Critical Roles of Pten in B Cell Homeostasis and Immunoglobulin Class Switch Recombination

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Abstract

Pten is a tumor suppressor gene mutated in human cancers. We used the Cre-loxP system to generate a B cell–specific mutation of Pten in mice (bPtenflox/flox mice). bPtenflox/flox mice showed elevated numbers of B1a cells and increased serum autoantibodies. Among B2 cells in bPtenflox/flox spleens, numbers of marginal zone B (MZB) cells were significantly increased while those of follicular B (FOB) cells were correspondingly decreased. Pten-deficient B cells hyperproliferated, were resistant to apoptotic stimuli, and showed enhanced migration. The survival kinase PKB/Akt was highly activated in Pten-deficient splenic B cells. In addition, immunoglobulin class switch recombination was defective and induction of activation-induced cytidine deaminase (AID) was impaired. Thus, Pten plays a role in developmental fate determination of B cells and is an indispensable regulator of B cell homeostasis.

Key words: PTEN • mutation • marginal zone B cells • class switch recombination • activation-induced cytidine deaminase

Introduction

PTEN is a tumor suppressor gene mutated in many human sporadic cancers (1) and in hereditary cancer syndromes such as Cowden disease and Bannayan-Zonana syndrome (2, 3). Functionally, PTEN is a dual protein and lipid phosphatase (4, 5). The major substrate of PTEN is phosphatidylinositol-3,4,5-triphosphate (PIP3),* a second messenger molecule produced through PI3K activation induced by growth factor stimulation. PIP3 activates the serine-threonine kinase PKB/Akt which is involved in anti-apoptosis, proliferation, and oncogenesis. PTEN negatively regulates cell survival by dephosphorylating PIP3.

In previous work, we showed that a null mutation of Pten in mice (Pten−/− mice; reference 6) is embryonic lethal. Using Pten−/− mouse embryonic fibroblasts (MEFs), we demonstrated that PKB/Akt was hyperactivated in the absence of Pten (7). Furthermore, Pten+/− mice frequently develop lymphoid hyperplasia, T cell lymphomas, and endometrial, prostatic, and breast cancers (6, 8, 9). Autoimmune disorders are also prevalent in Pten+/− mice (10). In T cell–specific Pten-deficient mice, we showed that CD4+ lymphomas and autoimmune disorders arise due to impaired thymic negative selection and peripheral tolerance (11). Since Pten mutations occur in human B cell malignancies (12–14), we investigated the role of Pten in B cell homeostasis.

*Abbreviations used in this paper: BM, bone marrow; CSR, class switch recombination; DC, digestion-circulation; FOB, follicular B; MEF, mouse embryonic fibroblast; MLN, mesenteric lymph node; MZB, marginal zone B; PEC, peritoneal cavity; PIP3, phosphatidylinositol-3,4,5-triphosphate; SDF, stromal cell–derived factor; TD, thymus-dependent; TI, thymus-independent antigen.
cell development and B cell–associated autoimmunity and oncogenesis.

B cells can be classified as either B1 or B2 cells. B1 cells occur mainly in the pleural and peritoneal cavities and are associated with the production of autoreactive antibodies (15). B2 cells are found chiefly in the periphery and comprise transitional T1 and T2 cells, and mature follicular B (FOB) cells and marginal zone B (MZB) cells. FOB cells form the follicular structures of the secondary lymphoid organs and are capable of recirculation. The much smaller MZB fraction resides in the spleen at the boundary between the red pulp and white pulp (16). These cells may be the first splenocytes to encounter blood-borne bacterial pathogens (16, 17). Splenic MZB cells, but not FOB cells, have high levels of surface immunoglobulin M (IgM) and the complement receptor CD21, and low levels of IgD and IgG23 (18, 19). Both MZB and FOB cells undergo immunoglobulin class switching in response to antigen stimulation and cytokines (20). Class switch recombination (CSR) requires the activity of the RNA editing enzyme AID (21) but the underlying mechanism is unknown.

To investigate the role of Pten in B cells, we generated \textit{bPten}^{flox/flox} mice using the Cre-loxP system. We report that Pten governs B cell subsets especially in B1, FOB, and MZB cells and is required for normal immunoglobulin class switching.

