The P110*-rescued BCR^{neg} B cells are thus resting, long-lived cells, living in their normal habitats in the peripheral immune system and subject to normal homeostatic control.

(Sasaki et al., 2006). Compound mutant mice of the genotypes *CD21-cre; B1-8f/J$_H$T* and *IKK2ca/+; CD21-cre; B1-8f/J$_H$T* developed normally, so that the analysis of the B cell compartment did not require the generation of fetal liver chimeras. As shown in Figure 4, IKK2ca expression led to only a very limited rescue of BCR^{neg} B cells in the spleen. This was also the case in other lymphoid organs (data not shown). Essentially all IgM^{neg} cells expressed GFP, indicating that the cells had activated the *IKK2ca* transgene. The inefficient rescue of IgM^{neg} cells by IKK2ca is not due to inefficient Cre-mediated recombination in this system, because 80% of mature B cells in *IKK2ca/+;CD21-cre* mice expressed GFP, indicative of efficient excision of the STOP cassette in the *IKK2ca* allele (Figure S3).

The inefficient rescue by IKK2ca suggests that the rescue of BCR^{neg} B cells by P110* is not mediated by NF-κB activation. Indeed, when we analyzed the expression of genes of the NF-κB pathway in such cells by quantitative RT-PCR, we saw little change in comparison to BCR^{pos} controls or BCR^{neg}
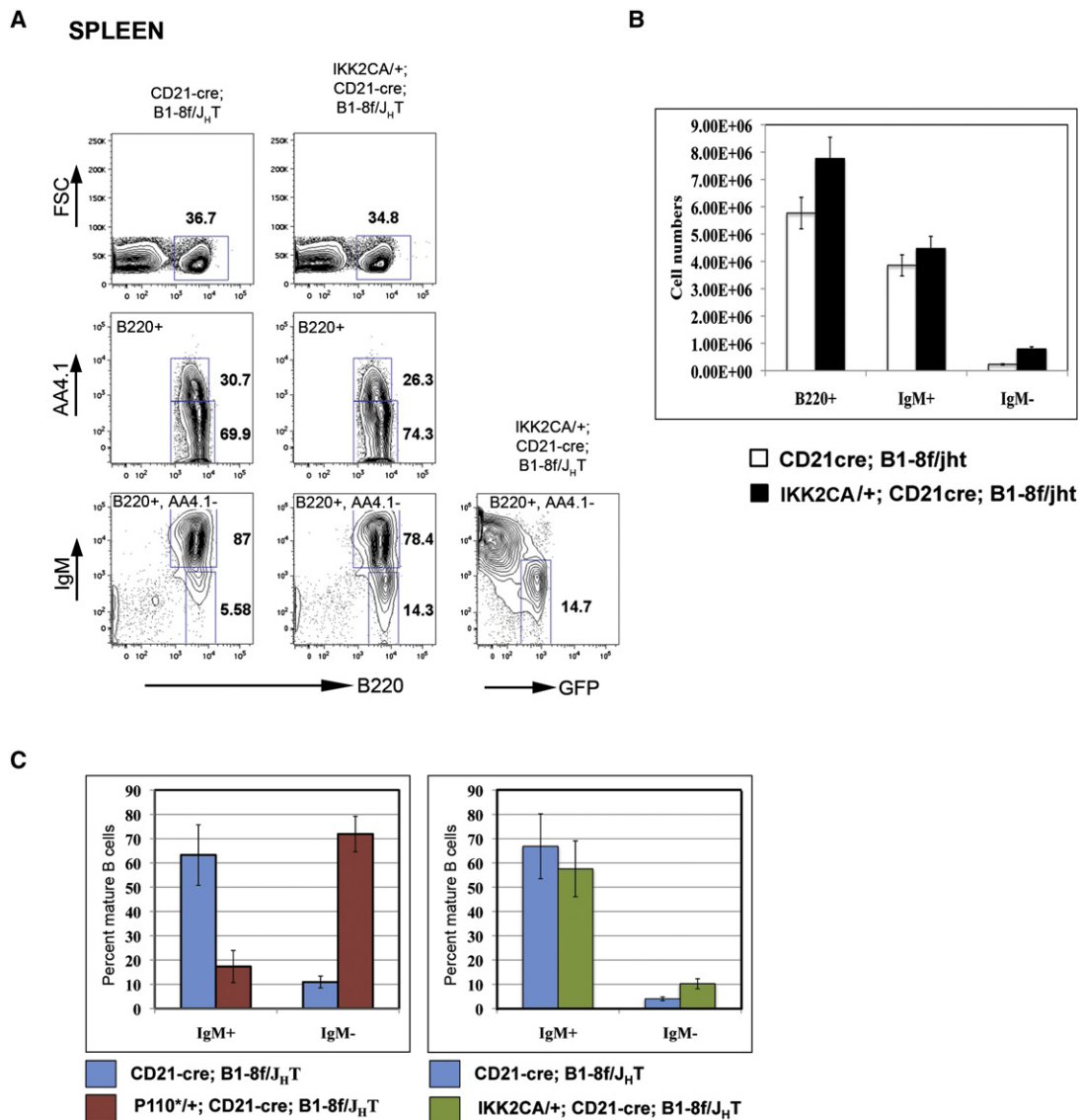
Figure 4. Expression of Constitutively Active IKK2 Causes Limited Rescue of IgM^{neg} B Cells

(A) Representative FACS analysis of lymphocytes from Spleens of *CD21-cre; B1-8f/J$_H$T* (left column) and *IKK2CA/+; CD21-cre; B1-8f/J$_H$T* mice. Numbers represent percentages of cells within the lymphocyte gate or of cells in the gate indicated in the FACS plot.

