European Journal of Immunology

SHORT COMMUNICATION

B-cell responses to B-cell activation factor of the TNF family (BAFF) are impaired in the absence of PI3K delta

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B-cell activating factor of the TNF family (BAFF) is critical for the survival and maturation of B cells. The molecular mechanisms by which BAFF regulates the survival of developing B cells are becoming better understood. Recent evidence has begun to emerge demonstrating a role for the PI3K/Akt signalling pathway in response to BAFF. However, the importance of the PI3K family for BAFF-signalling and the effects of loss of PI3K function on BAFF responses are still unknown. We therefore investigated the BAFF-mediated responses of B cells deficient for the PI3K catalytic subunit P110δ. We find that the loss of P110δ impairs the BAFF-mediated survival of cultured B cells demonstrating a direct role for this member of the PI3K family in regulating the survival of B cells in response to BAFF. P110δ was required for the growth of B cells in response to BAFF and was critical for the upregulation of the receptor for BAFF following BCR crosslinking. Our findings reveal an important role for p110δ in regulating B-cell responses to BAFF.

Key words: B-cell activating factor of TNF family · B cells · PI3K · Survival

Introduction

Signals generated by the B-cell activating factor of the TNF family (BAFF; also called BLyS, TNFSF13b, THANK, zTNF4 or TALL-1) upon binding to the BAFF receptor (BAFF-R, also known as BR3, TNFRSF13C, CD268) are necessary for the survival of some mature B-cell subsets. Genetic ablation of BAFF or mutational inactivation of BAFF-R leads to a decrease in the number of follicular (FO) and marginal zone (MZ) B cells illustrating the importance of this cytokine and its receptor for B-cell homeostasis [1, 2]. By contrast, transgenic mice overexpressing BAFF have an expansion of mature B cells and elevated levels of serum immunoglobulins, anti-DNA antibodies and immune complexes in the kidney [3, 4]. In humans, increased serum levels of BAFF have been demonstrated in diseases such as Sjogren's syndrome, rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis [5–8]. This suggests that BAFF may play an important role in the development of autoimmune disorders.

The molecular mechanisms by which BAFF exerts its pro-survival effects are not fully understood. BAFF regulates the processing of the NF-κB2 precursor protein p100 to its transcriptionally active derivative p52 and induces phosphorylation and degradation of the NF-κB inhibitor IκBα [9–11]. Active NF-κB can promote transcription of anti-apoptotic genes of the Bcl2 family and this may represent a component of BAFF-mediated survival. In addition, the nuclear accumulation of pro-apoptotic protein kinase Cδ (PKCδ) and the BCR-induced upregulation of pro-apoptotic Bim are both blocked by BAFF [12, 13]. BAFF also stimulates the pro-survival activities of the serine/threonine kinase Pim2 [14].

Recent evidence has shown that BAFF enhances the metabolic activity of B cells and induces cellular growth [15, 16]. When B cells are isolated and placed in culture in the presence of BAFF they increase their size and protein content over time [15]. The treatment of B cells with BAFF leads to the rapid phosphorylation of the serine/threonine kinase Akt as well as the induction of genes involved in the regulation of glycolysis and cell cycle progression [15]. Akt thus appears to be a critical component of

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BAFF-mediated survival and metabolic fitness. BAFF stimulation also results in activation of the mammalian target of rapamycin, an Akt target that regulates protein synthesis, cell growth and proliferation [14].

Akt activation is dependent upon PI3K and BAFF-induced activation of Akt was blocked by treatment of B cells with the PI3K inhibitor LY294002 [14, 15]. However, there are four class I PI3K proteins with the potential to signal in response to BAFF and the contributions of these to BAFF responses are unknown.

In this study we show that loss of the PI3K catalytic subunit P110δ impairs the BAFF-mediated survival and growth of cultured B cells. We also demonstrate the requirement of P110δ for the BAFF-mediated enhancement of B-cell proliferation upon BCR stimulation. Furthermore, we show that expression of BAFF-R is enhanced on mature B cells following BCR crosslinking by a P110δ pathway. Our findings reveal a role for p110δ in regulating B-cell responses to BAFF.

