Phospholipase C γ 2 Contributes to Light-Chain Gene Activation and Receptor Editing[∇]

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Phospholipase C γ 2 (PLC γ 2) is critical for pre-B-cell receptor (pre-BCR) and BCR signaling. Current studies discovered that PLC γ 2-deficient mice had reduced immunoglobulin λ (Ig λ) light-chain usage throughout B-cell maturation stages, including transitional type 1 (T1), transitional type 2 (T2), and mature follicular B cells. The reduction of Ig λ rearrangement by PLC γ 2 deficiency was not due to specifically increased apoptosis or decreased proliferation of mutant Ig λ ⁺ B cells, as lack of PLC γ 2 exerted a similar effect on apoptosis and proliferation of both Ig λ ⁺ and Ig κ ⁺ B cells. Moreover, PLC γ 2-deficient Ig^{HEL} transgenic B cells exhibited an impairment of antigen-induced receptor editing among both the endogenous λ and κ loci in vitro and in vivo. Importantly, PLC γ 2 deficiency impaired BCR-induced expression of IRF-4 and IRF-8, the two transcription factors critical for λ and κ light-chain rearrangements. Taken together, these data demonstrate that the PLC γ 2 signaling pathway plays a role in activation of light-chain loci and contributes to receptor editing.

During B-cell development, pro-B cells begin the process of immunoglobulin (Ig) heavy (H) gene rearrangement (1, 8, 38, 50). Successful rearrangement of Ig H-chain variable (V), diversity (D), and joining (J) gene segments leads to the formation of the pre-B-cell receptor (pre-BCR), which contains the newly generated H chain in complex with the Vpre-B/ λ 5 surrogate light (L) chain. Signals from the pre-BCR instruct pre-B cells to expand and halt further H-chain VDJ rearrangement to ensure allelic exclusion (15, 30, 39). Subsequently, the rapidly proliferating pre-B cells exit the cell cycle and undergo rearrangement of Ig L-chain V and J gene segments. The Ig L chain is encoded by the κ and λ loci. Rearrangements within the κ and λ L-chain loci occur independently with a sequential activation of the κ and λ loci (2, 10, 39, 72). In mice, the κ locus comprises 70 to 90 functional V κ and four functional J κ gene segments whereas the λ locus contains three functional V λ and three functional J λ segments (2, 55). It is believed that the kinetics and efficiency of gene segment rearrangements as well as the number of functional V κ and V λ gene segments largely determine the ratio of Ig κ ⁺ to Ig λ ⁺ mature B cells (2, 10, 14, 72). In mice, the ratio is 95% κ to 5% λ (14, 61). A successfully rearranged L chain in combination with the previously rearranged H chain generates a surface IgM form of the BCR. B cells with a functional BCR quickly progress into immature B cells and emerge from bone marrow (BM) into the spleen (16, 20). In the spleen, signals emanating from the BCR direct immature B cells to mature through transitional B cells of type 1 (T1) and type 2 (T2) stages and thereafter to long-lived

follicular B cells (34). Deletion of the BCR not only arrests B-cell development but also causes apoptosis (25, 27).

To establish B-cell tolerance, immature B cells expressing a self-reactive BCR are negatively selected during maturation by several distinct mechanisms, including clonal deletion, clonal anergy, and receptor editing (20, 42, 45, 49, 50, 56). The BCR signaling threshold appears to regulate the negative selection process. Highly self-reactive cells recognizing membrane-bound self antigens are eliminated by clonal deletion (17, 43), whereas self-reactive cells recognizing soluble self antigens become unresponsive to antigens, a state termed anergy (13). Moreover, self-reactive immature B cells can initiate new rearrangements of V genes, mainly at L-chain loci, to change receptor specificity by receptor editing (5, 7, 12, 48, 63). However, the mechanism that regulates the sequential activation of the κ and λ loci for recombination and the initiation of receptor editing in self-reactive B cells is not clear. Elevation of *RAG1* and *RAG2* mRNA levels has been observed during receptor editing in B cells (21, 36, 37). Although signals from the BCR have been shown to be involved (33, 67), the basis of this RAG up-regulation is not known. IRF-4 and IRF-8 are two members of the interferon regulatory factor (IRF) family of transcription factors whose expression can be induced by BCR engagement (9, 35, 62). Studies have implied important roles for both IRF-4 and IRF-8 in regulating L-chain rearrangements (4, 40, 46, 59). Importantly, lack of both IRF-4 and IRF-8 prevents rearrangements of Ig L-chain but not H-chain genes during B-cell development (31).

The BCR initiates signaling cascades via the two transmembrane molecules Ig α and Ig β and sequential activation of members of three distinct families of cytoplasmic protein tyrosine kinases, including Lyn, Syk, and Btk (22, 24, 64). Subsequent recruitment and tyrosine phosphorylation of the adapter protein, B-cell linker protein (BLNK), and transmembrane protein CD19

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facilitate activation of the lipid kinase phosphatidylinositol 3-kinase (22, 26, 44, 51). An important outcome of these events is activation of phospholipase $\text{C}\gamma 2$ ($\text{PLC}\gamma 2$), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate diacylglycerol and inositol 1,4,5-trisphosphate, both of which are required second messengers for cellular responses (53, 54). $\text{PLC}\gamma 2$ -deficient mice exhibit impaired early and late B-cell development, and $\text{PLC}\gamma 2$ -deficient B cells are unable to respond to antigens, demonstrating that $\text{PLC}\gamma 2$ plays an essential role in B-cell development and function (18, 68–70). Our current studies demonstrate that $\text{PLC}\gamma 2$ plays an important role in activation of the L-chain loci for recombination and receptor editing of self-reactive B cells.

