

A Crucial Role for the p110 δ Subunit of Phosphatidylinositol 3-Kinase in B Cell Development and Activation

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Abstract

Mice lacking the p110 δ catalytic subunit of phosphatidylinositol 3-kinase have reduced numbers of B1 and marginal zone B cells, reduced levels of serum immunoglobulins, respond poorly to immunization with type II thymus-independent antigen, and are defective in their primary and secondary responses to thymus-dependent antigen. p110 δ ^{-/-} B cells proliferate poorly in response to B cell receptor (BCR) or CD40 signals *in vitro*, fail to activate protein kinase B, and are prone to apoptosis. p110 δ function is required for BCR-mediated calcium flux, activation of phospholipaseC γ 2, and Bruton's tyrosine kinase. Thus, p110 δ plays a critical role in B cell homeostasis and function.

Key words: Akt • Btk • calcium • gene targeting • p110 δ

Introduction

B lymphocyte development, selection, and activation are critically dependent on signal transduction events mediated by the B cell antigen receptor (BCR)* (1). The BCR is tightly associated with nonpolymorphic subunits, CD79a and b, which contain immunoreceptor tyrosine-based activation motifs. Immunoreceptor tyrosine-based activation motifs serve to recruit tyrosine kinases of the Src and Syk families that initiate the signal transduction cascade by phosphorylation of multiple substrate proteins (2).

A number of studies have provided evidence for a role for phosphatidylinositol 3-kinase (PI3-K) and the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP-3)

in BCR signal transduction. PI3-K is activated downstream of the BCR for antigen (3, 4) and the CD19 coreceptor (5, 6). The class IA PI3-K's consist of three catalytic subunits p110 α , β , and δ that are encoded by distinct genes and interact with a family of adaptor proteins that regulate location and enzyme activity (7). Mice deficient in the p85 α or p85/p55/p50 α adaptor proteins (8, 9) of PI3-K display an immune defect similar to *xid* mice in which the Pleckstrin homology domain of Bruton's tyrosine kinase (Btk) can no longer bind PIP-3 (10). Btk, a member of the TEC family of tyrosine kinases, is thought to be a major effector of PI3-K in B cells. Btk acts, in part, to regulate calcium flux through the phosphorylation and activation of phospholipaseC γ 2 (PLC γ 2; reference 11). The regulation of Btk membrane association and activation by phosphoinositides plays an important role in regulating B cell responses (12).

B cells from mice deficient in the breakdown of PIP-3, such as those lacking the SH2 domain-containing inositol polyphosphate 5'-phosphatase 1, display accelerated development and are hyperresponsive to BCR stimulation (13–15). Furthermore, mice deficient in the phosphatase and tensin

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*Abbreviations used in this paper: BCAP, B cell adapter for PI3-K; BCR, B cell antigen receptor; Btk, Bruton's tyrosine kinase; IP3, inositol 3,4,5 trisphosphate; MZ, marginal zone; PI3-K, phosphatidylinositol 3-kinase; PIP-3, phosphatidylinositol 3,4,5 trisphosphate; PKB, protein kinase B; PLC γ 2, phospholipaseC γ 2; TD, thymus-dependent antigen; RF, recirculating follicular.

homolog gene that encodes a PIP-3 3' phosphatase develop B cell hyperplasia, lymphoma, and hypergammaglobulinaemia (16).

Each of the three class IA catalytic subunits of PI3-K are expressed in B cells, however their relative roles in B cell development and function are unknown. Mutant mice lacking p110 α (17) and p110 β (18) have previously been reported to have lethal phenotypes that precluded analysis of immune cell development and function. p110 δ is the most recently identified PI3-K catalytic subunit with expression reported to be highest in hematopoietic cells (19, 20). We have used gene-targeting to generate mice that lack p110 δ function. Our analysis of B cell development in these mice revealed an essential role for p110 δ in the development of B1 and marginal zone (MZ) B cells. Furthermore, B cell responses to thymus-dependent and -independent antigens required p110 δ function. Analysis of BCR signal transduction revealed an important role for p110 δ in the regulation of proliferation and calcium flux. These defects can be attributed to a failure to activate protein kinase B (PKB) and Btk.

Materials and Methods

Generation of p110 δ Knockout Mice. The structure of murine *pik3cd* genomic clones isolated from a 129/Sv genomic library has been described previously (21). The targeting vector consists of LoxP flanked neomycin and hygromycin-resistance cassettes cloned 7.5 kb apart into the EcoRV and XhoI sites respectively of the *pik3cd* genomic clone. This strategy was adopted in an attempt to generate a conditional allele of *pik3cd*. The targeting vector was transfected into PC3 mouse embryonic stem cells (22) and analyzed for the targeting event using Southern blotting of KpnI-digested DNA with probe A (a 700-bp EcoRI fragment). Correctly targeted clones were injected into blastocysts to produce chimaeric mice. The resulting chimaeras, which express the Cre enzyme in the male germline (22), were bred to obtain lines of mice harboring a *pik3cd* gene which had undergone Cre-mediated recombination and thus deleted exons 1–9 encoding the first 490 amino acids of p110 δ . These were detected by Southern blot analysis using KpnI-digested DNA and by PCR. All mice were bred at the Babraham Institute Small Animal Barrier Unit (SABU) and housed according to UK Home Office guidelines under project licence 80/1263. p110 δ -deficient mice were born in normal Mendelian ratios from heterozygous intercrosses and were fertile and healthy under SPF conditions.

Measurement of PIP-3 Levels. Lipid extracts were assayed from stimulated B cells after precipitation with 0.5 M trichloroacetic acid. A time resolved fluorescence resonance energy transfer ligand displacement assay was performed using the general receptor for phosphoinositides-1 Pleckstrin homology domain as a PIP-3-specific binding protein (23) (unpublished data).

Immunoprecipitation and Western Blot Analysis. Purification of splenic B cells, immunoprecipitation and Western blotting were performed using previously described methods (24). B cell purity was around 95% as assessed by flow cytometry of lymphocytes (unpublished data). Antibodies to Btk provided by V. Tybulewicz (National Institute for Medical Research, London, UK), antisera against amino acids 74–89 of murine p110 δ and against the COOH-terminal 20 amino acids of murine PLC γ 2 were from (Babraham Technix), anti-Vav-1 (24), phospho-Btk (25), have been described previously. Phospho-Ik β α , phospho-PKB, and

pan-PKB were from Cell Signaling Technology, p110 α (H-201), p110 β (S-19) Ik β α (C-21) were from Santa Cruz Biotechnology, Inc., anti-Bcl-XL was from BD Transduction Laboratories, anti-p85 α was from Upstate Biotechnology, anti-PLC γ 2 PY 759 antibody will be described previously (unpublished data).

