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The Effect of Deleting p110δ on the Phenotype and Function of PTEN-Deficient B Cells[1]

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Control of the intracellular levels of phosphatidylinositol-(3, 4, 5)-trisphosphate by PI3K and phosphatase and tensin homolog (PTEN) is essential for B cell development and differentiation. Deletion of the PI3K catalytic subunit p110δ leads to a severe reduction in B1 and marginal zone (MZ) B cells, whereas deletion of PTEN results in their expansion. We have examined the relationship between these two molecules by generating mice with a B cell-specific deletion of PTEN (PTEN^B) and a concurrent germline deletion of p110δ. The expanded B1 cell population of PTEN^B mice was reduced to normal levels in PTEN^B/p110δ mutant mice, indicating a critical role for the p110δ isoform in the expansion of B1 cells. However, numbers of MZ B cells in the PTEN^B/p110δ mutants was intermediate between wild-type and PTEN^B-deficient mice, suggesting an additional role for other PI3K catalytic isoforms in MZ differentiation. Furthermore, the defective class switch recombination in PTEN^B B cells was only partially reversed in PTEN^B/p110δ double mutant B cells. These results demonstrate an epistatic relationship between p110δ and PTEN. In addition, they also suggest that additional PI3K catalytic subunits contribute to B cell development and function. ***The Journal of Immunology*****, 2008, 180: 739–746.**

B cell differentiation, homeostasis, and proliferation are controlled by cell surface receptor-ligand interactions, which are integrated via multiple intracellular signaling pathways. One of the major signal transduction molecules used by B cells is the class IA subgroup of PI3K. These enzymes are recruited to receptors with phosphorylated YXXM motifs, such as those found on CD19 (1), whereupon they become activated and convert the lipid phosphatidylinositol-(4, 5)-bisphosphate (PIP_2)[5] to phosphatidylinositol-(3, 4, 5)-trisphosphate (PIP_3) (2). PIP_3 then acts to recruit other effector molecules, including phosphoinositide-dependent protein kinase-1 (PDK1), and subsequently protein kinase B (PKB) (3).

Class IA PI3Ks are comprised of a regulatory and catalytic subunit, of which there are multiple isoforms. There are three catalytic isoforms, p110α, p110β and p110δ. p110α and β are widely expressed; p110δ has a more restricted pattern with high levels reported in leukocytes (4, 5). There are three regulatory subunits: p85α (which includes two splice variants p55α and p50α), p85β and p55γ (6, 7). Mice deficient for p110δ, or expressing a catalytically inactive form of p110δ, have a severe reduction in marginal zone (MZ) B cell and B1 cell numbers. Furthermore, these mice show impaired responses to thymus-dependent and independent Ags. Additionally, these mutant B cells respond poorly to BCR cross-linking in vitro and their ability to activate PKB and other signaling pathways downstream of the BCR and CD19 complex is defective (8–11).

The contribution of the p110α and p110β catalytic isoforms to B cell development has not been assessed as genetic deletions of both of these subunits are embryonic lethal (12, 13) and conditional mutants have yet to be described. However, a role for these subunits can be inferred from the somewhat more severe B cell phenotype in p85α mutant mice. Mice in which the p85α regulatory subunit has been deleted have defective B1 and MZ B cell development but show further defects in the maturation of follicular B cells not evident in p110δ mutants (14–16). This phenotype suggests that the p110α and β catalytic subunits may also be required for B cell development and function.

The lipid phosphatase and tensin homolog (PTEN) opposes PI3K by catalyzing the conversion of PIP_3 into PIP_2 (17). PTEN is a tumor suppressor gene and is required for embryonic development (18, 19). The role of PTEN in B cell development has been studied using mice with a conditional deletion of PTEN in B cells (PTEN^B). These mice display significant increases in the number of MZ and B1 cells in the spleen (20, 21), respond poorly to thymus-dependent and independent immunogens, and display a defect in Ig class switch recombination. This latter defect has been attributed to impaired expression and function of activation-induced cytidine deaminase (AID) (22).

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[5] Abbreviations used in this paper: PIP_2, phosphatidylinositol-(4, 5)-bisphosphate; PIP_3, phosphatidylinositol-(3, 4, 5)-trisphosphate; PTEN, phosphatase and tensin homolog; PTEN^B, B cell-specific deletion of PTEN; PDK1, phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; MZ, marginal zone; WT, wild type; AID, activation-induced cytidine deaminase; FO, follicular; CSR, class switch recombination; BAFF, B cell activating factor of the TNF family.

Given the opposing catalytic activities of p110δ and PTEN, and the contrasting effects of their mutation on B cell development, we wanted to determine whether the deletion of p110δ would restore normal B cell development in PTEN^{B} mice. To this end, we generated mice with a combined germline deletion of p110δ and a B cell-specific *PTEN* deletion (PTEN^{B}) using CD19-cre. These double mutant mice have allowed us to address the contribution of p110δ, as well as other class IA catalytic isoforms, to the expansion of B1 and marginal zone B cells in PTEN^{B} mice. In addition, we have used the mutant mice to investigate role of the PI3K pathway as a modulator of class switch recombination.

Materials and Methods

Mice

P110δ$^{-/-}$ (8) and Eμ-bcl-2–36 (23) mice have been described previously. Mice deficient for PTEN in the B cell lineage were generated by crossing floxed *PTEN* (24) with CD19-cre (25). P110δ$^{-/-}$ and CD19$^{+/cre}$PTEN$^{fl/fl}$ mice were intercrossed to generate nine distinct mouse lines; wild-type (WT), heterozygote, or homozygous mutant for p110δ and PTEN. For studies using these genetic cohorts, all mice were CD19$^{+/cre}$. P110δ$^{-/-}$ and Eμ-bcl-2–36 mice were intercrossed to generate a p110δ$^{-/-}$bcl-2^{Tg} line. All mice were on a 129/Sv·C57BL/6 mixed background and were bred under United Kingdom Home Office license PPL 80/1736.