(B) Comparison of B cell numbers from *CD21-cre; B1-8f/J$_H$T* (open bars) and *IKK2CA/+; CD21-cre; B1-8f/J$_H$T* (filled bars) mice showing limited rescue of IgM^{neg} B cells by the *IKK2CA* transgene, mean and SD of four mice per genotype.

(C) Comparison of rescue of IgM^{neg} B cells by activation of PI3K signaling (left panel) or canonical NF-kB signaling (right panel) in the spleen. Data are plotted as percent of mature B cells (B220+, AA4.1-).

cells lacking P110* (Figure S4). Only *c-Rel* transcripts were about 2-fold downregulated in the latter cells compared to their BCR^{pos} counterparts, and this downregulation was not seen in the P110*-rescued BCR^{neg} cells. This result is in agreement with Matsuda et al. (2009), who reported that PI3K activity maintains the expression of the NF-kB proteins c-Rel and RelA.

We also analyzed the ability of two additional signaling molecules, which are rapidly activated upon BCR engagement, to rescue BCR^{neg} B cells, namely MEK1, a member of the Raf-MEK-Erk MAP Kinase pathway, and the Rho GTPase Rac1 (Genot and Cantrell, 2000). Activation of Rac1 upon BCR engage-

ment leads to rapid actin polymerization and cytoskeletal rearrangements, and ablation of Rac1 and 2 blocks B cell development (Walmsley et al., 2003). Activation of the MAP Kinase pathway and Rac1 was done with the help of newly constructed ROSA26 alleles, from which constitutively active MEK1, MEK1DD (Cowley et al., 1994), or dominant active Rac1, RacDA (Kobayashi et al., 1998) are expressed upon Cre-mediated excision of a STOP cassette. When we used these alleles in our experimental in vivo system, they failed to rescue BCR^{neg} B cells in the spleen, despite their ability to increase phospho-Erk levels and PAK1 binding activity, respectively, in splenic B cells (Figure S5; Bokoch, 2003).

Rescue of BCR[neg] B Cells by Pten Knockout

To corroborate that PI3K signaling is indeed the central survival signal in mature B cells downstream of the BCR, we assessed whether rescue of BCR^{neg} B cells can also be achieved by deletion of the Pten tumor suppressor, the key negative regulator of the PI3K pathway via its phosphatase directed conversion of PIP3 to PIP2 (Suzuki et al., 2008). Pten is expressed in the B cell lineage and known to play a role in B cell differentiation (Anzelon et al., 2003; Suzuki et al., 2003; Omori et al., 2006). Because Pten deletion by CD21-cre, like activation of P110*, produces lethality in the compound mutant mice (data not shown), chimeric mice were generated, whose lymphocytes had either the *CD21-cre; B1-8f/J$_H$T* or *Ptenf/Δ; CD21-cre; B1-8f/J$_H$T* genotype. The analysis of these mice revealed that Pten ablation rescued BCR^{neg} B cells almost as efficiently as the P110* transgene (Figure 5). Spleens from *Ptenf/Δ; CD21-Cre; B1-8f/J$_H$T* mice had more B cells (Figure 5A, top row), a decreased fraction of immature B cells (middle row) and a substantial increase in the percentage of mature IgM^{neg} B cells (bottom row) in comparison to controls deleting the BCR in the absence of Pten ablation. In the bone marrow, the Pten deleted, rescued IgM[neg] (B220 high) B cells constituted 17% of all lymphocytes (Figure 5B). Substantial fractions of rescued BCR^{neg} B cells were also found in lymph nodes (Figure 5C), and peritoneal cavity (Figure 5D). As in the case of P110*, there was no upregulation of the activation markers CD69 and MHC II in the rescued cells (Figure 5E). They were also again identical to their IgM[pos] counterparts in forward scatter analysis and expressed similar levels of the B cell markers B220, CD19, and CD21, whereas CD23 was downregulated about 2-fold (Figure S6). No Pten protein was detectable in the rescued cells by western blot (Figure 5F). Histological analysis of the spleens of the compound mutants revealed a normal organization of B cells into follicles and MZ B cells, with the majority of the cells being negative for IgM (Figure 5G).

The PI3K-FOXO Pathway Mediates Survival of BCR[neg] B Cells

The negative regulation of FOXO transcription factors through the serine/threonine kinase Akt, a key target of PI3K signaling, has recently emerged as a central regulator of B cell development and differentiation (Herzog et al., 2009; Chen et al., 2006; Yusuf et al., 2004). Analyzing in vivo generated BCR^{neg} B cells in the absence of any rescue, we indeed found upregulation of transcripts of the pro-apoptotic FOXO target genes *BCL2L11* (encoding the Bcl2 family member Bim) and *p27/Kip1* (Dansen and Burgering, 2008; Figure 6), and of two additional recently identified direct or indirect FOXO target genes, AICDA (encoding activation induced cytidine deaminase (AID)) and recombination activating gene 1 (RAG1; Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008; Figure 6). In all four cases, upregulation was reversed upon P110* rescue. These results are consistent with a role for FOXO in the control of BCR-mediated B cell survival and also indicate that P110* is active in the rescued cells, by controlling gene expression through FOXO transcription factors.

