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Protein Kinase Cδ Promotes Transitional B Cell-Negative Selection and Limits Proximal B Cell Receptor Signaling To Enforce Tolerance

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Protein kinase Cδ (PKCδ) deficiency causes autoimmune pathology in humans and mice and is crucial for the maintenance of B cell homeostasis. However, the mechanisms underlying autoimmune disease in PKCδ deficiency remain poorly defined. Here, we address the antigen-dependent and -independent roles of PKCδ in B cell development, repertoire selection, and antigen responsiveness. We demonstrate that PKCδ is rapidly phosphorylated downstream of both the B cell receptor (BCR) and the B cell-activating factor (BAFF) receptor. We found that PKCδ is essential for antigen-dependent negative selection of splenic transitional B cells and is required for activation of the proapoptotic Ca^{2+}-Erk pathway that is selectively activated during B cell-negative selection. Unexpectedly, we also identified a previously unrecognized role for PKCδ as a proximal negative regulator of BCR signaling that substantially impacts survival and proliferation of mature follicular B cells. As a consequence of these distinct roles, PKCδ deficiency leads to the survival and development of a B cell repertoire that is not only aberrantly autoreactive but also hyperresponsive to antigen stimulation.

Protein kinase Cδ (PKCδ) is a member of the novel protein kinase C (PKC) family of serine/threonine kinases, which has been implicated in maintaining immune homeostasis. PKCδ-deficient mice develop a severe autoimmune disease characterized by autoantibody production, glomerulonephritis, and robust B cell lymphoproliferation leading to splenomegaly and lymphadenopathy (1, 2). Several recent reports have identified mutations in PKCδ that appear to underlie autoimmune pathology in humans (3–5), supporting the notion that $PKC\delta^{-/-}$ mice represent a valuable mouse model of human disease. Although PKCδ clearly has a vital function in suppressing autoimmune disease in both mice and humans, the mechanisms by which PKCδ deficiency causes autoimmunity remain poorly defined.

Sequential checkpoints in B cell development are thought to progressively eliminate autoreactive B cell clones from the repertoire to prevent autoimmunity. It has been estimated that up to 75% of newly generated human B cells in the bone marrow are autoreactive (6, 7). Receptor editing and antigen-induced apoptosis eliminate some of these autoreactive clones, and only ~40% of the B cells that exit the bone marrow as transitional B cells and migrate to the spleen are still autoreactive. B cells arriving in the spleen as transitional 1 (T1) cells remain highly susceptible to antigen-induced apoptosis, and they undergo a second checkpoint of negative selection as they migrate toward the follicle to become transitional 2 (T2) cells. Approximately half of the remaining autoreactive B cell clones are eliminated at this transition between the T1 and T2 stages. Lupus patients often display defects in the T1-T2 checkpoint, and the increased autoreactivity in the repertoire that results as a consequence of this failure may contribute to disease pathogenesis (7–10).

The signaling properties of transitional B cells change substantially once they become T2 cells. T2 cells are much less sensitive to antigen-induced apoptosis than T1 cells, and instead, B cell receptor (BCR) engagement generates proliferative, antiapoptotic, and differentiation signals that promote positive selection into the follicular or marginal zone (MZ) B cell fate (11–15). Associated with selection into the follicular B cell compartment, engagement of self-antigen induces IgM but not IgD downregulation in a manner proportional to the affinity for the self-antigen. Therefore, surface IgM (sIgM) downregulation reflects the tuning of the responsiveness of B cells to self-antigens and is one of the hallmarks of anergic B cells (16–18).

T1 B cells are highly susceptible to BCR-mediated antigen-induced apoptosis, yet at the same time, tonic BCR signals are required for B cell survival throughout development (19). In addition, as T1 B cells transition into the T2 compartment, they upregulate surface expression of the B cell-activating factor (BAFF) receptor (BAFFr), and BAFF-dependent signaling also becomes crucial for the survival of T2, follicular, and MZ B cells (20, 21). Although BAFFr signaling has been shown to trigger the noncanonical NF-κB pathway (22, 23), a recent study demonstrated that the BAFFr coopts the BCR to enhance tonic BCR signals that promote survival, adding unexpected complexity to the regulation of B cell survival during development (24). Thus, BCR and BAFFr signaling appear to be connected.

Previous studies implicated PKCδ in B cell anergy (1), survival (25), and proliferation (2). More recently, we proposed a role for PKCδ in proapoptotic signaling during negative selection of B cells in the bone marrow (26). However, the role of PKCδ in peripheral B cell development and repertoire selection has not been defined, and it is unknown whether the different pathological aspects of the autoimmune phenotype in PKCδ-deficient mice and humans are secondary to its role in BCR or BAFF signaling, or both.

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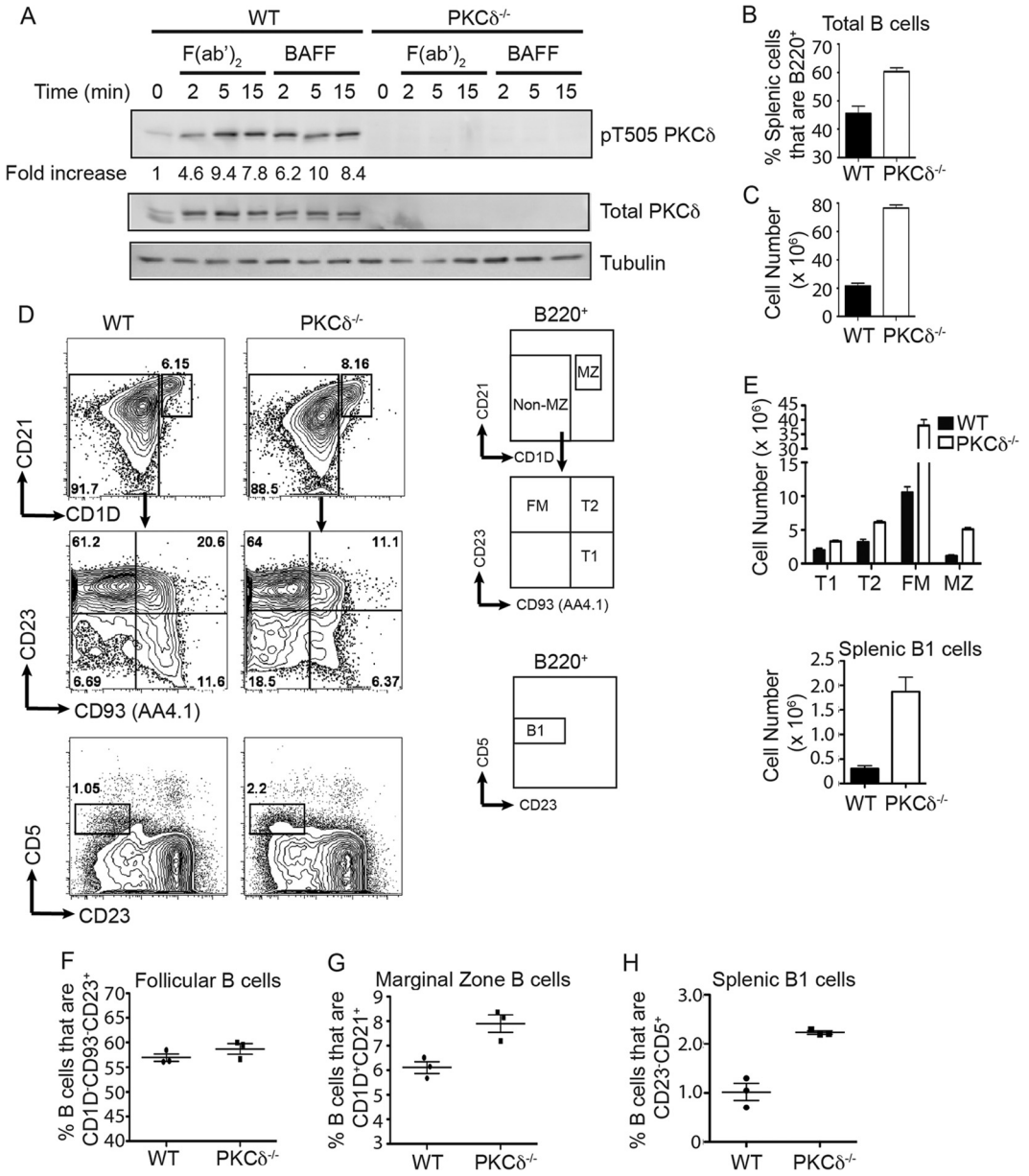