Immunofluorescence Staining of Tissue Sections. Spleens were harvested and immediately frozen by dipping in liquid nitrogen. Spleens were mounted in OCT and 8- μ m sections were cut, air-dried, and stored at -20°C until use. Sections were fixed in ice-cold acetone for 15 min, air-dried briefly, rehydrated in PBS and then blocked with 5% normal rat serum for 15 min. Sections were stained with FITC-conjugated MOMA-1 antibody (rat IgG2a; Serotec), and biotinylated anti-IgM (clone R6-60.2, rat IgG2a; BD BioSciences), or control FITC-conjugated rat IgG2a (BD BioSciences) for 1 h at room temperature. Sections were washed in PBS and stained with a 1:200 dilution of streptavidin-TRITC (Jackson ImmunoResearch Laboratories) for 1 h. Sections were washed and mounted in Aqua PolyMount (Polysciences, Inc.) and viewed with an Olympus BX-40 epifluorescence microscope using appropriate filters. Images were digitally captured using a high-resolution CCD camera (F-View) using analySIS[®] software (SIS, GmbH) and processed using Adobe Photoshop[®] v.7.0.

Antibody Responses. Staining of cells with fluorescent antibodies was performed as described previously (24). All antibodies were purchased from BD PharMingen except anti-IgM-Cy5 and anti-IgD-PE (Jackson ImmunoResearch Laboratories). Serum Igs in naive mice were determined by ELISA using antibodies purchased from BD PharMingen. For immune responses, 8-wk-old mice were injected intraperitoneally with either 5 μ g DNP-Ficoll in a solution of PBS or 50 μ g DNP conjugated to KLH in a solution of PBS. Serum antibody levels were determined by ELISA as described previously (24). The relative units of DNP-specific antibodies are shown as optical density values. A dilution series of the serum samples was measured and for each isotype a single dilution factor which fell in the linear part of the curve is represented for all time points. Serum dilution factors were as follows: thymus-independent responses 1:800 for IgM and 1:800 for IgG₃. For thymus-dependent responses, the dilutions were 1:800 for IgM, IgG_{2b}, and IgG₃, 1:1,600 for IgG₁, and 1:200 for IgG_{2a}.

B Cell Proliferation and Apoptosis Assays. Purified B cells were cultured for 72 h at an initial concentration of 10^6 cells per milliliter with the indicated doses of polyclonal anti-IgM or monoclonal anti-IgM (clone B7.6), monoclonal anti-CD40 (clone 3/23) and recombinant murine IL-4 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. Proliferation was measured by incorporation of [³H]thymidine following a 16-h pulse. For analysis of apoptosis, B cells were cultured in the above media but without antibodies or IL-4 and apoptosis determined by flow cytometry of permeabilized cells stained with propidium iodide as described previously (26).

Calcium Flux Analysis. Purified splenic B cells were loaded for 30 min at RT in the dark with 3 μ M Fluo-4 a.m. (Molecular Probes) at a density of 6×10^6 cells per milliliter in 0.5% BSA/PBS. The cells were washed in indicator free medium and then resuspended at 3×10^6 cells per milliliter in 0.5% BSA/PBS containing 1 mM CaCl₂. After a further incubation of 30 min to allow complete deesterification of intracellular Fluo-4 a.m. ester, the variations in absorbance were measured using a Perkin-Elmer LS55 Luminescence Spectrometer. [Ca²⁺]_i was calculated as described previously (27).

Results

Impaired BCR Stimulated PIP-3 Production in the Absence of p110 δ . To address the function of p110 δ in B cells, we used gene targeting to produce p110 δ null mice (Fig. 1, A and B). Thymocytes and B cells from these mice lacked p110 δ protein (Fig. 1 C and unpublished data). B cells from control and mutant mice expressed similar levels of p110 α and p110 β catalytic subunits, but showed a small reduction in the levels of the p85 α and P55/p50 α adaptor subunits (Fig. 1 D). To determine the impact of this mutation on BCR-induced PIP-3 production we employed a novel assay that permits determination of PIP-3 levels in primary cells, without the need for biosynthetic labeling. We observed that BCR-stimulated B cells from p110 δ -deficient mice produced little PIP-3 (Fig. 1, E and F). In control B cells, PIP-3 levels peaked after 1 min of BCR stimulation and returned to baseline by 10 min, in mutant mice no increases in PIP-3 levels were observed within 10 min of stimulation (Fig. 1 F). These data indicate p110 δ was responsible for most of the BCR-induced PIP-3 production.

B1 and MZ B Cells Require p110 δ . Flow cytometric analysis of the bone marrow of p110 δ ^{-/-} mice did not reveal any major blocks in B cell development (Table I). However, we observed a marked reduction in the B1 subset resident in the peritoneal cavity (Fig. 2 A and Table I). Furthermore, CD21^{hi} CD23^{lo} MZ B cells of the spleen were also significantly reduced in number (Fig. 2 B and Table I). This conclusion was further supported by noting the absence of the splenic B220⁺ CD1^{high} population in the mutants (unpublished data). Examination of frozen splenic tissue sections stained with anti-IgM and anti-MOMA-1 (which stains MZ metallophilic-macrophages), revealed that in the p110 δ mutant there were very few B cells beyond the marginal sinus (delineated by MOMA-1 staining), supporting the conclusion that the MZ B cell population was severely reduced. The number of conventional B2 B cells was significantly reduced in young p110 δ ^{-/-} mice (Table I). By contrast, older p110 δ ^{-/-} mice had only marginally reduced numbers of B2 cells (Table I). Subdivision