In the light of these results, we sought to provide direct genetic evidence for a role for FOXO in the rescue of BCR^{neg} B cells.

While FOXO transcription factors are encoded by four different genes (FOXO 1, FOXO 3, FOXO 4 and FOXO 6), at least two of which (FOXO 1 and FOXO 3) are expressed in B cells (Kerdiles et al., 2009), recent experiments indicate that FOXO1 is the dominant player in B cell differentiation (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008). We therefore used a conditional FOXO1 allele (Paik et al., 2007) to delete FOXO1 in mature B cells concomitantly with the BCR through the CD21-cre transgene. As can be seen in Figure 7A, FOXO1 deletion in mature B cells in *Foxo1f/Δ; CD21-cre; B1-8f/J$_H$T* mice led to a striking accumulation of IgM^{neg} B cells in the spleen, with most of the cells displaying a mature (AA4.1-) phenotype. Indeed, in these mice 90% of the mature splenic B cells were IgM[neg], with a strong reduction of the fraction of immature (AA4.1-) cells compared to controls. Efficient rescue of BCR^{neg} B cells was also seen in bone marrow and peritoneal cavity (Figure S7). Comparison of B cell numbers upon rescue by Pten or FOXO1 revealed that while in the case of Pten a larger fraction of IgM^{pos} cells persisted in the mice, the numbers of rescued IgM^{neg} cells were almost equivalent in the two mutants (Figure 7B).

However, the BCR^{neg} B cells rescued by FOXO1 deletion lacked a number of properties of normal B cells, quite distinct from what we had observed in the case of P110* and Pten. Histological analysis of the spleen from *Foxo1f/Δ; CD21-Cre; B1-8f/J$_H$T* revealed the efficient rescue of such cells in the follicles, but lack of MZ B cells (Figure 7C), which was also confirmed by flow cytometric analysis (Figure S8A). Furthermore, only a minor fraction of BCR^{neg} B cells was detected in lymph nodes (Figure 7D). This correlated with a strong decrease of L-selectin (CD62L) expression on the surface of these cells (Figure 7E). CD62L controls the migration of lymphocytes into lymph nodes, and in T cells FOXO1 indeed controls this process as well as CD62L expression (Kerdiles et al., 2009). In addition, the rescued FOXO1 deficient cells had drastically reduced levels of CD23 on their surface, and were slightly smaller than normal B cells or BCR^{neg} B cells rescued by Pten knockout or P110* (Figure S8B).

We gained some understanding of the abnormal properties of the rescued FOXO1 knockout cells when we analyzed FOXO1 protein levels in these cells and BCR^{neg} B cells rescued by P110* expression or Pten knockout. While FOXO1 was undetectable in the former cells (Figure S9A), in the cells rescued by P110* expression or Pten knockout FOXO1 levels were reduced to a level roughly equivalent to that in heterozygous FOXO1 knockouts, correlating with a slight downregulation of CD62L on the surface of the rescued cells, which was equally seen in BCR^{neg} B cells rescued by P110* (Figure 7E). This indicated that the PI3K pathway indeed controls FOXO1 levels in mature B cells and suggested that small changes of the concentration of FOXO1 in the cells have major effects on their physiology. Indeed, when we analyzed the rescue of BCR^{neg} B cells in a situation where the BCR was conditionally deleted in mature B cells on a FOXO1^{+/−} background, we found a 2- to 4-fold increase of BCR^{neg} B cells in both lymph nodes and spleen, compared to FOXO+/+ controls (Figure S9B).