PCR analysis of PTEN genotypes

Splenic B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec) before genomic DNA was extracted. The status of the *PTEN* locus was determined by PCR using the following primers; sense 5′-TGTTTTTGACCAATTAAAGTAGGCTGTG-3′ and antisense 5′-AAAAGTTCCCCTGCTGATGATTTGT-3′ to indicate a 340bp WT or 490bp floxed PTEN allele or sense 5′-TGTTTTTGAC CAATTAAAGTAGGCTGTG-3′ and antisense 5′-CCCCCAAGTCAAT TGTTAGGTCTGT-3′ to give a 750 bp recombined allele.

Population analysis by FACS

Single cell suspensions from the spleen and peritoneal lavarge were surface stained using various combinations of Abs conjugated to biotin, FITC, PE, or APC. Staining with biotinylated Abs was revealed by streptavidin-PE (Sigma-Aldrich). Cells were analyzed using a FACSCalibur flow cytometer and analyzed using FlowJo software (TreeStar). The following Abs were used: anti-B220 (RA3–6B2), anti-CD5 (53-7.3), anti-CD21 (7G6), anti-CD23 (B3B4), anti-CD24 (M1/69), polyclonal anti-IgM (all from Becton Dickinson), anti-IgD (11–26; Southern Biotechnology), and anti-CD1d (1B1; eBioscience). For detection of the *Bcl-2* transgene, cells were first stained for surface Ags, as described above, before fixation and permeablization with BD cytofix/cytoperm solution. Cells were then washed in 0.03% saponin before staining with anti-human Bcl-2-PE-conjugated Ab (BD Biosciences).

Western blot

For detection of p110δ, lymph node B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec). For detection of pPKB and total PKB B cells were purified from the spleen after complement lysis of T cells as previously described (26). Lysates from ~3×10^6 B cells per lane were separated by SDS-PAGE and transferred to nitrocellulose. Filters were immunoblotted using Abs to either p110δ (10) or to phosphoserine-473 of PKB (Cell Signaling Technology). Ab binding was revealed using HRP-conjugated anti-rabbit Ab (DakoCytomation) followed by ECL (Pierce). After pPKB detection membranes were then stripped in 62.5 mM Tris-HCl (pH 6.7), 2% SDS, 100 mM 2-ME, and reprobed with Ab to PKB (Cell Signaling Technology). The pPKB:PKB ratio was measured using Fuji AIDA software (Raytest).

Phospho-specific flow cytometry

Detection of phospho-specific proteins by flow cytometry has been previously described (27). In brief, splenic B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec), and fixed with 1% formaldehyde for 10 min at 37°C. Fixed cells were permeabilzed in 90% methanol overnight at −20ºC at 2×10^7 cells/ml. Cells (1×10^6/sample) were washed three times in PBS plus 2% FCS before incubation with 1 μg/ml either anti-PKB, anti-phosphothreonine-308 PKB (Cell Signaling Technology), or rabbit Ig (Jackson ImmunoResearch Laboratories) for 30 min at room temperature. Cells were washed three times before incubation with CyV-conjugated donkey anti-rabbit Ab for 30 min at room temperature before final washing and detection using a FACSCalibur flow cytometer. To distinguish follicular and marginal B cells, cells were stained with cell surface Abs as described above. Data were analyzed by taking the median of the PKB or phospho308-PKB signal and subtracting corresponding isotype control (a separate stain for each sample in the cohort). The specificity of this assay has been verified by demonstrating that the increase in phospho308-PKB signal after B cell stimulation with α-IgM was blocked by pretreatment with wortmannin (data not shown).

Cell culture

Lymph node B cells were purified by negative selection using a MACS B cell isolation kit (Miltenyi Biotec). B cells were labeled with CFSE (Molecular Probes) (28) before culture at 10^6 cells/ml in round-bottom 96-well plates in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10^{-5} M 2-ME for 4 days. For analysis of class switch recombination, cells were stimulated with 10 μg/ml monoclonal anti-CD40 (clone 3/23) and 5 ng/ml recombinant murine IL-4 (PeproTech). The pharmacologic inhibitor IC87114 was also added to some cultures at a concentration of 1 μM.

Quantitative PCR

RNA was extracted from cultured cells or sorted populations using TRIzol (Invitrogen Life Technologies) and converted to cDNA. Controls without reverse transcriptase were included for all samples. *AID*, *p110α*, *p110β*, *p110δ*, and *p110γ* mRNA was quantified using a Taqman probe (Applied Biosystems) and normalized to the expression of *β2M* (Applied Biosystems).

Statistics

Data presented throughout represent the mean and either the SD or SEM. For statistical analysis comparing the means of two groups, a two-tailed Student's *t* test was used. For more than two groups, a one-way ANOVA test was used. A Spearman's correlation test was used to determine the relationship between MZ B cell numbers and PKB phosphorylation status.

Results

Loss of p110δ reverses the effect of PTEN deficiency on B1 cell numbers

Previous analysis of mice deficient for p110δ revealed reduced numbers of B1 cells (8–10). By contrast, the conditional deletion of PTEN in B cells gave rise to an expanded B1 cell compartment (20, 21). To establish whether the expansion of B1 cells observed in the PTEN^{B} mice is p110δ-dependent, we generated mice in which the *p110δ* and *PTEN* genes were simultaneously inactivated in B lymphocytes. Examination of splenic and peritoneal B1 cell populations (defined as B220^{+}CD5^{+}) revealed that the increased proportion of PTEN-deficient B1 cells in both spleen and peritoneal cavity was reversed when p110δ was also absent (Fig. 1*A*). The effect of p110δ deficiency was more obvious from a comparison of the total B1 cell numbers in the spleen and peritoneum (Fig. 1*B*). These data demonstrate the importance of p110δ for the expansion of PTEN-deficient B1 cells.