MATERIALS AND METHODS

Mice. $\text{PLC}\gamma 2$ -deficient mice were generated previously (68) and have been backcrossed to the C57BL/6 background. Ig^{HEL} transgenic mice (C57BL/6 MD4), which bear rearranged Ig H- and Igk L-chain genes that encode a hen egg lysozyme (HEL)-specific BCR, and sHEL transgenic mice (C57BL/6 ML5), which express soluble HEL (sHEL), were obtained from the Jackson Laboratory. These transgenic mice were bred with heterozygous $\text{PLC}\gamma 2^{+/-}$ mice to ultimately generate wild-type Ig^{HEL} , $\text{PLC}\gamma 2$ -deficient Ig^{HEL} , wild-type Ig^{HEL} sHEL, and $\text{PLC}\gamma 2$ -deficient Ig^{HEL} sHEL mice. Mice used for the experiments were generally 8 to 12 weeks of age except where specifically indicated.

Induction of receptor editing in vitro. BM cells were isolated from the indicated mice and were depleted of red blood cells by lysis with Gey's solution (0.15 M NH_4Cl , 1 mM KHCO_3 , and 0.1 mM Na_2EDTA). The cells (2×10^6 cells/ml) were cultured in complete medium RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 ng/ml interleukin-7 (IL-7) (R&D Systems) for 5 days. Then, cells were washed extensively with phosphate-buffered saline (PBS) and recultured at 1×10^6 cells/ml in complete medium without IL-7 and in the presence or absence of HEL (500 ng/ml) (lysozyme-biotin-caproyl; lot no. 071k1567; Sigma). After a further 2-day culture, the cells were harvested for analysis of λ chain protein expression and λ chain gene rearrangements.

Splenocytes ($2 \times 10^6/\text{ml}$) from the indicated mice were cultured in complete medium in the presence or absence of freshly added HEL (500 ng/ml). After a 2-day culture, the cells were harvested for analysis of λ chain protein expression and λ chain gene rearrangements.

Detection of λ and κ chain gene rearrangements. Genomic DNA was isolated from the indicated cells and was quantified by semiquantitative PCR amplification of the β -actin gene (5' primer, ACTCTATGTGGGTGACGAG; 3' primer, CAGGTCCAGACGACGATGGC). The genomic DNA then was subjected to semiquantitative PCR analysis of endogenous λ or κ chain gene rearrangement. Primers employed in the PCR were the same as previously described (41, 73). Specifically, $\text{V}\lambda 1/2$ (AGAAGCTTGACTCAGGAATCTGCA) and $\text{J}\lambda 1$ (CAGGATCCTAGGACAGTCAGTTTGGT) primers were used to amplify $\lambda 1$ rearrangements, $\text{V}\lambda 2$ (ACTGGTCTAATCGGTGGTACCA) and $\text{C}\lambda 2$ (AGGAAGCTGCTGGCCATGAAGCTTGTG) primers were used to amplify $\lambda 2$ rearrangements, and $\text{V}\lambda x$ (GAGCTTAAGAAAGATGGAAGCCA) and $\text{C}\lambda 2$ primers were used to amplify λx rearrangements. The $\text{V}\kappa$ degenerate primer (GGCTGCAGSTTCAGTGGCAGTGGRTCW) and the reverse primer downstream of the $\text{J}\kappa 1$ coding region (GTTCTTTGCCCTTGGAGAGTGCCAGAATC) were used to amplify $\text{V}\kappa\text{-J}\kappa 1$ rearrangement (11, 21). PCRs were performed as previously described (47, 65). Briefly, the PCR was carried out in a 25- μl final volume containing 0.5 μl of deoxynucleoside triphosphate (10 mM), 1 μl of the primers (10 μM), 2.5 μl of $10\times$ PCR buffer, 5 μl of $5\times$ Q solution, 2.5 units of *Taq* enzyme (QIAGEN), and a serial dilution of template (25 to 1 ng). For detection of λ chain gene rearrangement, cycling conditions were 94°C for 4 min followed by 42 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 90 s and a final extension step at 72°C for 5 min. For detection of $\text{V}\kappa\text{-J}\kappa 1$ rearrangement, cycling conditions were 97°C for 45 s, 70°C for 1 min, and 72°C for 2.5 min for five cycles; followed by 94°C for 45 s, 70°C for 1 min, and 72°C for 2.5 min for another 29 to 32 cycles; and final extension at 72°C for 6 min.

PCR detection of recombination sequence (RS) rearrangement in the genomic DNA from the indicated cells was performed as previously described (19). Briefly, the primers within the $\text{J}\kappa$ intron (CTGACTGCAGGTAGCGTG GTCTTCTAG) and downstream of the recombination signal sequence (RSS) (TTGACTGTTTGCTACTTCAGCTCACTG) were used. Cycling conditions were 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 35 cycles. Products

were separated by electrophoresis in a 1% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P -labeled 3' primer. Genomic DNA was quantified by semiquantitative PCR amplification of the β -actin gene.

Flow cytometry. Single-cell suspensions of spleen and BM cells were treated with Gey's solution to remove red blood cells. Freshly isolated or cultured cells were resuspended in PBS supplemented with 2% fetal bovine serum and then stained with a combination of fluorescence-conjugated antibodies. CyChrome-conjugated anti-B220 (150452) and allophycocyanin-conjugated anti-IgM (17-5790-82) antibodies were purchased from eBioscience. Phycoerythrin (PE)-conjugated anti-IgD (1120-09L), PE-conjugated anti-Ig κ (1170-09), and PE-conjugated anti-Ig λ (1175-09L) antibodies were purchased from Southern Biotechnology. Fluorescein isothiocyanate (FITC)-conjugated anti-Ig λ (553434) antibodies were purchased from BD Biosciences. Samples were applied to a flow cytometer (LSRII; BD Biosciences), and data were collected and analyzed using CellQuest software (BD Biosciences). For all analyses, forward and side scatter gates were set to include viable cells and to exclude dead cells and debris.