While these results indicate that the control of FOXO1 by PI3K signaling is a central element of the BCR controlled B cell survival
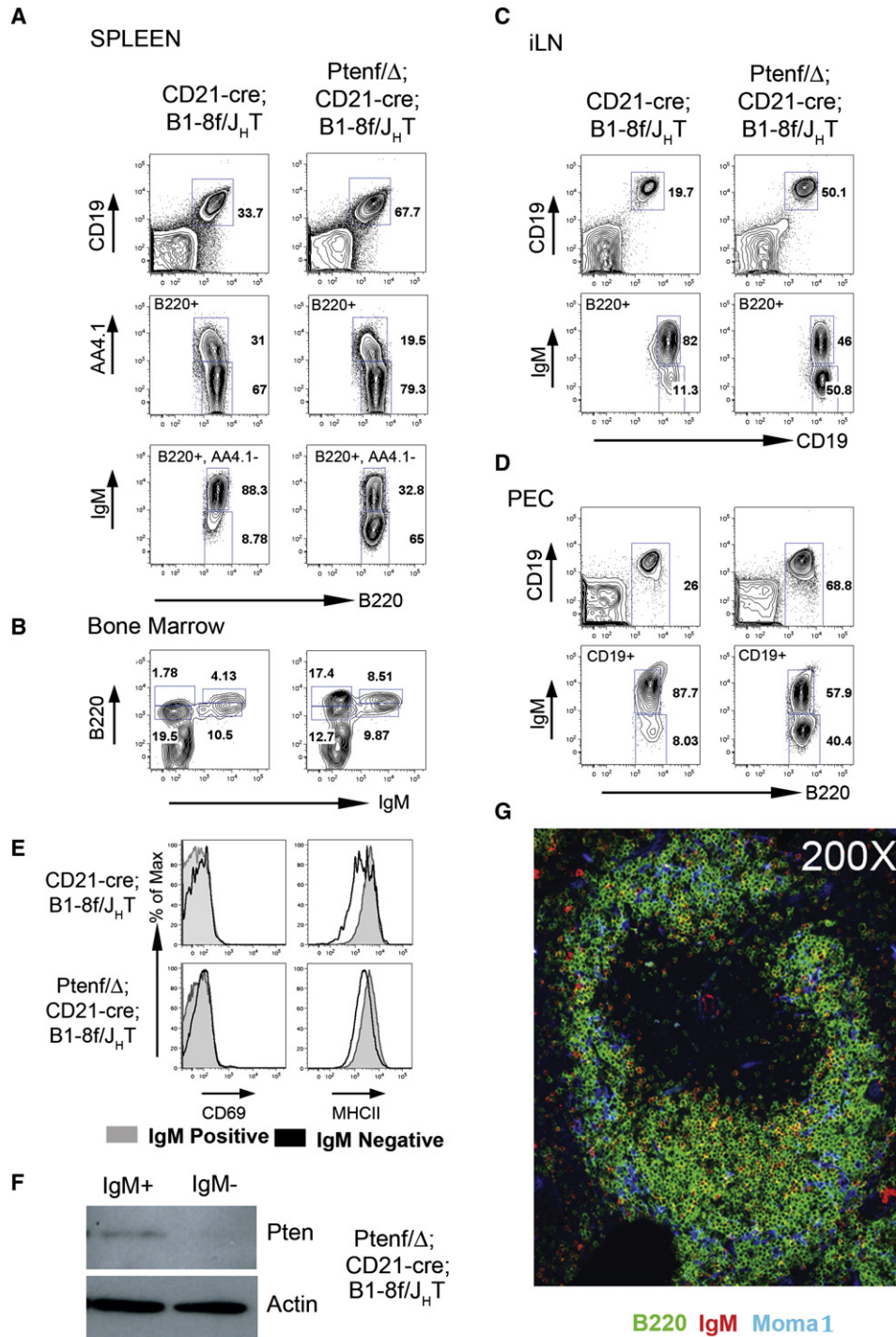
Figure 5. Deletion of Pten Rescues BCR^neg B Cells

Representative FACS analysis of lymphocytes from spleen (A), bone marrow (B), inguinal lymph node (iLN [C]) and peritoneal cavity (PEC [D]) from *CD21-cre; B1-8f/J_HT* and *PtenΔ/f; CD21-cre; B1-8f/J_HT* mice showing rescue of IgM^neg mature B cells by Pten deletion. Numbers represent percentages of cells within the lymphocyte gate or of cells in the gate as indicated in the FACS plot. (E) IgM^neg cells rescued by Pten deletion are not activated. Comparison of surface expression of activation markers CD69 and MHC class II on IgM^pos (gray histograms) and IgM^neg (black histograms) cells from the spleen of *CD21-cre; B1-8f/J_HT* and *PtenΔ/f; CD21-cre; B1-8f/J_HT* mice. (F) Western blot analysis for Pten (top panel) and Actin (bottom panel) in sorted IgM^pos and IgM^neg mature B cells from spleen of *PtenΔ/f; CD21-cre; B1-8f/J_HT* mice. (G) Immunofluorescent staining of sections of spleen showing a single follicle from *PtenΔ/f; CD21-cre; B1-8f/J_HT* mice. B220, marking all B cells is stained in green, IgM is in red and MOMA1, marking metallophilic macrophages is in blue.
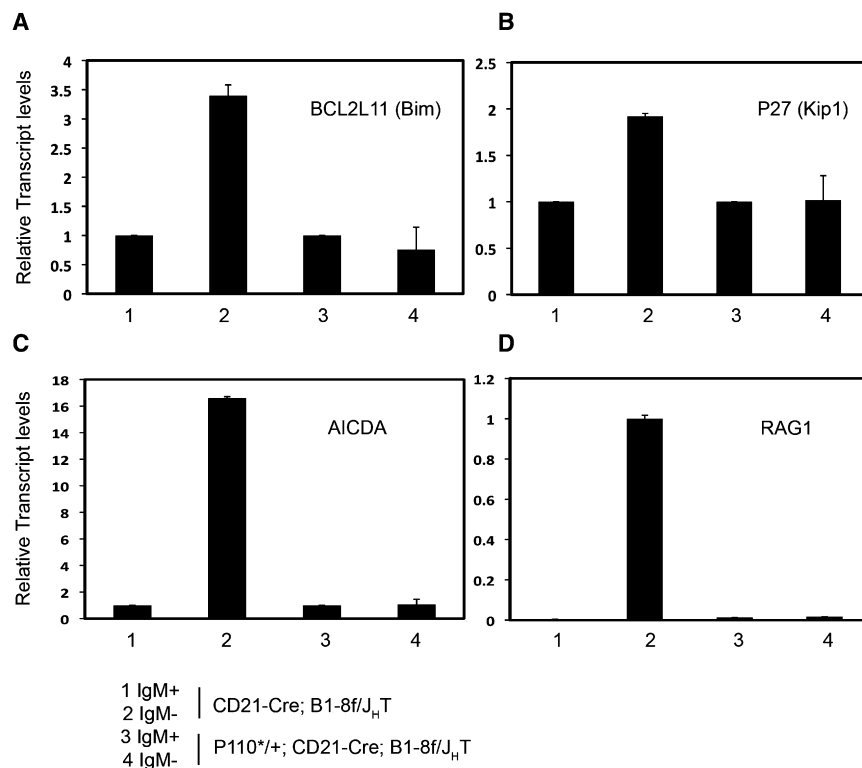
A

