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Protein Kinase C β Is Required for Lupus Development in Sle Mice

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Abstract

Objective—To evaluate the requirement for protein kinase C (PKC) in the development of lupus in mice, and to explore the potential of targeting PKC as a therapeutic strategy in lupus.

Methods—Congenic mice bearing the disease loci *Sle1* or *Sle1* and *Sle3*, which represent different stages of severity in the development of lupus, were crossed with PKC-deficient mice. The effect of PKC deficiency in lupus development was analyzed. In addition, the effects of the PKC-specific inhibitor enzastaurin on the survival of B cells from mice with lupus and human 9G4-positive B cells as well as the in vivo effect of enzastaurin treatment on the development of lupus in Sle mice were investigated.

Results—In Sle mice, PKC deficiency abrogated lupus-associated phenotypes, including high autoantibody levels, proteinuria, and histologic features of lupus nephritis. Significant decreases in spleen size and in the peritoneal B-1 cell population, reduced numbers of activated CD4 T cells, and normalized CD4:CD8 ratios were observed. PKC deficiency induced an anergic B cell phenotype and preferentially inhibited autoreactive plasma cells and autoantibodies in mice with lupus. Inhibition of PKC enhanced apoptosis of both B cells from Sle mice and human autoreactive B cells (9G4 positive). Treatment of Sle mice with the PKC-specific inhibitor enzastaurin prevented the development of lupus.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Drs. Chen and Sanz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Conclusion—This study identifies PKC δ as a central mediator of lupus pathogenesis, suggesting that PKC δ represents a promising therapeutic target for the treatment of systemic lupus erythematosus. Moreover, the results indicate the feasibility of using a PKC δ inhibitor for the treatment of lupus.

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the production of autoantibodies and damage to multiple organs. B cells represent important therapeutic targets in SLE, because they have several pathogenic functions, including autoantibody production and antigen presentation to T cells, resulting in differentiation of Th17 cells and proinflammatory cytokine production (1–3).

Both B cell receptor (BCR)– and BAFF-dependent signals are required for B cell differentiation and mature B cell maintenance (4–6). These signaling pathways are also critical for the enforcement of B cell tolerance (7–9). One of the major survival pathways mediated by BCR and BAFF is activation of the transcription factor NF- κ B, which mediates up-regulation of antiapoptotic proteins such as Bcl-2, Bcl-x_L, and A1, as well as down-regulation of proapoptotic proteins. However, exaggerated NF- κ B activation and prolonged B cell survival can lead to the generation of deleterious autoimmune responses, as shown in transgenic mouse models of Bcl-2 and Bcl-x_L over-expression (10).

Protein kinase C (PKC δ) is a serine/threonine kinase expressed in normal and malignant B cells (11,12). It plays an important role in mature B cell survival through the BCR-mediated NF- κ B activation pathway (13–15). The biochemical link between PKC δ and NF- κ B activation has been delineated in recent studies (14,16). Mice lacking PKC δ show defective B cell proliferation upon BCR crosslinking and impaired humoral responses. In medium with no exogenous stimuli, PKC δ -deficient B cells die more rapidly than B cells from control wild-type mice. The poor survival of PKC δ -deficient B cells is associated with an inability to activate NF- κ B signaling and its downstream targets Bcl-x_L and Bcl-2.

PKC δ is also involved in BAFF signaling pathways, which are critical for the survival of mature B cells. Moreover, the BAFF level controls the survival of autoreactive transitional and naive B cells (17,18). PKC δ is required for BAFF-controlled B cell metabolic fitness through phosphorylation of Akt (19). Therefore, the involvement of PKC δ in both the BCR and BAFF signaling pathways provides a molecular mechanism for the poor survival and impaired peripheral maturation of PKC δ -deficient B cells in vitro and in vivo.

Taken together, the available information suggests that PKC δ inhibition could regulate the survival of autoreactive B cells and control the development of lupus. However, the role of PKC δ in the survival of lupus B cells and the development of this disease has not been specifically investigated. To address this issue, we used the Sle congenic mouse model that has been extensively used in lupus studies (20,21). We demonstrate here that a deficiency in PKC δ abolishes all lupus-associated phenotypes, such as high levels of autoantibodies in serum and lupus nephritis, as indicated by reductions in proteinuria and in antibody deposition in the kidneys. Our results also indicate that PKC δ deficiency renders lupus B cells anergic and impedes BCR-mediated NF- κ B activation. Importantly, PKC δ deficiency abolishes the spontaneous germinal center (GC) formation and generation of autoreactive plasma cells that characterize SLE and have critical roles in the pathogenesis of this autoimmune disease. Furthermore, we demonstrate that in vivo treatment with the PKC δ -specific inhibitor enzastaurin in mice with lupus ameliorates disease development, thus identifying PKC δ as a promising therapeutic target for the treatment of SLE.