Results and discussion

BAFF-mediated B-cell survival and cell growth requires P110δ

It has been shown that treatment of B cells with BAFF led to tyrosine phosphorylation of a p85-associated protein, which was identified as P110δ by mass spectrometry [15]. To assess the requirement for P110δ in BAFF-mediated survival and growth, mature B cells from the lymph nodes of wild-type and P110δ$^{-/-}$ mice were placed in culture in the presence or absence of BAFF. Lymph node B cells were used in these studies as they contain a more homogeneous population of mature B cells than the spleen, which consists of a mixture of immature transitional, mature FO and MZ B-cell populations that show different responses to BAFF and express different levels of BAFF binding receptors [17, 18]. Furthermore, the MZ population is almost absent in P110δ mutants. Thus, the use of mature FO B cells from the lymph nodes allows a more accurate comparison of BAFF responses. P110δ-deficient B cells survived less well in the presence of BAFF with greatly reduced live cell numbers at 72 and 96 h (Fig. 1A). We also measured cell size in the presence of BAFF using flow cytometry. We observed no significant differences in cell size at the 24 and 48 h time points. When BAFF was absent from the culture, both wild-type and P110δ-deficient B cells showed a marked reduction in size at 72 and 96 h. In the presence of BAFF wild-type cells became larger while the size of P110δ-deficient B cells was significantly smaller (Fig. 1B). Taken together, these data indicate a role for P110δ in maintaining viability and growth in response to BAFF. The loss of P110δ appears to have an impact on these BAFF-mediated responses at later time points suggesting the role of P110δ may be more indirect and reflect a differential release of secondary mediators. The partial responsiveness of P110δ-deficient B cells suggests that P110δ independent mechanisms also regulate BAFF-mediated responses. Based on previous evidence this may reflect the activity of NF-κB/PIM2 pathways that appear to act independently of PI3K in response to BAFF stimulation [14]. It also suggests that other PI3K subunits may contribute to BAFF-mediated survival.

Loss of PTEN enhances BAFF-mediated growth

The phosphatase tensin homolog deleted on chromosome 10 (PTEN) directly opposes PI3K by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate. In cells that lack PTEN higher levels of phospho Akt have been detected indicating an elevated activity of this pathway. As previous studies have indicated PTEN deficiency leads to enhanced protection from apoptosis [19] we asked whether loss of PTEN would enhance responsiveness to BAFF stimulation. B cells from lymph nodes of control ($CD19^{+/cre}PTEN^{+/+}$) and PTEN conditional knockout mice ($CD19^{+/cre}PTEN^{LoxP/LoxP}$) [20] were cultured in the presence or absence of BAFF. PTEN-deficient B cells showed no difference in their capacity for survival in media alone or in response to BAFF (Fig. 1C). By contrast, PTEN-deficient B cells were consistently larger than control cells when cultured in media alone (Fig. 1D). When cultured in the presence of BAFF, PTEN-deficient cells reached a maximum size at 96 h and were significantly larger than control cells (Fig. 1D). This demonstrates that although loss of PTEN has no effect on BAFF-mediated survival it does result in enhanced BAFF-induced cell growth.

Evidence suggests that the loss of PTEN leads to hyperproliferation of B cells *in vitro* in the presence or absence of stimulation, but this is coupled with an increased rate of death due to inappropriate cell cycle entry [21]. This may be a reflection of the importance of PTEN for the regulation of cell cycle or its ability to prevent DNA damage and may account for the sensitivity of PTEN-deficient B cells in the survival assay [22–24].

BAFF-enhanced B-cell proliferation requires P110δ

Genetic deletion or mutational inactivation of P110δ results in impaired proliferation of BCR-stimulated B cells *in vitro* and loss of p110δ activity reduces entry into the cell cycle upon BCR stimulation [25–27]. BAFF does not induce proliferation of B cells *in vitro* by itself, yet the co-culture of BAFF and anti-IgM antibody elevates proliferation above that of anti-IgM stimulation alone [15, 28]. The impaired BAFF-mediated survival and growth of p110δ-deficient B cells prompted us to analyse the effect of BAFF on proliferation following BCR crosslinking. To this end, we labelled B cells from wild-type and P110δ$^{-/-}$ mice with CFSE and cultured them in media alone, or in the presence of anti-IgM or anti-IgM plus BAFF for 72 h. CFSE distribution indicated that stimulation of wild-type B cells with anti-IgM plus BAFF led to a significant increase in the number of cells that had entered to the cell cycle and undergone one or two divisions (Fig. 2A). B cells from p110δ-deficient mice proliferated less well in response to stimulation with anti-IgM alone. When cultured