FIG 1 PKCδ is phosphorylated downstream of the BAFF and B cell receptors and impacts B cell homeostasis throughout development. (A) Purified splenic B cells were stimulated with BAFF or anti-IgM F(ab′)$_2$ fragment for the indicated times. Cell lysates were prepared and immunoblotted with the indicated antibodies. The fold induction of T505 phosphorylation on PKCδ was quantified relative to tubulin loading in the same blot. (B) Single-cell suspensions of WT or PKCδ$^{-/-}$ spleens were stained with the indicated antibodies and analyzed by FACS to determine the percentages of splenic cells that are B220$^+$. (C) Total splenic B cell numbers. (D) Representative FACS plots showing the splenic T1, T2, follicular, marginal zone, and B1 B cell subsets. (E) Splenic B cell numbers in the indicated B cell subsets. (F) Percentages of splenic B cells that exhibit follicular markers. (G) Percentages of splenic B cells that exhibit marginal zone markers. (H) Percentages of splenic B cells that exhibit B1 B cell markers. The error bars indicate standard errors of the means (SEM).

Here, we find that PKCδ is rapidly phosphorylated downstream of both the B cell and the BAFF receptors and can integrate signals from both receptors. PKCδ deficiency uncouples BCR signaling from the induction of apoptosis in T1 B cells and impairs the activation of the proapoptotic Ca^{2+}-Erk pathway. Thus, the T1-T2 checkpoint in development is greatly impaired in these mice in a manner similar to that described in human systemic lupus erythematosus (SLE) patients. Moreover, we find that PKCδ additionally acts as a proximal negative regulator of BCR signaling by two distinct mechanisms, and as a consequence, PKCδ$^{-/-}$ B cells display enhanced tonic and antigen-induced BCR signals. Failure of negative selection of autoreactive T1 B cells in the PKCδ$^{-/-}$ mice thus enables the development of a mature B cell repertoire that is not only aberrantly autoreactive but also hyperresponsive to antigenic stimulation.

MATERIALS AND METHODS

Mice. PKCδ$^{-/-}$, MD4, ML5, and CD45HE mice were previously described (1, 16, 27). C57BL/6 and BoyJ mice were purchased from Jackson Laboratory. Mouse experiments were reviewed and approved by the In-

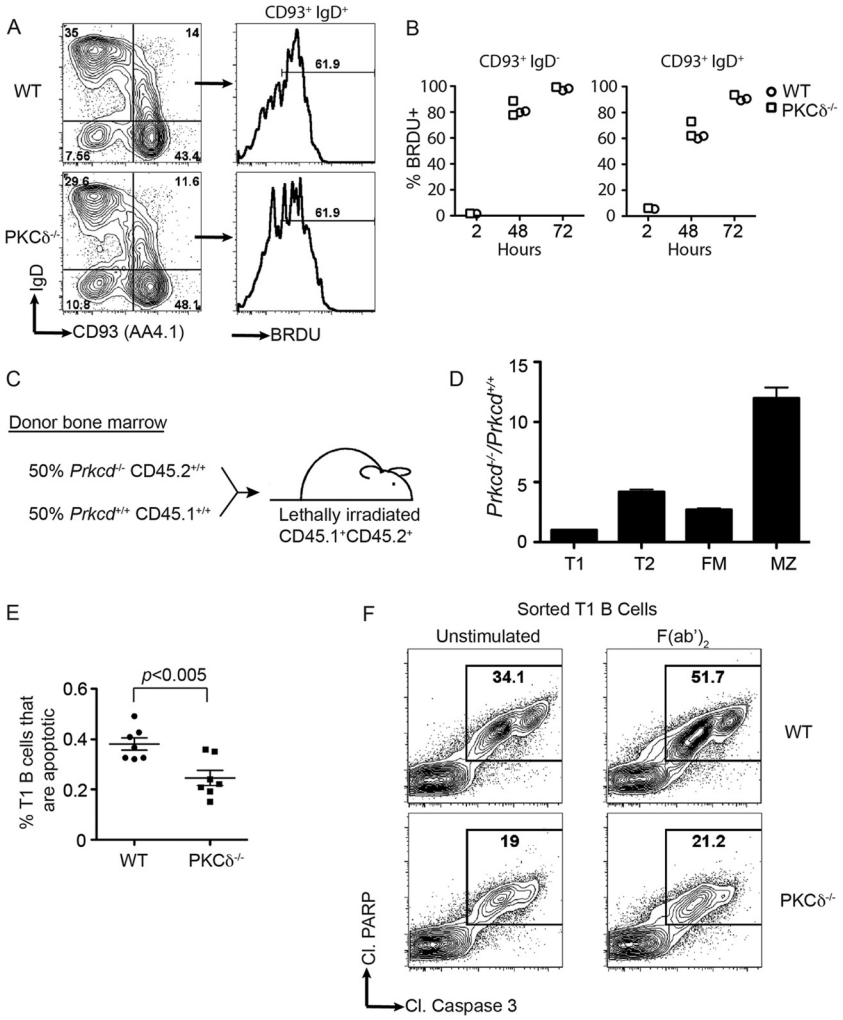


FIG 2 PKCδ$^{-/-}$ is required for B cell-negative selection at the T1-T2 transition. (A) Analysis of bone marrow from mice injected intraperitoneally with BrdU, harvested 48 h postinjection. (B) Percentages of BrdU^{+} cells in bone marrow precursor B cell populations (CD93^{+} IgD^{-}) and bone marrow transitional B cells (CD93^{+} IgD^{int}). (C) Scheme of experiment in which lethally irradiated mice were reconstituted with a 1:1 mixture of WT and PKCδ$^{-/-}$ bone marrow cells expressing different congenic markers, as indicated. (D) Spleens from chimeric mice shown in panel C were analyzed 6 to 8 weeks postreconstitution by FACS, and the ratios of the congenic markers were calculated at the indicated developmental stages. FM, follicular mature. (E) Percentages of T1 B cells that are positive for cleaved caspase 3 and cleaved PARP in WT or PKCδ$^{-/-}$ spleens. (F) Sorted T1 B cells from WT or PKCδ$^{-/-}$ mice were either left unstimulated or stimulated for 8 h with anti-IgM F(ab′)$_2$ fragment and subsequently stained for cleaved caspase 3 and cleaved PARP. The error bars indicate SEM.

stitutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco (UCSF).

Antibodies and reagents. Antibodies (Abs) to murine IgD (11-26), IgM (II/41), B220 (RA3-6B2), CD45.1 (A20), CD45.2 (104), CD1D (1B1), CD43 (S7), CD21 (7G6), CD23 (B3B4), CD93 (AA4.1), and cleaved poly-(ADP-ribose) polymerase (PARP) (F21-852) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP)-Cy5.5, PE-Cy7, Pacific Blue, allophycocyanin, or Alexa Fluor 647 were obtained from eBioscience, Biolegend, or BD Biosciences. DAPI (4′,6-diamidino-2-phenylindole) was from Roche. Anti-IgM F(ab′)$_2$ fragment, FITC-conjugated F(ab) monomeric fragment, and goat anti-rabbit IgG Abs conjugated to allophycocyanin were obtained from Jackson ImmunoResearch Laboratories. Phospho-Erk (number 197G2), PKCδ (number 2058), pT505-PKCδ (number 9374), and cleaved caspase 3 (number 9661) antibodies were obtained from Cell Signaling. Thapsigargin was from Calbiochem, BAFF from R&D, and carboxyfluorescein succinimidyl ester (CFSE) from Invitrogen. Bromodeoxyuridine (BrdU) labeling was performed according to the manufacturer's instructions using the BrdU labeling kit from BD.