The effect of PTEN on MZ B cells is partly reversed by the absence of p110δ

A further characteristic of the p110δ$^{-/-}$ and PTEN^{B} mice is their aberrant MZ B cell development, the MZ B cell population being markedly reduced in p110δ$^{-/-}$ mice (8–10) and significantly expanded in PTEN^{B} mice (20, 21). To determine the relative contribution of p110δ to the expansion of MZ B cells in PTEN^{B} mice, we analyzed the MZ B cell population in the mutant mice by flow cytometry.

CD23, which, together with CD21, is often used to distinguish follicular (FO) and MZ B cells, was found to be only weakly expressed by PTEN^{B} cells and was not useful for the demarcation of the FO and MZ subsets (21). Consequently, MZ B cells were defined as being IgM^{+}IgD^{low}CD1d^{high} (Fig. 2*A*) or CD21^{+}IgD^{low}CD1d^{high}

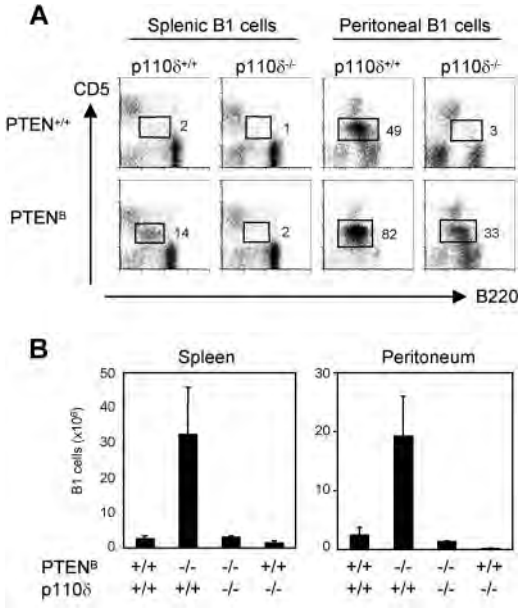

FIGURE 1. Splenic and peritoneal B1 cell development in p110δ and PTEN deficient mice. *A*, Flow cytometric analysis of B220 and CD5 expression on lymphocytes in the spleen and peritoneal cavity. B1 B cells were identified as $B220^{int}CD5^{int}$ and fall within the gated area. *B*, Histograms show the mean number and SD of B1 cells from a minimum of three mice in each experimental group.

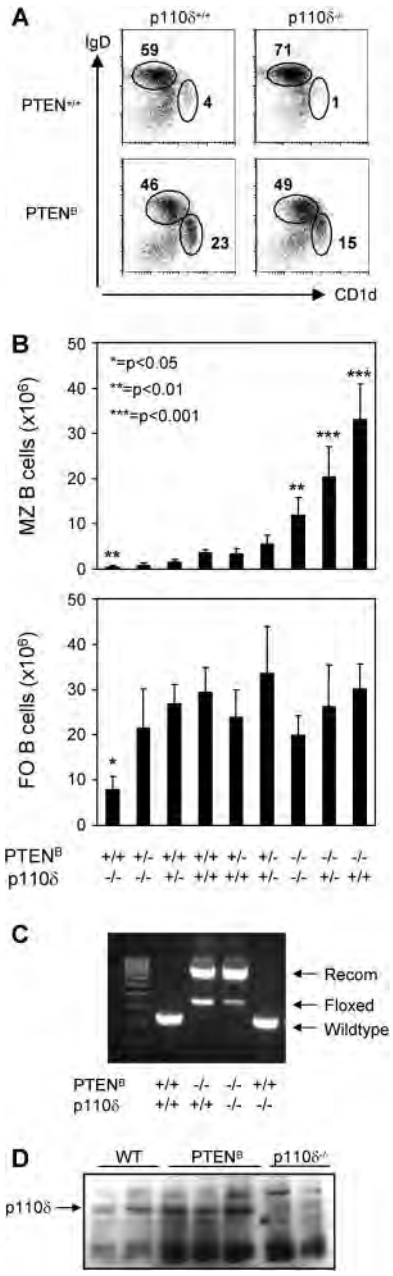

FIGURE 2. Follicular and marginal zone B cell development in p110δ and PTEN deficient mice. *A*, Flow cytometric analysis showing IgD and CD1d expression on IgM^+ splenic B cells. Marginal zone B cells are defined as $IgD^{low}/CD1d^{high}$ and follicular B cells as $IgD^{high}/CD1d^{low}$. The positions of the gates used to identify MZ and FO B cell subsets are indicated together with the percentage that each subset contributes to total lymphocytes. *B*, Number of follicular and marginal zone B cells in spleens of mice of the indicated genotypes. Histograms indicate the mean and SEM. Each group contains a minimum of three mice (maximum eleven). Statistical analysis was performed using a one way ANOVA test. Groups which differ significantly from WT are indicated (∗). *C*, PCR analysis of the *PTEN* locus from genomic DNA of purified splenic B cells showing WT, floxed, and Cre-recombined alleles. *D*, Immunoblot analysis of p110δ expression in B cells purified from lymph nodes of WT, $PTEN^B$, and $p110\delta^{-/-}$ mice.

