

Coupling Between B Cell Receptor and Phospholipase C- γ 2 Is Essential for Mature B Cell Development

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Abstract

Two signaling pathways known to be essential for progression from immature to mature B cells are BAFF receptor (BAFF-R) and the B cell receptor (BCR). Here, we first show that phospholipase C (PLC)- γ 2 is required for a BAFF-R-mediated survival signal. Then, we have examined the question of whether the reduced number of mature B cells in PLC- γ 2^{-/-} mice is caused by a defect in either BCR or BAFF-R signaling. We find that a PLC- γ 2 SH2 mutant, which inhibits coupling between BCR and PLC- γ 2, fails to restore B cell maturation, despite supporting BAFF-dependent survival. Therefore, our data suggest that the BAFF-R-mediated survival signal, provided by PLC- γ 2, is not sufficient to promote B cell maturation, and that, in addition, activation of PLC- γ 2 by BCR is required for B cell development.

Key words: cell differentiation • cell survival • B lymphocyte subsets • spleen • tumor necrosis factor

Introduction

Immature B cells that emigrate from the bone marrow to the periphery are referred to as transitional B cells (for reviews, see references 1–3). Accordingly, in the spleen, B cell development proceeds through the transitional 1 (T1), transitional 2 (T2), and mature follicular B cell stages (4). Signaling through the B cell receptor (BCR) is required for the development and maintenance of mature splenic B cells (5–8). Indeed, deletion of the Ig α cytoplasmic tail leads to a severe block in the generation of peripheral B cells (9). Moreover, loss of BCR expression by conditional IgM ablation aborts development from the transitional to mature B cell stage (10).

In addition to the BCR, signaling through BAFF receptor (BAFF-R) has been recently shown to be critical for B cell survival that regulates the number of follicular B cells (for reviews, see references 11–13). A/WySnJ mice have a mutated gene that encodes BAFF-R (14, 15). These mice, like BAFF-deficient mice (16, 17), have a developmental block at the T1 stage, suggesting that either differentiation to the T2 stage and/or survival of T2 cells require BAFF-R

signaling. Therefore, defective signaling emanating from either BCR or BAFF-R can cause a decrease in numbers of mature follicular B cells.

We and others have recently demonstrated that ablation of phospholipase C (PLC)- γ 2 results in reduced numbers of follicular mature B cells and poor responses to T-independent type 2 (TI-2) antigens (18, 19). Herein, we have investigated the mechanism by which PLC- γ 2 promotes B cell maturation in the spleen. Given that BAFF-R belongs to the same receptor family (TNF receptors) of CD40 (14), the possible link between BAFF-R and PLC- γ 2 can be speculated by the previous observations that CD40-mediated responses were significantly reduced in PLC- γ 2^{-/-} B cells (18, 19). Hence, if the BAFF-R, like CD40, utilizes PLC- γ 2, two alternative, but not necessarily mutually exclusive, possibilities could be considered: (a) defective BCR signaling by loss of PLC- γ 2 causes an decrease in numbers of mature B cells; (b) this decrease is mainly due to insufficient BAFF-R signaling in PLC- γ 2^{-/-} B cells. After establishing the link between BAFF-R and PLC- γ 2, we designed experiments to distinguish the above two possibilities. Our data suggest that activation of PLC- γ 2 by BCR is essential for development of mature B cells.

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Abbreviations used in this paper: BAFF-R, BAFF receptor; BCR, B cell receptor; BrdU, 5'-bromo-2'-deoxyuridine; PLC, phospholipase C.

Materials and Methods

Mice. Wild-type C57BL/6 mice were purchased from Clea Japan. RAG-2^{-/-} mice were purchased from Taconic. PLC- γ 2^{lox/lox} mice (18) were crossed with CAG-Cre transgenic mice (20) to obtain CAG-Cre/PLC- γ 2^{+/-} mice. Then these mice are crossed with C57BL/6 mice to generate PLC- γ 2^{-/-} mice.

Flow Cytometry. Cell surface expression of IgM and IgD was analyzed by FACSCalibur™ (Becton Dickinson) as described previously (18). FITC-conjugated anti-IgM was purchased from BD Biosciences. PE-conjugated anti-IgD was purchased from Southern Biotechnology Associates, Inc. Expression levels of EGFP were also monitored by FACSCalibur™. Viability of the cells was measured by staining dead cells with 1 μ g/ml propidium iodide (Sigma-Aldrich). Also apoptotic cells were detected using PE-conjugated Annexin V (BD Biosciences) according to the manufacturer's instruction. Experiments were performed at least three times and shown as mean \pm SD in Fig. 2 A and Fig. 4 A.

Preparation of T2 B Cells and Cell Culture. T2 B cells were purified from splenic B cells by cell sorting. Splenic B cells were prepared as described previously (18). Then 5×10^7 splenic B cells were stained with FITC-conjugated anti-CD21, PE-conjugated anti-HSA, and biotinylated anti-CD23 combined with streptavidin-CyChrome. T2 B cells (CD21^{hi}HSA^{hi}CD23⁺B220⁺) were sorted using EPICS ALTRA (Beckman Coulter). Staining with anti-CD21, anti-HSA, and anti-CD23 instead of anti-IgM and anti-IgD was performed to avoid contamination of marginal zone B cells and also in order to prevent potential activation signals through the BCR. Reanalysis of sorted T2 cell population revealed >90% purity. FITC-conjugated anti-CD21, PE-conjugated anti-HSA, biotinylated anti-CD23, and streptavidin-CyChrome were purchased from BD Biosciences.

In vitro cultures of T2 B cells were performed according to the previous reports (21, 22). In brief, $2\text{--}5 \times 10^5$ sorted T2 B cells were cultured in the growth medium consists of RPMI-1640 medium (GIBCO™; Invitrogen) supplemented with 10% FCS (Biological Industries), 50 μ M 2-ME (Sigma-Aldrich), 2 mM L-glutamine, 4 mM sodium pyruvate, and antibiotics (GIBCO™; Invitrogen). Recombinant human BAFF (Research Diagnostics Inc.) or anti-IgM F(ab')₂ (Jackson ImmunoResearch Laboratories) was added where indicated.

Retroviral or Lentiviral Transduction of Bone Marrow Cells and T2 B Cells. The cDNA encoding wild-type rat PLC- γ 2 or its SH2 mutant was cloned into both MSCV-based retroviral vector and HIV based lentiviral vector. These vectors contain EGFP cDNA as an expression marker downstream of the internal ribosomal entry site.

Culture supernatants containing retrovirus were generated using EcoPack2 packaging cell line (BD Biosciences). For activation of bone marrow cells, freshly isolated cells were cultured in the growth medium supplemented with 10 ng/ml SCF, 20 ng/ml IL-3 and 10 ng/ml IL-6 for 2 d. Activated bone marrow cells were transduced with retrovirus using RetroNectin Dish (TAKARA BIO) according to the manufacturer's instruction. 2 d after the infection, cells were collected and transferred into irradiated recipient mice as described below. Both GFP⁺ and GFP⁻ cells were transferred together without sorting in order to analyze GFP⁻ cells as an internal control. SCF, IL-3, and IL-6 were purchased from R&D Systems.

Culture supernatants containing the lentivirus were obtained using Virapower Lentivirus Kit (Invitrogen) according to the manufacturer's instruction. Collected supernatants were then centrifuged at 60,000 *g* for 2 h and the virus pellets were resus-

ended in culture medium at 100:1 volume. Freshly isolated T2 B cells were transduced with lentivirus as follows. Sorted T2 B cells were suspended in 250 μ l of growth medium containing polybrene (Sigma-Aldrich) at the concentration of 16 μ g/ml in 24-well culture dish, followed by addition of 250 μ l medium containing lentivirus. Then the culture plates were centrifuged at 30°C, 2,500 rpm for 90 min. After 48–72 h culture, transduction efficiencies were monitored by EGFP expression. For transduction using lentiviral vectors, ~60% of the cells were expressing EGFP routinely after 48 h when examined by infecting wild-type B cells.