Immunoblotting, FACS, and phosphoflow analyses. Immunoblotting, fluorescence-activated cell sorting (FACS), cell sorting, intracellular staining, and phosphoflow analyses were performed as previously described (26).

***Ex vivo* culture of lymph node cells.** CD45.1-PKCδ$^{-/-}$ or CD45.1^{+} wild-type (WT) lymph node cells were loaded with CFSE according to the manufacturer's instructions, mixed at a 1:1 ratio, and cultured in 96-well plates in complete Dulbecco's modified Eagle's medium (DMEM) with stimuli as indicated.

RESULTS

BCR- and BAFFr-induced phosphorylation of PKCδ. Phosphorylation of PKCδ on T505 within the activation loop increases its kinase activity (28–30). To determine whether PKCδ is phosphorylated in response to triggering of the BCR or BAFFr, we stimulated purified splenic B cells in a time course with anti-IgM F(ab′)$_2$ fragment or recombinant BAFF and assayed cell lysates for changes in PKCδ phosphorylation using a specific phospho-T505

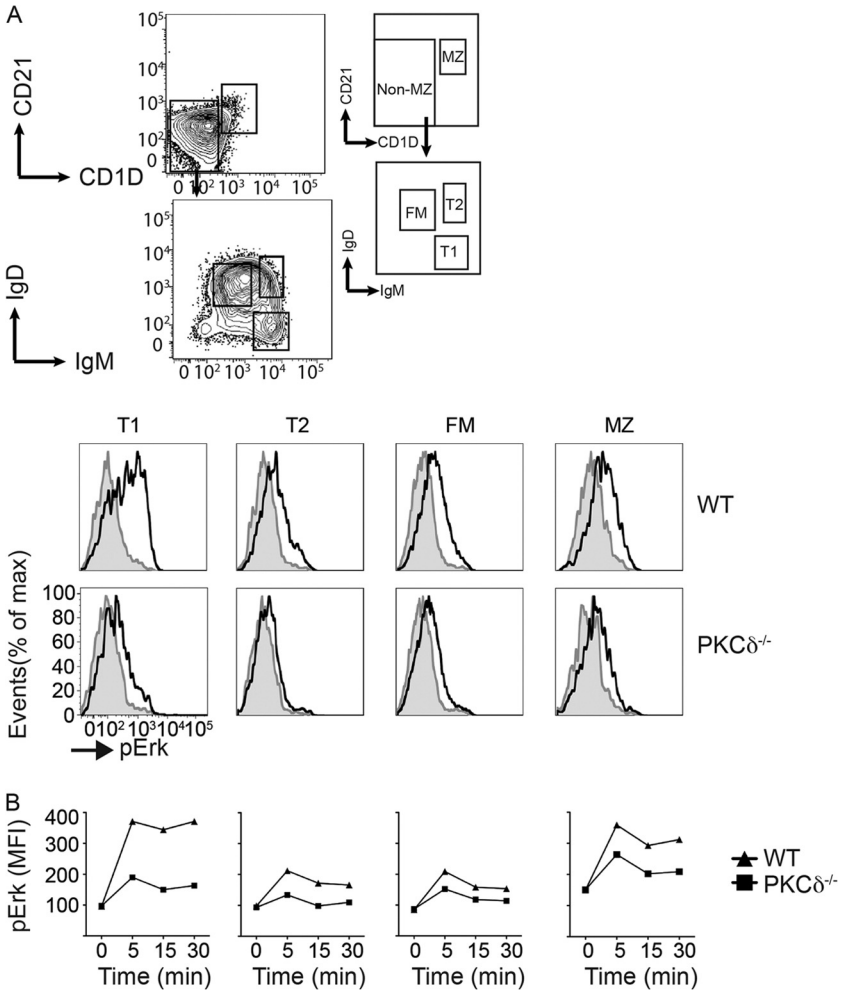


FIG 3 Impaired Ca^{2+}-Erk signaling in PKCδ$^{-/-}$ T1 B cells. (A) Gating strategy (distinct from that used for Fig. 1, because CD93 staining is incompatible with the permeabilization buffers used for phosphoflow) and representative histograms of phosphoflow analysis of pErk responses in different B cell subsets following 5 min of thapsigargin stimulation. (B) Quantification of the mean fluorescence intensities (MFI) of pErk responses in panel A.

antibody. T505 was robustly phosphorylated within 2 min of stimulation via either the BCR or the BAFFr, and this phosphorylation was accompanied by an induced electrophoretic mobility shift of total PKCδ (Fig. 1A).

Quantification of splenic B cell expansion in PKCδ$^{-/-}$ mice. Both BCR and BAFFr signaling contribute to setting the thresholds for tolerance checkpoints in the spleen, and they also influence the commitment of T2 B cells to a follicular or MZ fate. To determine the impact of PKCδ deficiency on fate decisions during B cell development, we first quantified the percentage and absolute number of B cells from each subset in the spleens of PKCδ$^{-/-}$ mice relative to WT mice. All subsets of B cells are significantly expanded in these mice (Fig. 1B to H). This expansion is already noticeable in the earliest T1 subset (defined as CD93^{+} CD23^{-} CD1D^{-} IgM^{high} IgD^{low}), where the cells are expanded between 1.5- and 2-fold in the PKCδ$^{-/-}$ spleens relative to WT spleens (Fig. 1E). Thus, the increased output of transitional B cells during development at least partially underlies the increased B cell numbers in the mice, with further expansion at later stages of development. Notably, MZ and splenic B1 B cell numbers are expanded to a greater extent than follicular B cells in PKCδ$^{-/-}$ spleens (Fig. 1F to H).

PKCδ is required for B cell-negative selection at the T1-T2 splenic checkpoint. The expansion of T1 cells in the spleens of PKCδ$^{-/-}$ mice (Fig. 1E) suggests that PKCδ$^{-/-}$ T1 B cells may be resistant to the T1-T2 negative selection checkpoint or, alternatively, that PKCδ deficiency increases the proliferation and output of B cells from the bone marrow. To address the latter possibility, we injected WT and PKCδ$^{-/-}$ mice with BrdU and measured its incorporation into developing bone marrow cells. It is known that WT pre-B cells proliferate vigorously and incorporate BrdU but cease to do so when the cells develop further and become quiescent. Therefore, the rate of BrdU incorporation in transitional B cells reflects the B cell output rate. We found that the kinetics of BrdU incorporation are comparable between WT and PKCδ$^{-/-}$ developing bone marrow B cells, with mice of both genotypes displaying similar percentages of BrdU-positive transitional B cells 48 h postinjection (Fig. 2A and B). Moreover, in both WT and PKCδ$^{-/-}$ mice, BrdU incorporation in transitional B cells was comparably delayed relative to earlier B cell precursors, indicating that PKCδ$^{-/-}$ transitional B cells are quiescent and the output of bone marrow B cells is not significantly altered in PKCδ$^{-/-}$ mice.