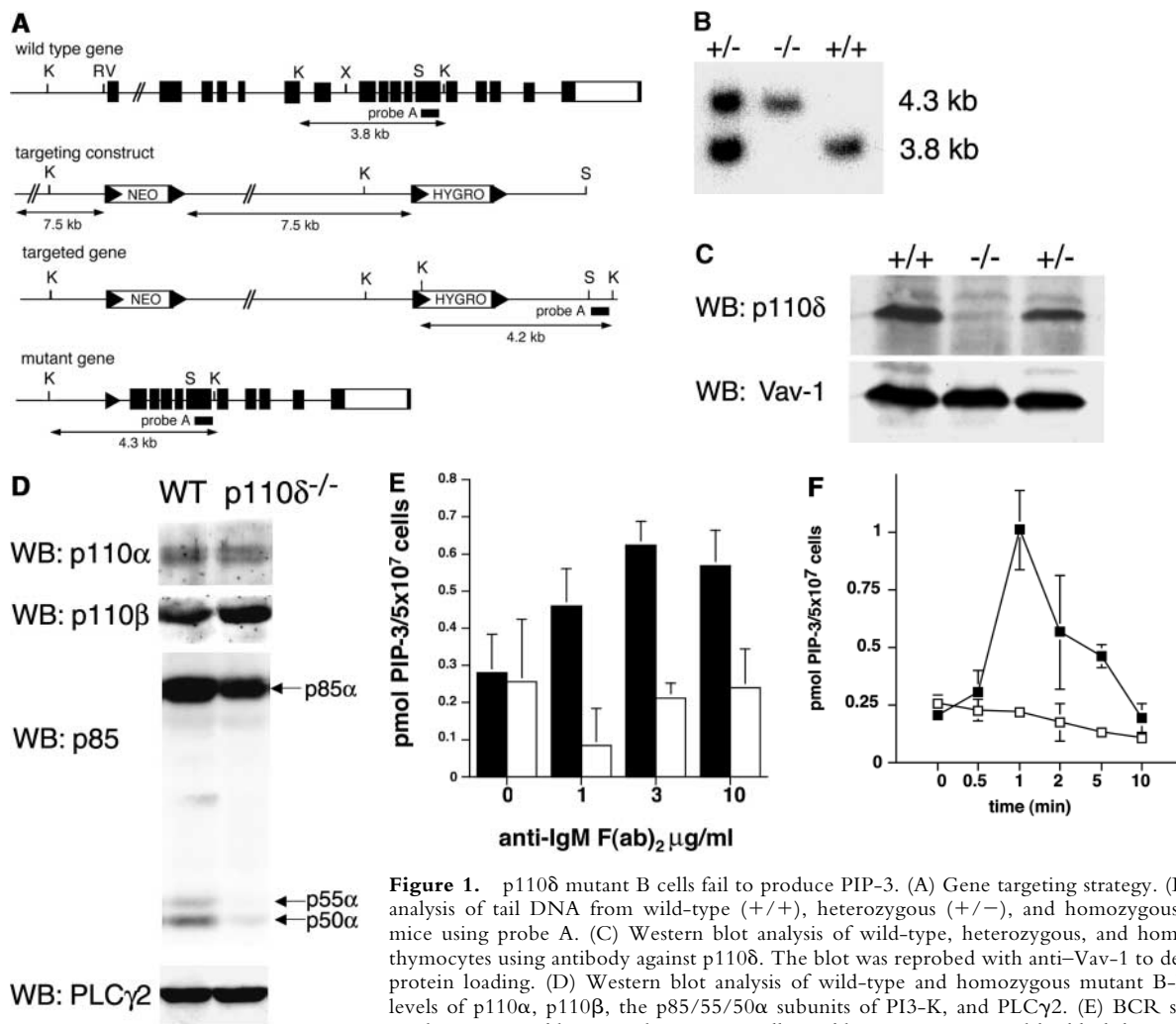


Figure 1. p110 δ mutant B cells fail to produce PIP-3. (A) Gene targeting strategy. (B) Southern blot analysis of tail DNA from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) mutant mice using probe A. (C) Western blot analysis of wild-type, heterozygous, and homozygous mutant thymocytes using antibody against p110 δ . The blot was reprobbed with anti-Vav-1 to demonstrate equal protein loading. (D) Western blot analysis of wild-type and homozygous mutant B-lymphocytes for levels of p110 α , p110 β , the p85/55/50 α subunits of PI3-K, and PLC γ 2. (E) BCR stimulated PIP-3 production in wild-type and mutant B cells. Wild-type is represented by black bars, mutant by white bars. (F) Time course of PIP-3 production in wild-type and mutant B cells stimulated with 10 μ g/ml anti-IgM F(ab)₂. In E and F, error bars represent the variance of triplicate determinations.

of splenic B cells using IgM and IgD staining revealed that p110δ^{-/-} mice had near normal numbers of IgM^{lo} IgD^{hi} B cells, which are mature recirculating follicular (RF) B cells (Fig. 2 B and Table I). The level of surface IgM staining on follicular B cells was not different between control and mutants mice (Fig. 2 B). Thymocyte development, and the number of CD4⁺, CD8⁺ T cells, and Mac1⁺ macrophages in the spleens of P110δ^{-/-} mice were not different from littermate controls (Table I and unpublished data).

Impaired Antibody Responses in p110δ^{-/-} Mice. In non-immunized P110δ^{-/-} mice the levels of serum IgM, IgG₁, IgG_{2a}, and IgG₃ and were significantly reduced, whereas IgG_{2b} and IgA were in the normal range (Fig. 3 A). To determine the ability of p110δ^{-/-} mice to mount humoral responses, we immunized p110δ^{-/-} mice with the T cell-independent type II antigen, DNP-Ficoll. The hapten-specific IgM response of p110δ^{-/-} mice after immunization was significantly reduced when compared with that of control

mice, similarly, the levels of DNP-specific IgG₃ produced were significantly lower in p110δ^{-/-} mice (Fig. 3 B). The primary response to thymus-dependent (TD) antigens was measured 7 d after administration of DNP-KLH. Wild-type littermate mice produced IgM, IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ antibodies against DNP (Fig. 3 C). By contrast, p110δ^{-/-} mice produced significantly less anti-DNP antibody of these subclasses. By 21 d after the primary immunization antigen specific Ig levels had generally fallen and reimmunization of control animals with DNP-KLH led to a secondary response. Although mice lacking p110δ produced antigen-specific Ig, there was a significant defect in the production of antigen-specific Ig of all subclasses tested (Fig. 3 C). Analysis of germinal center formation in the spleen after immunization revealed a reduction in the p110δ^{-/-} mice (Fig. 3 D). Those germinal centers that were found were often atypical in size or location (unpublished data). This defect was underscored by a reduction in the number of Peyer's patches on the small intestine from 7.75 ± 1.7 in control mice (*n* = 4) to 4 ± 1.2 in p110δ^{-/-} mice (*n* = 4). Furthermore, the Peyer's patches were smaller in the mutant mice and staining of B lymphocytes with GL7, an activation antigen expressed on germinal center B cells, also revealed a defect in p110δ^{-/-} mice (Fig. 3 E).