MATERIALS AND METHODS

Generation of PKC β -deficient *Sle* mice

The congenic mouse strains B6.*Sle1* and B6.*Sle1.Sle3* (22) and B6.PKC^{-/-} mice (13,23) were previously described. B6.*Sle1* mice were bred with B6.PKC^{-/-} mice to derive F1 mice. The F1 mice were mated to produce progenies, and the homozygotes at both the *Sle* and PKC^{-/-} loci were identified by polymerase chain reaction (PCR) using primers specific for *Sle1* (24) and PKC (23). The generated PKC^{-/-}-deficient *Sle* mice were referred to as *Sle1*.PKC^{-/-} mice and were used in our experiments. Similarly, B6.*Sle1.Sle3* mice were bred with B6.PKC^{-/-} mice, the homozygotes for *Sle1*, *Sle3*, and PKC^{-/-} were selected using PCR primers (24), and the resulting PKC^{-/-}-deficient *Sle1.Sle3* mice were referred to as *Sle1.Sle3*.PKC^{-/-} mice. For simplicity, B6 is henceforth omitted from the strain names, because all of the mice described had a B6 background. All animal procedures were approved by the Committee on Animal Resources at the University of Rochester.

Flow cytometric analysis

Single-cell suspensions were prepared from different tissues, and subsets of B cells and other types of cells were identified using a combination of antibodies, as previously described (25). All antibodies were purchased from eBioscience, except allophycocyanin-conjugated CD4 (BD Biosciences). Flow cytometry data were collected using FACSCalibur, FACSCanto II, or LSR II instruments (BD Biosciences) at the University of Rochester Flow Cytometry Core Facility and were analyzed using FlowJo software version 8.5.3 (Tree Star).

B cell purification, in vitro proliferation, and survival assays

For in vitro survival experiments, B cells purified using CD43 microbeads (Miltenyi Biotec) were either left untreated (control) or stimulated with 10 $\mu\text{g}/\text{ml}$ of anti-IgM F(ab)₂ (Jackson ImmunoResearch) or lipopolysaccharide (LPS; 5 $\mu\text{g}/\text{ml}$) for 22 hours. The cells were then stained and used for multicolor flow cytometric analysis.

To test the effect of the PKC^{-/-} inhibitor enzastaurin on the survival of human 9G4-positive B cells, peripheral blood was obtained from healthy donors according to protocols approved by the University of Rochester Medical Center Institutional Review Board. Peripheral blood mononuclear cells were isolated by a standard density-gradient centrifugation procedure. Naive B cells were purified using a Naive B Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's protocol. The purified naive B cells were treated with either DMSO (control) or enzastaurin (0.7 μM ; Selleck Chemicals) for 24 hours. The cells were stained with a 9G4 monoclonal antibody, using a Live/Dead Fixable Aqua Dead Cell Stain Kit (Invitrogen) to identify apoptotic cells in 9G4-positive cell populations.

Intracellular calcium measurements

The flux of Ca²⁺ was measured as previously described (26). Briefly, splenocytes (3 \times 10⁶/ml) were loaded with 1 μM Fura Red (Invitrogen) and then stained with phycoerythrin-conjugated anti-B220 antibodies in loading buffer. Changes in intracellular Ca²⁺ levels in B cells were analyzed using a BD FACSVantage SE system by measuring Fura Red fluorescence ratios in B220+ gated cells. A decrease in the fluorescence ratio indicates an increase in the intracellular Ca²⁺ concentration. Data were displayed as the relative ratio of intensities of Fura Red for each cell over time and were analyzed using FlowJo software (Tree Star).

Enzyme-linked immunosorbent assay (ELISA) and enzyme-linked immunospot (ELISpot) assay for autoantibodies

Serum immunoglobulins of various isotypes were analyzed by ELISA (27). Briefly, diluted sera were loaded onto precoated 96-well plates. Bound IgM or each IgG subtype was detected using alkaline phosphatase-conjugated goat anti-mouse IgM or IgG (SouthernBiotech) and an alkaline phosphatase substrate kit (Bio-Rad). Optical density at 405 nm was read on a BioTek Instruments microplate reader. IgG anti-double-stranded DNA (anti-dsDNA) antibody-secreting cells (ASCs) were detected by ELISpot assays, as previously described (28).