Passive Transfer of Bone Marrow Cells. For passive transfer of bone marrow cells, recipients were γ -irradiated at the dose of 10 Gy, followed by i.v. injection of 10⁶ donor bone marrow cells. For analysis of cell autonomous defect of PLC- γ 2^{-/-} bone marrow cells, RAG-2^{-/-} mice were injected i.v. with 5×10^6 bone marrow cells from wild-type or PLC- γ 2^{-/-} mice.

RT-PCR. Expression levels of BAFF receptor (BAFF-R) or PLC- γ 2 were analyzed by semi-quantitative RT-PCR. In brief, total RNA was extracted from 10⁶ cells using Trizol (Invitrogen). Obtained RNA was reverse transcribed using random hexamer (TAKARA BIO) with SuperScriptII (Invitrogen) according to the manufacturer's instruction. For amplification of BAFF-R, 5'-ACTGCGTGTCTGTGAGCTCTT-3' and 5'-TCTGGG-CCAGCTGTCTTGGT-3' were used as forward and reverse primers, and cycled 35 times at 94°C 1 min, 55°C 1 min, and 72°C 1 min. For amplification of mouse or rat PLC- γ 2, 5'-AGGCCAGTGCTGACCAGCTG-3' and 5'-CTGTGAGCCGCA-GCTCGAA-3' were used as forward and reverse primers, and cycled 35 times at 94°C 1 min, 55°C 1 min, and 72°C 1 min. To avoid detection of incomplete PLC- γ 2 mRNA expressed in cells from PLC- γ 2^{-/-} mice, forward primer was designed within the deleted exon (18). For amplification of bcl-2, 5'-TGCACCT-GACGCCCTTCCAC-3' and 5'-TAGCTGATTCGACCATT-TGCCTGA-3' were used as forward and reverse primers, and cycled 35 times at 94°C 1 min, 55°C 1 min, and 72°C 1 min. GAPDH was amplified as an internal control using 5'-CCAT-CACCATCTTCCAGGAG-3' and 5'-CCTGCTTACCAC-CCTTCTTG-3' as forward and reverse primers.

5'-Bromo-2'-deoxyuridine Labeling. 5'-Bromo-2'-deoxyuridine (BrdU) labeling was performed as reported previously (23). In brief, mice were fed with BrdU (Wako Pure Chemical Industries) in the drinking water at concentration of 1 mg/ml for 1 wk. Spleen cells were depleted of non-B cells as described previously (18). After staining with biotinylated anti-CD21 combined with streptavidin-CyChrome and PE-conjugated anti-HSA, BrdU labeled T1, T2, and mature B cells were detected using BrdU Flow Kit (BD Biosciences) according to the manufacturer's instruction.

Calcium Measurement and Protein Analysis. Calcium mobilization was measured as described previously (18) with several modifications. In brief, 5×10^7 spleen cells were loaded with 1.2 μ M Indo 1-AM (Sigma-Aldrich) at 37°C for 45 min. Cells were washed and stained with FITC-conjugated anti-CD21, PE-conjugated anti-HSA, and CyChrome-conjugated anti-B220. Then 10⁶ stained cells were stimulated with 2 μ g/ml recombinant human BAFF or 15 μ g/ml anti-IgM F(ab')₂. Continuous monitoring of fluorescence from the cell suspension was performed using BD-LSR (Becton Dickinson).

Stimulated splenic B cells (3×10^7) were solubilized and immunoprecipitated with anti-PLC- γ 2 (Santa Cruz Biotechnology, Inc.) as described previously (18). Immunoprecipitates were separated by 7.5% SDS-PAGE, blotted and detected by anti-phos-

photyrosine Ab, 4G10 (Upstate) using the enhanced chemiluminescence system (Amersham Biosciences).

Preparation of Nuclear Extracts. Stimulated cells (5×10^6) were washed with ice cold PBS and cell pellet was suspended in 800 μ l hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 5 μ g/ml antipain, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 0.5 μ g/ml pepstatin, and 0.5 mM PMSF) for 10 min on ice followed by addition of 50 μ l of 10% NP-40. After vortexing for 20 s, nuclei were prepared by centrifugation for 1 min at 15,000 rpm. Then the nuclear pellet was suspended in 50 μ l of high buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 5 μ g/ml antipain, 5 μ g/ml aprotinin, 5 μ g/ml leupeptin, 0.5 μ g/ml pepstatin, and 1 mM PMSF) and incubated for 20 min at 4°C with continuous brief mixing. The mixture was then centrifuged at 15,000 rpm for 5 min and the supernatants were subjected to EMSA.

EMSA. Nuclear extracts (5 μ g of protein) were preincubated in 28 μ l reaction volume containing 10 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 5% glycerol, 5 mM DTT, 250 μ g/ml poly dI-dC (Amersham Biosciences) for 10 min at 4°C. Then 2 μ l of 32 P-labeled oligonucleotide (20,000 cpm) were added and incubated for 20 min at room temperature. The samples were analyzed by electrophoresis in a 4% nondenaturing polyacrylamide gel using 0.5 \times TBE. Double stranded oligonucleotide containing NF- κ B binding site 5'-AGCTTCAACA-GAGGGACTTTCCGAGAGGCTCGAG-3' was used for κ B binding probe and double stranded oligonucleotide 5'-TGTCGAATGCAAATCACTAGAA-3' was used for Oct-1 binding probe.

Results

Reduction in the Number of Mature B Cells in PLC- γ 2 $^{-/-}$ Mice Is Cell Autonomous. Given the recent evidence that marginal zone (MZ) B cells in the spleen contribute to humoral immune responses (24), we first examined whether MZ B cells develop normally in PLC- γ 2 $^{-/-}$ mice. As shown in Fig. 1 A, the percentage of MZ B cells (CD21^{hi}CD23^{lo}B220⁺) was comparable in the spleens between PLC- γ 2 $^{-/-}$ and wild-type mice. Then, we addressed whether the reduced number of splenic follicular mature B cells observed in PLC- γ 2 $^{-/-}$ mice could result from a cell autonomous defect or a defect in stromal microenvironment that supports the differentiation of these cells. To distinguish between these possibilities, we performed a cell transfer experiment. Fig. 1 B shows that the transfer of bone marrow cells from wild-type mice into RAG-2 $^{-/-}$ recipients reconstituted normal number of IgM^{lo}IgD^{hi} mature B cells in the spleen. In contrast, bone marrow cells derived from PLC- γ 2 $^{-/-}$ mice transferred into the RAG-2 $^{-/-}$ recipients gave rise to a dramatic decrease in mature B cells, as seen in PLC- γ 2 $^{-/-}$ mice. Hence, we conclude that the mature B cell defect in PLC- γ 2 $^{-/-}$ mice is cell autonomous.