Impaired In Vitro Responses of p110δ^{-/-} B Cells. Purified splenic B cells from p110δ-deficient mice proliferated poorly in response to in vitro stimulation with polyclonal anti-IgM antibody, which is a potent B cell mitogen (Fig. 4 A). Supplementation of the media with IL-4 enhanced proliferation in both wild-type and p110δ^{-/-} B cells. p110δ-deficient B cells were also poorly responsive to combinations of monoclonal anti-IgM, CD40, and IL-4 that elicited high levels of proliferation in control B cells (Fig. 4 B). By contrast the proliferative response of the mutant B cells to LPS was normal (Fig. 4 B). PKB is a PI3-K effector that has been shown to be involved in growth control and the suppression of apoptosis (28). The activation of PKB is an early, dose dependent, event after BCR engagement in normal B cells, however we found PKB activation was severely impaired in B cells from p110δ^{-/-} mice (Fig. 4 C). While some PKB phosphorylation was evident in mutant B cells at the highest doses of antibody used for BCR stimulation, this phosphorylation was not sustained (Fig. 4 D), suggesting PKB was only weakly and transiently activated. When B cells from p110δ^{-/-} mice were cultured in serum containing media, the spontaneous level of apoptosis was increased when compared with wild-type (Fig. 4 E) suggesting survival pathways were defective. Survival signaling through the BCR requires the activation of NF-κB and subsequent induction of the prosurvival bcl-2 family member Bcl-xL (29). NF-κB activation requires the phosphorylation of a cytoplasmic inhibitor (IκB) by the multiprotein (IκB-kinase) IKK complex. This targets IκB for ubiquitination and subsequent degradation and permits nuclear translocation of the transcription factor (30). We measured activation of IKK after BCR stimulation using antibodies specific to phosphorylated serine 32 of IκBα. In control B cells there was a transient induction of phosphor-

Table I. Lymphocyte Populations in p110δ^{-/-} Mice

Tissue/cell type	Control	p110δ ^{-/-}
Bone Marrow ^a		
Fraction A–C	8.6 × 10 ⁵ (2.7)	7.5 × 10 ⁵ (1.5)
Fraction D	4.1 × 10 ⁶ (1.3)	3.1 × 10 ⁶ (0.8)
Fraction E	9.6 × 10 ⁵ (4.0)	7.2 × 10 ⁵ (1.4)
Fraction F	6.6 × 10 ⁵ (2.4)	9.6 × 10 ⁵ (4.2)
Spleen (8–10 wk old)		
Large Mac1 ⁺	1.5 × 10 ⁶ (0.2)	1.2 × 10 ⁶ (0.3)
CD4 ⁺	2.5 × 10 ⁷ (0.5)	2.5 × 10 ⁷ (0.9)
CD8 ⁺	1.3 × 10 ⁷ (0.3)	1.1 × 10 ⁷ (0.4)
CD21^{hi} CD23^{lo}	3.7 × 10⁶ (0.8)	0.6 × 10⁶ (0.05)^b
B220 ⁺	4.4 × 10 ⁷ (1.3)	3.1 × 10 ⁷ (0.7)
IgM ^{hi} IgD ^{lo}	3.6 × 10 ⁶ (1.1)	2.6 × 10 ⁶ (0.8)
IgM ^{hi} IgD ^{hi}	1.1 × 10 ⁷ (0.3)	1.1 × 10 ⁷ (0.2)
IgM ^{lo} IgD ^{hi}	2.6 × 10 ⁷ (0.8)	1.6 × 10 ⁷ (0.4)
Spleen (21-d old)		
IgM^{hi} IgD^{lo}	2.7 × 10⁶ (0.8)	10⁶ (0.1)^c
IgM^{hi} IgD^{hi}	9 × 10⁶ (1.4)	2.2 × 10⁶ (0.4)^c
IgM^{lo} IgD^{hi}	3.2 × 10⁶ (0.2)	0.8 × 10⁶ (0.2)^d
Peritoneum		
B1 total	10⁶ (0.2)	0.08 × 10⁶ (0.02)^c
B1a	2.4 × 10⁵ (0.2)	0.1 × 10⁵ (0.02)^c
B1b	1.9 × 10⁵ (0.3)	0.07 × 10⁵ (0.01)^c
B2	5.7 × 10 ⁵ (2.9)	4.9 × 10 ⁵ (3.6)

Values are given as mean with SD in parenthesis. *n* = 9 for determination of B cell numbers in the spleen of adult mice; *n* = 4 for all other determinations, except the mutant peritoneum where *n* = 3.

^aFractions assessed using the criteria of Hardy. Significant differences are highlighted in bold, significance assessed by Student's *t* test.

^b*P* < 0.02.

^c*P* < 0.001.

^d*P* = 0.02.

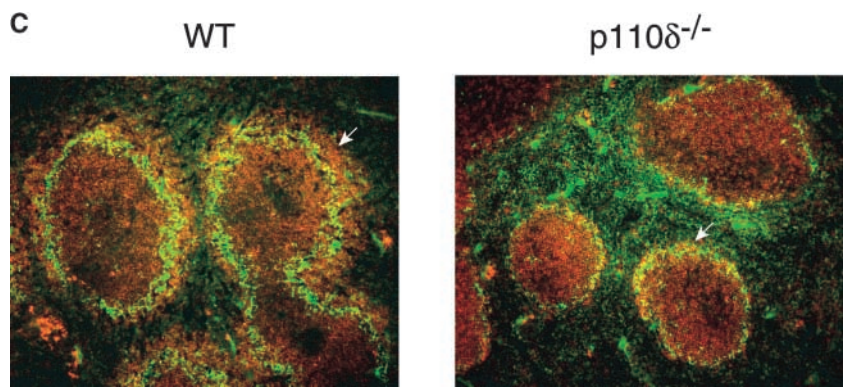
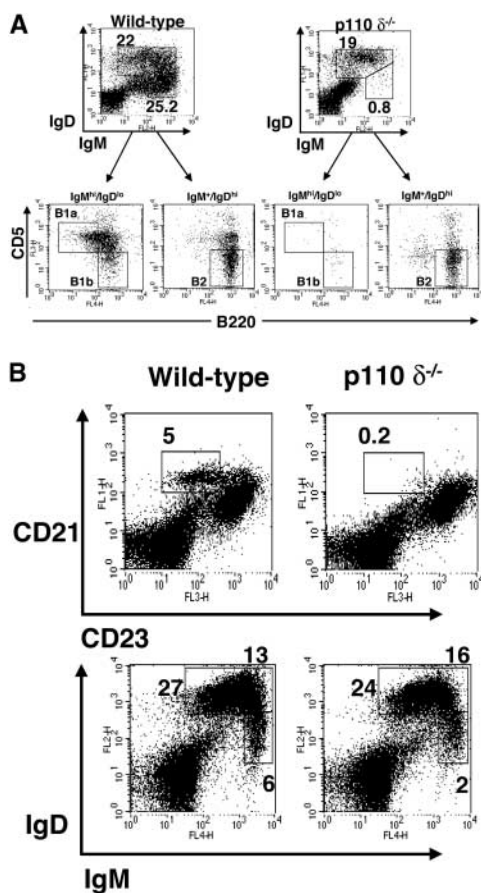