TUNEL assay. Single-cell suspensions of spleen and BM cells were stained with a combination of CyChrome-conjugated anti-B220 and PE-conjugated anti-Ig λ or PE-conjugated anti-Ig κ antibodies. Then, the cells were washed with PBS, fixed for 2 h in 4% paraformaldehyde in PBS at room temperature, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice, and labeled with FITC-conjugated terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) enzyme for 1 h in moist darkness at 37°C according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche). The degree of TUNEL positivity in the gated $\text{B220}^+ \text{Ig}\lambda^+$ or $\text{B220}^+ \text{Ig}\kappa^+$ subpopulation was analyzed by flow cytometry using CellQuest software.

BrdU incorporation assay. The *in vivo* 5-bromo-2'-deoxyuridine (BrdU) staining assay was performed as previously described (71). Briefly, mice were injected intraperitoneally with 1 mg BrdU in 200 μl PBS at 12-h intervals for 5 days. Single-cell suspensions of spleen and BM cells were stained with a combination of CyChrome-conjugated anti-B220 and PE-conjugated anti-Ig λ or PE-conjugated anti-Ig κ antibodies at 4°C for 30 min. The cells were washed with PBS, resuspended in ice-cold 0.15 M saline, and incubated on ice for 30 min with the addition of 95% ethanol. After washing, the cells were fixed with 1% paraformaldehyde containing 0.01% Tween 20 for 1 h at room temperature. Lastly, the cells were incubated with 50 Kunitz units/ml DNase at 37°C for 10 min, washed with PBS, and stained with FITC-conjugated anti-BrdU antibody (34783; BD Biosciences). The degree of BrdU positivity in the gated $\text{B220}^+ \text{Ig}\lambda^+$ or $\text{B220}^+ \text{Ig}\kappa^+$ subpopulation was analyzed by flow cytometry using CellQuest software.

Real-time quantitative RT-PCR analysis. Total RNA was isolated from the indicated cells, and first-strand cDNA was synthesized from the total RNA with the OneStep reverse transcription-PCR (RT-PCR) kit (210210; QIAGEN) using random primers (HFR704; Gibco) according to the manufacturer's protocols. Real-time PCR was performed to quantitate germ line transcription of $\text{V}\kappa$ and $\text{V}\lambda$ using the following primers (23, 57): κ germ line 5' primer, AGGAGGGT TTTGTACAGCCAGA; κ germ line 3' primer, TGGATGGTGGGAAGATG GAT; $\lambda 2$ germ line 5' primer, GCTGTGAGAGAACAGGACCA; $\lambda 2$ germ line 3' primer, CTCGGGGAAAAGTTGGAAT. Levels of $\text{V}\kappa$ and $\text{V}\lambda$ germ line transcription were normalized to β -actin abundance. The primers used for β -actin were as follows: 5' primer, CCACAGCTGAGAGGGAAATC, and 3' primer, CTTCTCCAGGGAGGAAGAGG. Real-time PCR was performed to analyze IRF-4 and IRF-8 gene expression using the following primers: IRF-4 5' primer, AGATTCCAGGTGACTCTGTG; IRF-4 3' primer, CTGCCCTGTGACAGTA TTTC; IRF-8 5' primer, GGGAAGTTTAAAGAGGGAGA; IRF-8 3' primer, GATCAGCTCCTCAATCTCTG. Levels of IRF-4 and IRF-8 transcript were normalized to CD19 abundance. The primers used for CD19 were as follows: 5' primer, AATCCACGCATTCAAGTCCAG, and 3' primer, GAGCCCTCTC GCTGTCTG. Real-time PCR was carried out in duplicate or triplicate at 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min in an iCycler-iQ (Bio-Rad) in 25- μl reaction volumes containing cDNA, primers, and IQ Supermix (170-8862; Bio-Rad). SYBR green was used as the detection reagent. Data were analyzed using standard curves generated for each sample by a series of four consecutive 10-fold dilutions (1×10^1 to 1×10^4) of the cDNA template and using iQ Cycler analyzing software and relative transcription calculated using the threshold cycle method. The specificity of the RT-PCR was controlled by the generation of melting curves, PCR efficiencies were $100\% \pm 15\%$, and correlation coefficients were 0.988 to 1.

RESULTS

$\text{PLC}\gamma 2$ -deficient mice have reduced Ig λ L-chain usage throughout B-cell maturation. To determine whether $\text{PLC}\gamma 2$

FIG. 1. Reduction of Ig λ chain expression in PLC γ 2-deficient B cells. (A) BM cells were isolated from wild-type (PLC γ 2^{+/+}) and PLC γ 2-deficient (PLC γ 2^{-/-}) mice and stained with a combination of anti-B220 and anti-Ig λ or anti-Ig κ antibodies. Percentages indicate Ig λ ⁺ or Ig κ ⁺ B cells in the gated live B220⁺ lymphoid population. Data are representative of 10 mice per genotype. (B) Statistical analysis of the percentages of Ig λ ⁺ B cells in the gated live BM B220⁺ lymphoid population from panel A ($n = 10$; *, $P < 0.01$). (C) Splenocytes were isolated from PLC γ 2^{+/+} and PLC γ 2^{-/-} mice and stained with a combination of anti-B220 and anti-Ig λ or anti-Ig κ antibodies. Percentages indicate Ig λ ⁺ or Ig κ ⁺ B cells in the gated live B220⁺ lymphoid population. Data are representative of 10 mice per genotype. (D) Statistical analysis of the percentages of Ig λ ⁺ B cells in the gated live splenic B220⁺ lymphoid population from panel C ($n = 10$; *, $P < 0.01$).

plays a role in L-chain gene rearrangement, we examined the expression of Ig λ and Ig κ L chains in PLC γ 2-deficient mice, which were generated previously (68). First, BM cells were stained with an antibody specific for the Ig λ or Ig κ L-chain constant regions in conjunction with anti-B220 antibody. The proportion of BM B cells that expressed Ig λ L chain was markedly reduced in PLC γ 2-deficient mice relative to wild-type mice (Fig. 1A and B). In contrast, the proportion of BM B cells that expressed Ig κ L chain was comparable or slightly increased in PLC γ 2-deficient mice relative to wild-type mice (Fig. 1A). Next, the expression of Ig λ or Ig κ L chain in splenic B cells was examined. Again, the fraction of splenic B cells expressing Ig λ was markedly reduced in PLC γ 2-deficient mice relative to wild-type mice (Fig. 1C and D), whereas the fraction of splenic B cells expressing Ig κ was comparable between PLC γ 2-deficient and wild-type mice (Fig. 1C). Therefore, PLC γ 2 deficiency reduces the subpopulation of B cells that express Ig λ .