PLC- γ 2 Is Required for Both BCR- and BAFF-R-mediated Survival of Immature B Cells. BCR ablation (10) or BAFF-R mutation (14, 15) results in cell death by apoptosis. To determine whether absence of mature B cell accumulation in PLC- γ 2 $^{-/-}$ mice is associated with cell death through a de-

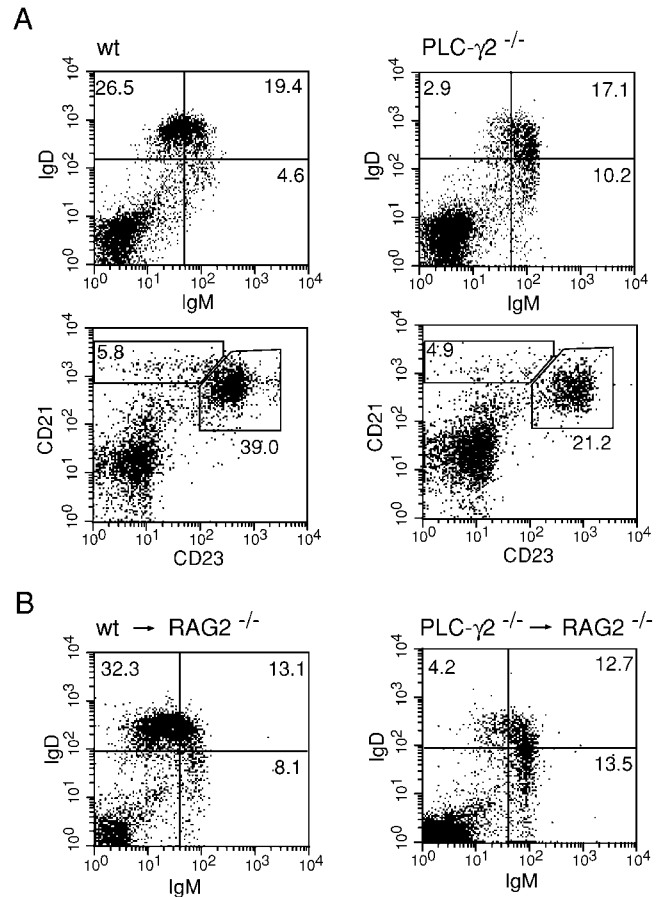


Figure 1. Reduction of mature B cell numbers in PLC- γ 2 $^{-/-}$ mice is cell autonomous. (A) Comparable number of marginal zone B cells in wild-type and PLC- γ 2 $^{-/-}$ mice. Total splenocytes from wild-type (left) or PLC- γ 2 $^{-/-}$ (right) mice were stained with indicated antibodies. Genotypes are as indicated above and cell surface markers are shown as coordinates. The numbers indicate the percentages of live cells in the lymphocyte gate. Panels are representatives of four experiments. Average numbers of total splenocytes in the wild-type and PLC- γ 2 $^{-/-}$ mice are $(5.8 \pm 0.5) \times 10^7$ and $(4.7 \pm 0.4) \times 10^7$, respectively. (B) FACS[®] analysis of reconstituted RAG-2 $^{-/-}$ mice. 5×10^6 bone marrow cells from wild-type (left) or PLC- γ 2 $^{-/-}$ (right) mice were transferred i.v. into RAG-2 $^{-/-}$ mice. 9 wk later, splenocytes were analyzed for the expression of IgM and IgD. Panels are representatives of two experiments. Average numbers of total splenocytes in RAG-2 $^{-/-}$ mice reconstituted with wild-type and PLC- γ 2 $^{-/-}$ bone marrow cells are 3.2×10^7 and 2.3×10^7 , respectively.

fect in BCR or BAFF-R signaling, we measured survival of immature B cells by using *in vitro* cultures in the presence of anti-BCR Ab or BAFF. As T2 B cells, among the splenic immature B cells, are thought to be the major target for generating a survival signal such as through BAFF-R (21, 25), T2 B cells from PLC- γ 2 $^{-/-}$ mice and controls were purified and cell death was measured by staining with propidium iodide. Isolated PLC- γ 2 $^{-/-}$ T2 B cells, after 48 h culture in the absence of exogenous added agonists, exhibited a two- to threefold decrease in the variability as measured by propidium iodide staining, compared with wild-type T2 B cells (Fig. 2 A).

Consistent with previous reports, both BCR (22, 26, 27) and BAFF-R (21, 25, 28) engagement promoted cell sur-

vival in T2 B cells from wild-type in a dose-dependent manner. In contrast, these effects could not be detected in T2 B cells from PLC- $\gamma 2^{-/-}$ mice (Fig. 2 A). Using annexin V-staining as another cell death marker, essentially the same data were obtained (unpublished data). The defect

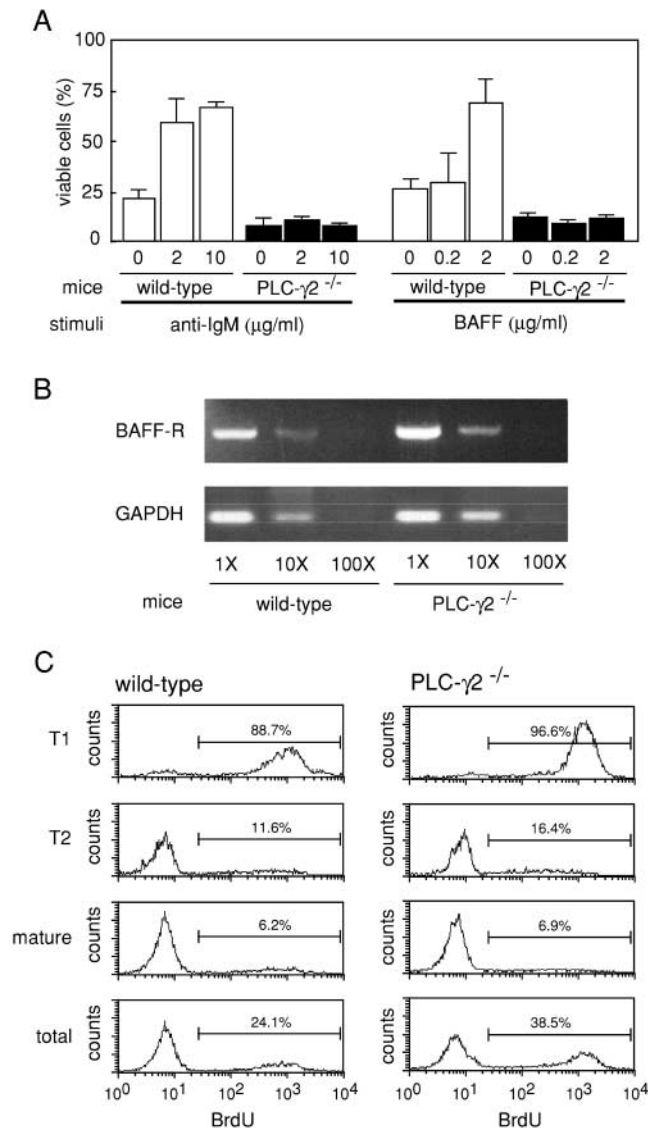


Figure 2. Reduction of viability in T2 B cells from PLC- $\gamma 2^{-/-}$ mice. (A) Viability of in vitro-stimulated wild-type or PLC- $\gamma 2^{-/-}$ T2 B cells. Sorted T2 B cells from wild-type or PLC- $\gamma 2^{-/-}$ mice were stimulated with indicated concentrations of anti-IgM F(ab')₂ or BAFF for 48 h. Cultured cells were stained with propidium iodide to distinguish live cells. (B) Expression of BAFF-R mRNA in T2 B cells from wild-type and PLC- $\gamma 2^{-/-}$ mice. Total RNA was extracted from sorted T2 B cells and RT-PCR was performed as described in Materials and Methods. (C) Increased B cell turnover in the spleen of PLC- $\gamma 2^{-/-}$ mice. BrdU incorporation by T1, T2, and mature splenic B cells from wild-type or PLC- $\gamma 2^{-/-}$ mice after 1 wk of BrdU administration in the drinking water was analyzed. Numbers indicate the percentages of BrdU-positive cells. Panels are representatives of three independent experiments using at least three mice each case. Average values of BrdU-positive cells in T1, T2, mature, and total B cells are (89.1 ± 0.4%, 13.1 ± 2.0%, 6.5 ± 0.5%, 24.9 ± 1.4%) for wild-type mice and (95.0 ± 1.6%, 15.4 ± 1.7%, 6.4 ± 1.6%, 36.4 ± 3.4%) for PLC- $\gamma 2^{-/-}$ mice, respectively.

in BCR- and BAFF-R-mediated survival of PLC- $\gamma 2^{-/-}$ T2 B cells is not due to low levels of expression of these receptors, as cell surface expression of IgM (unpublished data)