(data not shown). The effect of PTEN deletion on MZ B cell expansion was significantly suppressed when p110δ was simultaneously deleted. However, unlike the B1 cell population, the numbers of MZ B cells in the double mutant mice was significantly greater than in WT mice (Fig. 2*B*). This result suggests additional PI3Ks contribute to the expansion of MZ B cells in $PTEN^B$ mice. To investigate the relationship between p110δ and PTEN in MZ B cell development further, we analyzed mice that had a combination of WT, heterozygous, or mutant p110δ and PTEN alleles. We reasoned these mice would allow us to assess whether *PTEN* and *p110δ* gene dosage influenced MZ B cell development. The loss of a single allele of *PTEN* or *p110δ* did not result in a significant change in MZ B cell number, however it was possible to rank the different mice according to the number of MZ B cells they contained in their spleens (Fig. 2*B*). Furthermore, this ranking correlated with the amounts of phospho-PKB determined by Western blotting (see below).

We also analyzed the FO B cell population in these mice. Using the strategy for identifying MZ B cells we have adopted, the FO B cell population can be defined as $IgM^+IgD^{high}CD1d^{low}$ (or $CD21^+IgD^{high}CD1d^{low}$). Enumeration of FO B cells in the spleens of mice of the nine genotypes revealed normal numbers of FO B cells across all genotypes, with the exception of $p110\delta^{-/-}$ mice, which had somewhat reduced numbers (Fig. 2, *A* and *B*). In addition, no significant differences in the numbers of transitional splenic B cells were observed for any of the genotypes (data not shown).

To ensure that the absence of p110δ did not interfere with the Cre-mediated deletion of *PTEN*, the allelic status of the *PTEN* locus in purified B cells was determined by PCR. The recombined allele is the dominant PCR product in B cells from $PTEN^B$ and $PTEN^B \times p110\delta^{-/-}$ mice (Fig. 2*C*). These data suggest the effect of deletion of p110δ on B cell numbers in the double mutants is unlikely to be a consequence of a reduction in the efficiency of PTEN deletion. A small proportion of the genomic DNA remains floxed in B cells from mice of both genotypes, which is not unexpected as incomplete deletion with this CD19Cre transgene has been previously reported (25). To ensure that the absence of PTEN in B lymphocytes did not interfere with the expression of p110δ, we used immunoblot analysis to detect p110δ protein from purified

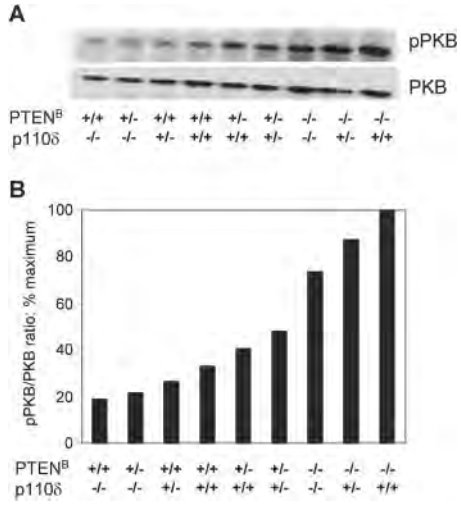

FIGURE 3. Immunoblot analysis of pPKB and PKB in B cells from PI3K mutant mice. *A*, Lysates of purified resting B cells were immunoblotted with anti-phospho 473PKB and anti-PKB as a control for loading. Gels were scanned on a Fuji Imager and pPKB levels normalized to PKB levels. *B*, The ratio of phospho 473PKB to total PKB was plotted as a percentage of the PTEN^{B} p110$\delta^{+/+}$ group. Data is representative of two gels.

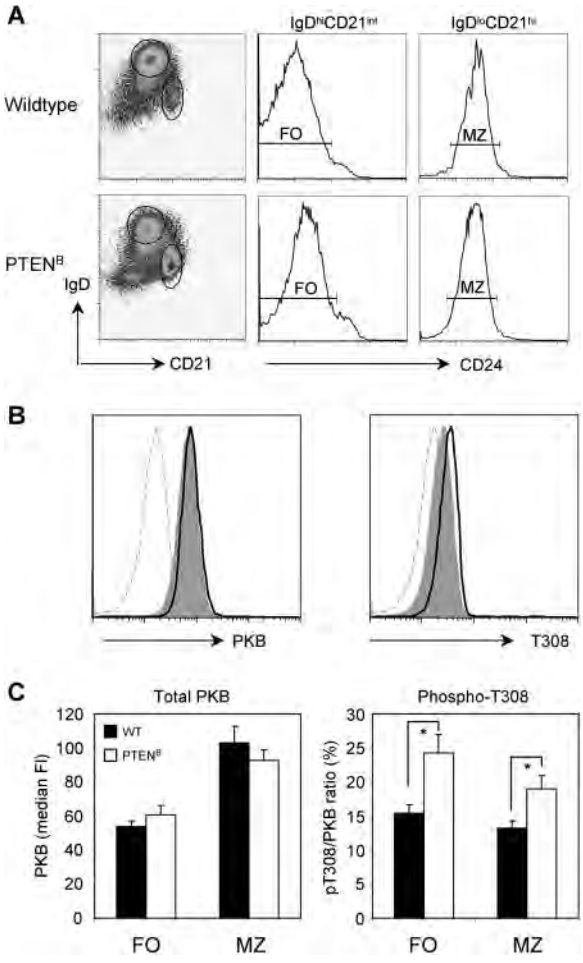

FIGURE 4. Phospho-flow analysis of pPKB and PKB in follicular and marginal zone B cells from WT and PTEN^{B} mice. *A*, Flow cytometric analysis showing IgD, CD21, and CD24 expression on MACS purified B cells from WT and PTEN^{B} mice. Marginal zone B cells are defined as IgD^{low}/CD21^{high}/CD24^{int} and follicular B cells as IgD^{high}/CD21^{int}/CD24^{low}. The positions of the gates used to identify MZ and FO B cell subsets are indicated. *B*, Flow cytometric analysis showing intracellular PKB and phospho-T308 PKB on FO B cells from WT (▨) and PTEN^{B} (solid black line) mice. Staining using the isotype control is also shown (grey dashed line). *C*, Graph showing the total intracellular PKB and phospho-T308 PKB levels detected in FO and MZ B cells from WT and PTEN^{B} mice. Phospho-T308 PKB levels were calculated as a proportion of total PKB. Each bar represents the mean and SEM from 5 to 7 individual mice. Statistical analysis was performed using a two-tailed Student's *t* test. *, $p < 0.05$.

lymph node B cells (Fig. 2*D*). Our results show that p110δ is detectable in both WT and PTEN^{B} cells, but as expected is absent in p110$\delta^{-/-}$ B cells.

