PI3K and Btk differentially regulate B cell antigen receptor-mediated signal transduction

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Phosphoinositide-3 kinase (PI3K) is thought to activate the tyrosine kinase Btk. However, through analysis of PI3K$^{-/-}$ and Btk$^{-/-}$ mice, B cell antigen receptor (BCR)-induced activation of Btk in mouse B cells was found to be unaffected by PI3K inhibitors or by a lack of PI3K. Consistent with this observation, PI3K$^{-/-}$ Btk$^{-/-}$ double-deficient mice had more severe defects than either single-mutant mouse. NF-κB activation along with Bcl-x$_L$ and cyclin D2 induction were severely blocked in both PI3K$^{-/-}$ and Btk$^{-/-}$ single-deficient B cells. Transgenic expression of Bcl-x$_L$ restored the development and BCR-induced proliferation of B cells in PI3K$^{-/-}$ mice. Our results indicate that PI3K and Btk have unique roles in proximal BCR signaling and that they have a common target further downstream in the activation of NF-κB.

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Phosphoinositide-3 kinase (PI3K) is a key enzyme producing phospholipid second messengers and has an important role in various signal transduction pathways[1,2]. PI3K family members are classified into three groups according to their structure and substrate specificity[2]. Among them, class I$_A$ heterodimeric PI3Ks consisting of a catalytic subunit (p110α, p110β, p110δ) and a regulatory subunit (p85α, p85β, p55γ) are involved in receptor-mediated signaling in the immune system. To precisely examine the functions of class I$_A$ PI3Ks, we and others generated PI3K$^{-/-}$ mice deficient for the gene encoding p85α, the most abundantly and ubiquitously expressed regulatory subunit of class I$_A$ PI3Ks[3–5]. Due to alternative splicing, p55α and p50α, in addition to p85α, are produced from the same gene[6,7]. Mice lacking only p85α (used here as P13K$^{-/-}$ mice) are viable[3,4], whereas mice lacking all alternatively spliced products are unable to survive after birth[5]. In the absence of PI3K, B cell development from pro-B cells to pre-B cells in the bone marrow is impaired and the number of mature B cells in the periphery is decreased[4,5]. In addition, mature B cell functions such as mitogen-induced proliferation *in vitro* are severely impaired[4].

Crosslinking of the surface B cell antigen receptor (BCR) evokes sequential activation of a variety of protein and lipid kinases including Src family kinases (Lyn, Fyn, Blk), Syk, Btk, Akt (also known as PKB) and PI3K[2,8–12]. Although activation of PI3K is observed upon BCR stimulation, signaling events upstream and downstream of PI3K are not well characterized. In B cells, Lyn, c-Cbl, CD19 and BCAP bind the p85α subunit of PI3K, suggesting that these molecules are upstream activators of PI3K. On the other hand, various proteins containing pleckstrin homology (PH) domains, such as Akt, phosphoinositide-dependent kinase 1 (PDK1) and Btk, are thought to function downstream of PI3K, because of the ability of their PH domains to bind phosphatidylinositol-(3,4)-bisphosphate (PIP$_2$) or phosphatidylinositol-(3,4,5)-trisphosphate (PIP$_3$), products of PI3K[13,14].

Btk, a Tec family kinase, is activated by tyrosine phosphorylation and has a critical role in BCR signaling[12,15–18]. Btk$^{-/-}$ mice, as well as mice with the *Xid* mutation (a natural mutation in the PH domain of Btk in which an arginine residue critical for the binding to PIP$_3$ is replaced by cysteine), show deficiencies in the development and activation of B cells. In humans, deficiency of Btk leads to X-linked Bruton's type agammaglobulinemia (XLA)[12,15,16]. Stimulation-dependent membrane localization of a Btk-PH domain-GFP chimeric protein in transient transfection systems has been demonstrated and such membrane recruitment is blocked by wortmannin, a PI3K inhibitor[19,20]. Overexpression of the p110 PI3K catalytic subunit in a B cell line results in Btk tyrosine phosphorylation[21]. It has been proposed from these observations that PI3K is responsible for the activation of Btk by bringing Btk to the plasma membrane through interactions between the PH domain of Btk and PIP$_3$[13,14], leading to tyrosine phosphorylation of Btk by other protein tyrosine kinases such as Syk. It was thus not surprising that PI3K$^{-/-}$ mice show a phenotype similar to that of Btk$^{-/-}$ or *Xid* mice[4,5].

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BCR stimulation also activates the serine-threonine kinase Akt[2,22]. Akt has crucial roles in anti-apoptotic signal transduction as well as cell cycle progression[23–25]. Activation of Akt prevents apoptosis in many cell types and its anti-apoptotic effect is blocked by wortmannin. One product of PI3K, PIP_2, is reported to associate with the PH domain of Akt to recruit the enzyme to the plasma membrane. Similarly, another PI3K product, PIP_3, recruits PDK1, which phosphorylates Akt to activate its kinase activity. Akt is the major downstream target of PI3K in many signal transduction pathways[2,23–26].

Here, we further investigated the role of PI3K in B cell signal transduction pathways and the functional relationship between PI3K and Btk, using PI3K$^{-/-}$ and Btk$^{-/-}$ mice. Contrary to our expectations, BCR-induced activation of Btk was unaffected by the lack of PI3K or by PI3K inhibitors. On the other hand, BCR-induced activation of Akt was normal in Btk$^{-/-}$ B cells, but was severely impaired in PI3K$^{-/-}$ B cells. Furthermore, PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice show more severe phenotypes than either single-deficient mouse. These biochemical and genetic data show that PI3K and Btk function independently in BCR signal transduction pathways. Among downstream events, activation of NF-κB and induction of Bcl-x_L and cyclin D2 were impaired in both PI3K$^{-/-}$ and Btk$^{-/-}$ single-deficient B cells. Forced expression of Bcl-x_L restored development and proliferative responses of B cells in PI3K$^{-/-}$ mice. Our results indicate that class I_A PI3K and Btk have clearly distinct roles in BCR signal transduction.