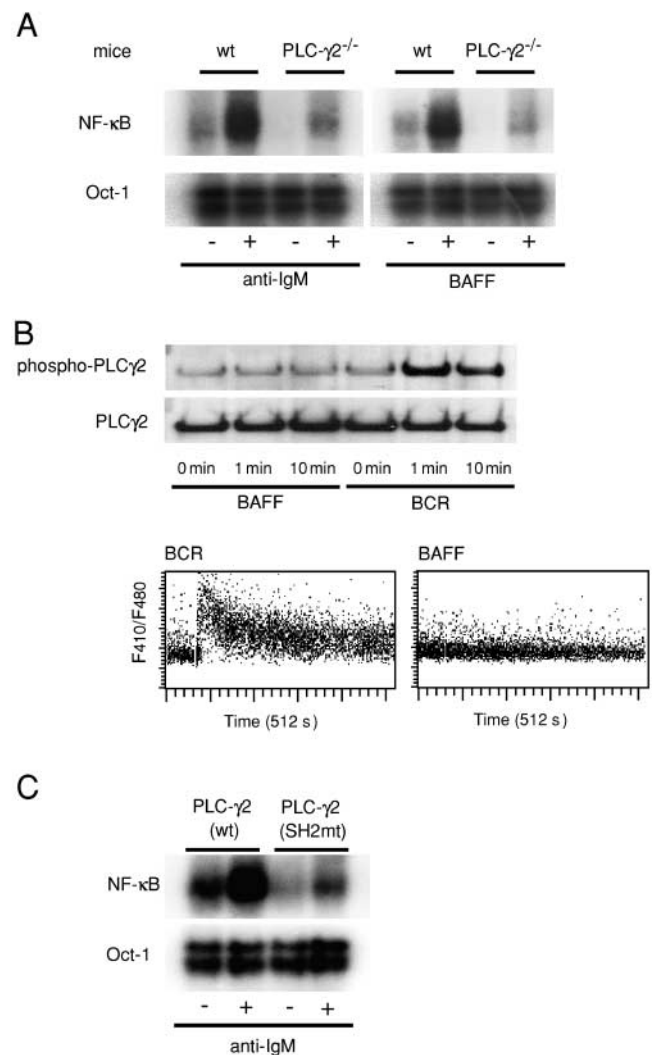


Figure 3. Impaired NF- κ B activation in BCR- and BAFF-R-stimulated PLC- $\gamma 2^{-/-}$ B cells. (A) Impaired nuclear translocation of NF- κ B in BCR- or BAFF-R-stimulated B cells from PLC- $\gamma 2^{-/-}$ mice. Splenic B cells from wild-type and PLC- $\gamma 2^{-/-}$ mice were stimulated with 10 μ g/ml anti-IgM F(ab')₂ for 4 h or with 2 μ g/ml BAFF for 12 h where indicated. Nuclear extracts were prepared from stimulated B cells (5 × 10⁶ cells/lane) and subjected to EMSA as described in Materials and Methods. Oct-1-binding is shown as a control. (B) Phosphorylation of PLC- $\gamma 2$ and calcium mobilization in BCR- and BAFF-R-stimulated B cells. Splenic B cells from wild-type mice (10⁷ cells/lane) were stimulated with either 10 μ g/ml anti-IgM F(ab')₂ or 2 μ g/ml BAFF for indicated periods. Obtained cell lysates were immunoprecipitated with anti-PLC- $\gamma 2$ Ab and analyzed by Western blotting with anti-phosphotyrosine antibody as described in Materials and Methods (top). The same membrane was stripped and reblotted with anti-PLC- $\gamma 2$ Ab. Splenic B cells from wild-type mice were stimulated with 15 μ g/ml anti-IgM F(ab')₂ or 2 μ g/ml BAFF and calcium mobilization was measured as described in Materials and Methods (bottom). (C) Impaired nuclear translocation of NF- κ B in BCR-stimulated B cells reconstituted with PLC- $\gamma 2$ SH2 mutant. PLC- $\gamma 2^{-/-}$ splenic B cells transduced with wild-type PLC- $\gamma 2$ (PLC- $\gamma 2$ (wt)) or its SH2 mutant (PLC- $\gamma 2$ (SH2mt)) were recovered and subjected to EMSA. Oct-1 binding is shown as a control.

and mRNA expression of BAFF-R (Fig. 2 B) were not altered between wild-type and PLC- $\gamma 2^{-/-}$ T2 B cells. Together, we conclude that PLC- $\gamma 2$ plays an important role in generating a survival signal emanating from both BCR and BAFF-R. To further validate this conclusion, we determined the percentage of BrdU-labeled B cells in the spleens from PLC- $\gamma 2^{-/-}$ mice administered with BrdU. When BrdU was fed for 1 wk, there was a marked increase in the population of BrdU labeled B220⁺ cells in PLC-

$\gamma 2^{-/-}$ mice compared with the wild-type mice (Fig. 2 C, bottom). Particularly, BrdU labeled T1 and T2 B cells were accumulated in the spleen of PLC- $\gamma 2^{-/-}$ mice, suggesting that B cell turnover is enhanced in PLC- $\gamma 2^{-/-}$ mice, consistent with the in vitro data described above.

BAFF-R-mediated Activation of NF- κ B Is Impaired in PLC- $\gamma 2^{-/-}$ B Cells. It has been reported that stimulation by both BCR and BAFF-R results in NF- κ B activation (5, 29). Thus, we next examined whether the nuclear translocation of NF- κ B upon BCR or BAFF-R engagement is dependent on PLC- $\gamma 2$. As reported, nuclear translocation of NF- κ B was clearly seen in BCR- or BAFF-R-activated wild-type B cells (Fig. 3 A). In contrast to wild-type, PLC- $\gamma 2^{-/-}$ B cells displayed the lower level of NF- κ B binding activity even before receptor stimulation. Moreover, translocation of NF- κ B was only marginally up-regulated in PLC- $\gamma 2^{-/-}$ B cells after BCR or BAFF-R stimulation (Fig. 3 A).

Although involvement of PLC- $\gamma 2$ in BAFF-R-mediated signaling is clear, the manner in which PLC- $\gamma 2$ contributes to this signaling appears to be distinct from the case of BCR signal. As shown in Fig. 3 B, in contrast to BCR cross-linking, BAFF-R stimulation did not induce significant phosphorylation of PLC- $\gamma 2$ in wild-type B cells. As a consequence, calcium was not mobilized by BAFF-R stimulation.

A PLC- $\gamma 2$ SH2 Mutant Cannot Completely Promote B Cell Development. The above results, given the absence of mature B cells in conditional IgM knockout mice (10) or A/WySnJ (mutant BAFF-R) mice (14, 15), suggest that the absence of mature B cells in PLC- $\gamma 2^{-/-}$ mice could arise either as insufficiency of BCR or BAFF-R signaling, or combination of both mechanisms. To dissect these possibilities, we considered that a PLC- $\gamma 2$ SH2 mutant could be useful. Both SH2 domains of PLC- $\gamma 2$ were previously shown to be essential for coupling the BCR to cellular responses such as calcium mobilization and NF- κ B activation (30, 31). Thus, we assumed that the PLC- $\gamma 2$ SH2 mutant could provide the BAFF-R-mediated signal, at least to some extent, despite shutting off the BCR signal. To verify whether this assumption is correct, we examined the BAFF-R-mediated survival of PLC- $\gamma 2^{-/-}$ T2 B cells reconstituted with the PLC- $\gamma 2$ SH2 mutant. For this recon-