We previously reported that PKCδ is required to sensitize developing bone marrow B cells to negative selection (26). If

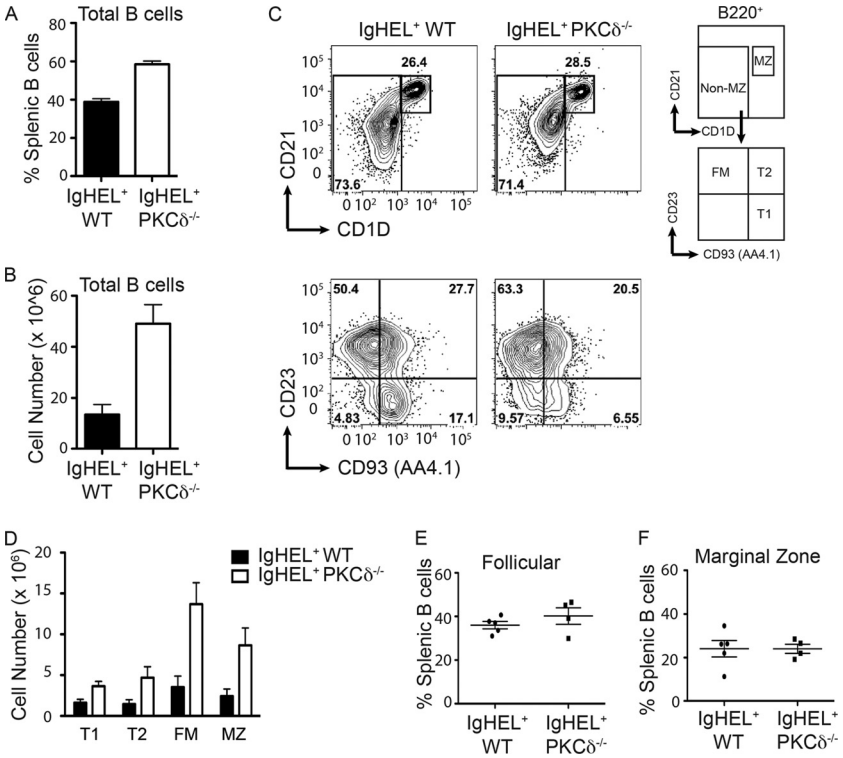


FIG 4 Antigen-dependent and -independent effects of PKCδ deficiency. Single-cell suspensions of WT IgHEL or PKCδ$^{-/-}$ IgHEL spleens were stained with the indicated antibodies and analyzed by FACS. (A) Percentages of splenic cells that are B220^{+}. (B) Total splenic B cell numbers. (C) Representative FACS plots showing the T1, T2, follicular, and marginal zone B cell subsets. (D) Quantification of cell numbers in the indicated splenic B cell subsets. (E) Percentages of splenic B cells that exhibit follicular markers. (F) Percentages of splenic B cells that exhibit marginal zone markers. The error bars indicate SEM.

the same is true in the splenic T1-T2 checkpoint, developing PKCδ$^{-/-}$ B cells should display a competitive advantage over developing WT B cells in a competitive setting, where WT autoreactive B cells would be deleted. We therefore performed competitive repopulation experiments where congenically marked WT and PKCδ$^{-/-}$ bone marrow cells were mixed at a 1:1 ratio and used to reconstitute lethally irradiated WT recipient mice (Fig. 2C). We analyzed the ratios of the two donor populations at different stages of B cell development. Indeed, developing PKCδ$^{-/-}$ B cells had a significant advantage over WT cells at the T1-T2 transition in the spleen (Fig. 2D). Additionally, in agreement with the noncompetitive assays shown in Fig. 1, we observed marked skewing of PKCδ$^{-/-}$ B cell commitment to the MZ fate and decreased commitment to the follicular fate in the competitive repopulations.

The competitive advantage of PKCδ$^{-/-}$ transitional B cells over the WT may reflect a defect in negative selection, enhanced proliferation of T2 cells, or both. To directly address whether PKCδ deficiency impairs negative selection, we first harvested splenocytes and performed immediate intracellular staining for cleaved caspase 3 and cleaved PARP to quantify the percentage of T1 B cells committed to apoptosis at the moment of harvest. Apoptotic cells are cleared very efficiently *in vivo* and do not accumulate. Nevertheless, our immediate FACS method allowed the detection of a sizeable and statistically significant reduction in the number of apoptotic PKCδ$^{-/-}$ T1 cells relative to WT cells (Fig. 2E). In a complementary approach, we sorted T1 B cells from WT or PKCδ$^{-/-}$ mice and stimulated them *ex vivo* via the BCR with anti-IgM F(ab′)$_2$ fragment, followed by the same intracellular

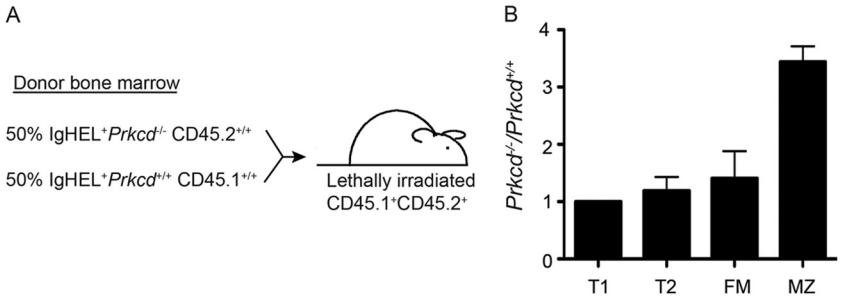


FIG 5 Competitive repopulation analysis of WT and PKCδ$^{-/-}$ IgHEL B cells in the absence of HEL. (A) Scheme of bone marrow reconstitution strategy. (B) Spleens from chimeric mice shown in panel A were analyzed 6 to 8 weeks postreconstitution by FACS, and the ratios of the congenic markers were calculated at the indicated developmental stages. The error bars indicate SEM.