B

C

D

1 IgM+ | CD21-Cre; B1-8f/J$_H$T
2 IgM- |
3 IgM+ | P110*/+; CD21-Cre; B1-8f/J$_H$T
4 IgM- |

Figure 6. P110*-Rescued IgM^{neg} Cells Do Not Upregulate FOXO Targets

Quantitative RT-PCR analysis of FOXO targets in sorted IgM^{pos} and IgM^{neg} mature B cells from spleens of *CD21-cre; B1-8f/J$_H$T* and *P110*/+; CD21-cre; B1-8f/J$_H$T* mice. Transcripts shown include BCL2L11 (A), P27/KIP1 (B), AICDA (C), and Rag1 (D). Lanes as indicated in the figure, mean and SD of triplicate experiment are shown.

moderate PI3K activity seen in mature B cells, and that, in the light of the genetic rescue experiments, this activity is critical for the survival of the cells.

DISCUSSION

Using concomitant BCR deletion and activation of candidate signaling pathways in mature B cells in vivo, we provide evidence that the PI3K signaling pathway is the critical survival determinant in mature, resting B cells downstream of the BCR. The central evidence for this conclusion is the efficient rescue of mature B cells losing their BCR by either transgenic expression of constitutively active PI3K or knockout of the inhibitor of PI3K signaling, Pten. In both cases, the rescue involves, as determined through the phosphorylation of the PI3K target Akt and further downstream components of the pathway, reconstitution of PI3K activity to levels only modestly above those in resting BCR^{pos} B cells, and much below PI3K activity induced by B cell activation. Our results also indicate that in resting mature B cells the PI3K-mediated phosphorylation of Akt largely depends on BCR expression.

The rescued BCR^{neg} cells resemble normal mature B cells almost perfectly: They are resting, long-lived cells that populate the same compartments in the peripheral immune system as wild-type B cells, with B2 and MZ B cells located in their respective habitats in spleen and LNs. (The experimental system precludes the analysis of B1 cells, which are not generated in B1-8f mice; Lam and Rajewsky, 1999). In both B cell follicles and the MZ, the BCR^{neg} cells intermix with B cells that have escaped BCR ablation, emphasizing their physiologically normal behavior. The cells also resemble normal mature B cells in terms of surface marker expression, with a few exceptions discussed below. The rescued BCR^{neg} cells comprise 60%–90% of the total peripheral B cells, whose overall number is similar to that seen in wild-type mice, with the exception of a slightly increased MZ B cell compartment. This indicates that the rescued cells are subject to the same numerical control as wild-type B cells. Indeed, the BCR^{neg} cells are as dependent on BAFF-BAFF-R interactions as wild-type B cells.