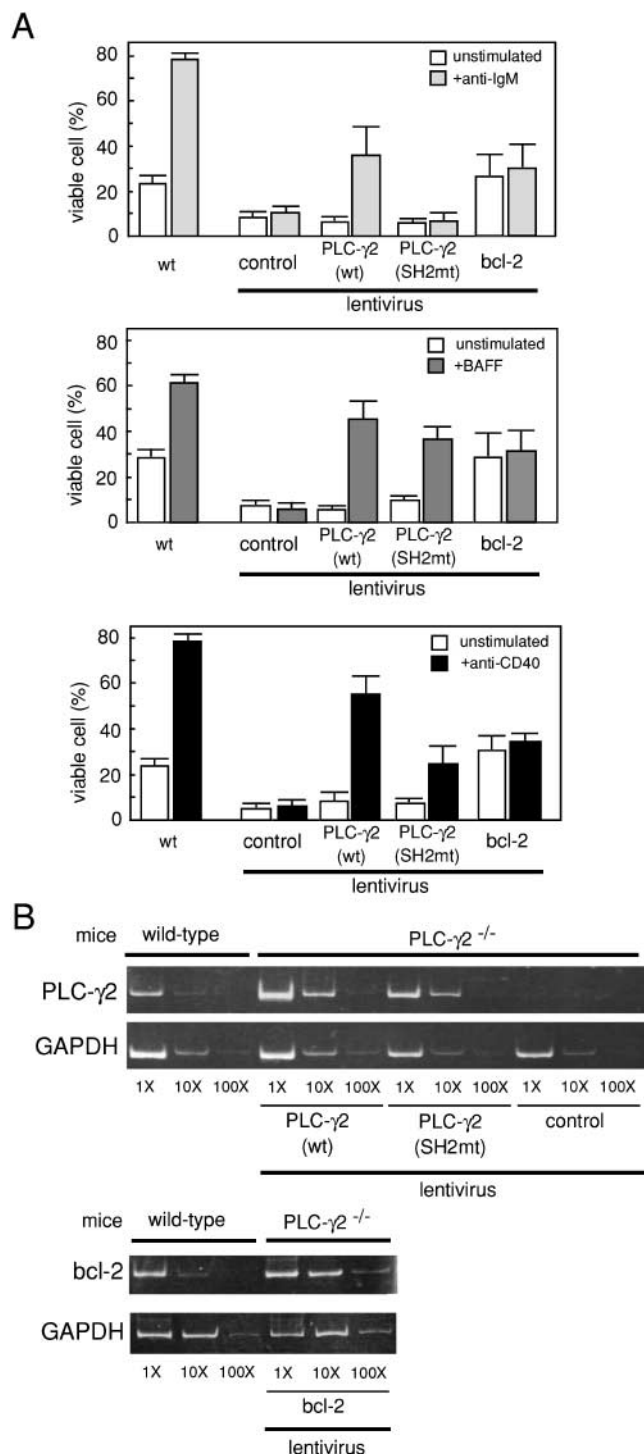


Figure 4. Restoration of BAFF-R-mediated survival of T2 B cells from PLC- $\gamma 2^{-/-}$ mice by a PLC- $\gamma 2$ SH2 mutant. (A) Viabilities of T2 B cells from PLC- $\gamma 2^{-/-}$ mice transduced with lentiviruses encoding either bcl-2, wild-type PLC- $\gamma 2$, or its SH2 mutant. T2 B cells from PLC- $\gamma 2^{-/-}$ mice were sorted as described in Materials and Methods. Obtained T2 B cells without contamination of marginal zone B cells were infected with lentiviruses encoding either bcl-2, wild-type PLC- $\gamma 2$, its SH2 mutant, or empty vector followed by stimulation with 10 μ g/ml anti-IgM F(ab')₂ (top), 2 μ g/ml BAFF (middle), or 2 μ g/ml anti-CD40 mAb (bottom) for 48 h. Infection efficiency achieved ~60%, routinely. The empty vector encoding only GFP was used as a negative control. Viabilities of cultured cells were analyzed by propidium iodide staining. (B) Expression levels of PLC- $\gamma 2$ mRNA and bcl-2 mRNA in transduced cells. To determine the expression levels of PLC- $\gamma 2$ mRNA (top) and bcl-2 (bottom) in transduced cells, RT-PCR was performed as described in Materials and Methods.

stitution, we employed a lentiviral-mediated gene transfer method, because this method does not require preactivation of T2 B cells to make them susceptible to gene transfer. As shown in Fig. 4 A (middle panel), the PLC- γ 2 SH2 mutant promoted the BAFF-mediated survival of T2 B cells, although \sim 25% less compared with wild-type PLC- γ 2. As expected, this mutant failed to promote survival of T2 B cells upon BCR engagement. As a positive control, when wild-type PLC- γ 2 was introduced, both BCR- and BAFF-R-mediated survival signals were restored. Survival

of CD40-activated T2 B cells was, at least to some extent, promoted by the PLC- γ 2 SH2 mutant, suggesting that PLC- γ 2 might be involved in not only BAFF-R-mediated signaling pathway but also in some of other TNF receptors in the similar way. Expression level of PLC- γ 2 mRNA in transduced cells was determined by RT-PCR (Fig. 4 B).

To examine the *in vivo* consequences of the PLC- γ 2 SH2 mutant, we performed complementation experiments with retroviruses carrying either wild-type PLC- γ 2 or its SH2 mutant. These PLC- γ 2 cDNAs were cloned into the