PKB phosphorylation in resting B lymphocytes from PI3K mutant mice

Analysis of PI3K activity by direct measurement of the lipid intermediates of this pathway in primary resting B cells represents a major and, as yet, unmet technical challenge. However, phosphorylation of serine-threonine kinase PKB, a major effector of the PI3K pathway, can be readily quantitated by immunoblotting with phosphospecific antisera. Therefore, we measured the proportion of PKB that was phosphorylated on serine 473 in purified B lymphocytes from all nine genotypes generated by the p110$\delta^{-/-}$ × CD19$^{+/cre}$PTEN$^{fl/fl}$ intercross (Fig. 3). The highest level of phospho-PKB was found in PTEN^{B} splenic B cells and the lowest in p110$\delta^{-/-}$ splenic B cells (Fig. 3*A*). To facilitate the comparison of phospho-PKB among B cells from mice of different genotypes, we used densitometry to determine signal intensity, normalized this to levels of total PKB, and plotted the results as a percentage of the level of signal in PTEN^{B} B cells (Fig. 3*B*). This revealed a graduated increase in phosphorylated PKB that paralleled the increase in MZ B cell numbers (Fig. 2*B*). The relationship between the numbers of MZ B cells and the PKB:phospho-PKB ratio was found to be highly significant as determined by a Spearman correlation test (r = 0.7714, $p < 0.0001$).

A shortcoming of using total splenic B cells for immunoblot analysis is that the cellular composition is dependent upon the genotype. To determine whether the results of the immunoblot assay indicated an intrinsic difference in PKB phosphorylation or were merely a reflection of B cell composition, we analyzed the phosphorylation status of PKB in FO and MZ B cells from WT and PTEN^{B} mice using flow cytometry (27). In these experiments, we used an Ab to phospho-T308 instead of phospho-S473 as we found it to be more sensitive for flow cytometry (data not shown). MZ B cells in PTEN^{B} and WT spleens were defined as CD21^{high}IgD^{int}CD24^{int}, and FO B cells were defined as CD21^{int}IgD^{high}CD24^{low} (Fig. 4*A*). MZ and FO B cells were then analyzed for total PKB and PKB phospho-T308 (T308) (Fig. 4*B*). Analysis of total PKB showed that the levels of PKB were equivalent in FO and MZ B cells irrespective of the *PTEN* genotype, but that the median fluorescence intensity was somewhat higher for MZ B cells than FO B cells (Fig. 4*C*). To account for this, phospho-T308 was normalized against total PKB levels for each sample. Analysis of phospho-T308 showed that the proportion of phosphorylated PKB in PTEN^{B} cells was higher for both the FO and MZ B cell populations (Fig. 4*C*). These results demonstrate that PTEN deficiency elevates PKB phosphorylation in both MZ and FO B cell subsets to a similar extent. The results of the immunoblot analysis are thus not a reflection of an increased number of MZ B cells with elevated PKB phosphorylation, but rather a consequence of the genetic manipulation of intrinsic PI3K activity.

The MZ B cell deficiency in p110δ$^{-/-}$ mice cannot be corrected by a Bcl-2 transgene

Our results indicate a positive correlation between PI3K activity and MZ B cell numbers in vivo. However, it is unclear whether PI3K provides MZ B cells or their precursors with an essential survival signal, a signal that directs MZ precursors to differentiate, or a combination of both of these. To address this, we assessed whether the MZ B cell deficiency of p110δ$^{-/-}$ mice could be overcome by extending the lifespan of B cells through introgression of the *bcl-2–36* transgene (29), as achieved in PLCγ2 knockout mice (30). In this experiment MZ B cells were defined as B220^{+}CD21^{high}CD23^{low} and FO B cells as B220^{+}CD21^{int} CD23^{high} (Fig. 5*A*). Despite the addition of the *bcl-2*, a significant reduction in MZ B cell numbers remained in p110δ$^{-/-}$ mice compared with WT, ($p < 0.001$) (Fig. 5*B*). The failure of the Bcl-2 transgene to rescue MZ B cell numbers was not due to a reduction in the expression of the transgenic Bcl-2 protein (Fig. 5*C*). We therefore conclude the MZ B cell deficiency in p110δ$^{-/-}$ mice cannot be reversed by artificially extending the lifespan of B cells.

The defect in class switch recombination in PTEN^{B} cells is partially reversed by the absence of p110δ

Previous studies of PTEN-deficient B cells have indicated high levels of PI3K activity are inhibitory for Ig class switch recombination (CSR) (21, 22), while pharmacological inhibition of p110δ increased CSR (22). Mindful that splenic B lymphocytes from PTEN^{B} and p110δ mutant mice are respectively enriched or depleted for MZ and B1 cells, we used lymph node B cells from these mice as these do not contain MZ and B1 B cells (data not shown).