Immunohistochemical analysis

Immunofluorescence analysis of frozen spleen sections was performed as previously described (29). IgG deposition was detected with fluorescein isothiocyanate-conjugated goat anti-mouse IgG, IgG2b, or IgG2c (Molecular Probes). Images were obtained using a Leica DMRXA microscope (Carl Zeiss Instruments) and analyzed using SlideBook software. Consecutive slides were stained with hematoxylin and eosin and periodic acid-Schiff at the University of Rochester Pathology Core Laboratory.

In vivo treatment

Two-month-old female *Sle1.Sle3* mice were administered enzastaurin (1.5 mg in 200 μ l 10% acacia in water/per mouse; Selleck Chemicals) 4 times weekly for a period of 8 weeks, by oral gavage. Analysis of the mice was performed as described above.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0. Two-group comparisons were analyzed using a Student's 2-tailed *t*-test. Data are presented as the mean \pm SD. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Effect of PKC β deficiency on lupus development in *Sle* mice

To investigate the role of PKC in lupus B cell survival and in lupus development, we took advantage of the availability of the congenic *Sle* mouse model of lupus (22) and PKC-deficient mice (13) and generated PKC-deficient *Sle* mice by separately crossing PKC^{-/-} mice with 2 congenic lupus mouse strains, *Sle1* and *Sle1.Sle3*. We designated the offspring mice as *Sle1*.PKC^{-/-} (homozygous for both the *Sle* locus and the PKC locus) and *Sle1.Sle3*.PKC^{-/-} (homozygous for *Sle1*, *Sle3*, and PKC). These 2 congenic strains were chosen because both *Sle1* and *Sle3* loci have an impact on B cells, and they represent 2 distinct stages of lupus severity (22,30). Compared with control mice, both *Sle1* and *Sle1.Sle3* mice displayed several features of systemic autoimmunity such as splenomegaly, increased activation of B cells and T cells, higher CD4:CD8 T cell ratios, and high antinuclear autoantibody (ANA) production. However, only *Sle1.Sle3* bicongenic mice can develop full-blown lupus nephritis (29).

One of the hallmarks of SLE in both humans and mice is the loss of tolerance to nuclear antigens, resulting in the production of ANAs such as antichromatin and anti-dsDNA (21). Moreover, high levels of serum anti-dsDNA autoantibodies, especially IgG2a and IgG2c, are associated with the severity of glomerulonephritis (GN) in mice with lupus (29). We thus examined the effect of PKC deletion on ANA production in *Sle* mice. As shown in Figure 1A, PKC deficiency resulted in a marked reduction in the levels of IgG1, IgG2b, and IgG2c anti-dsDNA and antihistone/anti-dsDNA autoantibodies in *Sle* mice. Notably, the levels of

all tested autoantibody isotypes except IgG3, for which few differences were observed (results not shown), were significantly lower in *Sle1.Sle3* PKC^{-/-} mice than in *Sle1.Sle3* mice with the wild-type PKC gene (Figure 1A), while total IgG levels were comparable in all 6 mouse strains used in this study (results not shown).

We also observed that PKC⁻-deficient Sle mice displayed reduced levels of IgM anti-dsDNA (3.1-fold decrease in *Sle1*.PKC^{-/-} mice versus *Sle1* lupus mice [$P=0.01$] and 9.5-fold decrease in *Sle1.Sle3*.PKC^{-/-} mice versus *Sle1.Sle3*.PKC^{-/-} mice [$P=0.01$]) and IgM antihistone/anti-dsDNA (2.5-fold decrease in *Sle1*.PKC^{-/-} mice and 6-fold decrease in *Sle1.Sle3*.PKC^{+/+} mice versus wild-type mice [$P=0.01$]), which is consistent with the notion that IgM autoantibodies play a role in the development of lupus nephritis (31). As expected, we observed that total IgM levels in PKC⁻-deficient Sle mice were much lower than those in Sle mice with wild-type PKC, in accordance with the observation that PKC^{-/-} mice have a low level of serum IgM due to a significant decrease in the peritoneal B-1 cell population (13).