Moreover, we further determined the effect of PLC γ 2 deficiency on the expression of Ig λ during the whole process of B-cell maturation. Based on the expression of IgM and IgD, B-cell maturation can be divided into several stages (29, 34).

Interestingly, the proportions of IgM⁺ IgD⁻ immature B cells, IgM⁺ IgD^{lo} transitional B cells, and IgM⁺ IgD^{hi} mature B cells that expressed Ig λ were all reduced in BM cells derived from PLC γ 2-deficient mice relative to wild-type mice, with a more severe reduction in Ig λ expression observed in B-cell subsets at earlier maturation stages (Fig. 2A and B). Similarly, a reduction of the proportion of cells expressing Ig λ was observed in IgM^{hi} IgD⁻ T1, IgM^{hi} IgD⁺ T2, and IgM^{lo} IgD⁺ mature splenic B cells from PLC γ 2-deficient mice relative to wild-type mice, with the reduction being more severe in the least-mature subpopulations (Fig. 2C and D). Taken together, these data demonstrate that PLC γ 2 deficiency results in reduced Ig λ usage throughout B-cell maturation.

Reduced Ig λ usage as a consequence of PLC γ 2 deficiency is not due to increased apoptosis or decreased proliferation of PLC γ 2-deficient Ig λ ⁺ relative to Ig κ ⁺ B cells. A possible explanation for the decreased frequency of Ig λ ⁺ B cells in PLC γ 2-deficient mice relative to wild-type mice is that PLC γ 2 deficiency specifically increases apoptosis or decreases proliferation of Ig λ ⁺ relative to Ig κ ⁺ B cells. To address this issue, we first used TUNEL staining to quantify the degree of apoptosis in Ig λ ⁺ and Ig κ ⁺ B cells from wild-type and PLC γ 2-

FIG. 2. Reduction of Ig λ chain expression during B-cell development in PLC γ 2-deficient mice. (A) BM cells were isolated from wild-type (PLC γ 2^{+/+}) and PLC γ 2-deficient (PLC γ 2^{-/-}) mice and stained with a combination of anti-B220, anti-IgM, anti-IgD, and anti-Ig λ antibodies. Based on expression of IgM and IgD, late BM B cells were defined as IgM⁺ IgD⁻ immature B-cell (I), IgM⁺ IgD^{low} transitional B-cell (II), and IgM⁺ IgD⁺ mature B-cell (III) subpopulations (top). The expression of B220 and Ig λ in the gated B-cell subpopulations (I, II, and III) was analyzed (bottom). Percentages indicate Ig λ ⁺ B cells in the gated B-cell subpopulation. Data are representative of eight mice per genotype. (B) Statistical analysis of the percentages of Ig λ ⁺ B cells in the gated B-cell subpopulation from panel A ($n = 8$; *, $P < 0.01$). (C) Splenocytes were isolated from wild-type and PLC γ 2-deficient mice and stained with a combination of anti-B220, anti-IgM, anti-IgD, and anti-Ig λ antibodies. Based on expression of IgM and IgD, splenic B cells were defined as IgM^{hi} IgD⁻ T1 B-cell (I), IgM^{hi} IgD⁺ T2 B-cell (II), and IgM^{lo} IgD⁺ mature B-cell (III) subpopulations (top). The expression of B220 and Ig λ in the gated B-cell subpopulations (I, II, and III) was analyzed (bottom). Percentages indicate Ig λ ⁺ B cells in the gated B-cell subpopulation. Data are representative of eight mice per genotype. (D) Statistical analysis of the percentages of Ig λ ⁺ B cells in the gated B-cell subpopulation from panel C ($n = 8$; *, $P < 0.01$).

FIG. 3. Comparable rates of apoptosis and proliferation of Ig κ ⁺ and Ig λ ⁺ B cells in wild-type and PLC γ 2-deficient mice. Freshly isolated BM cells (A) and splenocytes (B) from wild-type (PLC γ 2^{+/+}) and PLC γ 2-deficient (PLC γ 2^{-/-}) mice were stained with a combination of anti-B220 and anti-Ig λ or anti-Ig κ antibodies. Apoptotic cells were identified by TUNEL staining, and percentages indicate TUNEL-positive cells in the gated B220⁺ Ig κ ⁺ and B220⁺ Ig λ ⁺ BM or splenic B-cell populations. BM cells (C) and splenocytes (D) from wild-type (PLC γ 2^{+/+}) and PLC γ 2-deficient (PLC γ 2^{-/-}) mice that were injected with BrdU every 12 h for 5 days (5 days) were stained with a combination of anti-BrdU, anti-B220, and anti-Ig λ or anti-Ig κ antibodies. Mice that were not injected with BrdU served as the baseline control (0 day). The degree of BrdU-positive proliferating cells in the gated B220⁺ Ig κ ⁺ or B220⁺ Ig λ ⁺ BM or splenic B-cell populations was determined by flow cytometry, and percentages indicate BrdU-positive cells in the gated cells. The figure shown is representative of five independent analyses.

deficient mice. In freshly isolated BM, wild-type and PLC γ 2-deficient Ig κ ⁺ B cells exhibited comparable fractions of TUNEL-positive cells (Fig. 3A). Similarly, PLC γ 2-deficient and wild-type BM Ig λ ⁺ B cells also displayed comparable fractions of TUNEL-positive cells (Fig. 3A). Moreover, Ig κ ⁺

and Ig λ ⁺ B cells in the freshly harvested spleens of wild-type and PLC γ 2-deficient mice had comparable fractions of TUNEL-positive cells (Fig. 3B). Therefore, PLC γ 2 deficiency does not specifically increase apoptosis of Ig λ ⁺ B cells.