Lymph node B cells were stained with the division-tracking dye CFSE and stimulated in vitro using anti-CD40 and IL-4, using conditions which we had found to be optimal for promoting CSR. After 4 days of culture, cells were stained for surface IgG1 expression and a representative set of FACS profiles is shown in Fig. 6*A*. We found that although the proportion of PTEN^{B} B cells that had undergone CSR was significantly reduced compared with WT, CSR was normal in cultures of p110δ$^{-/-}$ B cells (Fig. 6*B*). These findings were also reflected in the absolute number of IgG1^{+} cells generated (Fig. 6*C*). The proportion and absolute number of IgG1^{+} cells generated in double mutant B cells cultures was found to be reduced compared with WT, however there was a significant increase in the number of IgG1^{+} cells in the double mutant cultures compared with PTEN^{B} ($p = 0.002$ using Student's *t* test), suggesting that the concurrent deletion of p110δ partially restores defective CSR in PTEN^{B} cells. Thus, although p110δ$^{-/-}$ B cells undergo CSR normally, the CSR defect of PTEN-deficient B cells is partially reversed when p110δ is simultaneously deleted.

Defective CSR in PTEN^{B} cells was due to the impaired expression of AID (21). We therefore compared the levels of AID in B cells cultured with anti-CD40 and IL-4 using real time PCR (Fig. 6*D*). As expected, AID induction in PTEN^{B} cells was significantly reduced compared with WT. Although AID mRNA levels in the double mutant B cells were increased compared with PTEN^{B} cells ($p = 0.006$ using Student's *t* test), they were also significantly reduced compared with WT. Although we observed no increase in the ability of p110δ$^{-/-}$ B cells to undergo CSR in vitro, the AID mRNA levels from cultured cells were significantly increased.

Our finding that removal of p110δ by itself did not enhance CSR is in contrast with a separate study by Omori et al. (22) based on data generated using the p110δ selective inhibitor IC87114. We note that the assays conducted by Omori et al. used IC87114 at a concentration of 10 μM, which we believe may have resulted in off-target suppression of other PI3Ks. Indeed, the IC_{50} of IC87114 in vitro is reported to be 0.07 μM, but IC87114 also cross-reacts with the PI3K catalytic subunits p110γ (IC_{50} of 1.24 μM) and p110β (IC_{50} of 1.82 μM) (31). To test this, we assessed the ability of B cells from the four genotypes to undergo CSR in the presence of an intermediate dose of IC87114 (Fig. 6*E*). When tested at 1 μM, we found no significant difference in the ability of dividing cells from WT, p110δ$^{-/-}$ or double mutant B cells to undergo CSR. However, treatment of PTEN-deficient B cells with 1 μM of IC87114 did yield an increased proportion of IgG1^{+} cells. This

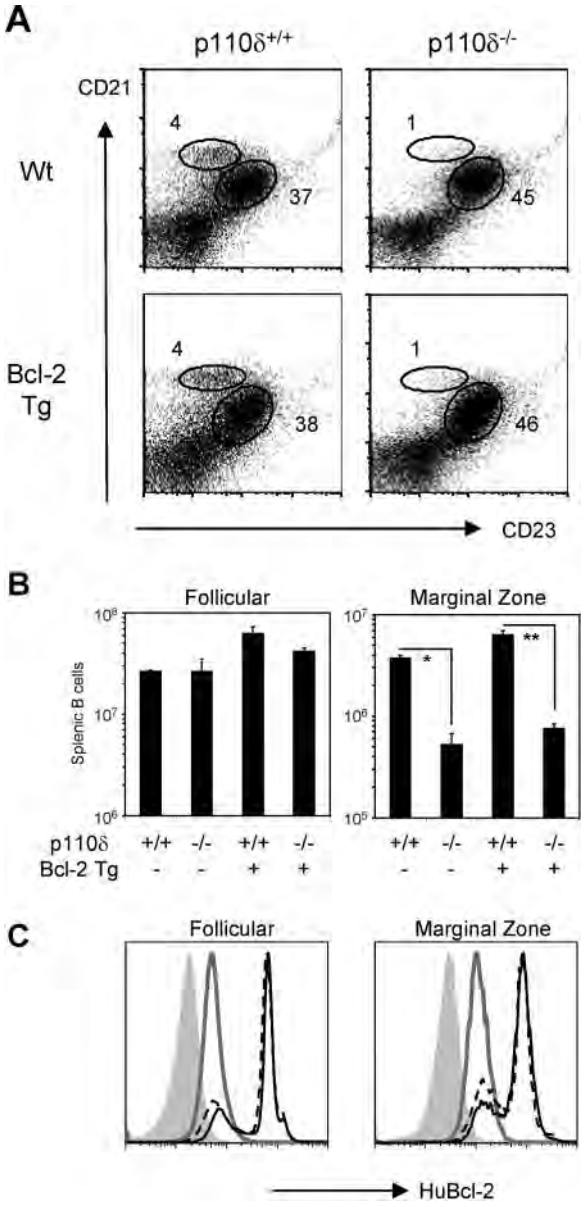

FIGURE 5. Effect of the *Bcl-2–36* transgene on B cell development in p110δ mutant mice. *A*, Flow cytometric analysis of splenic lymphocytes stained for CD21 and CD23. Marginal zone B cells were defined as CD21/35^{high}CD23^{low} and follicular B cells as CD21/35^{low}CD23^{high}. The gates used to identify these populations are indicated together with the percentage of each subset among total lymphocytes. *B*, Mean and SEM of follicular and marginal zone B cells pooled from two experiments with minimum of three mice/group. Statistical analysis was performed using a one way ANOVA test. *p* values are indicated where *, $p < 0.05$ and **, $p < 0.01$. *C*, Detection of the human Bcl-2 transgene by flow cytometry in FO and MZ B cells. (▨), Isotype control; (grey line), non-transgenic control; (black line), p110δ$^{+/+}$ Bcl-2 Tg; (black dashed line), p110δ Bcl-2 Tg.