Results

PI3K-dependent activation of Akt upon BCR stimulation

B cells from PI3K$^{-/-}$ mice used in this study expressed low amounts of p50α[4]. Expression of p85β and p55γ regulatory subunits was very low or undetectable in PI3K$^{-/-}$ and wild-type (WT) B cells (**Fig. 1a**). Expression of p110δ, the most abundantly expressed catalytic subunit in B cells, was reduced in the absence of these regulatory subunits (**Fig. 1a**). BCR-dependent activation of PI3K in the absence of p85α was examined by *in vitro* kinase assay, using phosphatidylinositol as a substrate to detect generation of phosphatidylinositol-3-phosphate (**Fig. 1b**). Total PI3K activity in tyrosine phosphorylated proteins was increased by BCR stimulation in WT and Btk$^{-/-}$ mice. In contrast, only a small amount of PI3K activity was observed upon BCR stimulation in PI3K$^{-/-}$ B cells (**Fig. 1b**; ~5% of WT activity), as previously reported[4]. The p50α regulatory subunit and possibly another class of PI3K likely contribute to this residual increase of PI3K activity in PI3K$^{-/-}$ B cells. On the contrary, activation of PI3K was unaffected in Btk$^{-/-}$ B cells.

Because Akt is widely accepted as a downstream target of PI3K in B cell signal transduction[2,22–26], we investigated BCR-mediated Akt activation by immunoblotting with specific monoclonal antibodies (mAbs) that detect phosphorylation at residues Thr[308] and Ser[473] of Akt, which is known to correlate with its kinase activity[27,28]. Phosphorylation of Akt on the Thr[308] and Ser[473] residues was increased upon BCR stimulation after 5 min in WT B cells, whereas phosphorylation of Akt

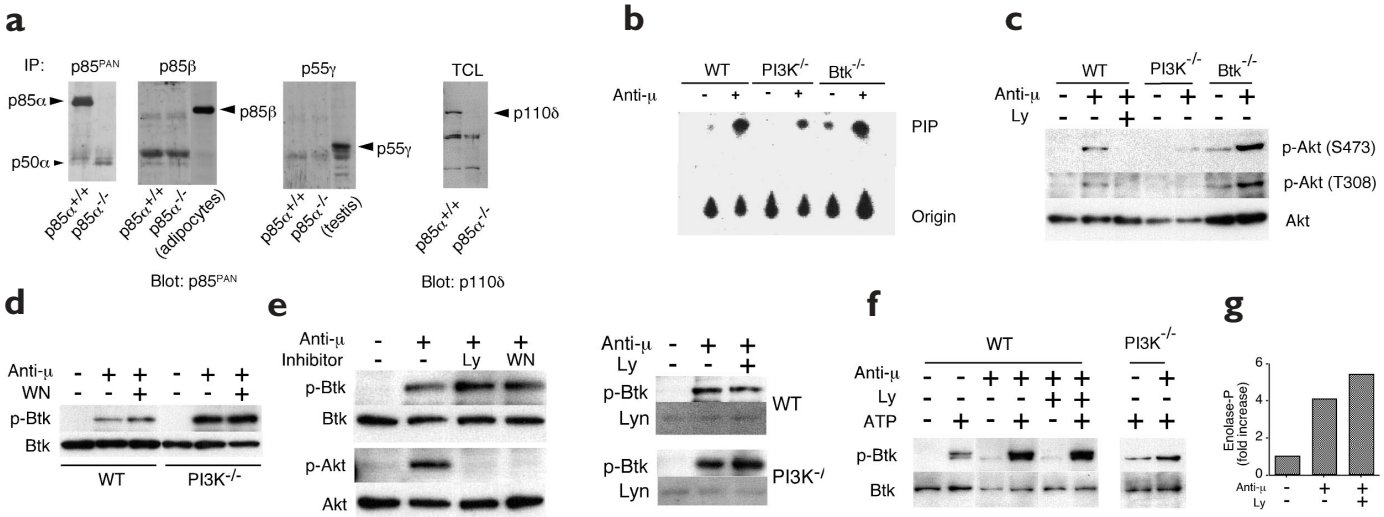

Figure 1. The Akt, but not the Btk, pathway is dependent on PI3K in B cells. (**a**) Expression of PI3K regulatory subunits in PI3K$^{-/-}$ (p85α$^{-/-}$) and WT (p85α$^{+/+}$) B cells. Postnuclear lysates of B cells derived from the indicated mice were immunoprecipitated with anti-p85^{PAN} and specific antisera for p85β and p55γ, then immunoblotted with anti-p85^{PAN}. Adipocytes and testis were used as positive controls for p85β and p55γ, respectively. Or, total cell lysates (TCL) prepared from PI3K$^{-/-}$ (p85α$^{-/-}$) and WT (p85α$^{+/+}$) B cells were immunoblotted with anti-p110δ. (**b**) PI3K activities in PI3K$^{-/-}$, Btk$^{-/-}$ and WT B cells. PI3K activities from BCR-stimulated B cells of the indicated genotypes were assayed. (**c**) PI3K-dependent activation of Akt. BCR-mediated activation of Akt in PI3K$^{-/-}$ and Btk$^{-/-}$ B cells was evaluated by immunoblotting with a specific antibody detecting phosphorylation at Thr[308] (p-Akt (T308)) and Ser[473] (p-Akt (S473)) residues of Akt. Membranes were re-blotted with anti-Akt (Akt). Data are representative of four independent experiments with similar results. (**d**) BCR-induced tyrosine phosphorylation of Btk in PI3K$^{-/-}$ B cells. WT and PI3K$^{-/-}$ B cells on a BALB/c background were stimulated with anti-IgM F(ab)'$_2$ (Anti-μ) in the presence or absence of 25 nM wortmannin (WN). Btk was then immunoprecipitated by anti-Btk and immunoblotted with 4G10 (p-Btk). Membranes were re-blotted with anti-Btk, 43-3B (Btk). (**e**) (Left) Effects of PI3K inhibitors on tyrosine phosphorylation of Btk. WT B cells were stimulated by BCR crosslinking (Anti-μ) in the absence or presence of 50 nM wortmannin (WN) or 25 μM Ly294002 (Ly). Btk was then immunoprecipitated and immunoblotted with 4G10 (p-Btk). Membranes were re-blotted with 43-3B (Btk). At the same time, cell lysates were examined for Akt phosphorylation by anti-phospho-Akt(S473) (p-Akt). Membranes were re-blotted with anti-Akt (Akt). (Right) Membrane fractions were prepared from WT and PI3K$^{-/-}$ B cells unstimulated or stimulated by BCR crosslinking (Anti-μ) in the absence or presence of 25 μM Ly294002 (Ly), and examined for tyrosine phoshorylation (p-Btk). Membranes were re-blotted with anti-Lyn (Lyn). Data in (**d**) and (**e**) are representative of three independent experiments with similar results. (**f,g**) BCR-induced activation of Btk. WT and PI3K$^{-/-}$ B cells on a BALB/c background were stimulated with or without 20 μg/ml of anti-IgM F(ab)'$_2$ (Anti-μ) at 37 °C for 3 min in the presence or absence of 10 μM Ly294002 (Ly). (**f**) Btk was immunoprecipitated and incubated with or without 100 μM ATP at 22 °C for 5 min followed by immunoblot analysis with 4G10. (**g**) Immunoprecipitates were incubated with acid-denatured enolase as an exogenous substrate in the presence of 100 μM ATP at 22 °C for 5 min. Btk activities are presented as the fold increase in the level of tyrosine phosphorylation of enolase. Data in (**f**) and (**g**) are representative of two independent experiments with similar results.

