The development of mature B lymphocytes requires the combined function of CD19 and the p110δ subunit of PI3K

Dorottya Kövesdi,† Sarah E. Bell and Martin Turner*
Laboratory of Lymphocyte Signalling and Development; The Babraham Institute; Babraham, Cambridge UK
†Current address: Department of Immunology; Eötvös Loránd University; Budapest, Hungary

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Abbreviations: MZ, marginal zone; WT, wild type; FO, follicular; BAFF, B cell activating factor of the TNF family; rag1/2, recombination activating genes; PI3-K, phosphoinositide 3 kinase

Introduction
The generation of mature B lymphocytes bearing a B-cell receptor (BCR) composed of surface immunoglobulin (Ig) heavy (HC) and light (LC) chains is controlled by an ordered developmental program initiated in the fetal liver or bone marrow.1 For some B lymphocytes the final stages of development may be completed in the bone marrow, while for others it is completed in peripheral lymphoid organs such as the spleen.2

The diversity of Ig is generated during the recombination of V(D)J gene segments in the Ig HC and LC loci. V(D)J recombination is catalyzed by the RAG-1 and RAG-2 proteins, the expression of which is limited to immature B and T lymphocytes. Stringent quality control by the pre-B-cell receptor composed of nascent HC and surrogate light chains mediates a checkpoint characterized by proliferative expansion, HC allelic exclusion and subsequent LC rearrangement to generate an immature B cell.3 Within immature B cells successful pairing of HC and LC mediates a checkpoint that results in LC allelic and isotype exclusion, survival and positive selection ultimately permitting developmental progression into the pool of mature B cells. These signals which are mediated by the BCR in the absence of high-affinity ligand have been termed “tonic” signals.4

In contrast, B cells expressing autoreactive BCRs engage ligand and are negatively selected but may escape deletion through continued RAG expression and further rounds of recombination at the LC loci in a process referred to as receptor editing.5

Signal transduction pathways initiated by the B-cell antigen receptor and the related pre-B-cell receptor regulate the developmental progress and activation of B cells.1 Amongst these pathways the phosphatidylinositol-3-kinase (PI3K) pathway has been shown to be of importance. PI3Ks mediate the phosphorylation of phosphoinositides at the D-3 position of the inositol ring leading to the production of a number of products including phosphatidylinositol-3,4,5-trisphosphate (PIP3). These lipids function as second messengers often by interacting with specific domains of proteins.

The class Ia PI3-Ks consist of three catalytic subunits p110α, β and δ that are encoded by distinct genes. These interact with a family of adapter proteins (e.g., p85α, p85β) that regulate the stability, location and enzymatic activity of p110 subunits. The related class Ib PI3K p110γ is regulated by p101 and p84 adapters.6,7

The activity of all class I PI3K is directly opposed by the 3’ lipid phosphatase PTEN and modified by the 5-phosphatase SHIP. The importance of PI3K in B cell biology is evident from the observation that germline mutation of p110δ or p85α to yield a null mutation or a kinase inactive isoform of p110δ results in...
reduced numbers of B1 and marginal zone (MZ) B cells and lowered responsiveness to thymus-dependent and independent antigens.\(^9,10\) Moreover, B-cell specific conditional inactivation (using CD19Cre) of the lipid phosphate PTEN leads to a profound defect in B-cell development characterized by the expansion of MZ and B1 B cells.\(^9\) Mice with PTEN-deficient B cells have elevated numbers of IgM⁺ plasma cells and reduced numbers of switched plasma cells, consequently there is a four-fold reduction in serum IgG1 levels.\(^11,12\)

It has been proposed that PI3K regulates class switching by suppressing the expression and activity of activation induced cytidine deaminase (AID) the essential enzyme of class switching and somatic hypermutation.\(^11,12\) Furthermore, FcγRIIB, a crucial negative regulator of BCR signaling regulates PIP3 levels through recruitment of PTEN and SHIP.\(^13\)