Figure 2. Lack of B1 and MZ B cells. (A) FACS[®] plot of scatter gated peritoneal cells stained with the indicated combinations of monoclonal antibodies. The gates in the top panel were used to generate the plots in the bottom panels which reveal the B1a, B1b, and B2 subsets. A proportion of B cells in the wild-type are B220^{high} CD5⁺, which are also missing in the mutant. (B) Splenic cells were stained with the indicated combinations of mAbs to reveal MZ and follicular B cell profiles. Numbers indicate the percentage of lymphocytes falling within the gate. These gates were used to calculate the numbers of cells in Table I. (C) Immunofluorescence performed on splenic cryosections using MOMA-1 (green) and anti-IgM (red) revealed a marked reduction in MZ B cells in p110 $\delta^{-/-}$ mice (right). The position of MZ B cells is arrowed. The results shown are representative of four mutant spleens examined. Original magnification: 200 \times .

ylation of $\text{I}\kappa\text{B}\alpha$ that was blocked by the PI3-K inhibitor wortmannin (Fig. 4 F). B cells from p110 δ -deficient mice did not show detectable phosphorylation of $\text{I}\kappa\text{B}\alpha$ after BCR stimulation (Fig. 4 F). Bcl-xL levels in freshly isolated B cells from mutant mice were lower than control cells (Fig. 4 G). Furthermore, when cultured for 24 h in the presence of antibodies to the BCR, Bcl-xL levels increased in control but not in p110 δ -deficient B cells (Fig. 4 G).

p110 δ Is Required for Normal Function of Btk and PLC γ 2. The mobilization of intracellular calcium (Ca^{2+}) after the addition of antibodies against IgM was reduced in p110 $\delta^{-/-}$ B cells at all doses of agonist tested (Fig. 5 A). This was evident in both the initial peak response and the sustained response. To further evaluate the level at which calcium mobilization was impaired we measured BCR induced production of inositol 3,4,5 trisphosphate (IP3), the second messenger that triggers calcium release from intracellular stores. BCR stimulation of IP3 production was reduced in p110 $\delta^{-/-}$ B cells (Fig. 5 B). This observation could reflect defective activation of PLC γ 2, as this lipase requires phosphorylation by Btk, a tyrosine kinase whose activity is dependent on PIP-3. Therefore, we measured the phosphorylation and activation of Btk using phosphorylation-site specific mAbs. In control mice Btk was phosphorylated on tyrosine 551 in response to BCR cross-linking (Fig. 5 C). This reflects phosphorylation in trans by Src and Syk family kinases (31–33). By contrast, phosphoryla-

tion of tyrosine 551 was not detected in p110 δ -deficient B cells. Tyrosine 223 is a major autophosphorylation site of Btk, its phosphorylation can thus be used as an indicator of whether Btk has become activated. B cells from p110 $\delta^{-/-}$ mice showed no detectable phosphorylation of Btk at position 223. These results suggest that Btk activation requires the function of p110 δ . Within PLC γ 2 tyrosines 753 and 759 have been identified as important Btk substrates (34, 35). To establish whether PLC γ 2 was phosphorylated by Btk on tyrosine 759 we employed a phosphospecific mAb. BCR stimulation of control B cells induced phosphorylation of PLC γ 2 on tyrosine 759, however there was little phosphorylation of this residue in stimulated B cells from p110 δ -deficient mice.

Discussion

B cells from mice deficient in p110 δ produce little PIP-3 after BCR engagement. This finding indicates that p110 δ is mainly responsible for the bulk of PIP-3 production downstream of the BCR and that the p110 α and β subunits, although expressed at normal levels in p110 δ -deficient B cells, cannot compensate for loss of p110 δ . This suggestion is further substantiated by the observations that the well characterized PI3-K effectors Btk and PKB are not, or only weakly, activated in response to BCR stimulation in p110 δ -deficient B cells. Our results do not exclude roles for