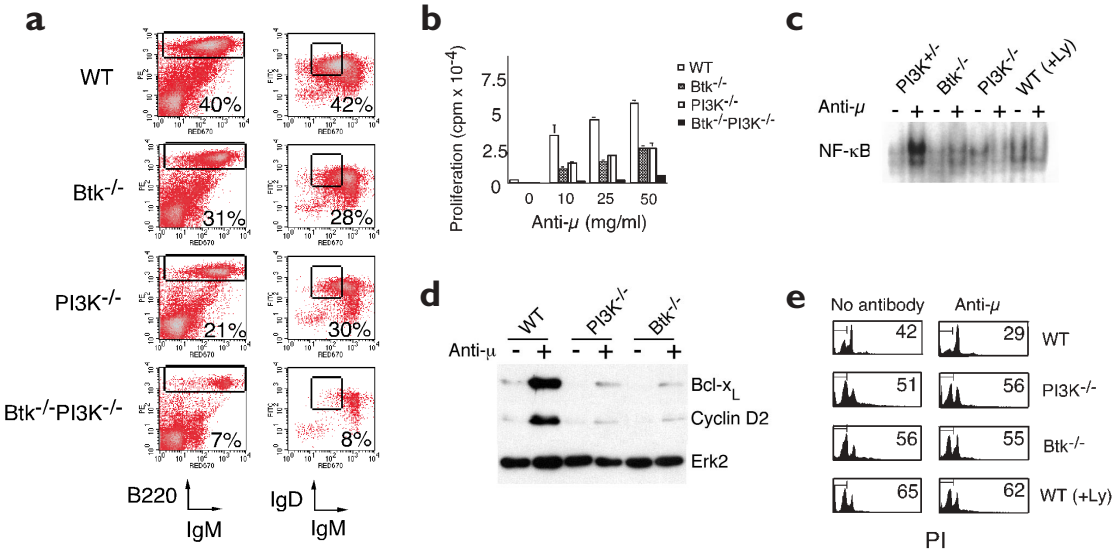

Figure 2. Phenotypes of PI3K and Btk double-deficient mice. (**a**) Splenocytes of indicated mice were stained with FITC-conjugated anti-IgD, PE-conjugated anti-B220 and biotinylated anti-IgM followed by Red670-conjugated streptavidin and examined by flow cytometry. IgM *versus* B220 profiles are shown on the left and IgM *versus* IgD profiles among B220$^+$ cells are shown on the right. Boxes in the left and right panels indicate total B cell and IgM^{lo}-IgD^{hi} circulating B cell fractions, respectively. (**b**) Proliferative responses of splenic B cells upon BCR stimulation *in vitro*. Proliferative responses are shown as [^{3}H]thymidine incorporation. Data are representative of two independent experiments with similar results. (**c**) NF-κB activation. PI3K$^{-/-}$ and Btk$^{-/-}$ B cells were stimulated with anti-μ in the absence or presence of Ly294002 (+Ly) for 4 h and nuclear extracts prepared. EMSA was carried out using ^{32}P-labeled NF-κB probe. (**d**) Induction of Bcl-x$_L$ and cyclin D2. Purified B cells of the indicated mice were stimulated with anti-μ for 16 h and evaluated for the expression of Bcl-x$_L$ and cyclin D2 by immunoblotting using specific antibodies. Membrane was re-blotted with anti-Erk2 (Erk2). (**e**) Apoptotic cell death in suspension culture. WT, PI3K$^{-/-}$ and Btk$^{-/-}$ B cells in the absence or presence of Ly294002 (+Ly) were incubated for 18 h and cell death was evaluated by DNA content analysis using propidium iodide. Numbers indicate the proportion of cells in the sub-G1 fraction (%) in cell cycle analysis. Data are representative of three independent experiments with similar results.

was severely blocked in the absence of PI3K (**Fig. 1c**). In contrast, BCR-mediated phosphorylation of Akt was unaffected in Btk$^{-/-}$ B cells (**Fig. 1c**), as shown previously[29]. Thus, BCR-mediated activation of Akt depends on PI3K, but not on Btk.

Activation of Btk is independent of PI3K

The phenotypic resemblance between PI3K$^{-/-}$ and Btk$^{-/-}$ mice in B cell developmental and activation defects suggests functional association between PI3K and Btk in BCR-mediated signal transduction[4,5]. If PI3K functions directly, and only, upstream of Btk, activation of Btk upon BCR stimulation would be expected to be impaired in PI3K$^{-/-}$ B cells. To this end, we examined the activation of Btk upon BCR stimulation (**Fig. 1d,e**).