Materials and Methods

\textbf{Generation of bPten}^{flox/flox} \textit{Mice.} \textit{Pten}^{flox/flox} mice (C57BL6/J background) were mated to \textit{CD19Cre} transgenic mice (C57BL6/J background; reference 22) in which expression of Cre is controlled by the endogenous promoter of the B cell–specific gene \textit{CD19}. Offspring carrying \textit{CD19Cre} and two copies of the floxed \textit{Pten} allele (\textit{CD19CrePten}^{flox/flox}), \textit{CD19Cre} plus one copy of the floxed \textit{Pten} allele (\textit{CD19CrePten}^{flox/+}), and \textit{CD19Cre} plus two copies of the WT \textit{Pten} allele (\textit{CD19CrePten}^{+/+}) were used in the analyses as homozygous mutant (\textit{bPten}^{flox/flox}), heterozygous mutant (\textit{bPten}^{flox/+}), and wild type (\textit{bPten}^{+/+}) mice, respectively.

\textbf{PCR Analysis of Pten Genotypes.} Genomic DNA from mouse tails was isolated and amplified by PCR following a published protocol (6). Sense primer (5'-GTCACCTAGGCTTTCTGAGCCTT-3') and antisense primer (5'-GAAACGGCCTTTAAACGACGTA-3') were used to detect the floxed \textit{Pten} allele; sense primer (5'-GTCACCTAGGCTTTCTGAGCCTT-3') and antisense primer (5'-GTGACATCAACATGACACTG-3') were used to detect the WT \textit{Pten} allele; and sense primer (5'-CTCCTCACCAGTTCTTTG-3') and antisense primer (5'-TTCATGATGGAACGACTGTGCG-3') were used to detect the \textit{CD19Cre} transgene. Amplified fragments of 512, 413, and about 500 bp, respectively, were obtained.

\textbf{Southern and Western Blots.} Genomic Southern blots were performed using a previously described probe and protocol (6). For Western blots, B cells (2 x 10^6) were either left untreated or stimulated with 10 μg/ml anti-IgM (ICN Biomedicals/Cappel). Total cell lysates were prepared and 10 μg lysate aliquots analyzed by Western blotting as described (7). Antibody directed against the NH2 terminus of Pten and anti-actin antibody were from Santa Cruz Biotechnology, Inc.; anti-phospho-PKB/Akt (Ser473) and anti-total Akt/PKB antibodies were from New England Biolabs, Inc. For PI3'K inhibition studies, an optimal amount of wortmannin (200 nM; Sigma-Aldrich) or LY294002 (50 μM; Sigma-Aldrich) as determined in pilot studies was added 15 min before stimulation.

\textbf{Flow Cytometric Analysis and Cell Purification.} Single cell suspensions were first incubated with anti-CD16/32 to minimize nonspecific staining. Cells were then stained with cocktails of various mAbs conjugated to FITC, PE, or biotin for 20 min at 4°C. Biotinylated mAbs were developed with streptavidin–Cy-Chrome (BD Biosciences). All mAbs, except PE-labeled anti-IgD (Southern Biotechnology Associates, Inc.), were purchased from BD Biosciences. Flow cytometric analysis was performed using a FACSCalibur™ (Becton Dickinson) with CELLQuest™ software (Becton Dickinson). Total spleen B cells were purified using B220 magnetic beads (Macs; Miltenyi Biotec). Splenic CD23/highCD21/low B cells and CD23/lowCD21/high B cells were purified using B220 magnetic beads followed by cell sorting with a FACSvantage™ (Becton Dickinson) after staining with anti-CD21/35-FITC and anti–CD23-PE antibodies (BD Biosciences).

\textbf{Histological Analysis of Splenic Sections.} For immunohistochemical staining, freshly dissected spleens were covered with Tissue–Tek OCT compound (Miles, Inc.) and quickly frozen in liquid nitrogen. Frozen sections (7-μm thick) were fixed in ice cold acetone and incubated in 3% H2O2 in 50% methanol for 30 min to inactivate endogenous peroxidase. Immunofluorescent staining was performed using MOMA-1 (Serotec) and anti–rat Alexa488 (Molecular Probes) antibodies followed by anti–B220-PE (BD Biosciences) staining. Immunohistochemical staining was performed using biotin-conjugated peanut agglutinin (PNA; Seikagaku Kogyo) followed by a Vectastain ABC Elite kit (Vector Laboratories).