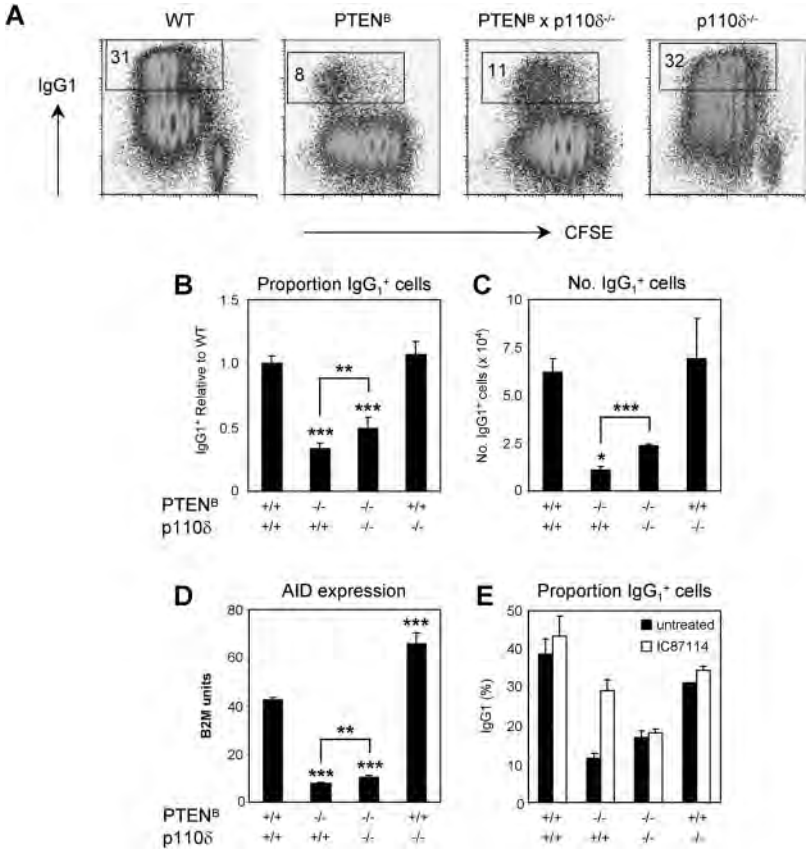

FIGURE 6. Class switch recombination in p110δ and PTEN mutant B cells. *A*, Flow cytometric analysis showing division linked IgG1 expression on purified lymph node B cells cultured for 4 days in the presence of anti-CD40 and IL-4. *B*, The proportion and (*C*) number of B cells expressing IgG1 in WT, PTEN^B, p110$\delta^{-/-}$, and double mutant cultures and (*D*) the levels of AID mRNA as determined by real time PCR. Each bar represents the mean and SEM from three to eleven individual mice. Statistical analysis was performed using a one-way ANOVA test. Significant differences compared with WT are indicated where $*$, $p < 0.05$ and $***$, $p < 0.001$. *E*, The proportion of WT, PTEN^B, p110$\delta^{-/-}$, and double mutant B cells expressing IgG1 in the presence and absence of 1 μM IC87114 after 4 days. Each bar represents the mean and SEM from three individual mice.

corresponds with our finding that B cells with a combined deletion of PTEN and p110δ have enhanced CSR compared with PTEN-deficient B cells. Taken together, our results are consistent with the suggestion that PI3K signaling regulates CSR, but p110δ is not a critical, nonredundant isoform required for this process.

P110 isoform expression in FO, MZ, and B1 cells

Our results indicated the potential for other catalytic subunits of PI3K to regulate B cell development and activation. To investigate this further, we analyzed the relative expression of the three class IA catalytic isoforms, p110α, p110β, and p110δ and the class IB catalytic isoform p110γ in sorted FO, MZ, and B1 cells using qPCR (Fig. 7). We found p110β expression to be very low for the three cell types analyzed. By contrast, p110α, p110δ, and p110γ mRNA was detectable in all cell types with none of the B cell subsets demonstrating exclusive expression of any isoform. Thus, the nonredundant role for p110δ in B1 cell development cannot be attributed to a lack of other catalytic isoforms being transcribed.

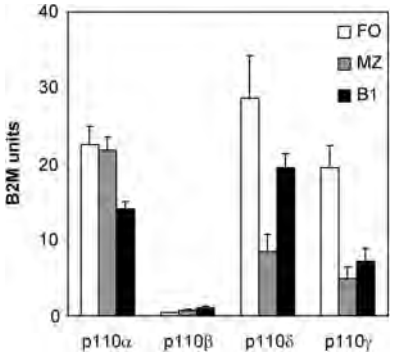

FIGURE 7. Expression of p110 catalytic isoforms in FO, MZ, and B1 B cells. Splenic FO and MZ B cells and peritoneal B1 cells were sorted from a pool of three to four mice. Levels of p110α, p110β, p110δ, and p110γ mRNA were determined by real time PCR and normalized to β2M. Each bar represents the mean and SEM of three independent biological replicates.

Discussion

The loss of PTEN in B cells leads to a marked increase in MZ and B1 cell populations as well as a reduction in the ability of FO B cells to undergo CSR. This phenotype highlights an important role for the PI3K pathway in regulating B cell development and differentiation. The class IA catalytic subunit p110δ is thought to be the dominant PI3K isoform involved in these processes, as the loss of p110δ leads to a severe reduction in B1 and MZ B cell numbers. However, the contribution of other catalytic subunits has been difficult to access as the p110α and p110β mutants are both embryonic lethal. We have addressed this issue by generating PTEN^B $\times$ p110$\delta^{-/-}$ double mutant mice and analyzing their FO, MZ, and B1 cell phenotypes as well as studying CSR in vitro.