Next, we compared the status of proliferation of Ig λ ⁺ and Ig κ ⁺

FIG. 4. Impairment of antigen-induced receptor editing in PLCγ2-deficient B cells. Antigen-induced receptor editing is impaired in PLCγ2-deficient BM-derived (A) and splenic (B) Ig^{HEL} B cells in vitro. BM cells from wild-type or PLCγ2-deficient Ig^{HEL} transgenic mice were initially cultured for 5 days with IL-7, and splenocytes were isolated from wild-type or PLCγ2-deficient Ig^{HEL} transgenic mice. The cells were then recultured without IL-7 and in the absence (medium) or presence (HEL) of HEL. After a further 2-day culture, the cells were harvested and stained with anti-B220 and anti-Igλ antibodies. Percentages indicate Igλ⁺ cells in the gated B220⁺ B lymphocytes. Data are representative of three independent experiments. Antigen-induced receptor editing is impaired in PLCγ2-deficient BM (C) and splenic (D) Ig^{HEL} B cells in vivo. BM cells from 21-week-old wild-type or PLCγ2-deficient Ig^{HEL} sHEL mice were stained with anti-B220, anti-IgM, and anti-Igλ antibodies. Fluorescence-activated cell sorting analysis with B220 and Igλ staining of IgM⁺ gated cells is shown. Splenocytes from 21-week-old wild-type or PLCγ2-deficient Ig^{HEL} sHEL mice were stained with anti-B220 and anti-Igλ antibodies. Percentages indicate Igλ⁺ cells in the gated B220⁺ B lymphocytes. Data are representative of three independent analyses. (E) Statistical analysis of the percentages of Igλ⁺ B cells in the gated live BM IgM⁺ B220⁺ lymphoid population from panel C ($n = 3$; *, $P < 0.01$). (F) Statistical analysis of the percentages of Igλ⁺ B cells in the gated live splenic B220⁺ lymphoid population from panel D ($n = 3$; **, $P < 0.05$).

B cells in wild-type and PLCγ2-deficient mice. Following administration of BrdU for 5 days, BM was harvested from wild-type and PLCγ2-deficient mice. As expected, both PLCγ2-deficient BM Igκ⁺ and Igλ⁺ B cells incorporated markedly less BrdU than did the corresponding subpopulation of wild-type B cells (Fig. 3C). Similarly, both PLCγ2-deficient Igκ⁺ and Igλ⁺ splenic B cells incorporated clearly less BrdU than did the corresponding subpopulations of wild-type B cells (Fig. 3D). Thus, PLCγ2 deficiency impaired in vivo proliferation of both Igκ⁺ and Igλ⁺ B cells to a similar extent (Fig. 3C and D) and did not specifically decrease proliferation of Igλ⁺ B cells. Taken together, we conclude that reduced Igλ usage by PLCγ2-deficient B cells is not due to increased apoptosis or decreased proliferation of PLCγ2-deficient Igλ⁺ B cells.

PLCγ2 plays a role in receptor editing. Receptor editing involves new Ig gene rearrangements, particularly at L-chain loci, and is accompanied by increased Igλ usage (5, 52, 63). The impaired Igλ usage by PLCγ2-deficient B cells prompted us to examine the role of PLCγ2 in receptor editing. PLCγ2-deficient mice were crossed with Ig^{HEL} transgenic mice, which

bear rearranged H- and Igκ L-chain genes encoding a BCR that specifically recognizes HEL (13). BM cells from wild-type or PLCγ2-deficient Ig^{HEL} transgenic mice were cultured for 5 days with IL-7 to expand the virtually uniform B-cell population that expresses the Ig^{HEL} BCR (data not shown). Consistent with a previous study (65), BM-derived (Fig. 4A) and freshly isolated splenic (Fig. 4B) Ig^{HEL} B cells from PLCγ2-deficient mice underwent receptor editing and rearranged endogenous L-chain genes in response to HEL stimulation in vitro, resulting in a detectable fraction of cells expressing Igλ. In contrast, following HEL stimulation, BM-derived (Fig. 4A) and freshly isolated splenic (Fig. 4B) Ig^{HEL} B cells from PLCγ2-deficient mice exhibited a small but constant reduction in the population of cells expressing Igλ compared to wild-type cells. Of note, both the wild-type and PLCγ2-deficient BM-derived (Fig. 4A) and freshly isolated splenic (Fig. 4B) Ig^{HEL} B cells exhibited low background expression of endogenous Igλ when cultured with medium alone. Therefore, PLCγ2 deficiency reduces antigen-induced expression of Igλ associated with receptor editing in vitro.

Importantly, the effect of PLC γ 2 deficiency on receptor editing was also confirmed *in vivo*. When wild-type Ig^{HEL} transgenic mice were bred with transgenic mice expressing sHEL, sHEL induced receptor editing and resulted in expression of Ig λ in BM and splenic B cells of 21-week-old mice (Fig. 4C to F). Strikingly, PLC γ 2-deficient Ig^{HEL} sHEL mice of the same age displayed a dramatic reduction in the fraction of BM and splenic B cells expressing Ig λ relative to wild-type controls (Fig. 4C to F). Thus, PLC γ 2 deficiency impairs antigen-induced expression of Ig λ , which is associated with receptor editing *in vivo*.