The equal rescue of BCR^{neg} B cells by constitutively active PI3K and Pten knockout implies that mature B cells display some PI3K activity irrespective of whether they express a BCR or not. Modulation of PI3K signaling thus appears to be the key
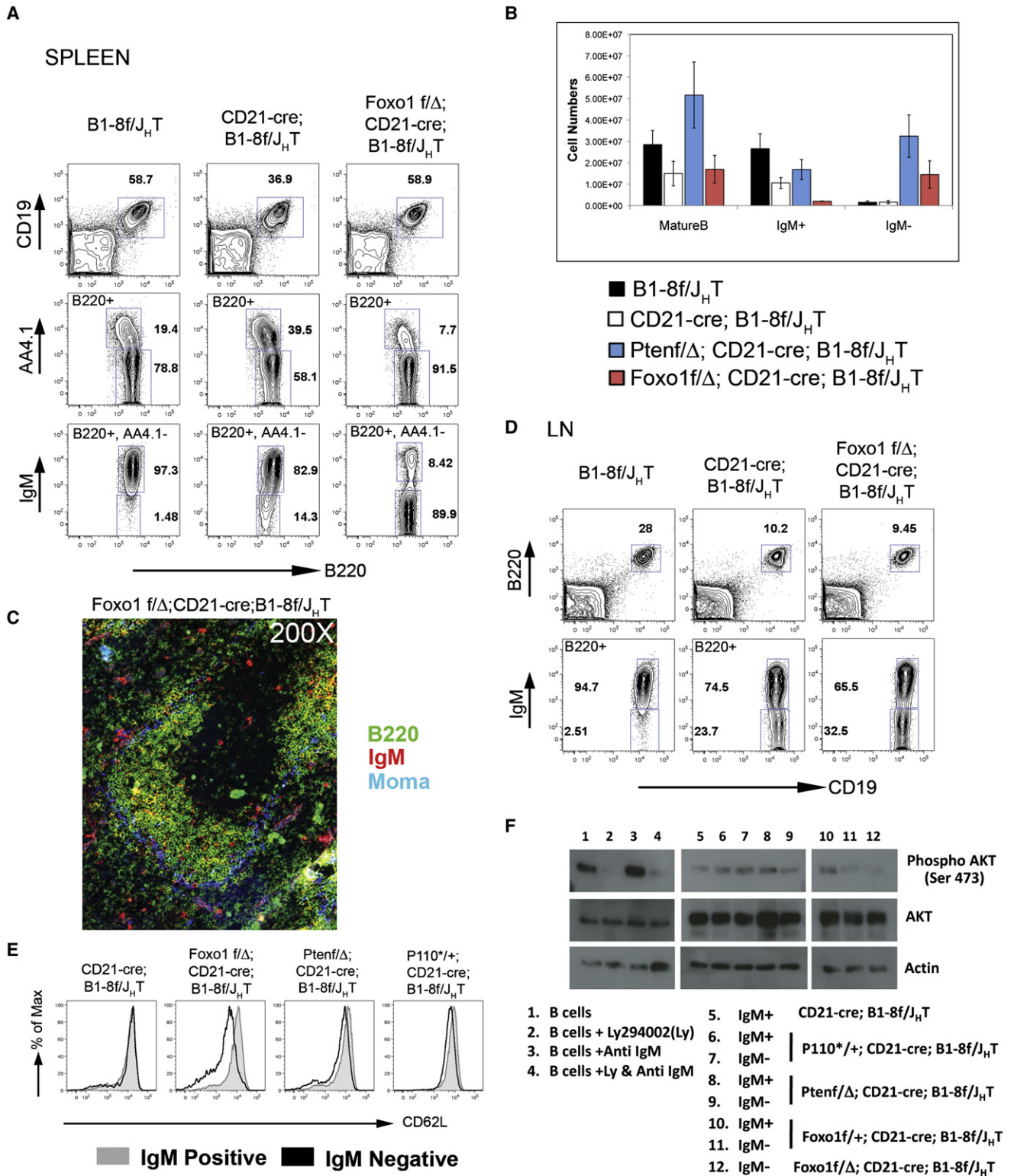
signal, they also reveal the subtlety of this control mechanism, which seems to require the modulation of this transcription factor within a narrow range.

Loss of PI3K-Mediated Akt Phosphorylation upon BCR Ablation and Its Reconstitution by P110* or Deletion of Pten

Sufficient BCR^{neg} cells could be isolated from *FOXO1f/Δ; CD21-cre;B1-8f/J$_H$T* mice to allow biochemical analysis. We focused on the activation of Akt through phosphorylation of serine 473, which is known to be under PI3K control. Accordingly, Akt S473 phosphorylation, detectable in resting and, more strongly, in activated mature B cells, was abolished by treatment of the cells with the PI3K inhibitor Ly294002 (Deane and Fruman, 2004; Figure 7F, lanes 1–4). In BCR^{neg} B cells rescued by partial or complete FOXO1 ablation, Akt phosphorylation on S473 was strongly reduced and indeed undetectable in 2/3 experiments (Figure 7F, lanes 10–12; Figure S10A). The data in Figure 7 and Figure S10A (repeat experiments) also show that the extent of Akt S473 phosphorylation induced in the cells upon rescue by P110* or Pten ablation is moderate and closer to that seen in resting mature B cells than B cells activated by BCR engagement or even just exposed to serum containing culture medium. We also did not see significantly increased phosphorylation of the Akt targets GSK3α and β in the rescued cells (Figure S10B). Finally, control and rescued cells did not exhibit any detectable phosphorylation of the mTOR target S6 kinase (S6K), which is strongly induced through this branch of the PI3K pathway upon B cell activation (Engelman et al., 2006; Figure S10C). We conclude that BCR expression mediates the

A SPLEEN

B

C Foxo1 f/Δ;CD21-cre;B1-8f/J$_H$T

B220
IgM
Moma

D LN

E

F

Figure 7. Survival of Resting B Cells through BCR Is Mediated by PI3K-FOXO Pathway

(A) FACS analysis of lymphocytes from spleens of *B1-8f/J$_H$T* (left column), *CD21-cre; B1-8f/J$_H$T* (middle column) and *Foxo1 Δ/f; CD21-cre; B1-8f/J$_H$T* mice (right column) showing rescue of IgM^{neg} mature B cells by deletion of Foxo1.

(B) Comparison of splenic B cell numbers of the mice as indicated in the figure, mean and SD of a minimum of 4 mice per genotype are shown.

(C) Immunofluorescent staining of splenic sections from *Foxo1 Δ/f; CD21-cre; B1-8f/J$_H$T* mice.

element in the control of mature B cell survival by the BCR. This notion is in accord with the general view that the quantitative control of the PI3K pathway determines its manifold roles in cellular physiology. Thus, heterozygous Pten knockout mice, which exhibit a 50% reduction in Pten protein levels in T cells (Di Cristofano et al., 1999; Xiao et al., 2008), develop autoimmunity, and T cells from these mice have a reduced response to Fas ligation. Monoallelic loss of Pten also contributes to tumor growth in the context of other somatic mutations, with Pten protein levels correlating with disease severity (Salmena et al., 2008). Along the same lines, the graded expression of FOXO transcription factors, which are targets of PI3K signaling, determines the balance between the proliferation and differentiation of B cell progenitors (Amin and Schlissel, 2008; Dengler et al., 2008; Herzog et al., 2008).