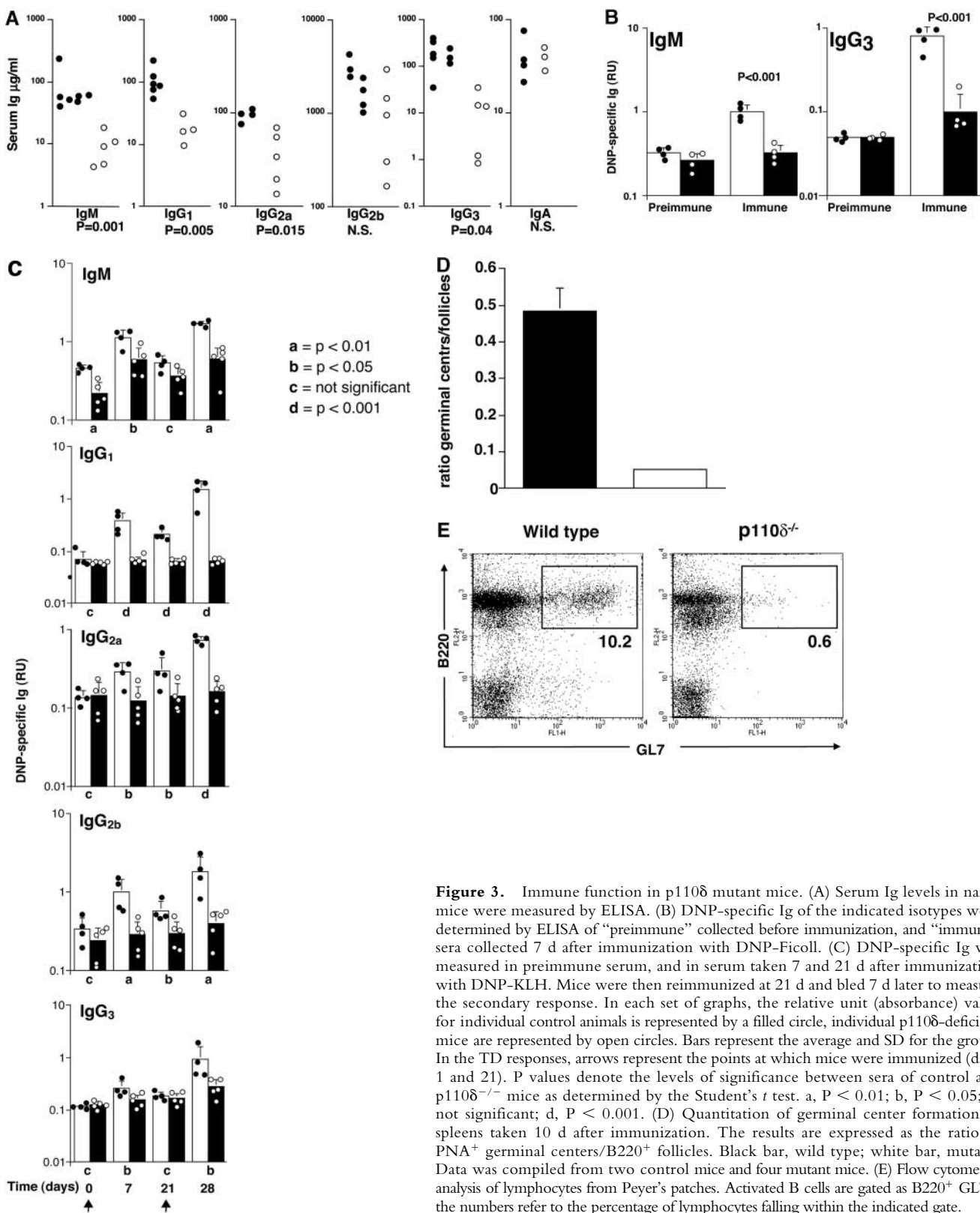


Figure 3. Immune function in $p110\delta$ mutant mice. (A) Serum Ig levels in naive mice were measured by ELISA. (B) DNP-specific Ig of the indicated isotypes were determined by ELISA of “preimmune” collected before immunization, and “immune” sera collected 7 d after immunization with DNP-Ficoll. (C) DNP-specific Ig was measured in preimmune serum, and in serum taken 7 and 21 d after immunization with DNP-KLH. Mice were then reimmunized at 21 d and bled 7 d later to measure the secondary response. In each set of graphs, the relative unit (absorbance) value for individual control animals is represented by a filled circle, individual $p110\delta$ -deficient mice are represented by open circles. Bars represent the average and SD for the group. In the TD responses, arrows represent the points at which mice were immunized (days 1 and 21). P values denote the levels of significance between sera of control and $p110\delta^{-/-}$ mice as determined by the Student's t test. a, $P < 0.01$; b, $P < 0.05$; c, not significant; d, $P < 0.001$. (D) Quantitation of germinal center formation in spleens taken 10 d after immunization. The results are expressed as the ratio of PNA⁺ germinal centers/B220⁺ follicles. Black bar, wild type; white bar, mutant. Data was compiled from two control mice and four mutant mice. (E) Flow cytometric analysis of lymphocytes from Peyer's patches. Activated B cells are gated as B220⁺ GL7⁺, the numbers refer to the percentage of lymphocytes falling within the indicated gate.

the $p110\alpha$ and β subunits in BCR signal transduction because weak phosphorylation of PKB could be detected in $p110\delta^{-/-}$ B cells after high levels of BCR cross-linking. Furthermore, the PI-3-K inhibitor wortmannin was able to

mediate additional inhibition of BCR-stimulated calcium flux in $p110\delta^{-/-}$ B cells (unpublished data). Our measurements of total cell PIP-3 do not take account of small, highly localized, concentrations of PIP-3 that may be gen-