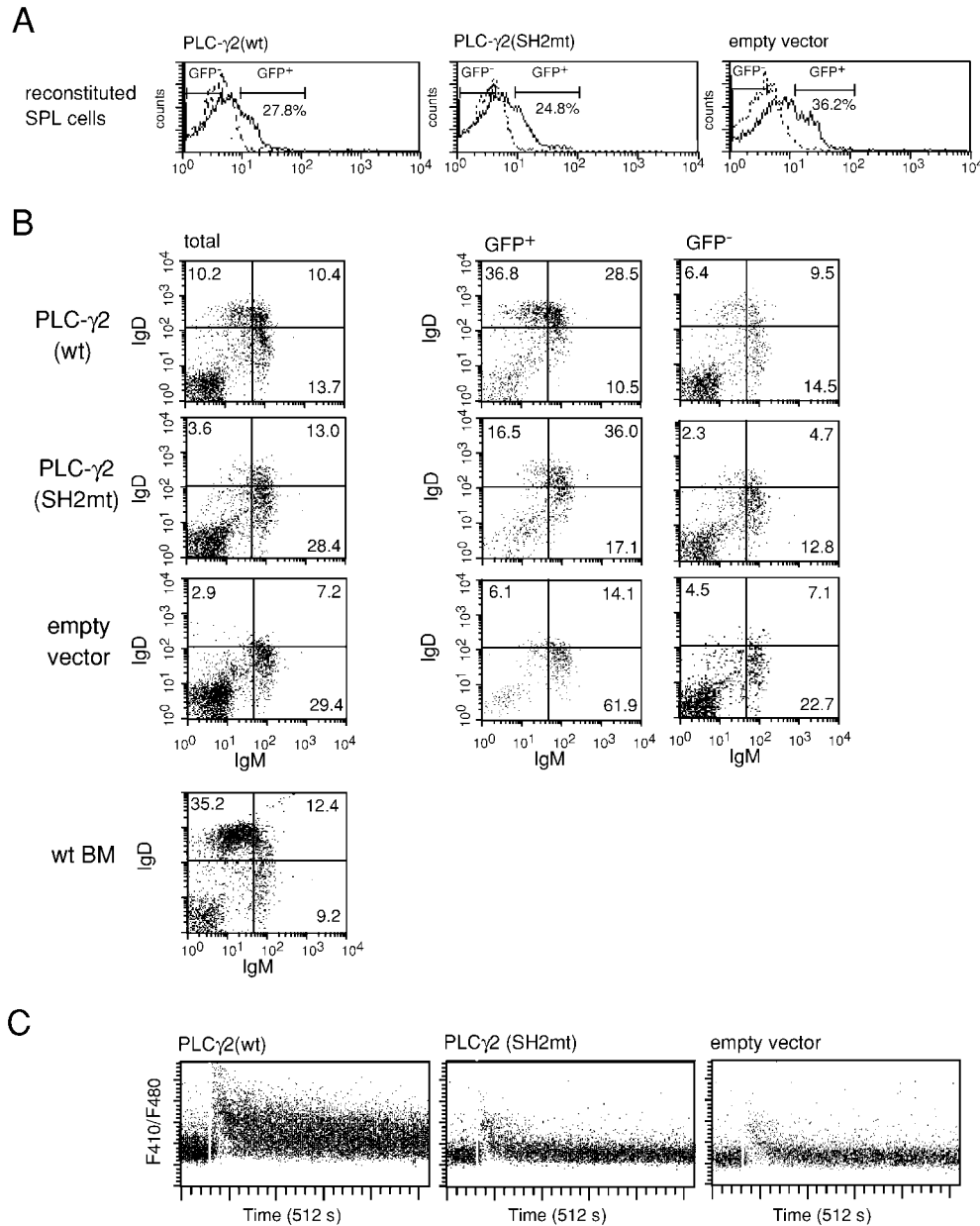


Figure 5. Impaired development of bone marrow cells from PLC- γ 2^{-/-} mice transduced with a retrovirus encoding PLC- γ 2 SH2 mutant. (A) Retroviral infection of bone marrow cells. Bone marrow cells from PLC- γ 2^{-/-} mice were infected with retroviruses encoding either wild-type PLC- γ 2 (wt), its SH2 mutant (PLC- γ 2 (SH2mt)), or empty vector (empty vector) as a negative control. After 2 d, 10⁶ cultured bone marrow cells were then injected *i.v.* to irradiated wild-type mice as described in Materials and Methods. After 9 wk, GFP expression levels in splenocytes from each reconstituted mice were analyzed. Dashed lines indicate the fluorescent levels of uninfected splenocytes. Solid lines indicate the fluorescent levels of splenocytes from reconstituted mice. Bars indicate the gates for analyzing GFP⁺ cells and GFP⁻ cells in B. (B) Expression of IgM and IgD in reconstituted splenocytes. Total splenocytes were analyzed without gating (left) or after gating GFP⁺ cells (middle) and GFP⁻ cells (right) as indicated in (A). Total splenocytes from the mice reconstituted with wild-type bone marrow cells were used as a positive control. The numbers indicate the percentages of live cells in the lymphocyte gates. Mean percentages of IgM^{lo}IgD^{hi}, IgM^{hi}IgD^{hi}, and IgM^{hi}IgD^{lo} cells calculated from three experiments are (9.5 ± 1.2%, 12.7 ± 2.5%, 14.5 ± 3.2%) for PLC- γ 2 transduced cells, (2.9 ± 1.2%, 15.9 ± 3.5%, 29.5 ± 2.4%) for PLC- γ 2 SH2 mutant transduced cells, (3.2 ± 0.9%, 8.2 ± 1.3%, 32.0 ± 3.1%) for control vector transduced cells, and (38.3 ± 4.2%, 15.0 ± 3.1%, 7.3 ± 1.9%) for splenocytes reconstituted with wild-type bone marrow cells, respectively. Panels are representatives of three experiments. Average numbers of total splenocytes in the mice reconstituted with wild-type PLC- γ 2, its SH2 mutant, empty vector infected bone marrow cells calculated from three experiments are (3.4 ± 0.3) × 10⁷, (3.1 ± 0.5) × 10⁷, and (2.8 ± 0.8) × 10⁷, respectively. (C) Calcium mobilization in retrovirally transduced B cells. Bone marrow cells from PLC- γ 2^{-/-} mice were transduced with retroviruses encoding either wild-type PLC- γ 2 (PLC- γ 2 (wt)), its SH2 mutant (PLC- γ 2 (SH2)), or empty vector (empty vector) and injected to irradiated wild-type mice. After 9 wk, reconstituted splenic B cells from each mice were stimulated with 15 μ g/ml anti-IgM F(ab')₂ and calcium mobilization was measured as described in Materials and Methods.

3.1%, 7.3 ± 1.9%) for splenocytes reconstituted with wild-type bone marrow cells, respectively. Panels are representatives of three experiments. Average numbers of total splenocytes in the mice reconstituted with wild-type PLC- γ 2, its SH2 mutant, empty vector infected bone marrow cells calculated from three experiments are (3.4 ± 0.3) × 10⁷, (3.1 ± 0.5) × 10⁷, and (2.8 ± 0.8) × 10⁷, respectively. (C) Calcium mobilization in retrovirally transduced B cells. Bone marrow cells from PLC- γ 2^{-/-} mice were transduced with retroviruses encoding either wild-type PLC- γ 2 (PLC- γ 2 (wt)), its SH2 mutant (PLC- γ 2 (SH2)), or empty vector (empty vector) and injected to irradiated wild-type mice. After 9 wk, reconstituted splenic B cells from each mice were stimulated with 15 μ g/ml anti-IgM F(ab')₂ and calcium mobilization was measured as described in Materials and Methods.

MSCV vector containing an internal ribosomal entry site, which results in the generation of a bicistronic mRNA encoding GFP at its 3' end. Bone marrow cells isolated from PLC- $\gamma 2^{-/-}$ mice were infected with these retroviruses and then injected into lethally irradiated mice. Efficiency of retrovirus infection ranged from 45% to 75% (unpublished data). After 9 wk, 25% to 40% of the spleen cells from reconstituted mice were GFP⁺ (Fig. 5 A). Representative data from three experiments are shown in Fig. 5 B. FACS[®] analysis of spleen cells demonstrated that wild-type PLC- $\gamma 2$, but not its SH2 mutant, was able to correct the maturation defect in PLC- $\gamma 2^{-/-}$ B cells. Although requirement for the PLC- $\gamma 2$ SH2 domains in development of IgM^{lo}IgD^{hi} mature population is clear, a reproducible increase, albeit small, of IgM^{hi}IgD^{hi} population was observed in mice reconstituted with the PLC- $\gamma 2$ SH2 mutant, compared with control mice. GFP⁻ cells showed similar expression pattern of IgM and IgD as PLC- $\gamma 2^{-/-}$ mice in every case. Collectively, these results suggest that the BAFF-R-mediated survival signal, provided by PLC- $\gamma 2$, contributes, in

part, to the survival of immature B cells, but is not sufficient to promote B cell maturation. Further development into mature B cells is therefore likely to require the BCR signal through PLC- $\gamma 2$. Indeed, shutting off the BCR signal by the PLC- $\gamma 2$ SH2 mutant was confirmed by both calcium and NF- κ B data (Figs. 5 C and 3 C).

When bcl-2 was introduced to PLC- $\gamma 2^{-/-}$ T2 B cells by lentiviruses, the viability was significantly increased in the absence of exogenous agonists (Fig. 4 A). This result is well consistent with the concept that Bcl-2 expression delays apoptotic cell death in many cell types including mature B cells that have lost their receptors by I μ deletion (10). Thus, to examine whether the survival signal emanating from BCR and BAFF-R is sufficient for B cell maturation, we performed a passive transfer experiment of PLC- $\gamma 2^{-/-}$ bone marrow cells infected with a retrovirus encoding bcl-2. RT-PCR analysis of the infected splenic B cells demonstrated that the expression level of bcl-2 was 5–10 times higher than that in control wild-type B cells (unpublished data). As shown in Fig. 6 B, transduction of PLC- $\gamma 2^{-/-}$