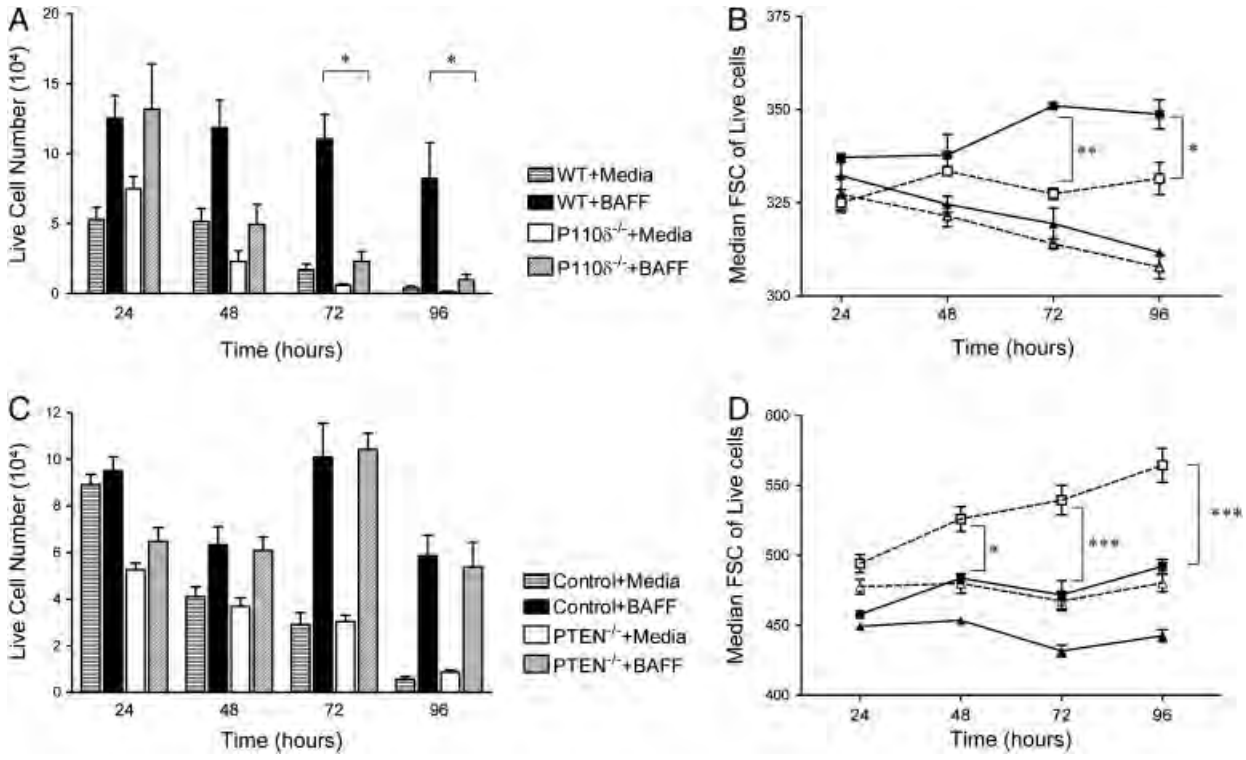

Figure 1. BAFF-mediated B-cell survival requires P110δ. Mature B cells purified from lymph nodes were cultured with media alone or media plus 200 ng/mL BAFF. (A) The live cell number at each time point is shown. (B) BAFF-mediated B-cell growth requires P110δ. Wild-type (filled symbols) and P110δ$^{-/-}$ (open symbols) B cells were cultured in media alone (triangles) or media supplemented with BAFF (squares). The size of viable B cells is displayed as the mean of the median forward scatter. In A and B all data points represent the mean ± SEM of five independent B-cell preparations. (C) Loss of PTEN has no effect on BAFF-mediated B-cell survival. The survival of mature lymph node B cells lacking PTEN ($CD19^{+/cre}PTEN^{LoxP/LoxP}$) is compared with control ($CD19^{+/cre}PTEN^{+/+}$) B cells following culture in media alone or media plus 200 ng/mL BAFF. (D) Loss of PTEN enhances BAFF-mediated increases in cell size. Control $CD19^{+/cre}PTEN^{+/+}$ (filled symbols) and $CD19^{+/cre}PTEN^{LoxP/LoxP}$ (open symbols) B cells were cultured in media alone (triangles) or media supplemented with BAFF (squares). The cell size of viable B cells is displayed as the mean of the median forward scatter. Data points in C and D represent the mean ± SEM of B cells from five $CD19^{+/cre}PTEN^{+/+}$ mice and eight $CD19^{+/cre}PTEN^{LoxP/LoxP}$ mice. Statistical analysis was carried out using the parametric one-way ANOVA test: ***$p<0.001$, **$p<0.01$, *$p<0.05$.