The present results demonstrate that modulation of the FOXO1 transcription factor is similarly crucial for the control of mature B cell survival by the BCR. However, the B cells rescued by FOXO1 ablation have a number of aberrant properties compared to the properties of normal mature B cells and BCR^{neg} B cells rescued by constitutive PI3K activity or Pten knockout. Therefore, the role of FOXO1 in the control of mature B cell survival by BCR-mediated PI3K signaling is subtler than modeled by our conditional loss-of-function experiment. Perhaps the FOXO transcription factors expressed in mature B cells play redundant and nonexclusive roles in this process.

In contrast to the efficient rescue of BCR^{neg} cells by manipulation of the PI3K signaling pathway we saw little rescue upon activation of the canonical NF-κB pathway in mature B cells losing their BCR. The IKK2ca allele used in these experiments induces strong canonical NF-κB signaling in mature B cells, making these cells BAFF independent and enhancing their response to mitogenic stimuli (Sasaki et al., 2006). The limited rescue of BCR^{neg} B cells by IKK2ca is in accord with our previous demonstration that transgenic Bcl2 rescues BCR^{neg} B cells only transiently (Lam et al., 1997). Taken together, these results indicate that canonical NF-κB activity, while essential for mature B cell persistence under conditions of cellular competition (Pasparakis et al., 2002), is unable to substitute for loss of the BCR in these cells and thus does not by itself represent the critical survival signal downstream of the BCR. This conclusion is in accord with the presence of a large compartment of mature, resting B cells in Bcl10 knockout mice, where BCR engagement does not elicit canonical NF-κB activity (Ruland et al., 2001; Xue et al., 2003). It is also supported by our findings that PI3K activation in mature B cells did not significantly impact the transcription of NF-κB components and target genes, except for an expected 2-fold upregulation of c-Rel mRNA; and that FOXO1 appears to be the major downstream target of PI3K-mediated mature B cell survival. With respect to the work of Stadanlick et al. (2008),

one might speculate that the postulated crosstalk between BCR-mediated canonical and BAFF-R-mediated NF-κB activity may apply to certain processes of B cell activation rather than tonic BCR signaling in resting B cells.

While our failure to rescue BCR^{neg} B cells by constitutively active Rac1 was not unexpected, it is noteworthy that this was also the case for MEK1DD, despite our demonstration that the expression of this molecule in mature B cells in vivo mediated substantial ERK phosphorylation. As the latter appeared to be in the same range as ERK phosphorylation in control B cells, this result does not support the possibility that BCR mediated MAP kinase activation either through PI3K signaling or independent of it (Brummer et al., 2002; Dye et al., 2007), is part of the survival signal downstream of the BCR.

How PI3K signaling through the BCR is initiated in resting mature B cells is not fully understood. If low affinity interactions of the BCR with self-antigens are involved in this process, PI3K signaling would likely be induced through the phosphorylation of the adaptor protein BCAP and the CD19 co-receptor by protein tyrosine kinases, with src family kinases and Syk playing a central role (Kurosaki, 1999). It is interesting in this context that the inactivation of a negative regulator of src family and other kinases, c-src tyrosine kinase (Csk), in developing T cells results in a quasi normal compartment of peripheral CD4+ T cells that lack T cell receptor expression (Schmedt et al., 1998). In a different model emerging from very recent evidence (Delgado et al., 2009), the antigen receptors in resting T and B cells are connected to the PI3K pathway by the GTPase TC21 and perhaps other members of the family, which directly recruits the catalytic subunit of PI3K to the non-phosphorylated immuno-tyrosine activation motifs (ITAMs) of these receptors. In this scenario, the "tonic," PI3K-mediated survival signal is independent of BCR engagement by an external ligand.

While the present data indicate a critical role of PI3K signaling in BCR-mediated mature B cell survival, they clearly do not define the survival signal in all its complexity. The slight alterations in CD23 and CD62L expression in the cells rescued by P110* or Pten ablation likely reflect the reduced FOXO1 levels in these cells, due to the modestly increased PI3K activity in the rescued, BCR^{neg} compared to BCR^{pos} control cells. However, PI3K likely signals B cell survival through additional targets, and we also cannot exclude minor contributions of other signaling pathways to this process. Additional candidate PI3K targets include the regulators of cell size and metabolism, mTOR (Edinger and Thompson, 2002), in line with the failure of FOXO1 deletion to rescue BCR^{neg} cell size.

Perhaps the biggest surprise in the present study was the finding that despite the ability of the BCR to activate a variety of different signaling pathways in parallel (Kurosaki, 1999), a single one of them seems to signal cellular survival.