More significantly, besides the dramatic decrease in the number of serum autoantibodies in PKC⁻-deficient Sle mice, the ability of PKC deficiency to alleviate SLE was further supported by both the absence of the immune complexes (Figure 1B) and the amelioration of histologic features of active GN, such as the glomerular enlargement and marked global mesangial and endocapillary proliferation that were present in *Sle1.Sle3* mice (Figure 1C). Additionally, consistent with previous reports, ~70% of the 8–10-month-old *Sle1.Sle3* mice developed high proteinuria levels (>100 mg/dl) (Figure 1D) (29). In contrast, the urinary protein concentration in age-matched PKC⁻-deficient Sle mice was normal (<30 mg/dl; $n=15$ mice of each genotype). Collectively, our results demonstrated that the absence of PKC significantly attenuates the serologic, clinical, and histologic phenotypes characteristic of the *Sle1* and *Sle3* loci.

Characteristics of PKC β -deficient Sle mice

Splenomegaly is a feature of murine lupus and is always associated with disease progression and severity (29). *Sle1* mice usually showed a moderate increase in spleen size compared with the control B6 mice, while *Sle1.Sle3* bicongenic mice displayed marked splenomegaly (Figure 2A). Strikingly, PKC deficiency resulted in a dramatic decrease in spleen size in Sle mice (Figure 2A). Consistent with the small size and weight of *Sle1*.PKC^{-/-} and *Sle1.Sle3*.PKC^{-/-} mice, the absolute cell numbers in the spleens of these mice were significantly decreased compared with those in the spleens of Sle mice (43×10^6 for *Sle1*.PKC^{-/-} mice versus 80.3×10^6 for *Sle1*.PKC^{+/+} mice [$P=0.05$] and 46×10^6 for *Sle1.Sle3*.PKC^{+/+} mice versus 90×10^6 for *Sle1.Sle3*.PKC^{-/-} mice [$P=0.0023$]).

Another characteristic of *Sle1* and *Sle1.Sle3* mice is expansion of the number of activated CD4⁺T cells and activated B cells (as judged by CD69 expression) and elevated CD4:CD8 T cell ratios (29,32). We observed that the loss of PKC prevented expansion of the populations of activated CD4⁺T cells and B220⁺ cells and restored the CD4:CD8 T cell ratio in Sle mice (Figures 2B–D).

One of the most striking phenotypes of PKC^{-/-} mice is a severe reduction in the frequency of peritoneal B-1 cells (13). Because expansion of the B-1 cell population has been associated with autoimmunity in both humans and mouse models (33,34), we analyzed peritoneal B cell populations (Figure 3). *Sle1* and *Sle1.Sle3* mice had a remarkable increase in the frequency of B-1a cells in their peritoneal cavities compared with B6 control mice (Figures 3A and B). This expansion was dramatically reduced in PKC⁻-deficient Sle mice.

A detailed flow cytometric analysis of spleen and bone marrow B cells from PKC⁻ deficient Sle mice did not reveal other obvious defects in B cell development, with one notable exception. PKC⁻ deficiency resulted in a several-fold decrease in the proportion of CD138⁺ plasma cells in spleen as well as bone marrow (Figure 4A, and results not shown). This decrease in the frequency of ASCs was also reflected by the results of functional ELISpot assays (Figure 4B), consistent with the earlier ELISA results showing greatly decreased serum levels of IgG anti-dsDNA antibodies in PKC⁻ deficient Sle mice (Figure 1A).

PKC⁻ deficiency resulted in the reversal of another SLE-associated feature of great interest and relevance to the overall disease process. PKC⁻ deficient Sle mice lacked the spontaneous GC formation that is characteristic of mice with lupus (Figure 4C). The diminished number of GC B cells (B220⁺GL7⁺CD95⁺) in PKC⁻ deficient Sle mice (Figure 4D) may contribute to the decreased de novo production of plasma cells in GC reactions.

Anergic B cell phenotype in PKC β -deficient Sle mice

We also assessed the functional status of PKC⁻ deficient Sle mice. Because PKC⁻ deficient B cells are characterized by drastically decreased proliferative responses to IgM-mediated BCR crosslinking (14,15), we sought to determine whether PKC⁻ deletion also leads to impaired BCR signaling in B cells from Sle mice. As shown in Figure 5A, deletion of PKC diminished the proliferation response of B cells from Sle mice to anti-IgM stimulation but not to LPS stimulation, which is mediated by a different receptor (Toll-like receptor 4). Consistently, up-regulation of activation markers such as CD69 and CD86 was attenuated in PKC⁻ deficient Sle mice (Figure 5A).