Recently we reported that immature B cells from mice lacking p110δ failed to properly suppress the expression of the RAG genes. As a consequence these B cells inappropriately initiated LC recombination resulting in elevated levels of antibody bearing λ LC, or, in the context of a BCR transgene loss of antigen specificity.\(^14,15\) It has subsequently been reported that the PI3K pathway suppressed RAG expression by means of PKB-mediated phosphorylation of FOXO transcription factors which promote RAG gene transcription.\(^16,17\)

CD19, together with CD21, CD81 and CD225 (Leu-13) is a vital component of the B lymphocyte antigen sensing pathway.\(^18\) CD19 functions by lowering the threshold for signal transduction following BCR crosslinking and integrates complement receptor signaling with that of the antigen-specific BCR through binding of C3d to CD21. CD19 mutant mice display defects in B-cell development which are very similar to mice with mutations in p110δ.\(^19,20\) These include reduced numbers of B1 and MZ B cells and defective responses to thymus-dependent and independent antigens.

The CD19 cytoplasmic tail contains nine tyrosines which have the potential to be phosphorylated and interact with multiple signaling molecules including rac GDP-GTP exchange factors of the Vav family, src family kinases, adapter proteins such as Grb2 and 3BP2 as well as lipid kinases of which the P85 subunit of PI3K has been shown to interact with tyrosines 482 and 513 of the CD19 cytoplasmic tail.\(^21-25\) Mice harboring a CD19 transgene in which two tyrosines are mutated to phenylalanine recapitulate the action of CD19 and p110δ. B cells from the lymph nodes of p110δ/CD19 double mutant mice showed a survival defect and reduced responsiveness to pro-survival cytokine BAFF despite normal NFκB2/p100 processing and elevated levels of Bcl-2. In addition these B cells show a strong increase in RAG-1 and RAG-2 expression above the levels found in single knockouts, which further confirm the combined action of CD19 and p110δ in B cell differentiation.

### Results

**Early B-cell development is intact in p110δ⁻/⁻CD19⁻⁻ double mutant mice.** Previous studies of mice lacking CD19 or p110δ have shown that B-cell development in the bone marrow is essentially normal. However, both PI3K and CD19 have been implicated in differentiation through the pre-BCR checkpoint. Therefore we determined the proportions and numbers of proB/preBI, preBII, immature and mature recirculating B cells in the bone marrow of p110δ⁻/⁻CD19⁻⁻ double mutant mice and compared this with wild-type and CD19 or p110δ single mutants (Fig. 1A). This analysis revealed the numbers of proB/preBII, preBII and immature B cells were not significantly different between the four groups of mice (Fig. 1B). By contrast, there was an obvious deficit in the B220<sup>high</sup>/IgM<sup>med</sup> fraction which primarily consists of mature recirculating B cells. This paucity of mature cells may reflect impaired development and/or a failure of mature B cells to recirculate through the bone marrow compartment.

**Peripheral B-cell development requires the combined action of p110δ and CD19.** To examine the developmental progression of p110δ⁻/⁻CD19⁻⁻ double mutant B cells further we examined the proportions and numbers of B cell subpopulations in the spleen. During B cell development surface IgM is the first form of membrane Ig to be expressed, however, as B cells mature, IgM levels decrease and IgD is increased so that FO B cells express high levels of IgD and low levels of IgM. By contrast, MZ B cells express low levels of IgD but retain expression of high levels of surface IgM. Examination of splenic B cell populations by surface IgM and IgD expression indicated that the proportions of FO and immature/MZ B cells was much reduced in the p110δ⁻/⁻CD19⁻⁻ double mutant (Fig. 2A), while in CD19 or p110δ single mutants only the IgM<sup>med</sup>/IgD<sup>low</sup> population that contains immature and MZ B cells was reduced.