with anti-IgM plus BAFF, the only significant increase in cell number was observed within the population of cells that had not entered into the cell cycle (Fig. 2A). When we considered the proportions of cells within each division, it was apparent a higher proportion of P110δ-deficient B cells than wild-type cells remained undivided when stimulated with BAFF and anti-IgM (Fig. 2B). Taken together, these results indicate that loss of P110δ leads to a partial impairment in BAFF-enhanced proliferation following BCR stimulation.

It has been shown that BCR stimulation increases expression of BAFF-R on developing mature B cells [29]. We measured the expression of BAFF-R on B cells from wild-type and P110δ$^{-/-}$ mice after culture with anti-IgM for 24 h. Anti-IgM resulted in a significant increase in BAFF-R expression as measured by flow cytometry on wild-type B cells (Fig. 3A and B). This anti-IgM-induced increase was blocked by co-culture with the PI3K inhibitor wortmannin, indicating a requirement of PI3K activity for BCR-induced BAFF-R upregulation. Stimulation of P110δ-deficient B cells with anti-IgM failed to increase BAFF-R expression over that seen in the absence of stimulation (Fig. 3A and B). BAFF-R expression levels were similar between p110δ-deficient cells stimulated with anti-IgM alone and anti-IgM plus wortmannin, illustrating the importance of the P110δ isoform in regulating BCR-induced BAFF-R upregulation. The defective upregulation of BAFF-R in the absence of P110δ may contribute to the reduced number of B cells following culture with anti-IgM and BAFF.

Concluding remarks

Our findings demonstrate a requirement for P110δ in the BAFF-mediated survival and growth of cultured B cells. The role of P110δ in regulating BAFF-dependent B-cell growth may be restricted to BAFF-mediated long-term survival as we observe impaired growth of BAFF treated B cells deficient for P110δ only at longer duration of culture. The enhancement of proliferation by BAFF was also defective in P110δ-deficient B cells. Taken together these findings indicate that the PI3K/Akt pathway is likely to be important for BAFF responses. Loss of PTEN had no effect on B-cell survival in response to BAFF, which may reflect