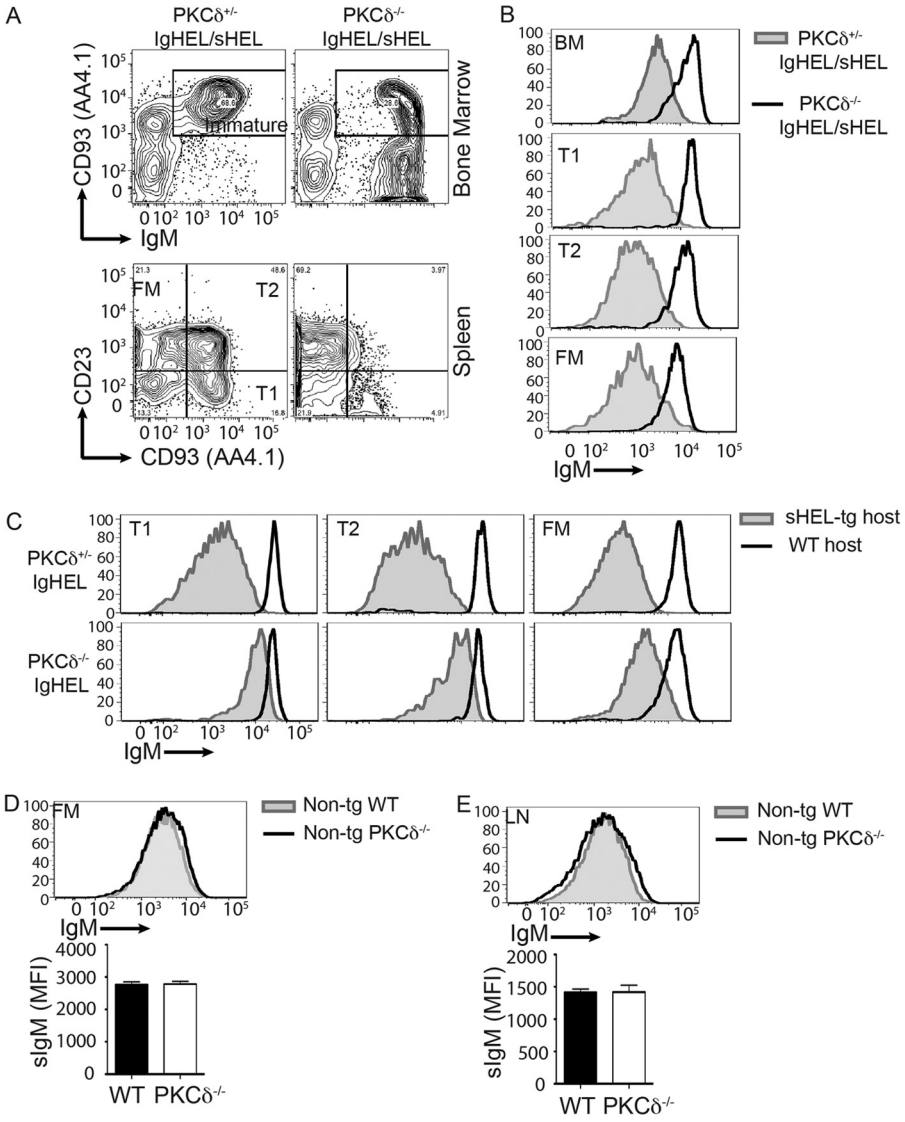


FIG 6 Consequences of antigen exposure for PKCδ$^{-/-}$ B cells. (A) FACS analysis of bone marrow B cells (top) or splenic CD1D-negative B cells (bottom). The gates shown correspond to immature bone marrow B cells and T1, T2, and follicular mature compartments in splenic B cells. (B) Surface IgM levels on the indicated bone marrow (BM) and splenic B cell subsets, as gated in panel A. (C) Surface IgM levels on splenic B cell subsets of noncompetitive chimeric mice. The genotypes of bone marrow donors are indicated on the left. tg, transgenic. (D and E) Histograms of surface IgM levels on WT or PKCδ$^{-/-}$ splenic follicular B cells (D) and lymph node B cells (E). The bar graphs show quantification of sIgM MFI (error bars, standard errors of the mean [SEM]; $n = 3$ mice of each genotype).

staining. As expected, WT T1 B cells are already highly susceptible to apoptosis under unstimulated conditions, and stimulation of WT T1 cells via the BCR further induces expression of cleaved caspase 3 and PARP. In sharp contrast, PKCδ$^{-/-}$ T1 B cells survived better in the unstimulated culture, and BCR-induced apoptosis was completely impaired in PKCδ$^{-/-}$ T1 B cells (Fig. 2F). This result demonstrates that PKCδ is required to couple BCR signaling to apoptosis in T1 B cells.

Our previous study identified selective activation of a proapoptotic Ca^{2+}-dependent Erk pathway that sensitizes bone marrow transitional B cells to negative selection (26). To address whether this mechanism is present in splenic tolerance checkpoints, we performed phosphoflow analysis for Erk activation in splenic cells stimulated with thapsigargin. Analogous to what we reported in bone marrow (26), we observed activation of the Ca^{2+}-Erk pathway in WT T1 cells, which was attenuated in other WT B cell subsets in the spleen. Moreover, activation of this pathway was severely impaired in PKCδ$^{-/-}$ T1 cells, as well as at later stages (Fig. 3A and B).

Antigen-dependent and independent effects of PKCδ deficiency. Although the competitive advantage of PKCδ$^{-/-}$ B cells at the T1-T2 transition suggests a role for PKCδ in transitional B cell-negative selection *in vivo*, it remains unclear if this advantage requires self-antigen. To address this, we bred the PKCδ$^{-/-}$ mice to IgHEL transgenic mice (MD4 mice) so that all B cells expressed a fixed BCR reactive to hen egg lysozyme (HEL). Because mice do not normally express HEL, B cell development in these mice proceeds in the absence of a self-antigen-driven selective environ-

ment. PKCδ$^{-/-}$ IgHEL transgenic mice displayed robust B cell lymphoproliferation and expansion of all splenic B cell subsets (Fig. 4A to F). Compared to WT IgHEL transgenic mice, the B cell expansion in the PKCδ$^{-/-}$ IgHEL animals was at least as severe as it was in the mice with an unrestricted B cell repertoire (Fig. 1E and 4D), despite overall lower cellularity in the spleens of IgHEL transgenic animals (Fig. 4B and D). Therefore, at least some features of the disease observed in these mice are antigen independent. However, both MZ and follicular B cells expanded equally in the PKCδ$^{-/-}$ IgHEL transgenic animals, suggesting that preferential MZ B cell expansion in the context of an unrestricted repertoire reflects antigen-dependent processes (Fig. 4E and F).

To test whether the competitive advantage of nontransgenic PKCδ$^{-/-}$ B cells at the T1-T2 transition (Fig. 2D) is due to failed negative selection, we reconstituted lethally irradiated WT mice with a 1:1 mixture of congenically marked WT IgHEL and PKCδ$^{-/-}$ IgHEL bone marrow (Fig. 5A). Because the recipient mice did not express HEL, development of the donor transgenic B cells proceeded in the absence of antigen-mediated selection. In the absence of self-antigen, we observed no significant advantage of PKCδ$^{-/-}$ IgHEL cells over WT IgHEL cells at the T1-T2 transition (Fig. 5B). Similar results were noted at the immature-transitional checkpoint in bone marrow (data not shown). Thus, eliminating self-reactivity is sufficient to reverse the advantage that PKCδ$^{-/-}$ transitional B cells have over WT B cells in the nontransgenic context (Fig. 2C and D). Together, the results from Fig. 2 and 5 demonstrate that PKCδ is required for antigen-dependent negative selection of transitional B cells in the spleen. Of note, in later stages of development, PKCδ$^{-/-}$ IgHEL B cells outcompeted WT IgHEL B cells in differentiation toward the MZ but not the follicular fate (Fig. 5B). This result contrasts with our observations in the noncompetitive setting (Fig. 4E and F) and suggests that, in the presence of competition, PKCδ deficiency favors the commitment of B cells to an MZ fate independent of self-antigen availability.

Consequences of self-antigen exposure for developing PKCδ$^{-/-}$ B cells. WT IgHEL/soluble HEL (sHEL) double-transgenic mice have long been used as a model for B cell anergy. B cells develop but are short-lived and unresponsive to stimulation and express much reduced levels of IgM on the surface compared to B cells from IgHEL transgenic mice that do not express HEL (16). PKCδ$^{-/-}$ IgHEL/sHEL double-transgenic mice were previously reported to display increased antigenic responses and sIgM levels relative to WT IgHEL/sHEL mice (1), but it has remained unclear how this phenotype relates to peripheral B cell development and selection. To assess the consequences of antigen exposure for B cell development, we generated PKCδ$^{-/-}$ IgHEL/sHEL double-transgenic mice both by breeding the IgHEL PKCδ$^{-/-}$ mice to the sHEL transgenic mice (Fig. 6A and B) and by reconstituting irradiated WT or sHEL transgenic mice with bone marrow from IgHEL littermate mice that were either PKCδ$^{+/-}$ or PKCδ$^{-/-}$ to generate noncompetitive chimeric mice (Fig. 6C). We analyzed sIgM levels in individual B cell subsets in the spleen and bone marrow of these mice. Notably, in WT double-transgenic B cells, we observed antigen-dependent reduction in the levels of sIgM even in the earliest immature population in the bone marrow (Fig. 6A to C), suggesting that antigen induces IgM downregulation in these immature IgHEL B cells. Alternatively, these B cells may not tolerate higher levels of IgM when the antigen is present and may be negatively selected. sIgM is then further downregulated at the T2-follicular transition in these WT cells. Strikingly, sIgM levels in PKCδ$^{-/-}$ IgHEL/sHEL immature bone marrow B cells are much higher than they are in WT IgHEL/sHEL mice, and they remain high throughout peripheral B cell development in the spleen, although a slight reduction is observed at the T2-follicular transition. These data demonstrate that the threshold of antigenic signaling required for sIgM downregulation is shifted in the absence of PKCδ.