\textbf{Analysis of Humoral Responses.} Serum Ig isotype concentrations were analyzed by ELISA as described (23). Abs and standard Igs were purchased from Southern Biotechnology Associates, Inc. For T cell–dependent immune responses, mice were immunized with 100 μg of alum-precipitated chicken γ-globulin (CG) coupled to 4-hydroxy-3-nitro-phenylacetyl (NP). For T cell–independent immune responses, mice were immunized with 100 μg of alum-precipitated Ficoll coupled to NP. In both cases, mice were bled at 7 and 14 d after challenge. Serum titers of NP-specific IgM, IgG1, and IgG3 were determined by ELISA as described (23). The measurement of serum anti-siDNA IgG and IgM antibodies was performed using ELISA as described (24). Statistical analyses were performed using the unpaired Student’s t test.

\textbf{Lymphocyte Activation in Culture.} Splenic B cells were purified using B220 microbeads and a Magnetic Cell Sorter (MACS; Miltenyi Biotec). B cells (2 x 10^6/well) were stimulated for 4 d with 50 μg/ml LPS alone or 50 μg/ml LPS plus 800 U/ml IL-4 in RPMI 1640 medium supplemented with 20% FCS, 2-mercaptoethanol (ME), penicillin, and streptomycin. Cells and culture supernatants were analyzed by flow cytometry and ELISA, respectively.

\textbf{RT-PCR.} Cells (5 x 10^5/ml) were stimulated in vitro for 2 d with 50 μg/ml LPS alone or LPS plus 800 U/ml of IL-4. Total RNA was extracted using TRIzol (GIBCO BRL) according to the manufacturer’s instructions. For PCR of germline transcripts, the following standard primers were used to obtain the indicated sizes of products: (μ) ImF and CmR, 245 bp; (γ3) Ig3F and Cg3R, 323 bp; (γ1) Ig1 and Cg1R, 429 bp. Post–switch transcripts were amplified using the following primer pairs: (γ3) ImF and Cg3R, 323 bp; (γ1) ImF and Cg1R, 353 bp. Germline and post–switch transcripts were amplified using 30 cycles of PCR. The primer sequences were as follows: ImF: 5’-CTCTGGCCTGGTATATTGGT-3’, CmR: 5’-GAAGACCATTGG-
GAAGGACTGACT-3', Ig3F: 5'-TGGGCAATGGATCT-GAACA-3', Cg3R: 5'-CTACAGGGAAGGCCTTGA-3', Ig1: 5'-GGCCCTCGACAGCTTTTGA-3', Cg1R: 5'-GGAAGGACTGACT-3'.

For amplification of the AID transcript, the primers pair of 5'-GAGGAGGATCGAAGTTCATGGA-3' and 5'-GGCTGAGGTAGGGTGTCATC-3' was used in 30 cycles of PCR. For amplification of MSH2 transcripts, the primers 5'-CTAAGGACGCCTAGCTTG-3' and 5'-TACTGGC- GAACCAGAAGAAG-3' were used. For amplification of the HPRT transcript, the primers 5'-GATAACGATGATGATGATGATGATGATGATGATG-3' and 5'-ACAGTACGCTTACGCTGATA-3' were used.

Digestion-Circularization-PCR. Digestion-circularization (DC)-PCR analysis was performed as described (25). Briefly, genomic DNA was isolated from B cells cultured in vitro for 4 d with LPS (50 μg/ml) and IL-4 (250 ng/ml; BD Biosciences). LPS (2.5 μg/ml; Sigma-Aldrich) or PDBu (20 ng/ml; ICN Biomedicals) in wells of plastic dishes at 37°C overnight, followed by washing with PBS. Anti-IgM (50 μg/ml; ICN Biomedicals/CAPP) was added to cultures. Cells were harvested on day 2 after a 12 h pulse with 1μCi [3H]thymidine (Amersham) per well.

Apyptosis of "Small Dense" B Cells. Anti-Ig antibody was immobilized by incubating PBS containing 100 μg/ml F(ab')2 fragments of affinity-purified goat anti-mouse IgM antibody (ICN Biomedicals) in wells of plastic dishes at 37°C overnight, followed by washing with PBS. “Small dense” B cells were prepared by depleting T cells from mouse splenocytes using anti-Thy1.2 FITC (Serotec), anti-CD4 RL172.5 (a kind gift of Dr. Kina, Kyoto University, Kyoto, Japan), anti-CD8 3.155 (American Type Culture Collection no.TIB211) and Low-Tox-M rabbit complement (Cedarlane), followed by fractionation using density gradient centrifugation through Percoll (BD Biosciences). Small dense B cells were then cultured in dishes containing immobilized anti-Ig antibody in RPMI 1640 medium supplemented with 10% FCS, 50 μM 2-ME, and 2 mM l-glutamine for up to 24 h. The percentage of viable cells was determined by trypan blue exclusion. Viability results were calculated as a comparison of the percentage of viable cells remaining after treatment relative to the viability of untreated cells cultured for the same length of time.