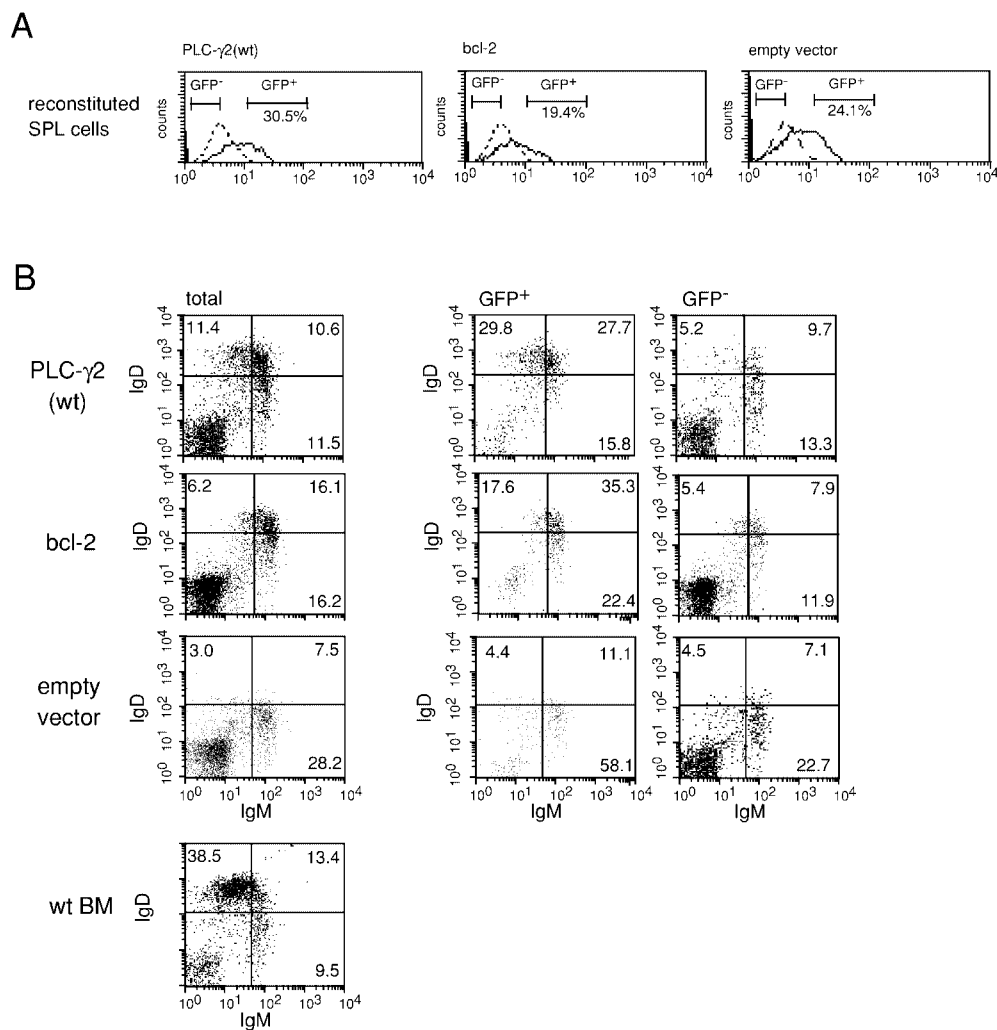


Figure 6. Impaired development of bone marrow cells from PLC- $\gamma 2^{-/-}$ mice transduced with a retrovirus encoding bcl-2. (A) Retroviral infection of bone marrow cells. Like in Fig. 5, bone marrow cells from PLC- $\gamma 2^{-/-}$ mice were infected with retroviruses encoding either wild-type PLC- $\gamma 2$ (PLC- $\gamma 2$ (wt)), bcl-2 (bcl-2), or empty vector (empty vector) as a negative control. Dashed lines indicate the fluorescent levels of uninfected splenocytes. Solid lines indicate the fluorescent levels of splenocytes from reconstituted mice. Bars indicate the gates for analyzing GFP⁺ cells and GFP⁻ cells in B. (B) Expression of IgM and IgD in reconstituted splenocytes. Reconstituted splenocytes were analyzed as in Fig. 4. The numbers indicate the percentages of live cells in the lymphocyte gates. Mean percentages of IgM^{lo}IgD^{hi}, IgM^{hi}IgD^{hi}, and IgM^{hi}IgD^{lo} cells calculated from three experiments are (11.2 \pm 1.4%, 11.3 \pm 2.3%, 13.5 \pm 3.4%) for PLC- $\gamma 2$ transduced cells, (6.9 \pm 2.1%, 18.7 \pm 3.1%, 15.5 \pm 1.2%) for bcl-2 transduced cells, (2.9 \pm 0.7%, 7.9 \pm 1.1%, 30.8 \pm 2.9%) for control vector transduced cells, and (35.8 \pm 2.9%, 7.9 \pm 1.1%, 2.9 \pm 0.7%) for splenocytes reconstituted with wild-type bone marrow cells. Panels are representatives of three experiments. Average numbers of total splenocytes in the mice re-

constituted with bone marrow cells transduced with retroviruses encoding either wild-type PLC- $\gamma 2$, bcl-2, or empty vector calculated from three experiments are (3.4 \pm 0.3) $\times 10^7$, (3.9 \pm 0.3) $\times 10^7$, and (2.8 \pm 0.8) $\times 10^7$, respectively.

precursor cells with *bcl-2* could not completely rescue the B cell development, suggesting that PLC- γ 2 mediates an additional signal, presumably a differentiation signal, from BCR to promote B cell maturation.

Discussion

Here, we have demonstrated that the loss of mature follicular B cells in PLC- γ 2^{-/-} mice is intrinsic to B cells. Thus, it is most likely that a cell surface receptor on B lymphocytes signals to activate PLC- γ 2 in order for development of mature B cells. In this regard, BAFF-R has recently emerged as an important receptor in transition from T1 B cells to mature B cells (11–13). Survival of PLC- γ 2^{-/-} T2 B cells following BAFF treatment was almost completely abolished. This could be explained by two possibilities: (a) BAFF-R utilizes PLC- γ 2 to exert its survival signal presumably through the NF- κ B pathway; (b) this defective survival signal reflects more general signaling perturbation induced by loss of PLC- γ 2. The first possibility seems to be more likely, because PLC- γ 2^{-/-} T2 B cells, like wild-type cells, proliferate after LPS treatment (18, 19).

The coupling mechanism between BAFF-R and PLC- γ 2 appears to differ from the case of BCR. In contrast to BCR stimulation, BAFF was not able to induce tyrosine phosphorylation of PLC- γ 2 and subsequent calcium mobilization (32), simply suggesting that PLC- γ 2 contributes to BAFF-R signaling in a lipase-independent manner. Given the importance of PLC- γ 2 SH3 domain, but not its lipase activity, in agonist-induced activation of calcium entry, PLC- γ 2 might play a structural role in BAFF-R signaling.

Our data suggest the importance of the BAFF-R-mediated survival signal, provided by PLC- γ 2, in expansion of immature B cells *in vivo*, as the PLC- γ 2 SH2 mutant (which supports BAFF-mediated survival) promoted an increase in IgM^{hi}IgD^{hi} immature, but not IgM^{lo}IgD^{hi} mature B cells. These findings, together with the evidence that our *in vitro* culture system, similar to a report by Batten et al. (21), could induce differentiation of T2 B to mature B cells in costimulation of anti-BCR Ab and BAFF (unpublished data), might support the proposed idea that BAFF primarily functions to survive immature B cells (21), rather than that BAFF plays a direct role in maturation from immature to mature B cells (28). Although we interpret expansion of immature B cells by the PLC- γ 2 SH2 domain as restoration of BAFF-R signal, our data cannot completely exclude the possibility that this PLC- γ 2 mutant mediates signaling emanating from an yet unidentified receptor, thereby manifesting this phenotype.

Based on the evidence that mature B cells, upon inducible deletion of BCR, undergo programmed cell death, it has been proposed that BCR generates a survival signal (10). Two models for explaining how BCR expression might protect B cells from apoptosis have been thought: first, receptor assembly on the cell surface might be sufficient to produce a constitutive survival signal; alternatively, the tonic signal might be produced by low-affinity interac-

tion between BCR and self-antigens. Although our experiments do not distinguish these two possibilities, cross-linking of BCR is thought to mimic some aspects of this BCR function, because it protects them from apoptosis. Hence, our data suggest that PLC- γ 2 mediates the survival signal emanating from the BCR by an SH2-dependent manner. PLC- γ 2-mediated hydrolysis of PIP₂ generates IP₃ and DAG, which trigger two parallel pathways that lead to a rise in cytoplasmic Ca²⁺ and activation of PKC β . Given the requirement for PKC β in BCR-mediated survival through IKK activation (27, 33), the defective BCR-mediated survival in PLC- γ 2^{-/-} T2 B cells could be explained by the PKC β axis.