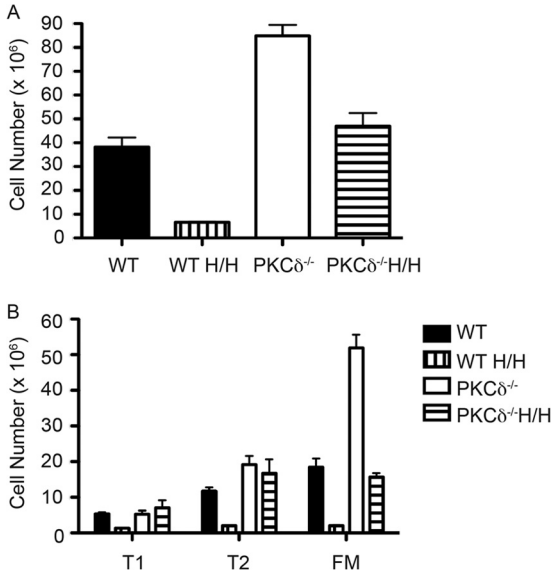


FIG 7 Quantification of splenic B cell subsets in CD45 H/H PKCδ$^{-/-}$ mice. Single-cell suspensions of WT IgHEL or PKCδ$^{-/-}$ IgHEL spleens were stained with the indicated antibodies and analyzed by FACS. (A) Total splenic B cell numbers. (B) Splenic B cell numbers in the indicated B cell subsets. The error bars indicate SEM.

We next investigated whether this difference in IgM levels was evident in non-BCR transgenic PKCδ$^{-/-}$ mice. Surprisingly, sIgM levels in both splenic follicular and lymph node B cells are virtually identical in nontransgenic WT and PKCδ$^{-/-}$ mice (Fig. 6D and E). Thus, in the context of a nonrestricted B cell repertoire, self-antigen reactivity in the PKCδ$^{-/-}$ B cell repertoire can compensate for the altered threshold of antigenic signaling required for sIgM downregulation, resulting in comparable sIgM levels in WT and PKCδ$^{-/-}$ B cells. These data suggest that the mature PKCδ$^{-/-}$ B cell repertoire is highly autoreactive.

PKCδ deficiency rescues transitional B cell numbers in CD45 H/H mice but only partially rescues mature B cells. The data from the IgHEL/sHEL double-transgenic mice in Fig. 6 demonstrate that PKCδ deficiency is sufficient to reverse at least some hallmarks of anergic B cells, albeit in the context of a single BCR specificity. We next investigated the role of PKCδ in mediating the B cell loss triggered by enhanced BCR signals in the context of an unrestricted B cell repertoire. CD45 H/H mice carry two copies of the CD45 H transgene and consequently express ~200% of WT CD45 levels with normal splicing. Because of the augmented CD45 expression, CD45 H/H mice display increased proximal BCR signal strength relative to antigen affinity, resulting in loss of peripheral B cells. Because this loss is seen at every developmental stage, the decreased output of early transitional B cells at least partially underlies their B cell lymphopenia (27).

We crossed PKCδ$^{-/-}$ mice with CD45 H/H mice to assess the

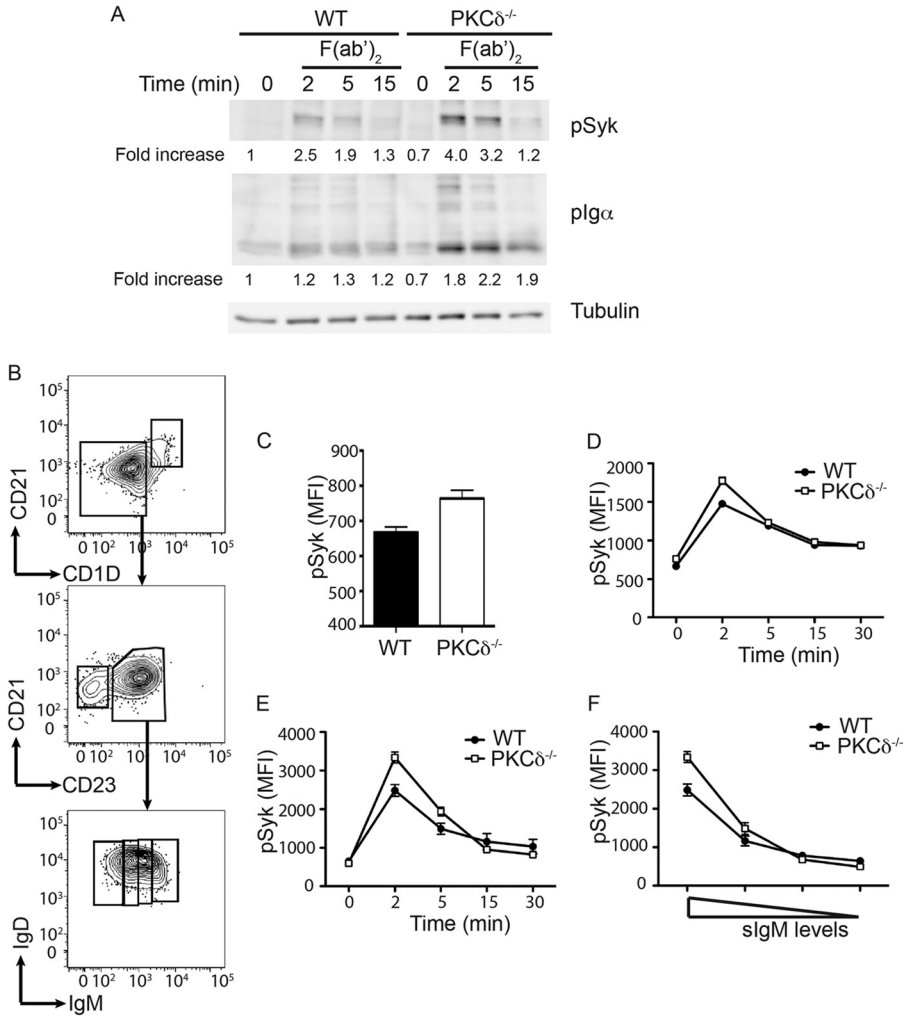


FIG 8 PKCδ is a negative regulator of proximal BCR signaling. (A) Lysates from purified lymph node B cells stimulated with anti-IgM F(ab′)$_2$ fragment, as indicated, were analyzed by immunoblotting with the indicated antibodies. The fold induction of Syk and Ig(α) phosphorylation was calculated relative to tubulin levels. (B) Representative gating strategy for phosphoflow analysis of pSyk responses in splenic B cells. (C) Mean fluorescence intensities of pSyk in unstimulated WT or PKCδ$^{-/-}$ T1 B cells. (D) Quantification of pSyk response in WT or PKCδ$^{-/-}$ T1 B cells, defined as B220$^+$ CD1D$^-$ CD21$^-$ CD23$^-$. (E) Quantification of pSyk response in T2 B cells, defined as B220$^+$ CD1D$^-$ CD21$^-$ CD23$^+$ IgM^{hi}. (F) Quantification of the peak of the pSyk response (at 2 min after stimulation) in CD23$^+$ splenic B cells relative to IgM levels. The error bars indicate SEM.

ability of PKCδ deficiency to rescue B cell survival in the context of exaggerated BCR signaling in an unrestricted repertoire. In PKCδ$^{-/-}$ CD45 H/H mice, splenic B cell numbers are significantly rescued, reaching numbers comparable to those seen in WT mice, but still lower than the B cell numbers observed in PKCδ$^{-/-}$ animals with WT CD45 levels (Fig. 7A). Therefore, while PKCδ deficiency partially rescues B cell numbers in the CD45 H/H mice, exaggerated BCR signaling is still able to curtail the aberrant expansion of PKCδ$^{-/-}$ B cells. We next determined the numbers of B cells in each splenic subset in these mice. CD45 H/H T1 and T2 B cell numbers are fully rescued by PKCδ deficiency to levels comparable to those observed in PKCδ$^{-/-}$ animals with WT CD45 levels (Fig. 7B). Therefore, PKCδ deficiency is sufficient to rescue CD45 H/H transitional B cells with excessive BCR signaling from negative selection. In contrast, follicular B cell numbers in the PKCδ$^{-/-}$ CD45 H/H mice are comparable to those in WT mice but substantially lower than those in PKCδ$^{-/-}$ mice, demonstrating that increased CD45 expression can curtail the expansion of mature PKCδ$^{-/-}$ B cells. These results demonstrate that, while PKCδ is critical for negative selection of transitional B cells, it is not essential for BCR-induced loss of mature autoreactive B cells.