B Cell Proliferation. Splenic B cells were purified using B220 magnetic beads and CD23<sup>low</sup>-CD21<sup>high</sup> or CD23<sup>high</sup>-CD21<sup>low</sup> cells were isolated by cell sorting. Purified cells (10<sup>5</sup>) were placed in wells of 96-well plates in RPMI 1640 medium containing 10% FCS. Anti-IgM (50 μg/ml; ICN Biomedicals/CAPP) in wells of plastic dishes at 37°C overnight, followed by washing with PBS. Anti-IgM (50 μg/ml; ICN Biomedicals/CAPP) was added to cultures. Cells were harvested on day 2 after a 12 h pulse with 1μCi [3H]thymidine (Amersham) per well.

Results

Generation of B cell-specific Pten-deficient Mice. B cell-specific Pten-deficient mice (bPten<sup>lox/flox</sup> mice) were generated by crossing CD19<sup>Cre</sup> transgenic mice (22) to mice homozygous for the floxed Pten allele (Pten<sup>lox/lox</sup> mice; reference 11). bPten<sup>lox/flox</sup> mice were born alive and appeared healthy. Genomic Southern blotting showed that, in the vast majority of mutant B cells, Cre-mediated recombination of loxP sites deleted much of the 6.0 kb Pten gene. (B) Western blot analysis of Pten protein from the indicated cells using Pten antibody (ICN Biomedicals). (C) Splenomegaly, lymph node swelling, and abundant peritoneal cells in bPten<sup>lox/flox</sup> mice. Results shown are the absolute numbers of splenocytes (Spleen), mesenteric lymph node cells (MLN), bone marrow cells (BM), and cells in the PEC from 6–8-wk-old bPten<sup>lox/+</sup> (n = 3), bPten<sup>lox/lox</sup> (n = 3) mice. Where appropriate in all figures, the results are expressed as the mean ± SEM of the indicated number of mice per group. Statistical differences in all cases were determined using the Student’s t test; *P < 0.05.
Pten was confirmed at the protein level by Western blotting using antibody recognizing the NH2 terminus of Pten (Fig. 1 B). The frequencies of gene deletion observed in B220+/CD21+/CD23+/ peritoneal cavity (PEC) cells and B220+ spleen cells were comparable (unpublished data). The health of 30 bPtenfl/fl mice and 30 control CD19 CrePten+/+ (bPten+/+) mice was monitored for over 12 mo. All mutant mice survived the observation period and no tumor formation was observed.

Altered B Cell Populations. bPten+/+ and bPtenfl/fl mice were killed at 6–8 wk of age, and B cell subpopulations in the spleen, mesenteric lymph nodes (MLNs), bone marrow (BM), and PEC were examined. Total numbers of splenocytes, MLN cells, and PEC cells in bPtenfl/fl mice were increased 2.0-fold, 1.9-fold, and 5.5-fold, respectively (Fig. 1 C). Although PTEN is expressed in WT pro-B cells were increased 2.0-fold, 1.9-fold, and 5.5-fold, respectively (Fig. 1 C). Although PTEN is expressed in WT pro-B cells and pre-B cells as well as in mature B2 cells, there were no obvious differences in either the total number of BM cells, or in numbers of pro-B cells (CD43+, B220+, IgM+) or pre-B cells (CD43+, B220+, IgM+) in the BM of WT and mutant mice (unpublished data).

The increased B cell number in the PEC was due to a 24-fold increase in CD5dullB220dull cells (Fig. 2 A, top panel; Fig. 2 B, left). These cells expressed Mac-1low, CD21+, CD23+, and HSA+, compatible with the surface phenotype of B1a cells (unpublished data; references 26 and 27). CD5dullB220dull cells were also increased 11-fold in the spleens of bPtenfl/fl mice compared with bPten+/+ spleens (Fig. 2 A, bottom panel; Fig. 2 B).

As shown in Fig. 2 C, IgM+IgD− B cells were also elevated in bPtenfl/fl spleens. Although CD5dull B220dull cells belong to the IgM+IgD− population, the increase in their numbers could account for only a part of this elevation. WT splenic IgM+IgD− B cells include MZB cells (CD21highCD23low), a population that was dramatically increased in bPtenfl/fl mice (Fig. 2, D, F, and G). FOB cells (CD21lowCD23high) were decreased not only in relative number (Fig. 2 D), but also in absolute number (Fig. 2 G). Immunohistochemical staining revealed that most of the B2 cells were located in marginal zones, while only a few appeared to be in follicular regions in the bPtenfl/fl spleen (Fig. 2 E). A similar skewing of cell numbers was noted in bPtenfl/fl lymph nodes (unpublished data).