Analysis of the double mutant mice establishes the critical role of p110δ in the expansion of B1 cells in PTEN^B mice. The other class IA PI3K subunits and p110γ cannot compensate for p110δ in this regard. By contrast, despite the negligible MZ B cell population in p110$\delta^{-/-}$ mice, MZ B cell numbers in the double mutant mice were significantly higher than WT. This finding indicates a clear requirement for other class I PI3K catalytic subunits in MZ B cell development. Although the identity of the relevant catalytic subunits has yet to be determined, we have previously reported that p110α but not p110β can associate with CD19 (11), a receptor implicated the regulation of the size of the MZ pool. Thus, it will be interesting to examine the role of p110α in determining the size of the MZ B cell pool.

Our analysis of mice with different combinations of *p110δ* and *PTEN*^{B} alleles allowed us to establish a correlation between increased PIP_3 activity (as inferred from PKB phosphorylation) and MZ B cell numbers. Our results suggest that the role of PI3K in the differentiation of MZ B cells may be something other than providing a survival signal as we were unable to recover the MZ population with a *Bcl-2* transgene, which has been previously shown to restore B cells numbers in PLCγ2$^{-/-}$ mice (30). PI3K is associated with numerous cell surface receptors that have been implicated in MZ or B1 cell development. The most well defined is the Ag receptor and its coreceptor CD19 (32, 33). In addition, Notch and BAFF receptors have also been found to play a role in the regulation of the MZ pool. Notch2 is preferentially expressed on peripheral B lymphocytes (34) and Notch2 mutant mice are deficient in their MZ and B1 B cell populations (34, 35). Although a direct link between PI3K and Notch signaling has yet to be demonstrated in B lymphocytes, Notch1 signaling in T cells and thymocytes has been found to be PI3K dependent (36, 37). PI3K signaling in B cells has also been shown to be activated by B cell activating factor of the TNF family (BAFF), which led to the rapid phosphorylation of PKB (38). BAFF is an important survival factor for B cells (39) regulating the numbers of FO and MZ, but not peritoneal B1, B cells (40, 41). Overproduction of BAFF has been shown to lead to an expansion of the FO and MZ B cells as well as splenic B1a cells (42, 43). Interestingly, the defect in MZ B cells arising from BAFF inhibition in TACI-Fc transgenic mice cannot be overcome by a *Bcl-2* transgene (44). Thus, BAFF-signaling may be defective in the absence of p110δ and elevated in PTEN-deficient B cells.

We found that the number of splenic FO B cells was normal in the PTEN^{B} and double mutant mice but reduced in the p110δ$^{-/-}$ mice, as reported previously (8). The absence of an increase in FO B cell numbers in PTEN^{B} mice suggests that PI3K signaling may not be involved in the selection of FO B cells from their transitional precursors. Instead, the reduced numbers of FO B cells in the spleens of p110δ$^{-/-}$ mice may reflect altered B cell recirculation, as the numbers of FO B cells in lymph nodes is not diminished (our unpublished data). Although the class IB PI3K is thought to be the dominant class of PI3K associated with chemotactic signaling responses in leukocytes, a role for p110δ in neutrophils (45, 46) and B cells in chemokine responses has been described (47). The mechanism by which p110δ is activated in response to chemokine signaling is yet to be elucidated, however it has been postulated that p110δ is activated via the integrin receptors, which are themselves up-regulated in response to chemokine signaling (45).

The roles of signaling pathways in regulating CSR and plasma cell differentiation are of particular interest given recent evidence that the BCR affinity directs plasma cell differentiation in vivo (48, 49). Previous studies using splenic B cells from PTEN^{B} mice indicated that PTEN-deficient B cells were defective in CSR. We found that PTEN^{B} B cells isolated from lymph nodes were also deficient in CSR. Notably, the ability to undergo CSR was significantly improved in the double mutant B cells and correlated with an increased in the levels of detectable AID cDNA. Thus, the concurrent deletion of p110δ in PTEN^{B} cells is probably sufficient to relieve the suppression of AID and allow CSR. Removal of p110δ did not enhance CSR despite p110δ$^{-/-}$ cells having elevated levels of *AID* mRNA. It may be that despite having elevated levels of *AID* mRNA p110δ$^{-/-}$, B cells are unable to respond efficiently to other stimuli required for maximal CSR. Indeed, it has been previously shown that in the presence of IL-4, but the absence of mitogenic stimuli, B cells, which over-express AID, display minimal CSR (50). A further possibility is that AID activity is regulated at the posttranscriptional level in B cells. This hypothesis is supported by the finding that in mice which express AID constitutively B cell abnormalities are not detected suggesting that B cells have mechanism by which AID can be negatively regulated (51).

The results presented in this study suggest a role for additional catalytic isoforms in MZ B cell development and CSR in FO B cells. Mice deficient for the p85α regulatory subunit, like the p110δ mutants, have a MZ and B1 B cell deficiency that cannot be rescued by the transgenic expression of Bcl-2 (14–16). However, p85α$^{-/-}$ mice also have reduced numbers of transitional and FO B cells, displaying an earlier block in B cell development than p110δ mutant mice. The primary roles for PI3K regulatory subunits are thought to be stabilization of the catalytic subunits (52) and regulating the association of PI3K with signaling complexes at the cellular membrane (53). Indeed, in p85α$^{-/-}$ B cells the PI3K enzymatic activity is only 5% of WT levels (14). Thus, it is possible that p110α contributes to B cell development in concert with p110δ. In addition, the B cell developmental block in p85α$^{-/-}$ mice may reflect functions of p85α which are independent of PI3K activity (54).

The data we have provided in this study clearly demonstrate an epistatic relationship between *p110δ* and *PTEN* for several aspects of B cell differentiation. Furthermore, our results suggest contributions from other PI3K catalytic isoforms in addition to p110δ are important for B cell development and activation. Uncovering the identity and role of additional PI3K subunits in B cell development and function will require the generation of conditional alleles and strategies for deletion of genes in the late stages of B cell differentiation.

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Disclosures

The authors have no financial conflict of interest.

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