PKCδ is a negative regulator of proximal BCR signaling. Our results with the IgHEL/sHEL system demonstrate that PKCδ is required for efficient IgM downregulation, which can be compensated for with an aberrantly autoreactive B cell repertoire (Fig. 6). IgHEL/sHEL double-transgenic PKCδ$^{-/-}$ B cells display enhanced responsiveness to antigenic stimulation (1), and the increased sIgM levels likely contribute to this break in anergy. Nevertheless, this does not preclude an additional role for PKCδ in BCR signaling independent of sIgM levels or antigenic strength.

To address this question, we purified lymph node B cells from non-BCR transgenic WT or PKCδ$^{-/-}$ mice, stimulated them *ex vivo* via the BCR using an anti-IgM F(ab′)$_2$ fragment in a time course, and examined cell lysates from these cells for the activation status of Igα and Syk. Lymph node B cells are a relatively homogeneous population of follicular recirculating B cells and, as men-

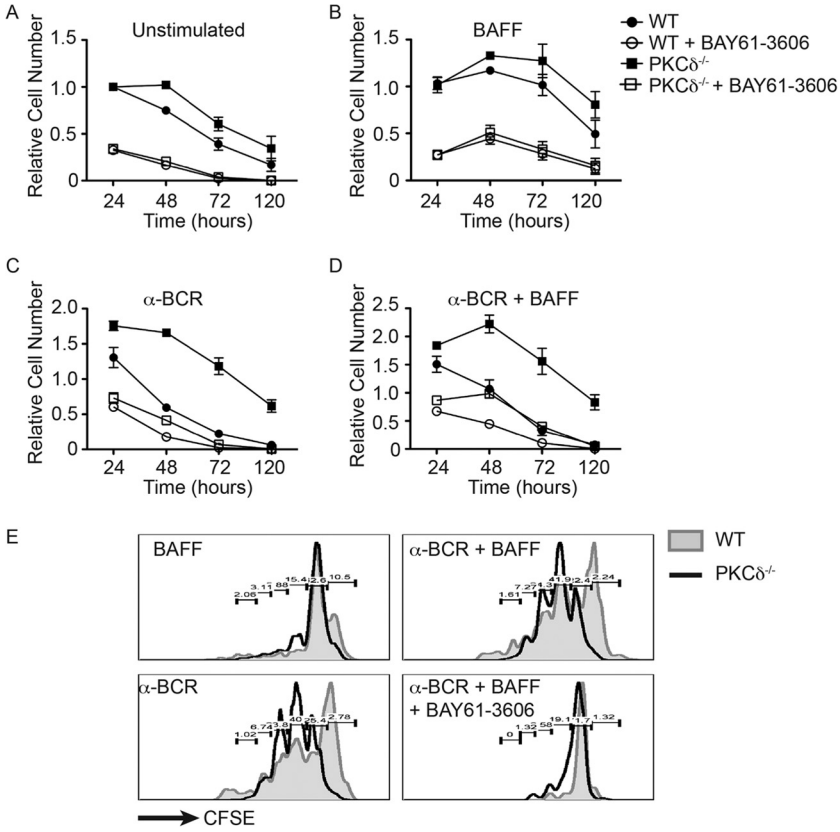
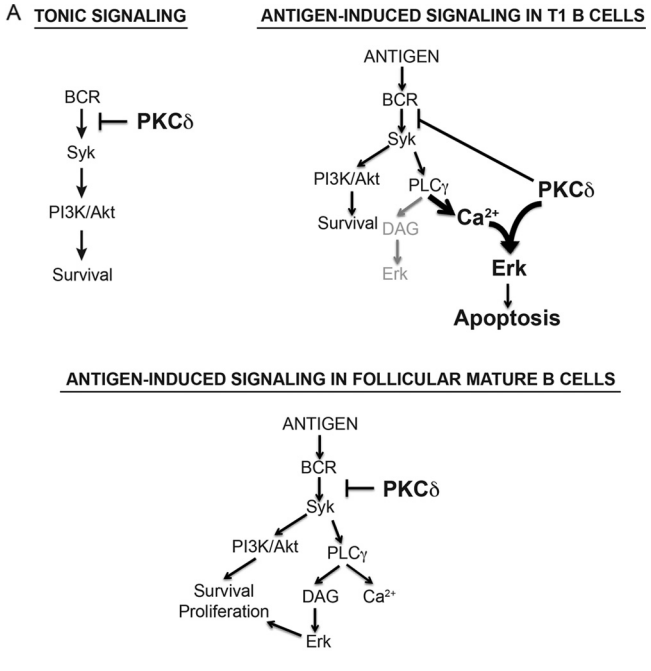


FIG 9 PKCδ deficiency augments B cell survival and proliferation in a Syk-dependent manner. CFSE-labeled lymph node cells from WT CD45.1$^+$ and PKCδ$^{-/-}$ CD45.1$^-$ mice were mixed at a 1:1 ratio and seeded on 96-well plates. The cells were left unstimulated or stimulated with either 40 ng/ml BAFF, 10 μg/ml anti-IgM F(ab′)$_2$ fragment, or both in the presence or absence of the Syk inhibitor BAY61-3606. The cells were harvested at the indicated time points and stained for B220, CD45.1, and DAPI. (A to D) The number of live B cells was normalized to the number of live B cells of each genotype in the unstimulated culture at the 24-h time point. The plots indicate relative cell numbers for each condition over 5 days in culture. (E) Representative CFSE FACS plots of the indicated cells after 5 days in culture. The error bars indicate SEM.

tioned above, sIgM levels are virtually identical in PKCδ$^{-/-}$ and WT lymph node B cells (Fig. 6E). Remarkably, phosphorylation of Syk and Ig(α) were greatly increased in PKCδ$^{-/-}$ cells (Fig. 8A), demonstrating that PKCδ is a negative regulator of proximal BCR signaling.

We next stimulated total splenic cells via the BCR using anti-IgM F(ab′)$_2$ fragment and examined pSyk responses in the different splenic B cell subsets by phosphoflow (Fig. 8B to F). This approach allowed us to monitor pSyk responses in different B cell subsets and to correlate these responses with the levels of sIgM expression. Interestingly, unstimulated PKCδ$^{-/-}$ B cells displayed enhanced basal Syk activation in T1 cells (Fig. 8C), indicating that transitional PKCδ$^{-/-}$ B cells have higher levels of tonic BCR signaling than WT B cells. Moreover, stimulated splenic PKCδ$^{-/-}$ B cells displayed increased Syk activation relative to WT cells. The excessive Syk activation was particularly pronounced in T1 B cells, as well as CD23$^+$ B cells, with high IgM levels (Fig. 8D and E). The increased Syk activation in PKCδ$^{-/-}$ B cells was less evident in IgM^{low} follicular B cells, suggesting that self-antigen-induced downregulation of sIgM in PKCδ$^{-/-}$ B cells efficiently mutes BCR responses (Fig. 8F). Together, these data demonstrate that PKCδ acts as a negative regulator of proximal BCR signaling, in addition to its role in coupling the BCR to apoptosis in transitional B cells.