First, purified B cells from PI3K$^{-/-}$ and WT mice were stimulated with a F(ab)'$_2$ fragment of anti-IgM and activation of immunoprecipitated Btk was evaluated by immunoblotting with the phosphotyrosine-specific mAb, 4G10. Contrary to our expectation, tyrosine phosphorylation of Btk induced by BCR crosslinking was unaffected in the absence of PI3K (**Fig. 1d**). Furthermore, addition of wortmannin had little effect on tyrosine phosphorylation of Btk in both PI3K$^{-/-}$ and WT B cells. Another PI3K inhibitor, Ly294002 also showed no effect on tyrosine phosphorylation of Btk (**Fig. 1e**, left). Both 50 nM wortmannin and 25 μM Ly294002, which inhibit all types of PI3Ks, did not block tyrosine phosphorylation of Btk, whereas these inhibitors completely block Akt activation in the same cells (**Fig. 1e**, left). Recruitment of phosphorylated Btk to the plasma membrane was also unaffected by inhibition of PI3K or by the lack of PI3K (**Fig. 1e**, right).

Next, we directly examined the kinase activity of Btk using an *in vitro* kinase assay system. BCR-induced activation of Btk activity, as examined by autophosphorylation of Btk, was unaffected in PI3K$^{-/-}$ B cells or by PI3K inhibitors (**Fig. 1f**). Likewise, Btk activation, as examined by phosphorylation of an exogenous substrate, enolase, was observed in the presence of Ly294002 (**Fig. 1g**). These results indicate that Btk can be activated in the absence of PI3K activity.

Phenotypes of PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice

To further examine if the activation of Btk can occur independent of PI3K in BCR signal transduction pathways, we used a genetic approach by comparing the phenotypes of single-deficient mice and PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice. If PI3K simply functions upstream of Btk by providing PIP$_3$ to the PH domain of Btk, the phenotype of double-deficient mice would be identical to that of PI3K or Btk single-deficient mice. On the other hand, if PI3K and Btk function independently in BCR signal transduction pathways, double-deficient mice should show a more severe phenotype. To this end, PI3K$^{-/-}$ and Btk$^{-/-}$ mice were crossed and analyzed. The number of mature (B220$^+$, IgM$^+$) splenic B cells in PI3K$^{-/-}$Btk$^{-/-}$ double-mutant mice was significantly ($P < 0.05$) less than that of each single-mutant counterpart (**Fig. 2a** and **Table 1**).

Table 1. Lymphocyte numbers in the spleen of Btk$^{-/-}$, PI3K$^{-/-}$ and PI3K$^{-/-}$Btk$^{-/-}$ mice

Genotype[a]	No. of B cells (× 10^6)	No. of IgM B cells (× 10^6)	No. of T cells (× 10^6)	B/T cell ratio
WT (n = 5)	28.3 ± 1.8	10.8 ± 1.0	28.7 ± 1.8	1.0 ± 0.1
Btk$^{-/-}$ (n = 7)	10.0 ± 2.4[b]	1.6 ± 1.5[e]	16.4 ± 2.0[h]	0.62 ± 0.17[i]
PI3K$^{-/-}$ (n = 5)	11.7 ± 4.8[b]	5.0 ± 3.4[e,f]	19.3 ± 5.8[h]	0.64 ± 0.28[j]
PI3K$^{-/-}$Btk$^{-/-}$ (n = 3)	6.2 ± 1.4[b,c,d]	1.0 ± 0.6[e,g]	22.5 ± 4.7	0.28 ± 0.06[j,k,l]

[a]Mice are on a mixed background between C57BL/6 and 129/Sv. Significance examined by Student-Newman-Keuls test: [b]$P < 0.01$ from WT; [c]$P < 0.05$ from Btk$^{-/-}$; [d]$P < 0.05$ from PI3K$^{-/-}$; [e]$P < 0.01$ from WT; [f]$P < 0.05$ from Btk$^{-/-}$; [g]$P < 0.05$ from PI3K$^{-/-}$; [h]$P < 0.01$ from WT; [i]$P < 0.05$ from WT; [j]$P < 0.01$ from WT; [k]$P < 0.05$ from Btk$^{-/-}$; [l]$P < 0.05$ from PI3K$^{-/-}$. Essentially the same results were obtained by statistical analysis using the Bonferroni correction method.

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The number of circulating ($B220^+$, IgM^{low}, IgD^{high}) B cells among mature ($B220^+$, IgM^+) B cells in the spleen of $PI3K^{-/-}Btk^{-/-}$ double-deficient mice was also significantly ($P < 0.05$) lower than that in $PI3K^{-/-}$ mice, but was similar to that of $Btk^{-/-}$ mice (**Table 1**). When B/T cell ratios were compared, double-deficient mice show significantly ($P < 0.05$) lower B/T ratios than do single-deficient mice.

We next investigated the proliferative response of double-deficient B cells. Although BCR-induced proliferation of splenic B cells was impaired in $PI3K^{-/-}$ or $Btk^{-/-}$ mice, the response of double-deficient B cells was even lower than that of single-mutant B cells (**Fig. 2b**). These genetic data support the biochemical evidence that PI3K and Btk function independently in B cell signal transduction pathways.

Impaired induction of NF-κB and Bcl-x_L

BCR stimulation activates the NF-κB pathway and both Akt and Btk are involved in NF-κB activation in B cells[30–33]. We thus investigated BCR-mediated activation of NF-κB in $PI3K^{-/-}$ and $Btk^{-/-}$ B cells. In normal B cells, the activity of nuclear NF-κB complexes containing p50 and c-Rel was increased upon BCR stimulation as revealed by electrophoretic mobility shift assay (EMSA) analysis (**Fig. 2c** and data not shown). On the contrary, activation of NF-κB was reduced in $PI3K^{-/-}$ B cells and in Ly294002-treated WT B cells, indicating that BCR-dependent NF-κB activation involves the PI3K pathway. BCR-mediated activation of NF-κB was also blocked in $Btk^{-/-}$ B cells (**Fig. 2c**), as previously reported[31,32]. Thus, BCR-dependent activation of NF-κB requires both PI3K and Btk.