Impaired antigen-induced rearrangement of endogenous Ig λ associated with PLC γ 2 deficiency *in vivo* was further confirmed at the level of genomic DNA. Genomic DNA was isolated from wild-type Ig^{HEL}, PLC γ 2-deficient Ig^{HEL}, wild-type Ig^{HEL} sHEL, and PLC γ 2-deficient Ig^{HEL} sHEL mice, and levels of endogenous Ig λ gene rearrangements were determined by semiquantitative PCR. Splenic B cells from wild-type mice served as a positive control, in which rearrangements of V-J λ 1, V-J λ 2, and V-J λ x were easily detectable (Fig. 5A). Embryonic stem (ES) cells served as a negative control, in which rearrangements of V-J λ 1, V-J λ 2, and V-J λ x were not detectable (Fig. 5A). As expected, both wild-type Ig^{HEL} and PLC γ 2-deficient Ig^{HEL} splenic B cells had barely detectable levels of rearrangements of endogenous V-J λ 1, V-J λ 2, and V-J λ x (Fig. 5A). Interestingly, whereas splenic B cells from wild-type Ig^{HEL} sHEL mice exhibited apparent rearrangements of endogenous V-J λ 1, V-J λ 2, and V-J λ x, such rearrangements were not detectable in PLC γ 2-deficient Ig^{HEL} sHEL splenic B cells (Fig. 5A).

Further, we examined the effect of PLC γ 2 deficiency on antigen-induced rearrangement of endogenous Ig κ , which is also associated with receptor editing *in vivo*. Ig^{HEL} transgenic mice have a rearranged V-J κ 2 transgene (60). Thus, we examined the rearrangement of endogenous V-J κ 1 in genomic DNA derived from wild-type Ig^{HEL}, PLC γ 2-deficient Ig^{HEL}, wild-type Ig^{HEL} sHEL, and PLC γ 2-deficient Ig^{HEL} sHEL mice by semiquantitative PCR. Both wild-type Ig^{HEL} and PLC γ 2-deficient Ig^{HEL} splenic B cells had low levels of rearrangements of endogenous V-J κ 1 (Fig. 5A). Notably, antigen-induced rearrangement of endogenous V-J κ 1 was reduced in splenic B cells derived from PLC γ 2-deficient Ig^{HEL} sHEL mice relative to wild-type controls (Fig. 5A). Moreover, RS rearrangement between an intron RS within the J κ -C κ intron and an RSS located downstream of C κ , a critical mechanism for the receptor editing of Ig κ (52), was reduced in splenic B cells derived from PLC γ 2-deficient Ig^{HEL} sHEL mice relative to wild-type controls (Fig. 5A). Therefore, PLC γ 2 contributes not only to Ig λ but also to Ig κ rearrangement.

Impaired antigen-induced rearrangements of Ig λ and Ig κ genes in PLC γ 2-deficient B cells were also confirmed at genomic DNA levels in an *in vitro* receptor editing experiment. BM cells from wild-type or PLC γ 2-deficient Ig^{HEL} transgenic mice were cultured for 5 days with IL-7 to expand the uniform B-cell population that expresses the Ig^{HEL} BCR. Following further stimulation with HEL or medium, genomic DNA was isolated from the cells and levels of endogenous gene Ig λ and Ig κ rearrangements were determined by semiquantitative PCR. BM B cells from wild-type mice served as a positive control, in which V-J λ 1 rearrangements were easily detectable (Fig. 5B). ES cells served as a negative control, in which V-J λ 1 rearrangements were not detectable (Fig. 5B). Both wild-type

and PLC γ 2-deficient BM-derived Ig^{HEL} B cells had low background levels of rearrangements of endogenous Ig λ and Ig κ genes when cultured with medium alone (Fig. 5B). Upon HEL stimulation, wild-type BM-derived Ig^{HEL} B cells underwent receptor editing such that rearrangements of endogenous V-J λ 1 and V-J κ 1 gene were easily detectable (Fig. 5B), consistent with a previous study (65). In contrast, PLC γ 2-deficient BM-derived Ig^{HEL} B cells displayed a barely detectable level of rearrangement of V-J λ 1 and a reduced level of rearrangements of V-J κ 1 following HEL stimulation (Fig. 5B). These genomic data further confirm that PLC γ 2 deficiency impairs activation of Ig λ and Ig κ locus. Taken together, our results demonstrate that PLC γ 2 plays a role in rearrangements of Ig λ and Ig κ , which are associated with receptor editing.

Ig L recombination requires accessibility of both the V region and J region to the recombination machinery, and germ line transcription of V λ and V κ positively correlates with their accessibility (3). Thus, we compared germ line transcription of V λ and V κ genes in wild-type and PLC γ 2-deficient pro/pre-B cells. B220^{low} IgM⁻ pro/pre-B cells were sorted out from BM of wild-type or PLC γ 2-deficient mice. The levels of V λ and V κ germ line transcription in these cells were quantitated by real-time PCR. The level of V λ germ line transcription was markedly decreased in PLC γ 2-deficient pro/pre-B cells compared to wild-type controls (Fig. 6A). Of note, the level of V κ germ line transcription was also decreased in PLC γ 2-deficient pro/pre-B cells compared to wild-type controls (Fig. 6A). Thus, consistent with an important role for PLC γ 2 in rearrangements of Ig λ and Ig κ , PLC γ 2 deficiency reduces the germ line transcription of both V λ and V κ genes, especially the former.