However, the survival signal, by itself, appears not to be sufficient, as *bcl-2* overexpression cannot completely rescue the B cell development in PLC- γ 2^{-/-} cells (Fig. 6). Hence, we propose that PLC- γ 2 conveys two signals, survival and differentiation, from BCR, both of which are sequentially or concomitantly required for mature B cell development.

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References

1. Meffre, E., R. Casellas, and M.C. Nussenzweig. 2000. Antibody regulation of B cell development. *Nat. Immunol.* 1:379–385.
2. Pillai, S. 1999. The chosen few? Positive selection and the generation of naive B lymphocytes. *Immunity.* 10:493–502.
3. Rajewsky, K. 1996. Clonal selection and learning in the antibody system. *Nature.* 381:751–758.
4. Loder, F., B. Mutschler, R.J. Ray, C.J. Paige, P. Sideras, R. Torres, M.C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.* 190:75–89.
5. Niuro, H., and E.A. Clark. 2002. Regulation of B-cell fate by antigen-receptor signals. *Nat. Rev. Immunol.* 2:945–956.
6. Kurosaki, T. 2002. Regulation of B cell fates by BCR signaling components. *Curr. Opin. Immunol.* 14:341–347.
7. Benschop, R.J., and J.C. Cambier. 1999. B cell development: signal transduction by antigen receptors and their surrogates. *Curr. Opin. Immunol.* 11:143–151.
8. Reth, M. 2001. Oligomeric antigen receptors: a new view on signaling for the selection of lymphocytes. *Trends Immunol.* 22:356–360.
9. Torres, R.M., H. Flaswinkel, M. Reth, and K. Rajewsky. 1996. Aberrant B cell development and immune response in mice with a compromised BCR complex. *Science.* 272:1802–1804.

10. Lam, K.P., R. Kuhn, and K. Rajewsky. 1997. In vivo ablation of surface immunoglobulin on mature B cells by inducible gene targeting results in rapid cell death. *Cell*. 90:1073–1083.
11. Mackay, F., and J.L. Browning. 2002. BAFF: a fundamental survival factor for B cells. *Nat. Rev. Immunol.* 2:465–475.
12. Sun, X.H., and P.W. Kincade. 2002. Gatekeeper mechanisms for immature B cells. *Nat. Immunol.* 3:898–899.
13. Rolink, A.G., and F. Melchers. 2002. BAFFed B cells survive and thrive: roles of BAFF in B-cell development. *Curr. Opin. Immunol.* 14:266–275.
14. Thompson, J.S., S.A. Bixler, F. Qian, K. Vora, M.L. Scott, T.G. Cachero, C. Hession, P. Schneider, I.D. Sizing, C. Mullen, et al. 2001. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. *Science*. 293:2108–2111.
15. Yan, M., J.R. Brady, B. Chan, W.P. Lee, B. Hsu, S. Harless, M. Cancro, I.S. Grewal, and V.M. Dixit. 2001. Identification of a novel receptor for B lymphocyte stimulator that is mutated in a mouse strain with severe B cell deficiency. *Curr. Biol.* 11:1547–1552.
16. Schiemann, B., J.L. Gommerman, K. Vora, T.G. Cachero, S. Shulga-Morskaya, M. Dobles, E. Frew, and M.L. Scott. 2001. An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science*. 293:2111–2114.
17. Gross, J.A., S.R. Dillon, S. Mudri, J. Johnston, A. Littau, R. Roque, M. Rixon, O. Schou, K.P. Foley, H. Haugen, et al. 2001. TACI-Ig neutralizes molecules critical for B cell development and autoimmune disease. Impaired B cell maturation in mice lacking BlyS. *Immunity*. 15:289–302.
18. Hashimoto, A., K. Takeda, M. Inaba, M. Sekimata, T. Kai-sho, S. Ikehara, Y. Homma, S. Akira, and T. Kurosaki. 2000. Cutting edge: essential role of phospholipase C- γ 2 in B cell development and function. *J. Immunol.* 165:1738–1742.
19. Wang, D., J. Feng, R. Wen, J.C. Marine, M.Y. Sangster, E. Parganas, A. Hoffmeyer, C.W. Jackson, J.L. Cleveland, P.J. Murray, and J.N. Ihle. 2000. Phospholipase C γ 2 is essential in the functions of B cell and several Fc receptors. *Immunity*. 13:25–35.
20. Sakai, K., and J. Miyazaki. 1997. A transgenic mouse line that retains Cre recombinase activity in mature oocytes irrespective of the cre transgene transmission. *Biochem. Biophys. Res. Commun.* 237:318–324.
21. Batten, M., J. Groom, T.G. Cachero, F. Qian, P. Schneider, J. Tschopp, J.L. Browning, and F. Mackay. 2000. BAFF mediates survival of peripheral immature B lymphocytes. *J. Exp. Med.* 192:1453–1466.
22. Su, T.T., and D.J. Rawlings. 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. *J. Immunol.* 168:2101–2110.
23. Pasparakis, M., M. Schmidt-Supprian, and K. Rajewsky. 2002. I κ B kinase signaling is essential for maintenance of mature B cells. *J. Exp. Med.* 196:743–752.
24. Martin, F., and J.F. Kearney. 2002. Marginal-zone B cells. *Nat. Rev. Immunol.* 2:323–335.
25. Hsu, B.L., S.M. Harless, R.C. Lindsley, D.M. Hilbert, and M.P. Cancro. 2002. Cutting edge: BlyS enables survival of transitional and mature B cells through distinct mediators. *J. Immunol.* 168:5993–5996.
26. Petro, J.B., R.M. Gerstein, J. Lowe, R.S. Carter, N. Shinnars, and W.N. Khan. 2002. Transitional type 1 and 2 B lymphocyte subsets are differentially responsive to antigen receptor signaling. *J. Biol. Chem.* 277:48009–48019.
27. Su, T.T., B. Guo, Y. Kawakami, K. Sommer, K. Chae, L.A. Humphries, R.M. Kato, S. Kang, L. Patrone, R. Wall, et al. 2002. PKC- β controls I κ B kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol.* 3:780–786.
28. Rolink, A.G., J. Tschopp, P. Schneider, and F. Melchers. 2002. BAFF is a survival and maturation factor for mouse B cells. *Eur. J. Immunol.* 32:2004–2010.
29. Claudio, E., K. Brown, S. Park, H. Wang, and U. Siebenlist. 2002. BAFF-induced NEMO-independent processing of NF- κ B2 in maturing B cells. *Nat. Immunol.* 3:958–965.
30. Ishiai, M., H. Sugawara, M. Kurosaki, and T. Kurosaki. 1999. Cutting edge: association of phospholipase C- γ 2 Src homology 2 domains with BLNK is critical for B cell antigen receptor signaling. *J. Immunol.* 163:1746–1749.
31. Petro, J.B., and W.N. Khan. 2001. Phospholipase C- γ 2 couples Bruton's tyrosine kinase to the NF- κ B signaling pathway in B lymphocytes. *J. Biol. Chem.* 276:1715–1719.
32. Patterson, R.L., D.B. van Rossum, D.L. Ford, K.J. Hurt, S.S. Bae, P.G. Suh, T. Kurosaki, S.H. Snyder, and D.L. Gill. 2002. Phospholipase C- γ is required for agonist-induced Ca²⁺ entry. *Cell*. 111:529–541.
33. Saijo, K., I. Mecklenbrauker, A. Santana, M. Leitger, C. Schmedt, and A. Tarakhovsky. 2002. Protein kinase C β controls nuclear factor κ B activation in B cells through selective regulation of the I κ B kinase α . *J. Exp. Med.* 195:1647–1652.