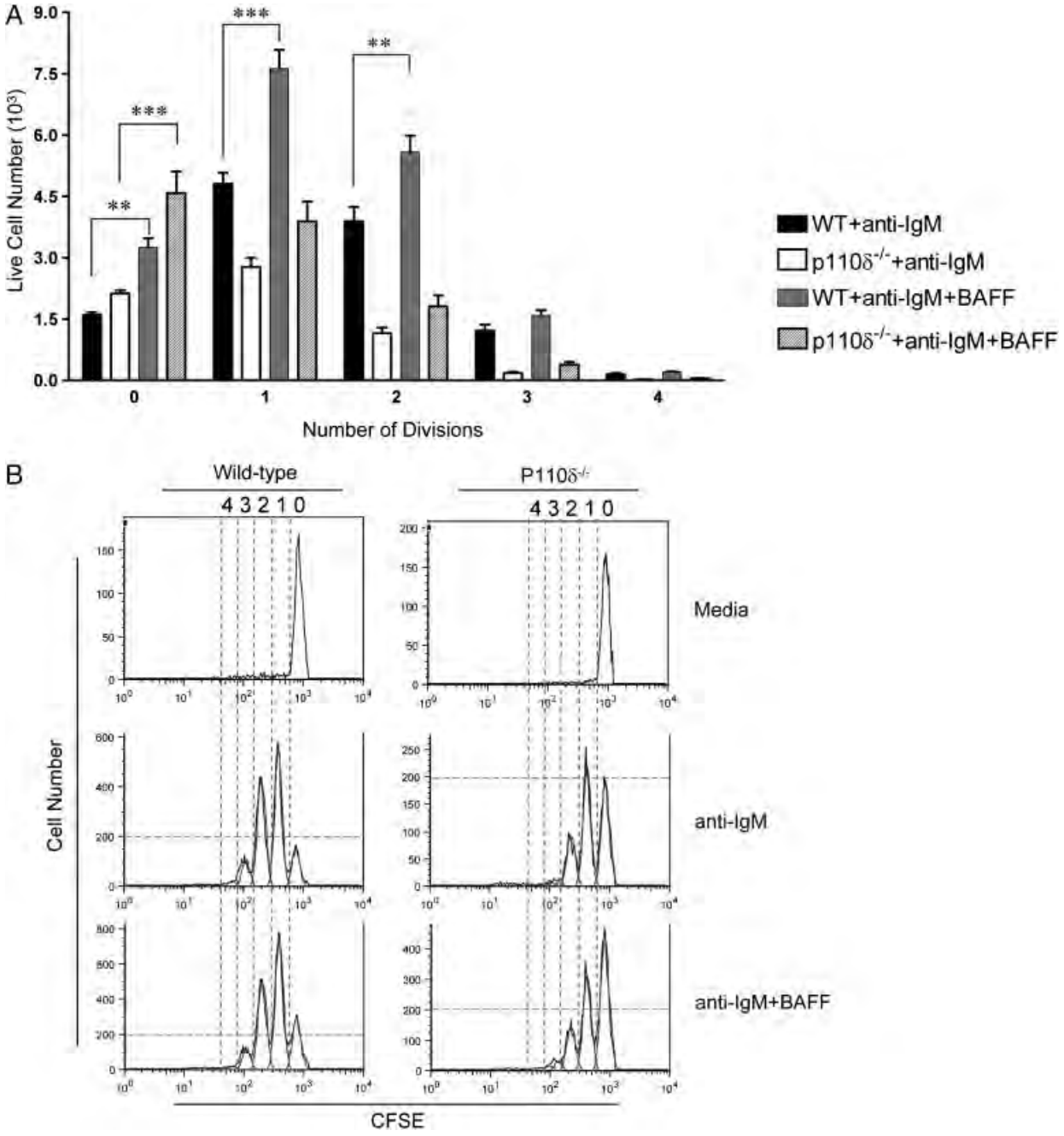

Figure 2. Optimal BAFF-enhanced B-cell proliferation requires P110δ. (A) Live cell number *per* cell division after 72 h of culture, only differences that are statistically significant are shown. (B) Histograms show the number of B cells that have undergone cell division as determined by CFSE dilution after 72 h culture in the presence of anti-IgM or anti-IgM plus BAFF. Gates represent each successive cell division, horizontal lines indicate 200 cell counts. Data points in A represent the mean ± SEM of values obtained from five individual mice, plots in B are representative of data from five individual mice. Statistical analysis was carried out using the parametric one-way ANOVA test: $^{***}p<0.001$, $^{**}p<0.01$.

other more complex changes to survival pathways in the absence of PTEN. However, PTEN loss resulted in a significant increase in cell size in the presence of BAFF, suggesting this component of BAFF-signalling may be enhanced by elevated levels of active Akt. Our results thus indicate the importance of the PI3K pathway and of P110δ in particular for BAFF-mediated B-cell survival and cell growth.

Materials and methods

Mice and cells

P110δ-deficient and B-cell-conditional PTEN-deficient mice have been previously described [20, 26]. All mice were maintained