NF-κB is known to have a role in the induction of Bcl-x_L and cyclin D2 upon BCR stimulation[34,35]. Bcl-x_L induction after BCR stimulation was impaired in $PI3K^{-/-}$ and $Btk^{-/-}$ B cells (**Fig. 2d**). Furthermore, induction of cyclin D2, indicative of cell cycle progression, was blocked in both $PI3K^{-/-}$ and $Btk^{-/-}$ B cells (**Fig. 2d**), consistent with the observed BCR-induced proliferative responses (**Fig. 2b**). These results suggest that NF-κB-Bcl-x_L and NF-κB-cyclin D2 pathways are common downstream targets of PI3K and Btk in BCR-mediated signal transduction.

As BCR-dependent induction of Bcl-x_L was impaired in both $PI3K^{-/-}$ and $Btk^{-/-}$ mice, one prediction was that these mutant B cells would be more susceptible to apoptosis than WT B cells. We thus examined apoptotic cell death in suspension culture of $PI3K^{-/-}$ and $Btk^{-/-}$ B cells with or without BCR stimulation. Apoptotic death after an 18-h incubation was evaluated by the proportion of cells in the sub-G1 fraction in cell cycle analysis using propidium iodide staining (**Fig. 2e**). We found that 40% of splenic B cells showed apoptosis after 18 h cultivation *in vitro* without stimulation, and such spontaneous cell death in suspension culture was enhanced in the absence of PI3K or Btk (**Fig. 2e**). Although BCR stimulation with anti-IgM $F(ab')_2$ fragment results in a partial rescue of WT B cells from apoptosis, BCR stimulation was unable to rescue $PI3K^{-/-}$ and $Btk^{-/-}$ B cells (**Fig. 2e**). These results indicate both $PI3K^{-/-}$ and $Btk^{-/-}$ B cells have an increased sensitivity to cell death, possibly because of the failure of BCR-mediated Bcl-x_L induction.

Forced expression of Bcl-x_L in $PI3K^{-/-}$ B cells

As shown above, the inability to induce Bcl-x_L may lead to the low B cell numbers as well as the low proliferative response of $PI3K^{-/-}$ and $Btk^{-/-}$ B cells, and may explain the phenotypic resemblance between $PI3K^{-/-}$ and $Btk^{-/-}$ mice. It has been shown that forced expression of Bcl-x_L in *Xid* mice restores B cell development and proliferative responses[36]. We thus examined the effect of overexpression of Bcl-x_L in $PI3K^{-/-}$ B cells by generating Bcl-x_L transgenic $PI3K^{-/-}$ mice. Equivalent numbers of mature B cells and circulating B cells were found in Bcl-x_L transgenic $PI3K^{-/-}$ mice and WT mice (**Fig. 3a** and **Table 2**). When B/T cell ratios were compared, it was also apparent that the transgenic expression of Bcl-x_L in $PI3K^{-/-}$ mice restored the relative lymphocyte composition to that found in WT mice.