In accordance with the previous observation that PKC⁻ deficient B cells exhibited poor survival in vitro in the absence of a stimulus (14,15,35), B cells from PKC⁻ deficient Sle mice had a shorter lifespan than those from Sle mice (Figure 5B). In contrast, B cells from B6.*Sle1.Sle3* mice showed prolonged survival and a stronger response to anti-IgM stimulation (Figures 5A and B). The B cells from B6.*Sle1* mice had a slightly higher rate of survival compared with B cells from the B6 control littermates. This attenuated BCR response and the shortened lifespan in PKC⁻ deficient Sle mice are reminiscent of anti-human erythroleukemia cell line and anti-dsDNA-transgenic B cells, which were rendered anergic following long-term stimulation with the corresponding self antigens.

Previous studies indicated that the poor survival of PKC⁻ deficient B cells correlates well with their inability to up-regulate BCR-mediated NF- κ B signaling (14,15). We therefore hypothesized that a deficiency in PKC uncouples BCR-mediated NF- κ B signaling in the B cells of Sle mice. To test this possibility, we cultured splenocytes from different mouse strains in medium, in the presence or absence of anti-IgM F(ab)₂ stimulation, for 24 hours. B cells from PKC⁻ deficient Sle mice failed to induce the expression of the antiapoptotic protein Bcl-x_L, an NF- κ B target (Figure 5C), or phosphorylation of I κ B in response to anti-IgM stimulation (results not shown). These observations suggest that the inability of PKC⁻ deficient cells to up-regulate BCR-mediated NF- κ B activation is responsible for the shortened lifespan of B cells from PKC⁻ deficient Sle mice.

Abnormal calcium signaling has been associated with anergic B cells. We thus investigated the effect of PKC⁻ deletion on calcium signaling in B cells from Sle mice. Both PKC^{-/-} mice and PKC⁻ deficient Sle mice showed a marked decrease in Ca²⁺ flux upon BCR stimulation (Figure 5D), further supporting the notion that PKC⁻ deficiency renders lupus B cells anergic.

Sensitivity of lupus B cells to PKC β inhibition

It was reported previously that, upon selective PKC β blockade, mature B cells respond to BCR engagement in a manner similar to that of immature B cells and undergo apoptosis, indicating that PKC β is a critical factor determining the fate of B cells in terms of apoptosis or survival (36). This observation, combined with our studies described above, raises the possibility of inhibiting mature autoreactive B cell survival through PKC β inhibition. We thus examined whether lupus B cells could be induced to undergo apoptosis by selective inhibition of PKC β with a pharmacologic agent (37). As shown in Figure 6A, enzastaurin, a well-tolerated PKC β -specific inhibitor, induced apoptosis of lupus B cells in the presence of BCR signaling, suggesting that lupus B cells depend on PKC β signaling for survival.

We next sought to determine whether the PKC β inhibitor enzastaurin exhibits a similar effect on human autoreactive B cells. We analyzed the effect of this intervention on cells expressing the 9G4 idiotype; 9G4-determined autoreactivity is censored in healthy subjects but is expanded in patients with SLE (38). Indeed, due to their autoreactivity, human 9G4-positive B cells were more sensitive to PKC β inhibition than were 9G4-negative B cells (Figure 6B), indicating that human autoreactive B cells are highly dependent on PKC β for survival.

Having shown that enzastaurin inhibited the survival of both Sle mouse B cells and human autoreactive B cells, we investigated the effect of enzastaurin on disease development in mice with lupus. We treated 2-month-old *Sle1.Sle3* mice, when they were disease free (22), with enzastaurin over an 8-week period. Compared with vehicle-treated control mice, *Sle1.Sle3* mice treated with enzastaurin showed a 43% decrease in spleen size (the average spleen weights of *Sle1.Sle3* mice with and without treatment were 159 mg and 279 mg, respectively; $n = 7$), and there was a significant reduction in the frequency of activated CD4⁺T cells (an average of 25.3×10^6 CD69⁺CD4⁺ T cells/spleen in control mice compared with an average of 15.2×10^6 CD69⁺CD4⁺ T cells/spleen in enzastaurin-treated mice). In addition, the mice treated with enzastaurin displayed remarkable decreases in the serum levels of anti-dsDNA and antihistone/anti-dsDNA autoantibodies (Figure 6C). More significantly, the severity of renal disease in enzastaurin-treated mice was diminished, as reflected by decreased GN (Figure 6D) and proteinuria (100% of the treated *Sle1.Sle3* mice remained free of proteinuria [<30 mg/dl; $n = 7$]). In contrast, 5 of 7 untreated mice had urea protein levels of >100 mg/dl. Taken together, these results showed that enzastaurin treatment prevented lupus development in mice, suggesting the possibility of targeting PKC β as a therapeutic strategy for lupus.