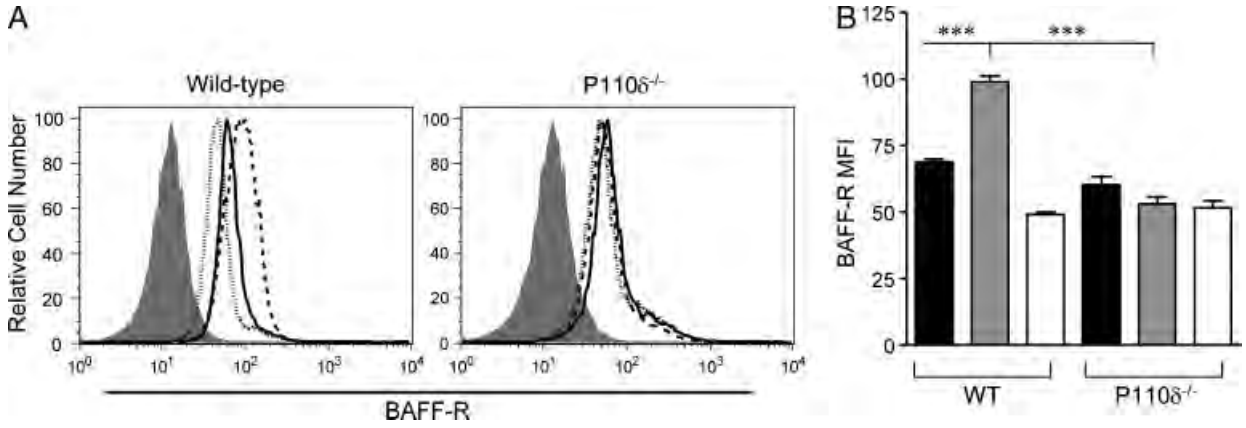

Figure 3. BCR-induced BAFF-R upregulation requires P110δ. (A) Levels of BAFF-R expression as determined by flow cytometry; continuous line, wild-type or P110$\delta^{-/-}$ B cells cultured in media alone; dashed line, wild-type or P110$\delta^{-/-}$ B cells cultured in the presence of 10 μg/mL anti-IgM; dotted line, wild-type B cells cultured in the presence of 10 μg/mL anti-IgM plus 100 nM wortmannin. Shaded histograms represent staining by an isotype control. One representative example of four independent B-cell preparations is shown. (B) Summary bar graphs showing the mean of the median fluorescence intensity of the four replicates ± SEM. Wild-type or P110$\delta^{-/-}$ B cells were cultured in media alone (filled bars), 10 μg/mL anti-IgM (gray bars) or 10 μg/mL anti-IgM plus 100 nM wortmannin (open bars). Statistical analysis was carried out using the parametric one-way ANOVA test: ***p<0.001, **p<0.01.

according to UK Home Office guidelines. B cells were purified from lymph nodes using the MACS B-cell isolation kit (Miltenyi) according to the manufacturer's protocol.

Cell culture and flow cytometry

Recombinant human BAFF was either purified from *Escherichia coli* as described [30] or purchased from Peprotech. Purified B cells were cultured at 1×10^6 cell/mL in RPMI supplemented with 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (culture media) in the presence or absence of either 2 μg/mL or 200 ng/mL BAFF. Cell viability was assessed following staining with 7AAD (Invitrogen) by flow cytometry using a FACScalibur flow cytometer (Becton Dickinson) and FlowJo (TreeStar) software. Live cell numbers were quantified using PKH67 reference microbeads (Sigma) and cell size was measured by forward light scatter. BAFF-R expression on cultured B cells was measured using a hamster anti-BAFF receptor antibody (P1B8) from Biogen [31]. Wortmannin was purchased from Sigma.

CFSE-labelling

Purified B cells from lymph nodes of wild-type and P110$\delta^{-/-}$ mice were loaded with CFSE as described [32]. In brief, B cells at a density of 1×10^7 cells/mL in PBS containing 5% FCS were incubated with 5 μM CFSE for 5 min at ambient temperature in the dark. Cells were then washed three times with PBS/5% FCS and cultured at a density of 2×10^6 cells/mL in culture media. In some wells anti-IgM $(Fab)_2$ fragment of goat polyclonal antibody (Jackson Immunoresearch) was added to a final concentration of 10 μg/mL and BAFF to a final concentration of 250 ng/mL. Following culture for 72 h cells were harvested and analysed by flow cytometry using TOPRO-3 for the exclusion of dead cells and PKH67 microbeads for the quantification of live cell number.

Acknowledgements: This work was supported by the Medical Research Council and the Biotechnology and Biological Sciences Research Council.

Conflict of interest: The authors have declared no financial or commercial conflict of interest.

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Abbreviations: **BAFF:** B-cell activating factor of the TNF family · **BAFF-R:** BAFF receptor · **FO:** follicular · **MZ:** marginal zone · **PTEN:** phosphatase tensin homolog deleted on chromosome 10

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Received: 16/6/2008
Revised: 28/8/2008
Accepted: 6/10/2008