To characterize more definitively MZ and FO B cells in the spleen we used flow cytometry to exclude CD93<sup>+</sup> immature B cells. CD93<sup>+</sup> cells were further subdivided into mature FO (CD21<sup>intermediate</sup>/CD23<sup>high</sup>) and MZ (CD21<sup>high</sup>/CD23<sup>low</sup>) B cells (Fig. 2A). As expected, the proportion of MZ B cells was much reduced in the spleens of in CD19 and p110δ single mutants. We observed a significant further reduction in the MZ population in the p110δ⁻/⁻CD19⁻⁻ double mutants which corresponds to an approximately ten-fold decrease in absolute number of MZ B cells when compared to the single mutants and a 100-fold decrease in comparison with the wild-type (Fig. 2B). Furthermore, there was an approximately ten-fold reduction in the number of mature FO B cells in the double mutants compared to the single mutants.
A substantial fraction of spleen B cells are immature B cells which have recently migrated from the bone marrow and retain expression of CD93. These cells, which have been called transitional immature B cells, can be further divided using the expression of IgM and CD23 into three subsets termed T1-T3 (see Fig. 2A). The most immature subset designated T1 is defined as IgM<sup>high</sup>/CD23<sup>low</sup>; T2 cells are defined as IgM<sup>high</sup>/CD23<sup>high</sup>; and T3 cells which may be precursors of mature cells and/or a population of cells undergoing negative selection against autoantibody BCRs are IgM<sup>low</sup>/CD23<sup>high</sup>. The spleens of p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant mice contained reduced numbers of all transitional subsets when compared with wild-type or single knockout mice (Fig. 2B). Although the reduction is not significantly different, this might suggest that lack of p110<sup>δ</sup> and CD19 affects B cells at all stages of B-cell development subsequent to the generation of immature B cells in the bone marrow.

Immunofluorescence analysis of tissue sections from the spleen with antibodies to MOMA-1, which recognises MZ macrophages, and IgM confirmed the absence of the MZ B-cell subset in p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant mice (Fig. 3A). Although the B-cell follicular areas were readily visualized using antibodies to IgM and IgD, the intensity of staining was different between p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant and wild-type mice (Fig. 3A).

Staining sections with anti-IgM and anti-IgD from wild-type mice resulted in a clear distribution of MZ and FO B cells. While MZ B cells expressed very high levels of IgM and they are low for IgD (blue cells), mature FO B cells are highly positive for IgD and express lower amounts of IgM on their surface (mostly red cells). On sections from the double mutant mice staining with the same antibodies resulted in a purple colour, which means that these B cells are double positive for IgM and IgD and the expression levels of these molecules are similar (IgM<sup>high</sup>/IgD<sup>high</sup>). These results are consistent with the FACS data and demonstrate that those fully matured B cells (type I FO B cells), which are IgM<sup>low</sup>/IgD<sup>high</sup> are missing from the double mutant mice.

It was also apparent that the B-cell follicles were of smaller size in the p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant mice. To quantify this we visualized staining with anti-laminin antibody which binds to the basement membrane of follicular structures thus marking the margins of the follicle (Fig. 3A). Examination of multiple sections from three p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant mice revealed that, on average, the size of the follicular structures was reduced by approximately 70% compared to wild-type (Fig. 3B). This is consistent with the reduction in the overall numbers of B cells within the spleen.

To further analyze B-cell development at the periphery cervical, axillary and inguinal lymph nodes were dissected, mixed and analyzed. The number of B220<sup>+</sup> lymph node cells was similar between wild-type and CD19 or p110<sup>δ</sup> single mutants but was reduced by almost ten-fold in p110<sup>δ</sup>-/-CD19<sup>-/-</sup> double mutant mice (Fig. 4A and B). Further examination of these B cells revealed them to be surface IgD positive with intermediate expression of IgM.
mutant B cells had a somewhat greater tendency to die. However, culture in the presence of BAFF promoted the survival of wild-type but not the double mutant B cells (Fig. 5A). As BAFF regulation of NFκB2 processing is a necessary component of its survival signaling we examined the levels of p100 and p52 in lymph node B cells by western blotting. Lysates of p110δ−/−CD19−/− double mutant B cells contained processed NFκB2 which was at least as abundant as that found in wild-type cells. Indeed, it was apparent that the mutant cells contained less unprocessed NFκB2 p100 protein suggesting this aspect of BAFF signaling was independent of CD19 or p110δ. By contrast, the levels of c-Rel were much reduced in the same samples suggesting this component of the NFκB pathway was defective.

(Fig. 4C). Surface levels of CD23, CD24 and CD93 were similar to wild-type lymph node B cells (Suppl. Fig. 1B).