DISCUSSION

In the present study, we demonstrate that PKC β is required for disease development in the Sle mouse model of lupus. Remarkably, PKC β deficiency reversed all tested lupus phenotypes including spontaneous formation of GCs, production of autoantibodies, splenic cellular abnormalities, activation of B cells and T cells, and expansion of CD4:CD8 T cell ratios. Of critical importance, PKC β deficiency dramatically alleviated clinical and histologic manifestations of GN, one of the main organ targets of SLE and the major determinant of disease outcome in human SLE. Also of note, PKC β deficiency induced a preferential reduction in the number of SLE-related anti-dsDNA and antihistone antibodies and anti-dsDNA ASCs relative to total IgG.

Taken together, the observations that GC formation is inhibited in PKC β -deficient Sle mice and that pharmacologic inhibition of PKC β preferentially increased apoptotic death in murine lupus and human autoreactive B cells suggest that PKC β blockade preferentially

targets activated B cells that actively participate in ongoing autoimmune reactions and therefore represents a promising avenue for the therapeutic targeting of B cells in SLE.

Given that PKC δ regulates both the BCR and BAFF signaling pathways, which are able to potentiate each other and are implicated genetically and functionally in the pathogenesis of SLE (1,2,18), the beneficial effects of PKC δ inhibition would include the enforcement of tolerance in developing immature autoreactive B cells, induction of anergy in mature B cells, and suppression of GC formation and development of auto-reactive plasma cells. All of these actions are supported by our data, with the exception of the functionality of early tolerance checkpoints, which were not formally analyzed in this work.

How does PKC δ exert such a great impact on lupus development? One possible explanation is that the absence of PKC δ function may induce B cell anergy, which is a major mechanism of B cell tolerance (39). Similar to the role of PKC δ in T cells, PKC δ has been reported to play a critical role in determining whether B cells are tolerant or activated (36). Consistent with this function, B cells from PKC δ -deficient Sle mice display typical anergic features previously identified in auto-reactive immunoglobulin-transgenic mice, such as a shortened lifespan, lack of proliferative responsiveness to BCR stimulation, failure to up-regulate activation markers, and impaired calcium mobilization after BCR engagement.

Effective antigen stimulation of B cells requires activation of the NF- κ B pathway, which is critical for B cell survival, and PKC δ is required for BCR-mediated NF- κ B signaling in B cells. Consistent with the notion that the NF- κ B pathway is not induced in tolerant cells, we observed that PKC δ deficiency results in the uncoupling of BCR-induced NF- κ B activation in lupus B cells. We observed that Bcl-x_L, a downstream target of NF- κ B, was not up-regulated in PKC δ -deficient B cells from Sle mice. The inability to up-regulate antiapoptotic genes upon BCR stimulation is likely to be responsible, at least in part, for the shortened lifespan of PKC δ -deficient B cells and may contribute to the reversal of lupus features observed in PKC δ -deficient Sle mice.

PKC δ deficiency may also contribute to amelioration of T cell-mediated autoimmunity by compromising the antigen presentation and costimulatory functions of B cells (1,40,41). Indeed, we observed that PKC δ -deficient B cells were not able to efficiently up-regulate the critical T cell-costimulatory molecules CD80 and CD86 in response to BCR stimulation (data not shown). Consistent with these results, PKC δ -deficient mice displayed an impaired response to T cell-dependent antigens (trinitrophenyl [TNP]-ovalbumin), because the serum level of IgG anti-TNP in PKC δ -deficient Sle mice was only ~30% of the IgG level in lupus mice 4 weeks after immunization (data not shown). The relevance of these findings for lupus is also highlighted by the observations that human lupus B cells display marked up-regulation of CD86 expression, and that anti-CD80 antibody treatment induces a dramatic decrease in anti-dsDNA production and reduced kidney pathology in mice with lupus (42).

One striking phenotype of PKC δ -deficient Sle mice is a dramatic decrease in the production of peritoneal B-1 cells (Figure 3), similar to what was previously observed for PKC δ -knockout mice. Although the role of B-1 cells in lupus is controversial, multiple studies indicate that B-1a cells may be involved in the development of lupus through the production of autoantibodies (33). Additionally, accumulations of large numbers of B-1 cells in peritoneal and, to a lesser extent, spleen cells have been shown to be associated with mouse models of lupus, including Sle mice (34,43). Deletion of B-1 cells by hypotonic shock reduced disease severity in (NZB \times NZW)F1 mice with lupus (44). B-1 cells could also contribute to lupus manifestations in the following ways: autoantibody production, interleukin-10-mediated B cell proliferation (45), and enhanced antigen-presenting capabilities (46).