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Figure 2. Pten deficiency alters B1a, MZB, and FOB B cell subsets. (A and B) Accumulation of B1a cells in bPtenfl/fl mice. Increased numbers of CD5dullB220dull cells in the PEC and spleen of 6–8-wk-old bPtenfl/fl mice were apparent when analyzed by either flow cytometry (A) or total cell counts (B). (C) Increased IgM+IgD− cell numbers in the bPtenfl/fl spleen. Levels of surface IgM and IgD were determined by flow cytometry. (D–F) Alterations to B cell subsets. Flow cytometric (D and F) and total cell count (G) analyses were used to evaluate mature IgM+CD21highCD23low cells (putative MZB cells; D and F) and IgM+CD21lowCD23high cells (putative FOB cells; D), and transitional IgM+CD21lowCD23low cells (putative T1 cells; F) and IgM+CD21highCD23high (putative T2 cells; F) among splenic B cells of 6–8-wk-old bPtenfl/fl and bPten+/+ mice. Immunohistochemical analysis (E) using MOMA1 (green) and B220 (red) antibodies shows a dramatic increase in MZB cells among B2 cells in bPtenfl/fl mice, while the number of FOB cells is markedly reduced. For B and E, results are expressed as the mean ± SEM for 6 mice per group. For A, C, D, E, and F, one result representative of seven independent experiments is shown.
population was significantly reduced (Fig. 2 G). The overall segregation of T cell and B cell zones was not impaired in the splenic white pulp, MLN, or Peyer’s patches of bPtenflox/flox mice (unpublished data). Thus, B cell–restricted Pten deficiency results in discrete alterations to the B1a, MZB, and FOB subsets of B lymphocytes.

**Impaired Humoral Immunity.** To determine whether the altered B cell populations in the mutant mice affected humoral immunity, we assessed serum Ig levels in bPtenflox/flox mice at 8 weeks of age. As shown in Fig. 3 A, marked decreases in most IgG subclasses and IgA were observed in bPtenflox/flox mice compared with bPten+/+ mice. In contrast, serum IgM levels in bPtenflox/flox mice were elevated fourfold over normal.

We then examined the humoral responses of bPten+/+ and bPtenflox/flox mice immunized with either the thymus–dependent (TD) antigen NP-CG (nitro-phenylacetyl-chicken γ-globulin) or the thymus-independent antigen (TI) type II NP-Ficoll. Production of antigen-specific IgG in response to TD antigen was dramatically decreased in bPtenflox/flox mice (Fig. 3 D) as was germinal center formation (Fig. 3, B and C). Production of antigen–specific IgG in response to TI-II antigen was also severely impaired in the absence of Pten (Fig. 3 E). Thus, an absence of Pten impairs both TD and TI IgG responses.

**Reduction of CSR.** Because of the altered serum Ig profile observed in bPtenflox/flox mice, we examined isotype switching in vitro in bPtenflox/flox B cells. bPten+/+ and bPtenflox/flox B cells were cultured for 4 d in the presence of the nonspecific B cell stimulator LPS with or without IL-4. Trypan blue exclusion analysis confirmed that the viability of stimulated cells of both genotypes was not significantly different (unpublished data). Stimulated cells were surface-stained with anti-IgG1 or anti-IgG3 antibody and subjected to flow cytometric analysis. LPS plus IL-4, but not LPS alone, induced switching to IgG1 in WT cells (Fig. 4 A). Prolonged stimulation of WT cells with LPS alone induced switching to IgG3 which was down-regulated by

**Figure 3.** Reduced antigen-specific IgG production and germinal center formation in bPtenflox/flox mice. (A) Serum immunoglobulin levels of unimmunized 8-wk-old mice. Serum IgM was increased in bPtenflox/flox mice while most IgG subclasses and IgA were decreased compared with the WT. (B and C) Germinal center formation. After injection of TD-antigen (NP-CG), the number of germinal centers was markedly reduced in bPtenflox/flox mice as determined by H&E staining (B, top panel); PNA staining (B, bottom panel); and total counts (C). For C, the total number of germinal centers per maximum section was plotted. Magnification in B, ×10. (D) Production of TD antigen–specific IgGs. Serum levels of specific IgGs induced by injection of NP-CG were significantly decreased in bPtenflox/flox mice. (E) Production of TI antigen–specific Igs. Serum levels of specific Igs induced by injection of TI antigen (NP-Ficoll) were also strikingly diminished in bPtenflox/flox mice. Results are expressed as the mean ± SEM for six mice per group.
the addition of IL-4. These aspects of isotype switching were reduced in bPten\textsuperscript{lox/lox} B cells, a result confirmed by ELISA analysis of culture supernatants (Fig. 4 B).