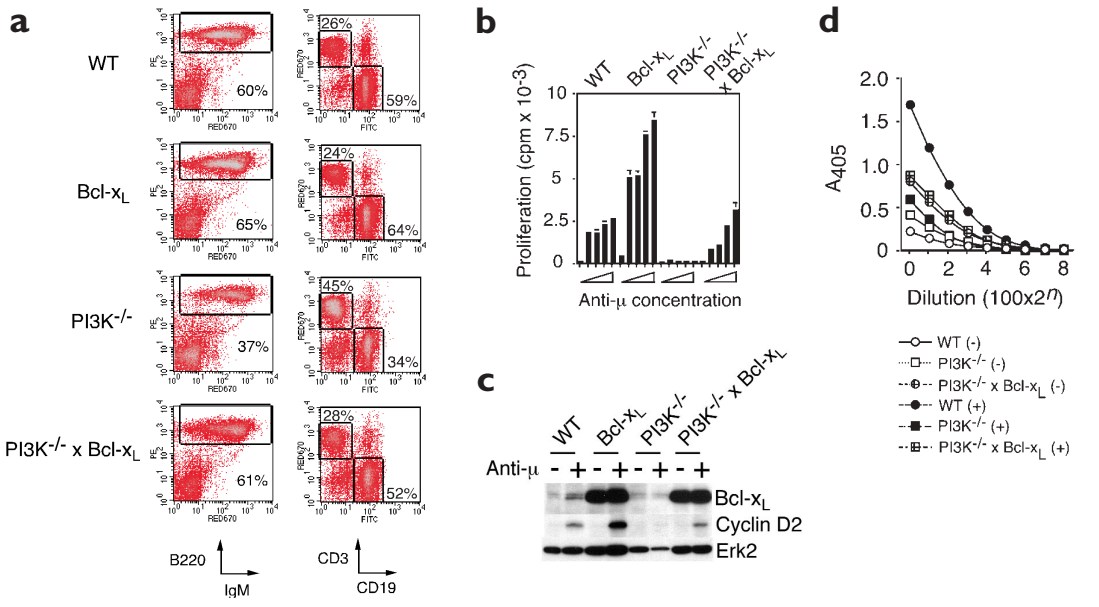

Figure 3. Restoration of B cell numbers and proliferative response of $PI3K^{-/-}$ mice by transgenic expression of Bcl-x_L. (**a**) Splenocytes of indicated mice were stained with a combination of PE-conjugated anti-B220 and biotinylated anti-IgM followed by Red670-conjugated streptavidin, or FITC-conjugated anti-CD19 and biotinylated anti-CD3 followed by Red670-conjugated streptavidin, and examined by flow cytometry. IgM *versus* B220 profiles are shown on the left and CD19 *versus* CD3 profiles to examine the ratio of B and T cells are shown on the right. Boxes in the left panels indicate B cell fractions. Top and bottom boxes in the right panels indicate T and B cell fractions, respectively. (**b**) Purified B cells of the indicated mice were examined for their proliferative responses following BCR stimulation as in **Fig. 2b**. Concentrations of anti-μ were 0, 5, 10, 25 and 50 μg/ml from left to right for each group. (**c**) Induction of cyclin D2 by forced expression of Bcl-x_L. Purified B cells of the indicated mice were stimulated with anti-BCR for 18 h and examined for the expression of Bcl-x_L and cyclin D2. Note the constitutive expression of Bcl-x_L in Bcl-x_L transgenic B cells. Membrane was re-blotted with anti-Erk2 (Erk2). (**d**) T lymphocyte-independent antibody production of indicated mice using DNP-Ficoll was examined as described[3]. The immune sera (+) were analyzed at day 7 for DNP specific total immunoglobulin by ELISA and titers were shown as absorbance at 405-nm wavelength (A_{405}). Preimmune sera (-) were used as controls. Data are representative of two independent experiments with similar results.

Finally, we examined the functions of B cells in Bcl-x_L transgenic PI3K$^{-/-}$ mice. PI3K$^{-/-}$ B cells were incapable of proliferating in response to BCR stimulation[4,5], but the proliferative response of Bcl-x_L transgenic PI3K$^{-/-}$ B cells was similar to that of WT B cells (**Fig. 3b**). Consistent with these results, transgenic expression of Bcl-x_L increased the expression of cyclin D2 in PI3K$^{-/-}$ B cells (**Fig. 3c**). These results indicate that the lack of Bcl-x_L induction is a common defect in PI3K$^{-/-}$ and Btk$^{-/-}$ B cells leading to the similar phenotypes seen in PI3K$^{-/-}$ and Btk$^{-/-}$ mice. On the other hand, T cell-independent antibody production in response to dinitrophenyl (DNP)-Ficoll, which is impaired in PI3K$^{-/-}$ mice[4], was not restored by introduction of the Bcl-x_L transgene (**Fig. 3d**), indicating that the expression of Bcl-x_L alone is insufficient for the restoration of some of the functional defects observed in PI3K$^{-/-}$ mice.

Table 2. Restoration of splenic B cell numbers in PI3K$^{-/-}$ mice by Bcl-x_L expression

Genotype[a]	No. of B cells ($\times 10^6$)	No. of IgM^{low} B cells ($\times 10^6$)	No. of T cells ($\times 10^6$)	B/T cell ratio
WT (n = 6)	34.2 ± 9.1	17.3 ± 5.3	27.1 ± 9.9	1.32 ± 0.32
Bcl-x_L tg (n = 4)	56.4 ± 16.7[b]	28.7 ± 9.3[e]	29.5 ± 9.2	1.99 ± 0.44
PI3K$^{-/-}$ (n = 7)	21.0 ± 6.7[b,c]	6.3 ± 2.2[f,g]	35.6 ± 3.2	0.60 ± 0.15[i,j]
PI3K$^{-/-}$ × Bcl-x_L tg (n = 7)	48.2 ± 10.0[d]	14.3 ± 4.6[g,h]	36.1 ± 11.2	1.42 ± 0.43[k]

[a]Mice are on a C57BL/6 background. Significance examined by Student-Newman-Keuls test: [b]$P < 0.05$ from WT; [c]$P < 0.01$ from Bcl-x_L tg; [d]$P < 0.01$ from PI3K$^{-/-}$; [e]$P < 0.05$ from WT; [f]$P < 0.01$ from WT; [g]$P < 0.01$ from Bcl-x_L tg; [h]$P < 0.05$ from PI3K$^{-/-}$; [i]$P < 0.01$ from WT; [j]$P < 0.01$ from Bcl-x_L tg; [k]$P < 0.01$ from PI3K$^{-/-}$.

Discussion

Contrary to the current model, in which PI3K acts directly upstream of Btk, tyrosine phosphorylation and subsequent activation of kinase activity of Btk was unaffected by the lack of PI3K or by PI3K inhibitors. There have been a few hints previously that this might be the case. BCR-induced Btk activation is blocked only marginally by wortmannin at 50 nM in the B cell line J558Lμm3[37]. Overexpression of the p110 catalytic subunit of PI3K in fibroblasts as well as in the B cell line A20B results in tyrosine phosphorylation of Btk. In this case as well, tyrosine phosphorylation of Btk is only modestly blocked by wortmannin, even at 100 nM, implying the presence of a PI3K-independent pathway for Btk activation[21]. A recent study further shows that tyrosine phosphorylation of Btk is unaffected in B cells deficient for p110δ, the most abundantly expressed catalytic isoform of class I_A PI3K[38]. Phenotypes of PI3K$^{-/-}$Btk$^{-/-}$ double-deficient mice were consistent with these observations. We repeatedly observed higher amounts of tyrosine phosphorylation of Btk in PI3K$^{-/-}$ B cells than in WT B cells. Likewise, PI3K$^{-/-}$ B cells showed higher kinase activity than WT B cells. The reason for the hyperactivation of Btk in PI3K$^{-/-}$ B cells is unknown at present.

The fact that a point mutation within the PH domain of Btk (in which an arginine residue critical for the binding to PIP_3 is replaced by cysteine) leads to *Xid* also supports the current model[12,15,16]. In the DT40 chicken B cell system, targeted disruption of Btk results in impaired activation of phospholipase C-γ2, which is restored by transfection of WT Btk, but not Btk with the *Xid* mutation[39]. In our hands, however, the mutant Btk protein produced from the gene carrying the *Xid* mutation was unstable and degraded rapidly when expressed in cells by gene transfer (data not shown). It is possible that the defect caused by the *Xid* mutation is not due to the inability to bind PIP_3, but to the degradation of the mutant protein. It was theoretically possible that Btk functions upstream of PI3K, but the fact that PI3K was activated in the absence of Btk excluded this possibility.