In addition to participating in BCR-mediated NF- κ B activation, PKC δ has a crucial role in BAFF-mediated Akt activation, by phosphorylating Akt at Ser⁴⁷³ (19). Because it is well documented that the bioenergetic state of T cells correlates directly with their survival (47), and because BAFF controls B cell survival by regulating B cell metabolic fitness (19), we speculate that PKC δ deficiency would impair the fitness of lupus B cells, resulting in poor survival of these cells. Moreover, BAFF is critical for the survival of autoreactive B cells, and excess BAFF induces clinical autoimmunity (48). Therefore, future studies will be aimed at understanding both the effects of BAFF stimulation in PKC δ -deficient B cells and the potential clinical benefit of PKC δ deficiency in BAFF-mediated autoimmunity. Our initial studies have already indicated that, consistent with a previous report (19), anti-IgM stimulation of B cells does not have any effect on Akt phosphorylation (data not shown) irrespective of PKC δ status, suggesting that PKC δ -mediated Akt Ser⁴⁷³ phosphorylation is BAFF specific. Further analysis of the role of BAFF in PKC δ -deficient lupus B cells is under way.

In addition to B cells, other cells, such as T cells and macrophages, play important roles in autoimmune diseases. Deletion of PKC δ in those non-B cells may also contribute to the amelioration of lupus in PKC δ -deficient Sle mice.

In summary, our results illustrate that PKC δ plays a critical role in disease development in a mouse model of SLE, and that PKC δ deficiency induces striking clinical, serologic, and histologic improvement. Furthermore, we demonstrate that inhibition of PKC δ with the pharmacologic inhibitor enzastaurin can preferentially impair the survival of human autoreactive B cells (9G4 positive). Although our experiments were performed only in an Sle mouse model, and exploration with other mouse models of lupus remains to be carried out, the collective evidence presented in this report strongly supports the notion that targeting PKC δ may represent a promising treatment of human SLE. PKC δ inhibitors for treatment of SLE may be used either as single agents or in combination with the recently approved anti-BAFF inhibitors (49,50) or other small-molecule drugs such as a Syk inhibitor.

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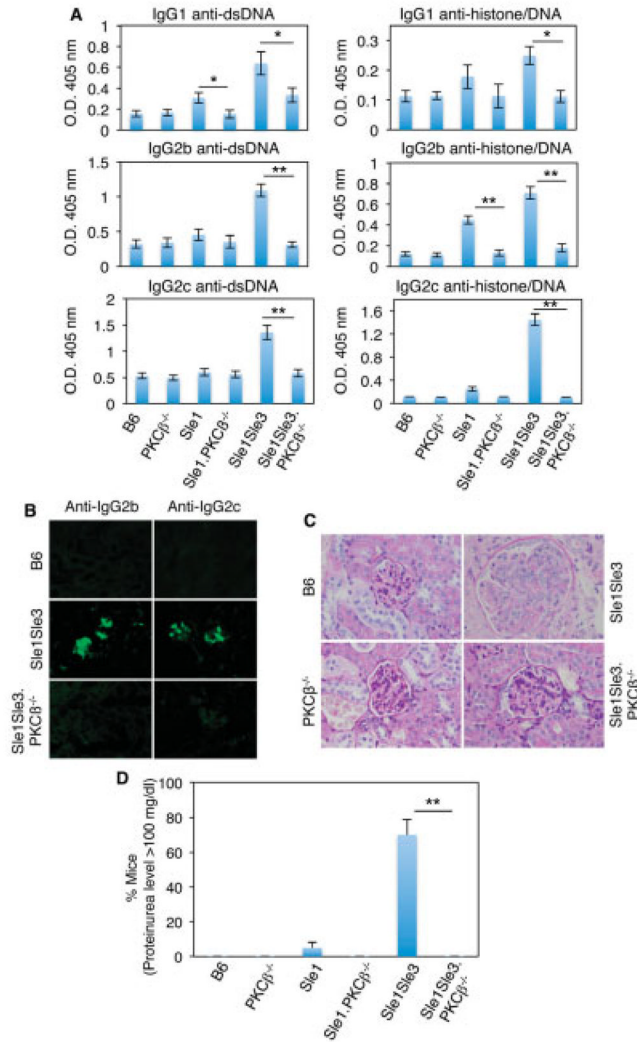


Figure 1.