(D) FACS analysis of lymphocytes from lymph nodes of the mice as indicated in the figure showing limited rescue of IgM^{neg} B cells in the lymph node upon deletion of Foxo1.
(E) Comparison of surface expression of CD62L on IgM^{pos} (gray histogram) and IgM^{neg} (black histogram) B cells from the lymph nodes of mice as indicated in the figure.
(F) Western blot analysis of sorted IgM^{pos} and IgM^{neg} B cells from mice as indicated in the figure (Lanes 5–12) for Phospho- Akt (Ser473, Top panel), Akt (middle panel) and Actin (bottom panel). Lanes 1–4 represents mature B cells sorted from *B1-8f/J$_H$T* mice and contains two fold less protein than lanes 5–12. For a detailed description of this experiment, see the Experimental Procedures section.

Furthermore, it is through modulation of the activity of the PI3K pathway, a pathway that is of central importance for the homeostasis and survival of higher cells in general (Engelman et al., 2006), that BCR deficient mature B cells can be rescued. While a contribution of an ER stress response upon loss of BCR expression remains a possibility, our results consistently support the concept that the survival of mature B cells depends upon BCR-mediated activation of the PI3K pathway. We have shown in a separate study that BCR-mediated PI3K activation also controls the proliferative response of mature B cells to mitogens through Toll like receptors (Otipoby, Waisman et al., unpublished). We speculate that the BCR has subverted the PI3K signaling pathway in order to integrate the control of cellular survival and responsiveness to pathogens through innate and cognate immune receptors. The exact mechanism of this subversion remains to be elucidated. It will also be interesting to investigate, to which extent PI3K signaling controls the maintenance of B cell lymphomas, which in the human mostly derive from mature B cells and seem to often depend on BCR expression.

EXPERIMENTAL PROCEDURES

Genetically Modified Mice

To generate tissue-specific P110*-transgenic mice we cloned a *loxP*-flanked *neo^R*-stop cassette into a modified version of pROSA26-1. We then added a cDNA encoding P110*, a *frt*-flanked *IRES-eGFP* cassette and a bovine polyadenylation sequence (*R26Stop^{FL}P110**). B6 ES cells (Artemis) were transfected, cultured, and selected as previously described for Bruce 4 ES cells (Sasaki et al., 2006). Pten conditional knockout mice were purchased from the Jackson Laboratory (stock number 006068). Other mouse strains used in this study include *Cd21-cre* (Kraus et al., 2004), *B1-8f* (Lam et al., 1997), *J_HT* (Gu et al., 1993) and *Foxo1f* (Paik et al., 2007). Mice were analyzed at the age of 8–12 weeks. Fetal liver chimeras were generated by transferring fetal liver cells from 14.5 DPC embryos into sub-lethally irradiated (600rads) Rag2/common gamma chain knockout mice purchased from Taconic farms (stock number 004111-MM). Chimeric mice were analyzed 10–12 weeks after transfer. All mice were bred and maintained in specific pathogen free conditions. Mouse protocols were approved by the Harvard University Institutional Animal Care and Use Committee and by the Immune Disease Institute.

Flow Cytometry

Single-cell suspensions prepared from various lymphoid organs were stained with the following monoclonal antibodies: anti-CD19 (ID3), anti-CD23 (B3B4), anti-CD1d (1B1), anti-CD95 (Fas), anti-CD69 (H1.2F3), anti CD62L (MEL-14), anti B220 (RA3-6B2), anti-CD93 (AA4.1), anti-MHCII (all from eBioscience), anti-CD21 (Biolegend) and Goat anti mouse IgM (Fab fragment, Jackson Immunoresearch). All samples were acquired either on a FACSCalibur or FACSCanto II (BD PharMingen), and results analyzed using Flow-Jo software (Tree star inc).

BrdU Labeling and Staining

Mice were fed with drinking water containing 0.8mg/ml BrdU (Sigma) for 20 days. Splenocytes were purified and stained using the FITC BrdU flow kit (BD bioscience) according to manufacturers instructions.

Cell Sorting, qRT-PCR, and Immunoblotting

CD19+, AA4.1-, IgM +/− splenic mature B cells were sorted on a FACSVantage (BD biosciences). Total RNA was isolated from the sorted cells using Trizol (Invitrogen) and cDNA was synthesized using Superscript III cDNA synthesis kit (Invitrogen). qPCR reactions were performed on a Step One Plus real time PCR system using SYBR Green PCR Core reagents according to manufacturers instructions (Applied Biosystems). Primer sequences are listed in Table S1. Immunoblotting was carried out as previously described (Xiao et al., 2008). For details, refer Supplemental Experimental Procedures.

Histology

Tissues fixed with 10% formalin (Sigma) were stained with hematoxylin and eosin (H&E). For immunofluorescence analysis, spleens were frozen in OCT (Sakura Finetek) in liquid nitrogen and staining was done as described previously (Oliver et al., 1999). Antibodies used for staining include FITC anti-B220 conjugate (BD Biosciences), Alexa 488-RS3-1 (Specific for the IgMa allotype) and anti-Moma1.

In Vitro Cell Survival Assay

1×10^6 Mature B cells (B220+, AA4.1-) were cultured in 0.5ml B cell medium (complete DMEM containing 10% FCS) with or without 200ng/ml recombinant mouse BAFF (R&D systems). The FCS was previously tested for it's potential to induce CD69 and MHC class II expression that are sensitive markers for B cell activation. Viable cells were determined at each time point using the Guava -PCA96 cell counter (Guava technologies Inc).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 10 figures, and one table and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)01115-5.

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