Syk is required for the enhanced survival and proliferation of PKCδ$^{-/-}$ B cells. To address the functional relevance of enhanced Syk signals in PKCδ$^{-/-}$ B cells, we utilized a Syk inhibitor in a coculture of CFSE-labeled, congenically marked WT and PKCδ$^{-/-}$ lymph node cells for a 5-day period *ex vivo*. These cocultures ensured that both WT and PKCδ$^{-/-}$ B cell populations were stimulated under identical conditions within the same well. First, the cells were left unstimulated or stimulated with BAFF, anti-IgM F(ab′)$_2$ fragment, or both. As expected (25), unstimulated PKCδ$^{-/-}$ B cells displayed a survival advantage relative to WT B cells (Fig. 9A, solid symbols). BAFF stimulation promoted the survival but not the proliferation of both WT and PKCδ$^{-/-}$ B cells, and the PKCδ$^{-/-}$ cells retained a significant survival advantage over WT B cells (Fig. 9B, solid symbols, and E). B cells in the cultures stimulated via the BCR or via both the BCR and BAFF proliferated vigorously (Fig. 9C to E), with the PKCδ$^{-/-}$ B cells proliferating substantially more than the WT B cells (Fig. 9E). We next assessed the effect of 2-[7-(3,4-dimethoxyphenyl)-imidazo[1,2-c]pyrimidin-5-ylamino]nicotinamide dihydrochloride (BAY61-3606), a specific Syk inhibitor (31), on these cocultures. Remarkably, inhibition of Syk with BAY61-3606 completely abrogated the survival advantage of unstimulated PKCδ$^{-/-}$ B cells, and B cells of both genotypes died at equivalent rates (Fig. 9A, open symbols). BAY61-3606 also abrogated the survival advantage of PKCδ$^{-/-}$ B cells in the BAFF cultures, again indicating that this advantage is Syk dependent (Fig. 9B, open symbols). As expected, BCR-induced proliferation was Syk dependent for both



B

Summary of effects of PKCδ deficiency	
In T1 B Cells	In Follicular Mature B Cells
↓ Ca^{2+}-Erk signaling ↓ Antigen-induced apoptosis	↑ Tonic BCR Signaling ↑ BCR-induced signaling ↑ BAFF-dependent survival ↑ BCR-induced proliferation ↑ Autoimmune responses
↑ Tonic BCR Signaling ↑ Survival ↑ B cell output and autoreactivity	

FIG 10 Model of PKCδ roles during B cell development and activation. (A) In T1 B cells, PKCδ is required to couple BCR signaling to apoptosis to facilitate antigen-dependent negative selection. Additionally, PKCδ acts as a negative regulator of proximal BCR signaling in all B cell subsets, thereby curtailing prosurvival tonic signaling, as well as antigen-driven BCR signaling and proliferation in mature B cells. (B) Summary of the effects of PKCδ deficiency on T1 or follicular mature B cells.

WT and PKCδ$^{-/-}$ B cells (Fig. 9C to E). These results demonstrate that the negative regulation of BCR signal strength by PKCδ has profound functional consequences for survival signals driven by both tonic BCR signaling and BAFFr signaling and on BCR-induced proliferative responses.

DISCUSSION

Our findings reveal pleiotropic roles for PKCδ in BCR signaling that impact B cell function in unique ways at different developmental stages. First, PKCδ is required to couple antigen engagement to negative selection in developing transitional B cells to enforce early tolerance checkpoints. Second, PKCδ acts as a negative regulator of BCR signaling throughout B cell development and in mature B cells. As a consequence of these two distinct roles, PKCδ deficiency results in a B cell repertoire that is not only highly autoreactive but also hyperresponsive to stimulation, leading to robust autoimmune pathology (Fig. 10).

We show that PKCδ is required for efficient IgM downregulation in the IgHEL/sHEL double-transgenic system. However, in an unrestricted repertoire, the sIgM levels of mature PKCδ$^{-/-}$ B cells are equivalent to those on WT mature B cells, demonstrating that an unrestricted repertoire is able to compensate for this defect in IgM downregulation. Although the affinity of sHEL for IgHEL is extremely high, sHEL is a monomeric antigen, and as such, it presumably fails to induce clustering of the BCR. Therefore, it is likely that multimeric self-antigens, such as membrane-bound self-antigens, play an important role in compensating for the loss of PKCδ and inducing IgM downregulation in nontransgenic mice. These observations, together with the defects observed at the T1-T2 checkpoint, suggest that the overall PKCδ$^{-/-}$ mature B cell repertoire is more autoreactive than it is in WT mice.

It is likely that the impaired IgM downregulation in PKCδ$^{-/-}$ double-transgenic B cells at least partially underlies the previously reported enhanced responsiveness of these B cells to antigenic stimulation (1), thereby contributing to frank autoimmune disease. Nevertheless, our phosphoflow data demonstrate that in B cells with equivalent sIgM levels, BCR signals are stronger in PKCδ$^{-/-}$ B cells than in WT B cells at early time points after stimulation (prior to IgM internalization). Therefore, our data suggest two independent mechanisms by which PKCδ deficiency contributes to enhanced BCR signaling, namely, IgM downregulation and negative regulation of proximal BCR signals. Combined, the effects of PKCδ deficiency converge to increase BCR signal strength in PKCδ$^{-/-}$ B cells and likely facilitate the onset of autoimmune responses in the mice.

Increased BCR signal strength is also observed in mice lacking components of the inhibitory Lyn/CD22/SHP-1 pathway, but in these mouse models, the increase in BCR signal strength is accompanied by exaggerated negative selection (32–34). In contrast, in PKCδ$^{-/-}$ mice, BCR signaling is uncoupled from apoptosis, thus allowing autoreactive cells that would normally be deleted to develop into the T2 stage. Indeed, this key difference explains the distinct outcomes for tolerance induction in these different mutant strains in the context of the sHEL/IgHEL system: while mutations of the Lyn/CD22/SHP-1 pathway turn anergy into deletion in the double-transgenic mice, PKCδ deficiency does not enhance deletion but rather allows the B cells to develop and subsequently leads to loss of anergy. The requirement for PKCδ for coupling the BCR to apoptosis in T1 cells can be at least partially attributed to its role in activating proapoptotic Ca^{2+}-dependent Erk signaling (3, 26, 35). In addition, tonic signaling from the BCR is also enhanced in PKCδ$^{-/-}$ transitional B cells, and the recent finding that the BAFFr acts at least in part by coopting the BCR signaling machinery (24) implies that PKCδ deficiency may mimic at least some features of BAFF stimulation by enhancing tonic BCR signaling. This notion also explains why the B cell lymphoproliferation and the preferential expansion of MZ PKCδ$^{-/-}$ B cells do not require self-antigen in a competitive setting, as positive selection of T2 cells into the MZ compartment is thought to be driven by low-affinity self-antigens and favored by excess BAFF signaling (14). Moreover, our observation that PKCδ is rapidly and robustly phosphorylated downstream of both the BCR and the BAFFr strongly suggests that PKCδ may integrate signals from both of these receptors to fine tune the antigen response during B cell development.

The T1-T2 checkpoint in peripheral B cell development is often defective in lupus patients (7–10). PKCδ$^{-/-}$ mice represent a lupus mouse model in which this checkpoint is severely impaired, and its relevance in human disease is underscored by three recent reports of human patients with mutations in PKCδ that underlie juvenile-onset SLE, autoimmune lymphoproliferative syndrome (ALPS), and B cell deficiency with autoimmunity, with some of these patients displaying defective B cell apoptosis and hyperpro-

liferation of transitional B cells (3–5). The striking resemblance of the disease in these patients to the phenotypes we have characterized in the PKCδ$^{-/-}$ mice highlights the relevance of these mice as a model of human disease that warrants further investigation.

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A.L. designed and did experiments, analyzed the data, and wrote the manuscript. J.Z. designed and did experiments, analyzed data, and revised the manuscript. T.L. provided technical assistance. M.L. generated the PKCδ$^{-/-}$ mice. A.W. designed experiments, provided reagents and support, and revised the manuscript. J.P.R. designed experiments, supervised the research, revised the manuscript, and provided support.

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