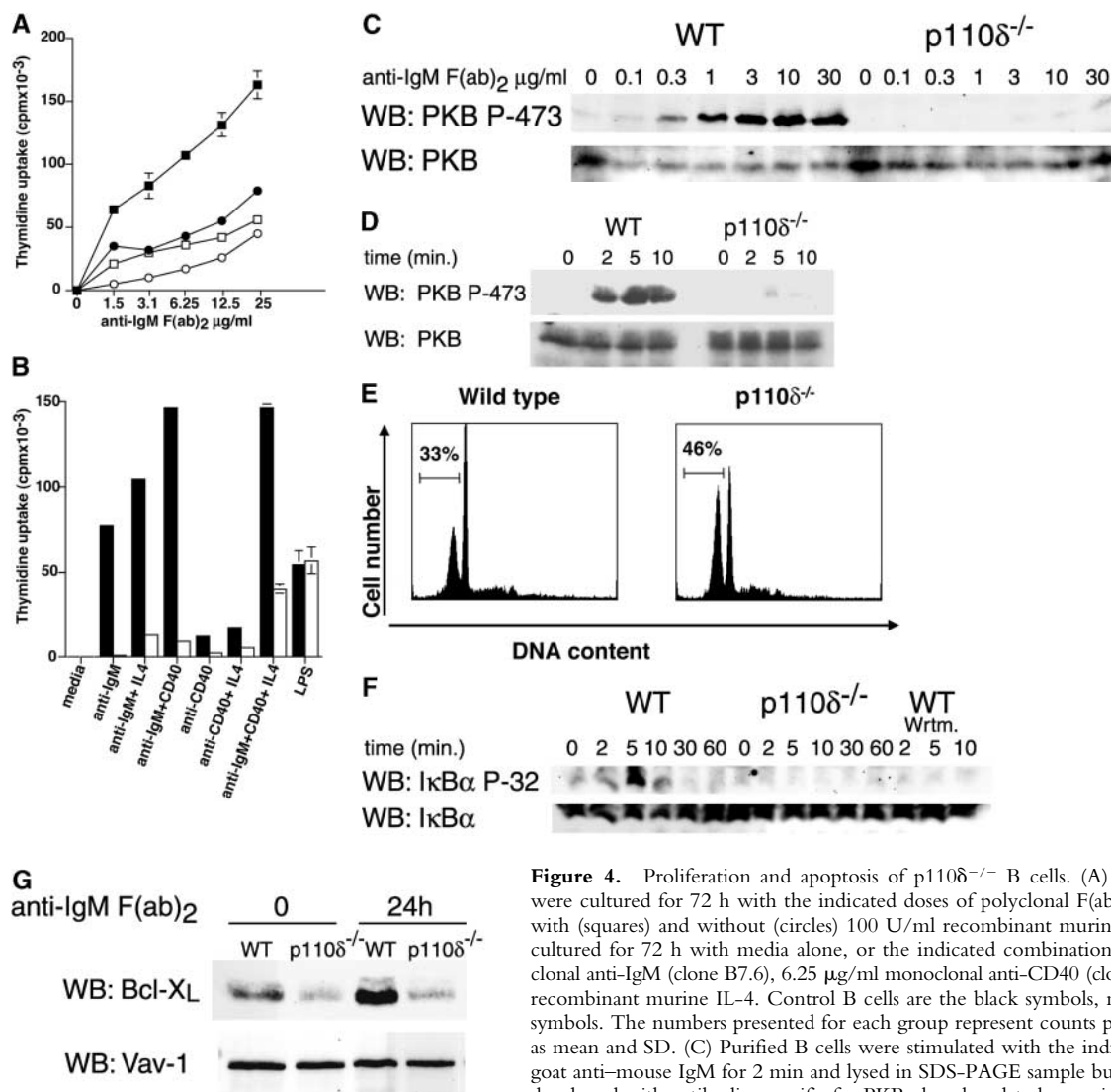


Figure 4. Proliferation and apoptosis of p110δ^{-/-} B cells. (A) Purified splenic B cells were cultured for 72 h with the indicated doses of polyclonal F(ab)₂ goat anti-mouse IgM with (squares) and without (circles) 100 U/ml recombinant murine IL-4. (B) B cells were cultured for 72 h with media alone, or the indicated combinations of 6.25 μg/ml monoclonal anti-IgM (clone B7.6), 6.25 μg/ml monoclonal anti-CD40 (clone 3/23), and 100 U/ml recombinant murine IL-4. Control B cells are the black symbols, mutant B cells the white symbols. The numbers presented for each group represent counts per minute (cpm) plotted as mean and SD. (C) Purified B cells were stimulated with the indicated amounts of F(ab)₂ goat anti-mouse IgM for 2 min and lysed in SDS-PAGE sample buffer. Western blots were developed with antibodies specific for PKB phosphorylated on serine 473 then stripped and reprobed with a pan-PKB antibody (Cell Signaling Technology). (D) Time course of PKB phosphorylation on serine 473 after stimulation with 10 μg/ml F(ab)₂ goat anti-mouse IgM. (E) B cells were cultured for 24 h in RPMI 1640 plus 10% serum and apoptotic cells identified by flow cytometric analysis of DNA content using propidium iodide staining. Data are representative of B cells from three mice of each genotype. (F) Defective IκBα serine 32 phosphorylation, B cells were stimulated as in D and whole cell lysates blotted with phosphospecific antibody, top panel, the blot was then stripped and reprobed with antibody to IκBα. On the right-hand side of the panel wild-type B-lymphocytes were treated with 100 nM wortmannin (Wrtm.) before stimulation. (G) Bcl-xL levels were determined in freshly isolated B cells and in B cells that had been stimulated for 24 h with 20 μg/ml F(ab)₂ goat anti-mouse IgM.

phosphorylation on serine 473 after stimulation with 10 μg/ml F(ab)₂ goat anti-mouse IgM. (E) B cells were cultured for 24 h in RPMI 1640 plus 10% serum and apoptotic cells identified by flow cytometric analysis of DNA content using propidium iodide staining. Data are representative of B cells from three mice of each genotype. (F) Defective IκBα serine 32 phosphorylation, B cells were stimulated as in D and whole cell lysates blotted with phosphospecific antibody, top panel, the blot was then stripped and reprobed with antibody to IκBα. On the right-hand side of the panel wild-type B-lymphocytes were treated with 100 nM wortmannin (Wrtm.) before stimulation. (G) Bcl-xL levels were determined in freshly isolated B cells and in B cells that had been stimulated for 24 h with 20 μg/ml F(ab)₂ goat anti-mouse IgM.

erated (36). Methodological limitations preclude our measuring the spatio-temporal accumulation of PIP-3 in primary B cells, but such measurements will greatly increase our understanding of PI3-K function in B cell signaling. We have also noted a difference in B cell phenotype between p110δ-deficient mice and mice deficient in the p85α or p85/p55/p50α adaptor subunits of PI3-K (8, 9). Unlike p85α and p85/p55/p50α-deficient mice, the spleens of adult p110δ-deficient mice contain normal numbers and proportions of IgM^{lo} IgD^{hi} RF B cells. These observations presumably reflect the participation of the p110α or β subunits, coupled via the p85α adaptor, into a pathway that regulates the maturation of RF B cells. Alternatively, there may exist additional functions for the p85α subunit that are

independent of the catalytic subunits. The functional inter-relationships between the catalytic and regulatory subunits are poorly understood in complex systems, as exemplified by the phenotype of P85α-deficient mast cells. These cells display reduced expression of p110α but normal levels of p110β and δ (37). Intriguingly, c-kit signaling is impaired in P85α-deficient mast cells, whereas signaling through the high affinity IgE-receptor (that is also dependent on PI3-K activation) is normal (37, 38). Taken together with studies using manipulated cell lines (39), these data argue for selective roles for the individual p110 catalytic subunits.

Our results show that p110δ is required for the development and/or survival of the B1 and MZ B cell subsets. A number of previous studies have highlighted similarities be-