BAFF-mediated B-cell survival in p110δ−/−CD19−/− mutant mice is defective but independent of NFκB2 and Bcl-2. As a first step towards characterising the survival capacity of B cells lacking p110δ and CD19 we cultured B cells purified from lymph nodes in media alone with the pro-survival cytokine BAFF for 48 and 72 hours. For this and subsequent experiments we chose to study lymph node B cells, as this source of B cells lacks transitional and MZ populations in both wild type and p110δ−/−CD19−/− double mutant mice and appears similar between wild-type and p110δ−/−CD19−/− double mutant mice in terms of cell surface marker expression. When cultured in media alone p110δ−/−CD19−/− double mutant B cells had a somewhat greater tendency to die. However, culture in the presence of BAFF promoted the survival of wild-type but not the double mutant B cells (Fig. 5A). As BAFF regulation of NFκB2 processing is a necessary component of its survival signaling we examined the levels of p100 and p52 in lymph node B cells by western blotting. Lysates of p110δ−/−CD19−/− double mutant B cells contained processed NFκB2 which was at least as abundant as that found in wild-type cells. Indeed, it was apparent that the mutant cells contained less unprocessed NFκB2 p100 protein suggesting this aspect of BAFF signaling was independent of CD19 or p110δ. By contrast, the levels of c-Rel were much reduced in the same samples suggesting this component of the NFκB pathway was defective.
Bcl-2 is an important component of the pro-survival signal in mature B cells and bcl-2 levels increase when immature B cells undergo maturation possibly as a consequence of exposure to BAFF and via "tonic" pro-survival signals from surface immunoglobulin. Previous studies using Vav-deficient mice have implicated diminished expression of bcl-2 as a contributing factor to the survival defect of Vav-deficient B cells. Therefore we used intracellular flow cytometry with specific antibodies to examine bcl-2 expression in transitional and mature B cells from the spleen (Fig. 5C and D). T1 cells from both wild-type and p110δ−CD19− double mutant mice expressed low levels of bcl-2. Consistent with previous results the levels of bcl-2 were higher in wild-type T2, T3 and FO cells (Fig. 5D). The level of bcl-2 was even higher in p110δ−CD19− double mutant B cells. These data indicate the function of p110δ and CD19 is not required for the expression of bcl-2 during B-cell maturation. Elevated expression of bcl-2 was also observed upon examination of its steady state mRNA level using quantitative real-time PCR on cDNA prepared from p110δ−CD19− double mutant lymph node B cells. The same samples were also examined for expression of c-Rel and the pro-apoptotic BIM, which has been implicated as a gene suppressed by the PI3K pathway. These data showed that the level of c-Rel mRNA was reduced two-fold, while BIM expression was only marginally decreased in double mutant B cells relative to wild-type (Fig. 5E). Thus elevated BIM levels cannot account for the lack of mature B cells in p110δ−CD19− double mutant mice.

**CD19 and p110δ synergistically regulate Rag expression.** We have previously shown that the immature B cells which develop in the absence of p110δ fail to repress both RAG-2 and recombination at the light chain loci. It has also been demonstrated that CD19 is required for the suppression of RAG-gene expression and light chain isotype exclusion in immature B cells. To assess the effect of combined loss of p110δ and CD19 on light chain isotype exclusion we analyzed bone marrow...
To examine RAG-gene expression in p110δ-/-CD19-/- double mutant cells we used quantitative real-time PCR on cDNA prepared from MACS purified lymph node B cells. Both RAG-1 and RAG-2 mRNA were found to be elevated in p110δ and CD19 single mutant B cells (Fig. 6C). This was further enhanced for both RAG-1 and RAG-2 in p110δ-/-CD19-/- double mutant lymph node B cells (Fig. 6C). These data suggest RAG genes may continue to be expressed in double mutant B cells beyond the immature stage.