Recruitment of phosphorylated Btk to the plasma membrane was also unaffected by PI3K inhibitors or in PI3K$^{-/-}$ B cells. Recent studies have raised the possibility that Btk is recruited to the plasma membrane through a mechanism independent of PIP_3 generation. Identification of an adapter protein, BLNK (also known as SLP65), and its involvement in Btk activation support this alternative possibility[17,18]. In fact, BLNK is phosphorylated by Syk and provides Btk with docking sites to bring them into close proximity. Btk is then activated by tyrosine phosphorylation after binding to BLNK upon BCR stimulation. At the same time, BLNK is recruited to the plasma membrane upon BCR stimulation by binding to the BCR complex, which leads to the recruitment of Btk to the plasma membrane[40]. Involvement of such molecular mechanisms of recruiting Btk to the plasma membrane should be evaluated for a better understanding of the role of Btk in BCR signaling. Although PI3K and Btk likely function independently in B cell signal transduction pathways and have unique roles in proximal BCR signaling, we do not exclude the possibility that the interaction between PIP_3 and the PH domain is more critical for the activation of Btk, and possibly other Tec family kinases, in other cell types with different receptor systems.

BCR-mediated activation of Akt was completely blocked in the absence of PI3K. Activation of Akt is a multi-step reaction involving the generation of PIP_2 and PIP_3, which recruit Akt and PDK1, respectively, to the plasma membrane[22,23]. Although the role of Btk in Akt activation is controversial in the chicken DT40 B cell system[22,41], Akt activation, as revealed by phosphorylation of two critical residues in primary Btk$^{-/-}$ B cells, was unaffected. Thus, activation of Akt is dependent on class I_A PI3K containing p85α, but is independent of Btk in mouse primary B cells. Akt binds and activates IKK to induce degradation of IκB and activation of NF-κB[42]. Btk is also required for the activation of NF-κB in B cells[31,32]. Because activation of Akt does not depend on Btk in mouse B cells, it is likely that both Btk-dependent and Akt-dependent distinct pathways are required for activating NF-κB in B cells.

Induction of both Bcl-x_L and cyclin D2 involves NF-κB–mediated transcriptional activation. For example, overexpression of dominant-negative NF-κB inhibited CD40-mediated Bcl-x_L induction[43] and transgenic mice expressing a constitutively active, membrane-anchored Akt showed elevated activation of NF-κB and Bcl-x_L[44]. Bcl-x_L is a major anti-apoptotic protein that is induced upon BCR stimulation[35]. Consistent with these observations, PI3K$^{-/-}$ B cells and Btk$^{-/-}$ B cells showed increased apoptosis compared with WT B cells. Previously, we observed little significant difference in viability between PI3K$^{+/-}$ and PI3K$^{-/-}$ B cells upon BCR stimulation, as measured by annexin V staining[4]. However, we noted that annexin V staining is higher on B cells than on other cell types[45] and is not a sensitive method for measuring apoptotic B cells. As shown here, propidium iodide staining seems to be a better method to evaluate apoptosis in B cells. The lack of Bcl-x_L as well as cyclin D2 induction may be the cause of the phenotypic similarity between PI3K$^{-/-}$ and Btk$^{-/-}$ mice. In fact, forced expression of Bcl-x_L as a transgene restored B cell development and proliferative responses similar to what has been observed in *Xid* B cells[36]. These results also support our conclusion that the NF-κB-Bcl-x_L pathway is a

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common target of PI3K- and Btk-dependent distinct signaling pathways in B cell activation. As observed in *Xid* mice, however, T cell-independent antibody production was not restored by introduction of the Bcl-x_L transgene, indicating that the expression of Bcl-x_L alone is insufficient for the restoration of some of the functional defects caused by the lack of Btk and PI3K.

Biochemical and genetic approaches revealed that class I_A PI3K and Btk constitute functionally distinct signaling pathways proximal to the membrane, but share a common downstream target, the NF-κB-Bcl-x_L pathway, in BCR-mediated signal transduction. The lack of activation of the NF-κB-Bcl-x_L pathway likely leads to the similarity of phenotypes in $PI3K^{-/-}$ and $Btk^{-/-}$ mice. The mechanisms that coordinate the PI3K-Akt and Btk pathways in the activation of NF-κB remain to be determined.

Methods

Mice. PI3K-deficient mice[3,4] were backcrossed to C57BL/6 or BALB/c mice for more than seven generations before intercrossing heterozygous mice[46,47]. Mice on a C57BL/6 background were used unless otherwise mentioned. $Btk^{-/-(y)}$ mice on a (C57BL/6 × 129/Sv) mixed background were purchased from The Jackson Laboratory (Bar Harbor, ME). Because Btk is encoded on the X chromosome, Btk-deficient female and male mice have the $Btk^{-/-}$ and $Btk^{-/y}$ genotypes, respectively. Hence, we designate Btk-deficient mice as $Btk^{-/-}$ mice. $PI3K^{-/-}Btk^{-/-}$ double-deficient mice were generated by crossing $PI3K^{-/-}$ and $Btk^{-/-}$ mice to generate F2 mice carrying the $PI3K^{-/-}Btk^{-/-}$ genotype. Bcl-x_L transgenic mouse line #87 on a C57BL/6 background has been described[35,48]. In this transgenic mouse line, human Bcl-x_L protein is driven by the SV40 promoter and Eμ enhancer and is abundantly expressed in B cells. Bcl-x_L transgenic and $PI3K^{-/-}$ mice were crossed to generate $PI3K^{-/-}$ mice expressing the Bcl-x_L transgene in $PI3K^{-/-}$ B cells. All mice were maintained at Taconic (Germantown, NY) or in our animal facility under specific pathogen-free conditions. All experiments were performed in accordance with our Institutional Guidelines.