Isotype switching depends on transcription of class-specific mRNAs in which the μ exon is spliced onto the 5′ exon of another C\textsubscript{H} gene (28). Specific immunoglobulin transcripts can be identified by RT-PCR using primers specific for each C\textsubscript{H} gene (29). We stimulated bPten\textsuperscript{lox/lox} spleen cells with LPS plus IL-4 and observed that, while transcription of germline transcripts was intact, there was a dramatic reduction in μ-C\textsubscript{H}3 and μ-C\textsubscript{H}1 post-switch transcripts (Fig. 4 C). These results imply that Pten deficiency leads to a defect in CSR.

CSR depends in part on the activity of AID, a member of the RNA-editing cytidine deaminase family. AID was recently reported to regulate CSR (21) and is activated by LPS in vitro as well as by antigens in vivo. In bPten\textsuperscript{lox/lox} mice, AID expression was markedly reduced (Fig. 4 C). In

Figure 4. Reduction of CSR associated with impaired induction of AID in Pten-deficient B cells. (A and B) Defect in IgG1 and IgG3 production. B cells from 8-wk-old bPten\textsuperscript{lox/lox} and control mice were stimulated with LPS, or LPS plus IL-4, and production of IgG1 and IgG3 on the cell surface (A) and in culture supernatants (B) was analyzed by flow cytometry and ELISA, respectively. For B, results are expressed as the mean ± SEM for three mice per group. (C) Reduction in post-switch transcripts. IgG1 (bottom panel) and IgG3 (top panel) post-switch transcripts were analyzed by RT-PCR. Induction of AID expression was almost absent in bPten\textsuperscript{lox/lox} B cells. (D) Impaired switching at the DNA level. μ-γ1 DC-PCR products were profoundly diminished in stimulated mutant B cells. (E and F) CSR in isolated CD21\textsuperscript{hi}/CD23\textsuperscript{lo} (MZB) and CD21\textsuperscript{lo}/CD23\textsuperscript{hi} (FOB) cells. Production of IgG1 and IgG3 postswitch transcripts and AID expression were reduced in both populations of bPten\textsuperscript{lox/lox} B cells. Data shown are representative of three independent experiments.
contrast, the expression of MSH2, a mismatch repair gene also important for CSR (30, 31), was not significantly different in bPten<sup>++</sup> and bPten<sup>fl/fl</sup> B cells.

To directly examine DNA rearrangement in the Ig locus, we performed digestion-circularization (DC) PCR of DNA obtained from splenic B cells stimulated with LPS plus IL-4. As shown in Fig. 4 D, µ-γ1 DC-PCR products were amplified in DNA from stimulated splenic bPten<sup>++</sup> B cells but diminished in DNA from stimulated bPten<sup>fl/fl</sup> B cells. This result demonstrates that Pten deficiency leads to a failure in CSR. To rule out the possibility that the observed defect in CSR was due to differences in the relative numbers of particular cell populations, we examined Ig production and CSR in purified CD21<sup>high</sup>CD23<sup>low</sup> (MZB) cells and CD21<sup>low</sup>CD23<sup>high</sup> (FOB) cells by ELISA and RT-PCR. Production of IgG1 and IgG3 in response to stimulation with LPS plus IL-4 was impaired in both CD21<sup>high</sup>CD23<sup>low</sup> and CD21<sup>low</sup>CD23<sup>high</sup> cells of bPten<sup>fl/fl</sup> mice (Fig. 4 E). Similarly, the synthesis of post-switch transcripts and AID expression were reduced equally in bPten<sup>fl/fl</sup> CD21<sup>high</sup>CD23<sup>low</sup> and CD21<sup>low</sup>CD23<sup>high</sup> cells compared with the WT (Fig. 4 F). These data demonstrate that Pten is indispensable for CSR, presumably because Pten regulates the induction of AID expression.