PLC γ 2 deficiency impairs BCR-induced expression of IRF-4 and IRF-8. IRF-4 and IRF-8 are transcription factors whose expression can be induced by BCR engagement (9, 35, 62). Both transcription factors play essential roles in rearrangement of Ig L-chain genes during B-cell development, and their deficiencies block rearrangement of κ and λ chain genes (31). Thus, we examined the effect of PLC γ 2 deficiency on BCR-mediated induction of IRF-4 and IRF-8 expression. BM cells from wild-type or PLC γ 2-deficient Ig^{HEL} transgenic mice were cultured for 5 days with IL-7 to expand the Ig^{HEL} BCR-expressing uniform B-cell progenitors. Cross-linking of the BCR dramatically induced expression of IRF-4 and IRF-8 in wild-type BM-derived Ig^{HEL} B cells (Fig. 6B). In contrast, BCR-induced expression of IRF-4 and IRF-8 was markedly impaired in PLC γ 2-deficient BM-derived Ig^{HEL} B cells (Fig. 6B). In addition, we examined the effect of PLC γ 2 deficiency on BCR engagement-induced expression of IRF-4 and IRF-8 *in vivo*. Splenic B cells were isolated from wild-type or PLC γ 2-deficient Ig^{HEL} transgenic mice that also express sHEL, and expression of IRF-4 and IRF-8 in these cells was determined by real-time RT-PCR. The expression of IRF-4 and IRF-8 was markedly reduced in PLC γ 2-deficient Ig^{HEL} sHEL B cells compared to wild-type Ig^{HEL} sHEL splenic B cells (Fig. 6C). Thus, PLC γ 2 deficiency severely impairs antigen-induced expression of IRF-4 and IRF-8 both *in vitro* and *in vivo*.

DISCUSSION

Although previous studies have shown that PLC γ 2 plays an important role in early B-cell development at the pro-B to

FIG. 5. Impairment of antigen-induced receptor editing among the endogenous λ and κ loci in PLC γ 2-deficient Ig^{H/EL} B cells. (A) Impairment of antigen-induced receptor editing among the endogenous λ and κ loci in PLC γ 2-deficient splenic Ig^{H/EL} B cells in vivo. Genomic DNA was isolated from the splenocytes derived from wild-type Ig^{H/EL}, PLC γ 2-deficient Ig^{H/EL}, wild-type Ig^{H/EL} sHEL, or PLC γ 2-deficient Ig^{H/EL} sHEL mice. Subsequently, the genomic DNA was subjected to a semiquantitative PCR analysis of the β -actin gene; of endogenous λ 1, λ 2, λ x, and J κ 1 rearrangements, and of RS rearrangement. Genomic DNA of splenic B cells from wild-type mice served as a positive control, whereas genomic DNA from ES cells served as a negative control. Data are representative of three independent experiments for λ rearrangements and two independent experiments for J κ 1 and RS rearrangements. (B) Impairment of antigen-induced rearrangements of endogenous λ and κ chain in PLC γ 2-deficient BM-derived Ig^{H/EL} B cells in vitro. BM cells from wild-type or PLC γ 2-deficient Ig^{H/EL} transgenic mice were cultured for 5 days with IL-7 to expand into a B-cell population that expresses the Ig^{H/EL} BCR. Following further stimulation with (HEL) or without (medium) HEL for 2 days, genomic DNA was isolated from the cells and subsequently subjected to a semiquantitative PCR analysis of the β -actin gene and of endogenous λ 1 and J κ 1 rearrangements. Genomic DNA of BM B cells from wild-type mice served as a positive control, whereas genomic DNA from ES cells served as a negative control. Data are representative of three independent experiments for λ rearrangements and two independent experiments for J κ 1 rearrangement. WT, wild type.

pre-B transition and is essential for maturation of T2 to follicular B cells (68–70), our current studies discovered that PLC γ 2 deficiency impairs rearrangements of Ig λ and Ig κ L-chain genes, resulting in an impairment of antigen-induced receptor

editing. Although the impairment of Ig κ rearrangement is not obvious in nontransgenic PLC γ 2-deficient mice, it is clearly detected in the PLC γ 2-deficient Ig^{H/EL} sHEL transgenic mice. Previous studies have demonstrated that rearrangements of

FIG. 6. Impairment of germ line transcription of light-chain genes and BCR-mediated expression of IRF-4/IRF-8 in PLC $\gamma 2$ -deficient B cells. (A) Reduction of germ line transcription of λ and κ chain in PLC $\gamma 2$ -deficient pro/pre-B cells. The pro/pre-B (B220^{low} IgM⁻) cells were sorted from BM of 4-month-old PLC $\gamma 2^{+/+}$ and PLC $\gamma 2^{-/-}$ mice. Then, total RNA was isolated from the cells and subjected to real-time RT-PCR analysis of germ line transcription of λ and κ chain. Data are a combination of results from two independent experiments. (B) BCR-induced expression of IRF-4 and IRF-8 is impaired in PLC $\gamma 2$ -deficient BM-derived Ig^{HEL} B cells in vitro. BM cells were isolated from wild-type (PLC $\gamma 2^{+/+}$ Ig^{HEL}) and PLC $\gamma 2$ -deficient (PLC $\gamma 2^{-/-}$ Ig^{HEL}) Ig^{HEL} mice and were initially cultured with IL-7 for 5 days. The cells were then stimulated with (α -IgM) or without (medium) anti-IgM for 2 h. Total RNA was isolated from the cells and subjected to real-time RT-PCR analysis of IRF-4 and IRF-8 gene expression. The figures shown are representative of three independent experiments. (C) Impairment of BCR-induced expression of IRF-4 and IRF-8 in PLC $\gamma 2$ -deficient splenic Ig^{HEL} B cells in vivo. Splenic B cells were isolated from wild-type and PLC $\gamma 2$ -deficient Ig^{HEL} sHEL mice. Total RNA was isolated from the cells and subjected to real-time RT-PCR analysis of IRF-4 and IRF-8 gene expression. The figure shown is representative of four independent experiments.