Discussion

We have shown here that peripheral B cells require the combined functions of CD19 and p110δ for their normal development. MZ and B1 B cell numbers are reduced in the absence of either signaling molecule but only in the double mutants is the size of the FO B cell compartment significantly reduced. Peripheral B cell maturation has been recognized in recent years as an active process of selection giving rise to mature B cells with distinct anatomical locations, survival properties and responses to antigen. Selection into these mature B cell subsets requires a signal from the BCR which is integrated with signals from BAFF and Notch receptors to control lineage choice.34 Continued BCR signaling together with exposure to BAFF is then required for the development and maintenance of the MZ and FO B cells subsets.35-37

The PI3K pathway plays an important role in the generation of the BCR derived survival signal.38 However the identity of the PI3K catalytic subunits and the signaling platforms upon which the BCR dependent survival signal depends has not been resolved. Our results could reflect a role for p110δ and CD19 in the BCR dependent survival signal. Several lines of evidence have already implicated CD19 in the process of B cell selection and survival.27,28 CD19-/- B cells have a shorter in vivo lifespan.39 Mice lacking CD19 together with SLP65/BASH/BLNK have an impairment of B cell development in the bone marrow, most likely as a consequence of defective signaling by the pre-BCR.40 More recently mice lacking CD19 and BCAP, an adapter protein able to interact with PI3K, were found to manifest a reduction in immature B cells in the bone marrow and a further loss of mature B cells in the spleen.41 B cell development in the bone marrow of mice deficient in p110δ and CD19 was shown to be relatively unperturbed as the size of the pre-BII and immature B cell compartments were not significantly smaller than that of wild-type mice. However all subsequent stages of B cell development were less well represented. B cells with the surface characteristics of mature cells most closely resembling IgMhigh/IgDlow type II FO cells were clearly present in p110δ-/-CD19-/- double mutant mice.2 In wild-type mice this long-lived population has been shown to develop independently of antigen and the tyrosine kinase brk, a well-characterized effector of the PI3K pathway.42 Our data further suggest that this population can develop in the absence of either CD19 or p110δ. By contrast, IgMlow/IgDhigh immature and recirculating B cells for the expression of kappa and lambda light chains (Fig. 6A and Suppl. Fig. 2). Bone marrow immature IgM+ B cells were chosen for the final conclusion as they were present in similar numbers in all four genotypes. Although there was an additional decrease in the kappa/lambda ratio in double mutant recirculating B lymphocytes the differences compared to the single mutants were not statistically significant (Suppl. Fig. 2). As this population of B cells may have been subject of selection at the periphery we can only conclude, that B cells from the p110δ or CD19 single knockouts display increased numbers of lambda positive cells yielding a decrease in the kappa/lambda ratio, but at the stage of immature B cells we could not detect any further increase in the lambda isotype in double mutant mice (Fig. 6B).
type I FO cells were difficult to identify in p110δ-/-CD19-/- double mutant mice; this is most easily appreciated from our analysis of lymph node B cells in the double mutant. Type I FO B cells may therefore require CD19 and p110δ in addition to relatively strong BCR stimulus and btk for their correct development.42

Here we have shown an elevated level of RAG gene expression in lymph node B cells that lack both CD19 and p110δ. BCR signals generated in the absence of antigen engagement, in addition to being vital for B cell survival, are important for the termination of RAG-gene expression thus ensuring allelic exclusion.28 Indeed, L-chain allelic exclusion fails when signaling components of the BCR such as the tyrosine kinases syk and lyn are missing.43,44 Previously it has been shown that pharmacological inhibition of PI3K can lead to the induction of RAG-gene expression.45 It remains unclear from our study whether RAG gene expression was extinguished and then reactivated or whether it failed to be shut
down as B cells matured. Our results which were obtained using lymph node B cells with the characteristics of mature type II FO B cells suggests the basal BCR signaling required for repression of RAG genes has both a p110δ and CD19-dependent component. The more striking phenotype in the double mutant suggests an independent role for each of these molecules.