**Autoantibody Secretion.** Pten deficiency has been previously associated with autoimmunity (10, 11), and B cell–specific Pten-deficient mice have increased numbers of autoantibody-producing B1 cells (15). We therefore examined serum autoantibody titers of bPten<sup>fl/fl</sup> mice at 6–8 wk and 6–8 mo of age. Both age groups of mutant mice produced significantly greater amounts of anti-ssDNA IgM Ab compared with bPten<sup>++</sup> mice in both absolute and relative (% ssDNA/total IgM) terms (Fig. 5). While the absolute amount of anti-ssDNA IgG Ab was not increased significantly in bPten<sup>fl/fl</sup> mice, the relative amount of IgG autoantibody (% ssDNA/total IgG) was elevated. The observed impairment of CSR may partially mitigate the elevation of IgG autoantibodies in bPten<sup>fl/fl</sup> mice.

**Resistance to Apoptosis, Enhanced Proliferation, and Increased Migration.** We next subjected isolated MZB and FOB populations to various apoptotic, proliferative, and migratory stimuli. “Small dense” bPten<sup>fl/fl</sup> B cells treated in vitro with immobilized anti-IgM were significantly more resistant to apoptosis than bPten<sup>++</sup> small dense B cells (Fig. 6 A), suggesting that the increase in MZB cells in bPten<sup>fl/fl</sup> mice might be due at least in part to enhanced resistance to apoptosis.

To examine the proliferation of peripheral B cells, purified splenic CD21<sup>high</sup>CD23<sup>low</sup> (MZB) cells and CD21<sup>low</sup>CD23<sup>high</sup> (FOB) cells were stimulated in vitro as indicated in Fig. 6 B. Both populations from bPten<sup>fl/fl</sup> mice showed enhanced proliferation compared with the WT in response to stimuli such as anti-IgM, anti-CD40, LPS, or PDBu (phorbol-12, 13-dibutyrate) plus ionomycin. Thus, hyperproliferation contributes to the increased numbers of MZB cells in bPten<sup>fl/fl</sup> mice.

Dammers et al. have reported that MZB cells are derived from a subset of FOB cells by migration (32), although the origin of MZB cells remains controversial. If an absence of Pten enhanced the migration of FOB cells such that more of them became MZB cells, one would expect to see decreased numbers of FOB cells and correspondingly increased numbers of MZB cells, just as we observe in bPten<sup>fl/fl</sup> mice. To test FOB migration, we used transwell migration.

![Figure 5. Autoantibody secretion by Pten-deficient B cells. Concentration of serum anti-ssDNA autoantibodies of the IgM class (top panel) and IgG class (bottom panel) in 6–8-wk-old mice (left panel) and 6–8-mo-old mice (right panel) as determined by ELISA. Pten-deficient mice in both age groups produced significantly greater amounts of anti-ssDNA IgM Ab in both absolute and relative (% ssDNA/total IgM) terms. While the absolute amount of anti-ssDNA IgG Ab was not increased in the mutant mice, the relative amount of IgG autoantibody was elevated. Results are expressed as the mean ± SEM for eight mice per group.](image-url)
assays to measure the induction of directed cellular migration of purified splenic B cells in a gradient of the chemokine SDF-1α (stromal cell–derived factor-1α). As shown in the left panel of Fig. 6 C, the migration of Pten-deficient B cells was consistently greater than that of controls, even in the absence of SDF-1α.

To clarify which cell population, MZB or FOB, was responsible for the enhanced splenic B cell migration, the percent change in these cell populations before and after migration was calculated (Fig. 6 C). In bPten+/+ mice, CD21hiCD23lo cells were more mobile than CD21lo CD23hi cells, consistent with a previous report (33). In contrast, CD21loCD23hi cells from bPtenfloxflox mice were much more mobile than either CD21loCD23hi cells or CD21hiCD23lo cells from bPten+/+ mice. These data suggest that the reduction in the FOB population in bPtenfloxflox mice can be attributed to the enhanced migration properties of these cells.

Activation of PKB/Akt. Regulation of PKB/Akt activation by Pten is critical for normal apoptosis in MEF and for proliferation/apoptosis in T cells (7, 11). We therefore analyzed the phosphorylation of PKB/Akt in bPtenfloxflox splenic B cells. After stimulation with anti-IgM, densitometric analysis showed that phosphorylated PKB/Akt was significantly elevated in bPtenfloxflox B cells compared with bPten+/+ B cells (Fig. 7 A). Furthermore, phosphorylation was completely abolished in both bPten+/+ and bPtenfloxflox B cells by the addition of an optimal amount of either of the PI3K inhibitors wortmannin or LY294002. As shown in Fig. 7 B, the abnormal activation of PKB/Akt was observed in both B cell subsets in bPtenfloxflox spleens. Thus, in both MZB and FOB cells, as in T cells and MEF, PKB/Akt is activated via a PI3K-mediated pathway that is subject to negative regulation by Pten.