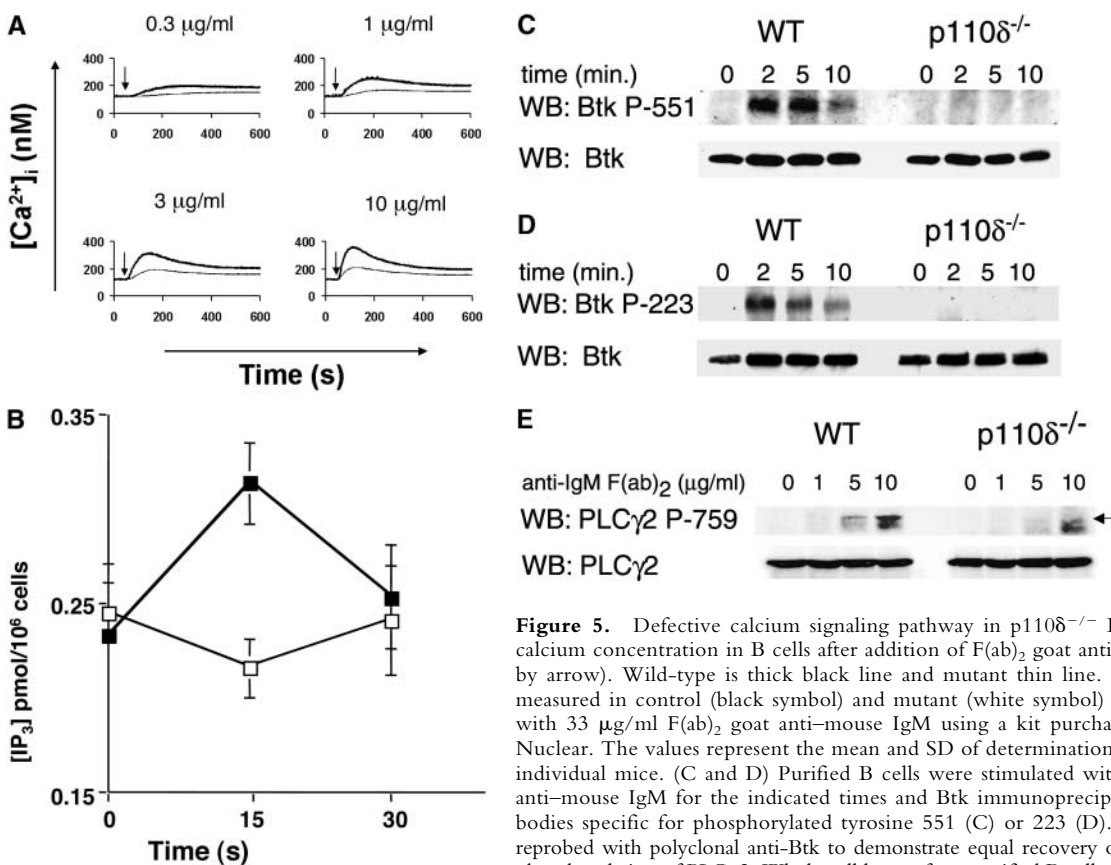


Figure 5. Defective calcium signaling pathway in $p110\delta^{-/-}$ B cells. (A) Intracellular calcium concentration in B cells after addition of $F(ab)_2$ goat anti-mouse IgM, (indicated by arrow). Wild-type is thick black line and mutant thin line. (B) IP_3 production was measured in control (black symbol) and mutant (white symbol) B cells after stimulation with $33 \mu\text{g/ml}$ $F(ab)_2$ goat anti-mouse IgM using a kit purchased from New England Nuclear. The values represent the mean and SD of determinations from B cells from four individual mice. (C and D) Purified B cells were stimulated with $10 \mu\text{g/ml}$ $F(ab)_2$ goat anti-mouse IgM for the indicated times and Btk immunoprecipitates blotted with antibodies specific for phosphorylated tyrosine 551 (C) or 223 (D). Blots were stripped and reprobed with polyclonal anti-Btk to demonstrate equal recovery of proteins. (E) Defective phosphorylation of PLC γ 2. Whole cell lysates from purified B cells stimulated for 1 min with the indicated amounts of $F(ab)_2$ goat anti-mouse IgM were blotted with antibody specific to phosphorylated tyrosine residue 759 of PLC γ 2. The blot was then stripped and reprobed with antisera specific to PLC γ 2 to confirm loading. The arrow indicates the position of phospho-PLC γ 2.

the indicated amounts of $F(ab)_2$ goat anti-mouse IgM were blotted with antibody specific to phosphorylated tyrosine residue 759 of PLC γ 2. The blot was then stripped and reprobed with antisera specific to PLC γ 2 to confirm loading. The arrow indicates the position of phospho-PLC γ 2.

tween these subsets, which appear to play important roles in immunity through the production of natural antibodies, and by being able to rapidly respond to antigenic challenge (40). Indeed, the ability to mount T cell-independent type II antigen responses has been attributed to MZ B cells (41). Our finding that the levels of IgM and IgG $_3$ in the serum of naive mice were significantly reduced and that antibody responses to DNP-Ficoll were severely impaired is consistent with the properties of these cells. We found that $p110\delta$ was required for the generation of normal numbers of B2 B cells, particularly in young mice, but not their maturation. Taken together with the increased tendency of $p110\delta^{-/-}$ B cells toward apoptosis this observation may reflect increased turnover of B2 cells. Confirmation of this will require further experimentation. The response to TD antigen, which is principally mediated by B2 cells, was significantly impaired in both the primary and secondary immune response. Although we observed a modest primary antigen-specific IgM response, there was no detectable class switching to IgG $_1$ and severely impaired switching to the other IgG subtypes. Consistent with this, germinal center formation was profoundly impaired. The phenotype of $p110\delta^{-/-}$ mice shows similarities with $CD19^{-/-}$ mice that also lack B1 and MZ B cells, but have apparently normal development of B2 cells which display impaired function (42–44). However, splenic B cells from $CD19^{-/-}$ mice are less severely impaired in their ability to activate PKB (45, 46) and

Btk (47) than are $p110\delta^{-/-}$ B cells. It will be interesting to determine whether CD19 employs $p110\delta$ as a signal transducer. The defective proliferation of $p110\delta^{-/-}$ B cells to anti-CD40 is also a feature shared with $CD19^{-/-}$ B cells (48) and suggests that the mutant B cells may be unable to respond to T cell help. CD40 ligation on B cells activates PI3-K (49), and CD40-mediated B cell proliferation is blocked by PI3K inhibitors or $p85\alpha$ deficiency (8, 9). Preliminary experiments suggest CD86 upregulation is also defective in $p110\delta^{-/-}$ B cells (unpublished data), therefore cognate T–B interactions required for the normal humoral response may be defective. It will also be interesting to determine whether the ability of $p110\delta^{-/-}$ T cells to provide help is defective. $p110\delta$ was not required for mitogenic responses to all stimuli, as $p110\delta^{-/-}$ B cells proliferated normally in response to LPS which also activates PI3-K (50) and requires the function of $p85\alpha$ to exert its mitogenic effect (8, 9). Our results thus suggest that catalytic subunits other than $p110\delta$ may be mediating the mitogenic LPS signal. IL-4-mediated proliferation is also sensitive to PI3-K inhibitors and requires $p85\alpha$ function (8, 9). $p110\delta$ does not appear to be required for IL-4 costimulation of proliferation induced by anti-IgM, as this was generally of a similar magnitude in both wild-type and $p110\delta^{-/-}$ B cells. Furthermore, preliminary experiments determining the ability of IL-4 to mediate survival of cultured B cells has not revealed a difference between wild-type and $p110\delta^{-/-}$ B cells (unpublished data).

PI3-K signaling has been intimately linked with cell survival in a number of systems by virtue of its ability to regulate PKB (28). p110 δ -deficient B cells were impaired in their ability to survive after in vitro culture and expressed less of the antiapoptotic protein Bcl-xL. After activation through the BCR p110 δ -deficient B cells failed to appreciably activate PKB and also displayed defective phosphorylation of I κ B α . In addition, the increased expression of Bcl-xL that follows BCR stimulation required the function of p110 δ . Taken together, these results are consistent with the suggestion that p110 δ is important for the activation of survival pathways in B cells.

A number of studies have described mice lacking components of the BCR signaling pathway that share B cell developmental phenotypes. These include *xid* mice as well as mice deficient in Btk, B cell linker protein, PLC γ 2, Vav-1/Vav-2, and B cell adaptor for PI3-K (BCAP) (51). The similarities in phenotypes between these mice, taken together with evidence of physical association, has led to the suggestion that these molecules act as a molecular machine or "signalosome" (52, 53). One model for BCR activation of calcium flux places emphasis on Btk-mediated phosphorylation and activation of PLC γ 2. Activation of Btk is dependent on PI3-K (12, 54), as PIP-3 regulates Btk function by regulating both the location (55, 56) and the catalytic activity of Btk (57). Our data are consistent with such a model as we found that Btk was not phosphorylated and activated as assessed using phosphospecific antibodies. In addition, we found PLC γ 2 phosphorylation and activation was defective in p110 δ ^{-/-} B cells. A recent study also implicated p110 δ in the regulation of PLC γ 2 function in Fc ϵ RI-stimulated RBL-2H3 cells by injecting p110 δ -specific inhibitory antibodies (58). Besides activation of Btk, additional PIP-3-mediated mechanisms may contribute to PLC γ 2 activation as PIP-3 has been reported to bind directly to and activate PLC γ 2 (58–60). Taken together, our results suggest that p110 δ is the key PI3-K catalytic subunit required for the function of the signalosome in B cells.

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