BAFF/BAFF-R interaction activates the alternative NFκB pathway through the phosphorylation of p100 that results in its proteolytic cleavage to p52. We have demonstrated previously that B cells deficient for p110δ are defective in BAFF-mediated survival. In the experiments reported here we see a less marked effect on survival. This may be a consequence of two important differences in the way the experiment was performed. Firstly in this study, the B cells were purified by negative depletion, whereas positive selection was used in the earlier study. Secondly a more nutrient rich medium was used in this study which proved to be comparatively superior in overall cell viability of the cultured cells. However, B cells lacking both CD19 and p110δ showed an increased propensity to die when cultured in vitro even in the presence of BAFF. We have also shown that freshly isolated double mutant cells contained abundant processed NFκB2 indicating that this aspect of BAFF signaling may not require either p110δ or CD19. Furthermore, Bcl-2 levels were increased as the B cells matured and were found to be higher than normal in the double mutant B cells. It has been published recently that sustained BCR-induced nuclear c-Rel activity depends on de novo c-Rel gene transcription and translation, and that this process occurs in T2 and mature FO B but not in T1 cells. Given that Btk deficiency blocks c-Rel induction in mature B cells, the reduced c-Rel level found in the double mutant cells could be a result of the missing CD19 and PI3K mediated signaling. Although we have not determined the expression of other anti/pro-apoptotic factors, there is a possibility that c-Rel regulated A1 or Bcl-xL could contribute to the loss of mature B cells.

BAFF-mediated growth and survival of naïve B lymphocytes in vitro also requires downstream elements of the PI3K pathway such as the activation of protein kinase B and the mTOR complex which may be defective in p110δ/CD19−/− double mutant B cells. As mentioned previously BCAP is an adapter protein for PI3K and has a strong contribution to the maintenance of mature B cells. BCAP deficiency is associated with alteration of NFκB activity, particularly with reduced c-Rel levels. The late-stage B cell developmental defect in the spleen of BCAP deficient mice can be restored by c-Rel reintroduction. The similar phenotypes of BCAP deficient and p110δ/CD19 double mutant mice implicate again that the reduced level of c-Rel level might be at least in part responsible for the perturbed pool of mature B lymphocytes.

Another intriguing possibility is that mature B cells continue to induce L chain rearrangements and that this process, which involves the generation of double-strand DNA

Figure 6. Elevated Rag expression but no further increase to lambda isotype switching in p110δ−/−CD19−/− double mutant mice. (A) Bone marrow cells from mice of the indicated genotypes were stained with antibodies recognizing B220, IgM, Igkappa and Iglambda. The gating strategy is the same as in Figure 1A for immature (B220intIgM+) B cells. Numbers on the plots indicate the percentage of lymphocytes falling into kappa or lambda positive gates from immature B cells. One representative plot from three mice of each genotype is shown. (B) Ratio of the number of kappa-expressing cells to lambda-expressing cells in the immature B-cell population (B220intIgM+) in the bone marrow as indicated by the gates in (A). Statistical analysis was performed using a one-way ANOVA test and significant differences are indicated as *(p < 0.05). (C) Levels of Rag-1 and Rag-2 mRNA in purified lymph node B cells were determined by quantitative PCR and plotted relative to wild-type which is set as one. Each bar represents the mean ± SEM of five independent biological replicates from p110δ−/−, CD19−/− or p110δ−/−CD19−/− mutant mice. Statistical analysis was performed using one-way ANOVA test and significant differences are indicated as ****(p < 0.001).
breaks, promotes a DNA damage response and mediates the loss of Rag expressing peripheral B cells. It was recently shown that Foxo1 directly regulates the transcription of the RAG-1-RAG-2 locus during B cell development. Thus PI3K may also contribute to the suppression of RAG-gene transcription via activation of PKB, which has an inhibitory effect on FOXO transcriptional activity. The regulatory role of PI3K may be further increased by CD19 acting in concert with PI3K signaling or through other, PI3K-independent, pathways. Finally, since p110δ deficiency is not limited to B cells we cannot exclude a B cell extrinsic role of p110δ in the phenotype of p110δ/CD19 double mutant mice we report here.

Materials and Methods

Mice. Mutant mice harboring null mutations in CD19 and p110δ described previously were backcrossed to C57BL/6 for at least five generations before inter-crossing to generate double mutant mice. All mice were maintained according to UK Home Office guidelines.

B-cell purification. For the detection of gene expression B cells were purified from lymph nodes by B220 positive selection using MACS CD45R Microbeads (Miltenyi Biotech) according to the manufacturer’s instructions. For the survival experiments B cells were purified from lymph nodes by negative selection using the B-cell isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions.