Discussion

To continue our studies of the important tumor suppressor Pten, we have generated and characterized B cell–specific Pten-deficient mice. To our surprise, bPtenfloxflox mice have shown no signs of B cell malignancies, although most T cell–specific Pten-deficient mice develop T cell lymphomas (11), and Pten mutations occur in human sporadic B cell malignancies (12–14). We have initiated the crossing of bPtenfloxflox mice into the p53 null genetic background to better assess the onset of B cell malignancy in the absence of Pten.

In this study, bPtenfloxflox mice showed an increase in MZB cells and a decrease in FOB cells, suggesting that Pten is important for the maintenance of normal B cell subsets in the spleen. It has been proposed that MZB cells may be derived from a subset of FOB cells that migrates...
B cell anergy also enters the red pulp. CD21^highCD23^low B cells also accumulated in bPten^+/+CD21^hiCD23^lo and CD21^hiCD23^lo mice. However, the CSR defect in bPten^+/+ mice could be CD3-independent. We are undertaking studies of the transcriptional regulation of the CSR gene that led to the publication of Act in Pten-deficient B cells. (A) Top panel: expression of phospho-PKB/Akt (top band) and total PKB/Akt (bottom band; control). Increased phosphorylation of PKB/Akt is evident in extracts of mutant B cells, and this phosphorylation is dependent on PI3K activation as determined by the addition of the PI3K inhibitors wortmannin (wort) and LY294002 (LY). Bottom panel: densitometric quantitation of phospho-PKB/Akt levels relative to total cellular PKB/Akt. (B) Top panel: expression of phospho-PKB/Akt in MZB and FOB populations. Increased phosphorylation of PKB/Akt can be seen in Western blot analyses of extracts of isolated CD21^hiCD23^lo and CD21^hiCD23^lo cells. Bottom panel: densitometric quantitation of phospho-PKB/Akt levels relative to total cellular PKB/Akt. Results shown are representative of three independent experiments.

Several lines of evidence in this study indicate that CSR is impaired in bPten^+/+ mice. First, MZB and B1 cells are important for TI responses (33, 46), but even though these populations were elevated in bPten^+/+ mice, the production of antigen-specific IgG in response to TI-II antigen was profoundly decreased. Second, bPten^+/+ MZB and FOB cells showed defective class switching at the cellular level. Third, Ig germline transcripts were intact in bPten^+/+ B cells but the expression of AID, an essential factor for CSR, was diminished. Little is presently known about the regulation of AID gene expression and the link between Ptn and AID. It may be germane that mice deficient for SHIP, a phosphatase whose substrate is also PIP3, have intact CSR (47). This result implies that the defect in bPten^+/+ mice could be CD3-independent. We are undertaking studies of the transcriptional regulation of the AID gene to address how Pten might directly or indirectly influence its expression.

MZB cells are presumed to have a critical role in host defense against bacterial pathogens (16, 17). However, in preliminary experiments, no differences were observed between bPten^+/+ and bPten^+/+ mice subjected to lethal Staphylococcus aureus infections. It is possible that, even though they have greater numbers of the anti-bacterial MZB cells, the CSR defect in bPten^+/+ B cells leads to inadequate host defense.

bPten^+/+ mice display marked elevations in B1 cell numbers and serum levels of autoantibodies, particularly those of the IgM isotype. These B1 cells were CD21^highCD23^low and even though the activation of intracellular signaling pathways mediated by PKB/Akt and Btk was intact (unpublished data), FOB cells are required to form germinal centers, and the reduction in this cell population in bPten^+/+ mice may account for the observed defect.

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and autoantibody secretion. Curiously, despite elevated levels of autoantibodies, our bPten\^{flx/flx} mice have survived for over a year without showing definite histological abnormalities characteristic of autoimmune disease. This result stands in contrast to the development of autoimmune disorders in mice heterozygous for the null Pten mutation (10) and in T cell–specific Pten–deficient mice (11). Impaired CSR may derail the onset of autoimmune disease in bPten\^{flx/flx} mice.

In conclusion, we have demonstrated that Pten deficiency alters B1, MZB, and FOB cell subsets in mice. Moreover, Pten deficiency causes an impairment of immunoglobulin isotype switching. Pten is thus an important regulator of B cell development and homeostasis in the immune system.

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