Ig κ and Ig λ occur independently with different kinetics, such that the κ locus is activated and the λ locus is not subsequently activated until rearrangements of κ genes fail to yield a functional Ig κ L chain (2, 10, 39, 72). A different regulation of the activation of these two loci for recombination might explain that differential effect of PLC $\gamma 2$ deficiency on the κ versus the λ locus. PLC $\gamma 2$ provides an important signal involved in the activation of both the λ and κ loci, although the signal seems to be more essential for the activation of λ . Interestingly, the two signaling molecules that are upstream of PLC $\gamma 2$ in BCR signaling, the adapter molecule BLNK and the tyrosine kinase Btk, have also been shown to play roles in the activation of the L-chain loci (6, 19). Similar to deficiency of PLC $\gamma 2$, lack of BLNK impairs the activation of both Ig λ and Ig κ loci, leading to an impairment of antigen-induced receptor editing (19). In contrast, Btk deficiency has no effect on antigen-induced receptor editing, although it severely reduces the expression of

Ig λ chain (6). One possible explanation is that PLC $\gamma 2$ and BLNK initiate additional Btk-independent signals that are required for receptor editing.

The ratio of Ig κ^{+} versus Ig λ^{+} mature B cells is ultimately determined by the kinetics and efficiency of activation of κ and λ loci, the number of functional V κ and V λ gene segments, and the survival and proliferation rates of B cells that have undergone L-chain gene rearrangement (28, 72). Although PLC $\gamma 2$ is known to be involved in the survival and proliferation of transitional and mature B cells (68, 70), our analysis of apoptosis and proliferation rates in Ig λ^{+} and Ig κ^{+} B-cell populations derived from wild-type and PLC $\gamma 2^{-/-}$ mice demonstrates that PLC $\gamma 2$ deficiency does not specifically increase apoptosis or decrease proliferation of Ig λ^{+} B cells relative to Ig κ^{+} B cells. Therefore, the reduced usage of Ig λ relative to Ig κ in non-transgenic PLC $\gamma 2$ -deficient B cells is not due to a shortened life span or decreased proliferative capacity of Ig λ^{+} B cells

relative to Ig κ ⁺ B cells. Instead, the reduced usage of Ig λ by PLC γ 2-deficient B cells appears to be due solely to the absence of signals from PLC γ 2 that differentially regulate activation of the Ig λ and Ig κ loci. Consistently, overexpression of the anti-apoptotic molecule Bcl-2 protects Btk-deficient B cells from apoptosis but does not alter the frequency of Ig λ expression in the mutant B cells (6).

Binding of self antigen to self-reactive B cells can initiate new Ig gene rearrangements, especially at L-chain loci, to replace a self-reactive receptor with a new receptor (5, 7, 12, 48, 63). The BCR signaling pathway that specifically regulates receptor editing is not clear, although BLNK has been shown to play an important role in receptor editing (19). Although development of anergy is a dominant phenotype in the Ig^{HEL} sHEL transgenic mode, this model can be used to study receptor editing (66). Previous studies have demonstrated that sHEL is able to induce autoreactive Ig^{HEL} B cells to undergo receptor editing in vitro (65, 66). Consistent with these previous studies, we found that both BM culture-derived and primary splenic B cells from wild-type Ig^{HEL} mice undergo sHEL-induced Ig λ expression in vitro. However, sHEL-induced expression of Ig λ is severely impaired in PLC γ 2^{-/-} Ig^{HEL} B cells. Expression of sHEL as a "self antigen" in mice that express Ig^{HEL} might be expected to induce editing of the "autoreactive" Ig^{HEL} receptor and consequent expression of endogenous Ig λ in vivo. Whereas a previous study failed to detect elevated levels of Ig λ expression in B cells from young (<12 weeks of age) Ig^{HEL} sHEL double-transgenic mice in vivo (65), we constantly observed Ig λ expression in B cells from older (>13 weeks of age), but not younger (data not shown), Ig^{HEL} sHEL double-transgenic mice in vivo. Importantly, Ig λ expression was much lower in B cells from older PLC γ 2-deficient mice than in B cells from wild-type Ig^{HEL} sHEL double-transgenic mice. Taken together, our results show that PLC γ 2 signaling downstream of BCR engagement plays a role in receptor editing both in vitro and in vivo.

IRF-4 and IRF-8 are two members of the IRF family of transcription factors that are essential for down-regulation of both surrogate L-chain expression and rearrangement of Ig κ and Ig λ chain genes during B-cell development (32). In the absence of IRF-4 and IRF-8, rearrangement of both Ig κ and Ig λ chain genes is blocked (32). We show in the present studies that the PLC γ 2 pathway plays a critical role in BCR-induced expression of IRF-4 and IRF-8. Nevertheless, although PLC γ 2 deficiency severely impairs up-regulation of both transcription factors, it more severely impairs rearrangement of Ig λ than Ig κ chain genes in nontransgenic mice. One possible explanation for this observation is that high levels of IRF-4 and IRF-8 may be required for Ig λ chain gene rearrangement whereas low levels of IRF-4 and IRF-8 may be sufficient for Ig κ chain gene rearrangement. Thus, the complete absence of IRF-4 and IRF-8 caused by targeted gene disruption would be expected to block rearrangement of both Ig κ and Ig λ chain genes, whereas reduced expression of IRF-4 and IRF-8, for example as a consequence of PLC γ 2 deficiency, would be expected to affect rearrangement of Ig λ more than that of Ig κ . In fact, a recent study has demonstrated that IRF-4 is expressed in a graded manner in differentiating B cells and that different concentrations of IRF-4 regulate expression of distinct genes that coordinate isotype switching during plasma cell differentiation (58).

Moreover, both IRF-4 and IRF-8 are essential for germ line transcription of V κ and V λ (31). Levels of germ line transcription of V κ and V λ positively correlate with their accessibility to the recombination machinery (3). The impaired expression of IRF-4 and IRF-8 as a consequence of PLC γ 2 deficiency should contribute to the reduction of the germ line transcription of V κ and particularly V λ genes. Nonetheless, further studies are required to fully understand the mechanism by which the PLC γ 2 pathway regulates L-chain gene rearrangement and receptor editing.

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