Flow cytometry. Single-cell suspensions from bone marrow, spleen and lymph nodes were surface-stained using various combinations of antibodies conjugated to biotin, fluorescein isothiocyanate (FITC), R-phycocerythrin (PE), allophycocyanin (APC) or Cy5. Staining with biotinylated antibodies was revealed by streptavidin-PE-Cy5.5 (Caltag Laboratories). Cells were analyzed using LSR II flow cytometer (Becton Dickinson) and FlowJo software (Treestar, Ashland, OR). The following antibodies were used: anti-c-kit (CD117, ACK45), anti-CD25 (7D4), anti-B220 (RA3-6B2), anti-CD21 (7G6), anti-CD23 (B3B4), anti-kappa (187.1), anti-lambda (R11-153) (all from BD/Pharmingen); anti-IgD (11-26) from Southern Biotechnology (Birmingham, AL); polyclonal anti-IgM from Jackson ImmunoResearch (West Grove, PA). Anti-CD93 conjugated to APC was from eBioscience (San Diego, CA). For intracellular detection of Bcl-2, cells were first stained with surface markers, fixed with Cytofix/Cytopermin buffer (Becton Dickinson) for 20 min on ice and washed twice with BD Perm/Wash solution. Subsequently, cells were stained with anti-Bcl-2 PE (Becton Dickinson) in Perm/Wash solution for 30 min at 4°C.

Cell viability assay. Purified lymph node B cells were cultured at 5 x 10⁴ cells/ml in IMDM supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (culture medium) in the presence or absence of 200 ng/ml BAFF (Peprotech). Cell viability was assessed following staining with DAPI (Invitrogen) by flow cytometry. Live cell numbers were quantified by reference to AccuCount blank particles (Spherotech Inc., II).

Immunohistology. Spleens from wild-type and p110δ-/-; CD19-/- mutant mice were frozen in OCT compound above dry ice and stored at -80°C. Cryo-sections (10 μm) were fixed in acetone and blocked with 10% normal rat serum in PBS followed by the incubation with the primary antibody for 2 h at ambient temperature. Secondary reagents included anti-rabbit Ig AlexaFluor488 and streptavidin-AlexaFluor555 (both from Invitrogen). The Moma-1 antibody was purchased from Vector Laboratories (Burlingame, CA 94010) and polyclonal Laminin Ab (ab11575) was from Abcam (Cambridge, UK). Sections were mounted in Vectashield medium (Vector Laboratories) containing DAPI. Images were captured with a FV1000 confocal microscope (Olympus) and processed using Olympus Fluoview software. Follicle size was calculated using ImageJ software (National Institutes of Health, Bethesda, MD) using the area measurement function.

Western blotting. For western blot analysis purified lymph node B cells were processed as described previously. Lysates from 2 x 10⁶ purified cells were separated by SDS-PAGE and transferred to nitrocellulose. Membranes were immunoblotted using antibodies to NFkB p100/p52 (a kind gift from Steve Ley (National Institute for Medical Research, London), c-Rel (Santa Cruz Biotechnolgy, Santa Cruz, CA) and β-actin (Sigma). Binding was revealed by rabbit IgG TrueBlot (eBioscience) or HRP-conjugated anti-mouse Ig antibody (Dako, Ely, UK) followed by ECL (Pierce) detection.

Analysis of gene expression. RNA was extracted from sorted B220 positive lymph node B cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and converted to cDNA for real-time PCR analysis as described previously. Controls without reverse transcriptase were included in the experiments. Bcl-2, Bim, c-Rel, Rag-1, Rag-2 mRNA was quantified using primers and FAM-labelled probes from Applied Biosystems (Taqman assays on demand) according to the manufacturer’s instructions. Variations in cDNA input were normalized using β2m (Applied Biosystems) as a reference gene and the expression level of the gene of interest was calculated relative to wild-type.

Statistics. For comparison between 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 performed. P values are indicated and defined in figure legends.

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/KovesdiSNS1-2-Sup.pdf
References

32. Lieber MR, Yu K, Raghavan SC. Roles of nonhomologous DNA end joining, V(D)J recombination, and class switch recombination in chromosomal translocations. DNA Repair 2006; 5:1234-45.

www.landesbioscience.com Self/Nonsel 153