Reagents. Antibodies to cyclin D2, Erk2, Btk and Lyn were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–Bcl-x_L was obtained from Transduction Laboratories (Lexington, KY). A mAb to Btk, 43-3B[49], was a generous gift from S. Tsukada (Osaka University, Osaka, Japan). Anti-$p85^{PAN}$ was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Akt, anti-phospho-Akt(S473) and anti-phospho-Akt(T308) were from Cell Signaling Technology (Beverly, MA). Specific antisera for p85β and p55γ have been described[7]. Anti-phosphotyrosine antibody (4G10) was a gift from T. Roberts (DFCI, Boston, MA). PI3K-specific inhibitors, wortmannin and Ly294002, were purchased from Calbiochem (La Jolla, CA).

Flow cytometric analysis. Fluoroscein isothiocyanate (FITC)-conjugated anti-mouse IgM, FITC-conjugated anti-mouse IgD, FITC-conjugated anti-CD19, phycoerythrin (PE)-conjugated anti-B220, biotinylated anti-mouse IgM and biotinylated anti-CD3 were purchased from PharMingen (San Diego, CA). Binding of biotinylated mAbs was detected with streptavidin-Red670 (GIBCO BRL, Grand Island, NY). One to two million cells were stained with designated antibodies in PBS with 2% fetal calf serum (FCS) and subjected to analysis on a FACScan using the CELLQuest program (Becton Dickinson, San Jose, CA).

Cell stimulation and immunoblotting. B cells were purified from total splenocytes using anti-B220-coated magnetic beads and AutoMACS (Miltenyi Biotech, Sunnyvale, CA). Purity of the cells was >95%. We resuspended $2–7 \times 10^7$ purified B cells in 1 ml of culture medium and preincubated them for 15 min at 37 °C with or without inhibitors. Cells were then stimulated with F(ab)'$_2$ fragment of goat polyclonal antibody to mouse IgM (anti-IgM F(ab)'$_2$, 40 μg/ml; Jackson ImmunoResearch, West Grove, PA) and incubated at 37 °C for the indicated time. Cells were collected, lysed in a lysis buffer solution (1% NP-40, 50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 50 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na-vanadate) and immunoprecipitated with the indicated antibodies or directly applied to SDS-PAGE and transferred to polyvinyldifluoride (PVDF) membranes. Reactive proteins were visualized with ECL Chemiluminescent substrates (NEN, Boston, MA). To examine phosphorylation of Btk in the membrane fraction, cells were lysed with 300 μl of hypotonic buffer solution (10 mM HEPES, pH 7.9, 10 mM NaF, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM benzamidine, 2 mM EGTA, 2 mM DTT, 1 mM vanadate, 1 mM PMSF, 1% aprotinin) using a Dounce homogenizer. Lysates were centrifuged at 10,000*g* for 30 s, and the supernatant was further centrifuged at 100,000*g* for 30 min to obtain S100 (supernatant) and P100 (pellet). P100 was subjected to immunoblot analysis with 4G10 and anti-Lyn.

PI3K activity. Activation-induced PI3K activity in B cells was estimated as PI3K activity among tyrosine phosphorylated proteins[3]. After BCR stimulation, cell lysates were immunoprecipitated with 4G10 and subjected to *in vitro* PI3K assay. Briefly, immunoprecipitate was incubated with phosphatidylinositol and γ-[^{32}P]ATP for 15 min at room temperature, and the chloroform extract was separated by thin-layer chromatography.

Btk activity. Splenic B cells (6×10^7) were stimulated with or without 20 μg/ml of anti-IgM F(ab)'$_2$ at 37 °C for 3 min in the presence or absence of PI3K inhibitors, and lysed in an extraction buffer solution (20 mM Tris, pH 7.4, 2 mM EGTA, 12.5 mM β-glycerophosphate, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 2 mM DTT, 1 mM PMSF, 1 mM vanadate, 1% aprotinin) containing 0.2% Triton X-100. For immunoprecipitation, 15 μg of an anti-Btk was coupled to protein A Sepharose at 4 °C overnight. The beads were washed once with extraction buffer solution and incubated with precleared total cell lysates for 1 h at 4 °C. Subsequently, the beads were washed twice with extraction buffer solution containing 1% Triton X-100 and once with extraction buffer solution alone, followed by incubation with or without 100 μM ATP at 22 °C for 5 min. Samples were subjected to immunoblot analysis with 4G10. Or, immunocomplex was incubated with 5 μg of acid-denatured enolase as an exogenous substrate in the presence of 100 μM ATP at 22 °C for 5 min.

EMSA. Preparation of nuclear extract and EMSA were carried out as described[31,32]. Briefly, 10 μg nuclear extract was incubated with 20 fmol ^{32}P-labeled NF-κB probe (Santa Cruz). The DNA-protein complexes were resolved on a native 5% polyacrylamide gel, dried and exposed to an x-ray film for autoradiography. Identity of the band was confirmed by anti-p50 (Santa Cruz)-induced supershift (data not shown).

Cell proliferation and cell cycle analysis. Purified B cells (0.5 to 1×10^5/well) were treated with the indicated concentrations of anti-IgM F(ab)'$_2$ in culture medium containing 2 ng/ml rIL-4 (Pepro Tech EC_{LTD}, London, England) in 96-well plates for 72 h. [^{3}H]Thymidine (3.7×10^4 Bq (1 μCi)/well) was added to the cultures during the last 16 h and uptake of radioactivity was measured by liquid scintillation counter. For cell cycle analysis, splenic B cells were activated with anti-IgM F(ab)'$_2$ *in vitro* for 18 h, fixed with 70% ethanol and treated with RNaseA (1 mg/ml). Fixed cells were stained with 50 μg/ml propidium iodide for 3 h at room temperature and analyzed on a FACScan (Beckton Dickinson).

Antibody production. Mice were pre-bled and immunized intraperitoneally with 100 μg DNP-keyhole lympet hemocyanin (KLH; LSL, Tokyo, Japan) in a 1:1 emulsion with Freund's complete adjuvant (Sigma), or 10 μg DNP-Ficoll in PBS at day 0. The serum was analyzed at day 7 for DNP specific total immunoglobulin by ELISA.

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Competing interests statement

The authors declare that they have no competing financial interests.

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