Protein kinase C (PKC) deficiency prevents lupus development in Sle mice. **A**, Levels of serum IgG anti-double-stranded DNA (anti-dsDNA) and antihistone/anti-dsDNA autoantibodies from the indicated mouse strains (8–10 months old), as determined by enzyme-linked immunosorbent assay. For analysis of anti-dsDNA IgG and antihistone/anti-dsDNA, sera were diluted 100-fold and 200-fold, respectively. Values are the mean ± SD of 3 mice per group. **B**, Frozen kidney sections from 5-month-old mice, stained with fluorescein isothiocyanate-labeled anti-IgG2b or anti-IgG2c. **C**, Paraffin-embedded kidney sections from the same mice as in **B**, stained with periodic acid-Schiff reagent. Enlarged glomeruli that are typical of *Sle1.Sle3* mice were not observed in PKC⁻ deficient Sle mice. Images in **B** and **C** are representative of 2 independent experiments. **D**, Proteinuria levels, as measured with Uristix strips. Values are the mean ± SD of 8–10 mice per strain. * = $P < 0.05$; ** = $P < 0.01$. In **B** and **C**, original magnification × 20 and × 40, respectively.

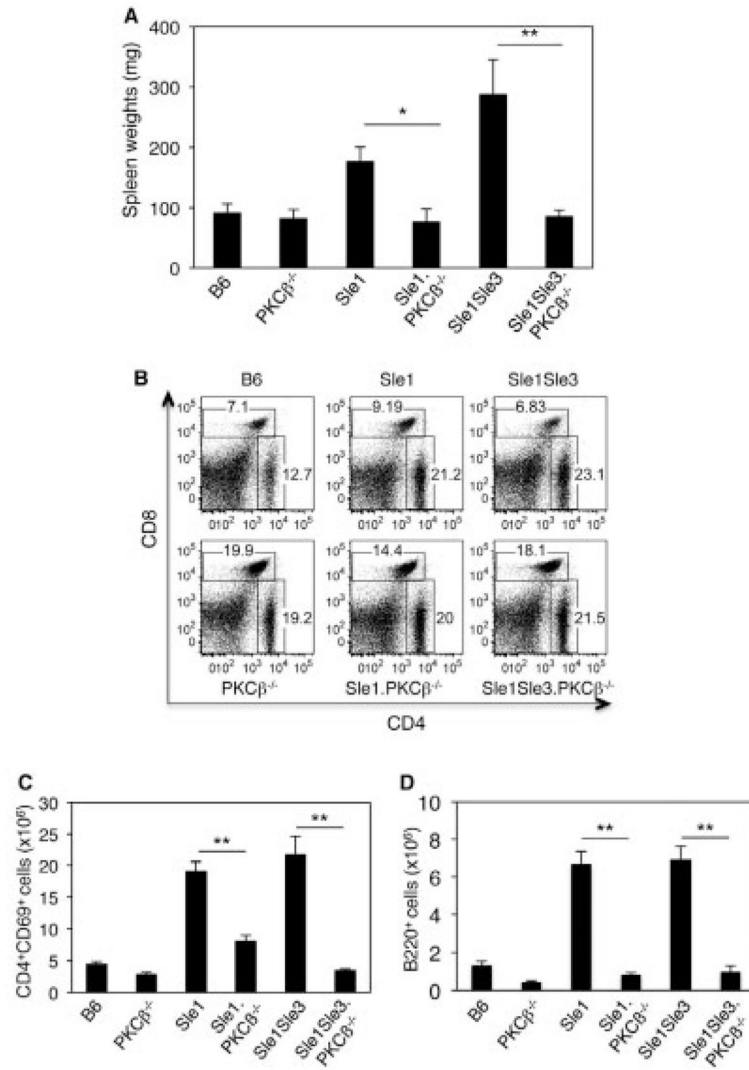


Figure 2. Protein kinase C (PKC) deficiency reverses splenomegaly and restores the CD4:CD8 T cell ratio in Sle mice. **A**, Spleen weights in the indicated mouse strains at age 8 months. **B**, Representative results of flow cytometric analysis of spleen T cells from 5–6-month-old mice. Cells were stained with CD4 and CD8 antibodies. **C** and **D**, Absolute numbers of activated CD69⁺ cells in the CD4⁺ T cell population (**C**) and in the B220⁺ cell population (**D**). Results shown in **B**, **C**, and **D** were obtained from the same group of mice. Bars in **A**, **C**, and **D** show the mean \pm SD of 5 mice per group (**A**) and 3 mice per group (**C** and **D**). * = $P < 0.05$; ** = $P < 0.01$.

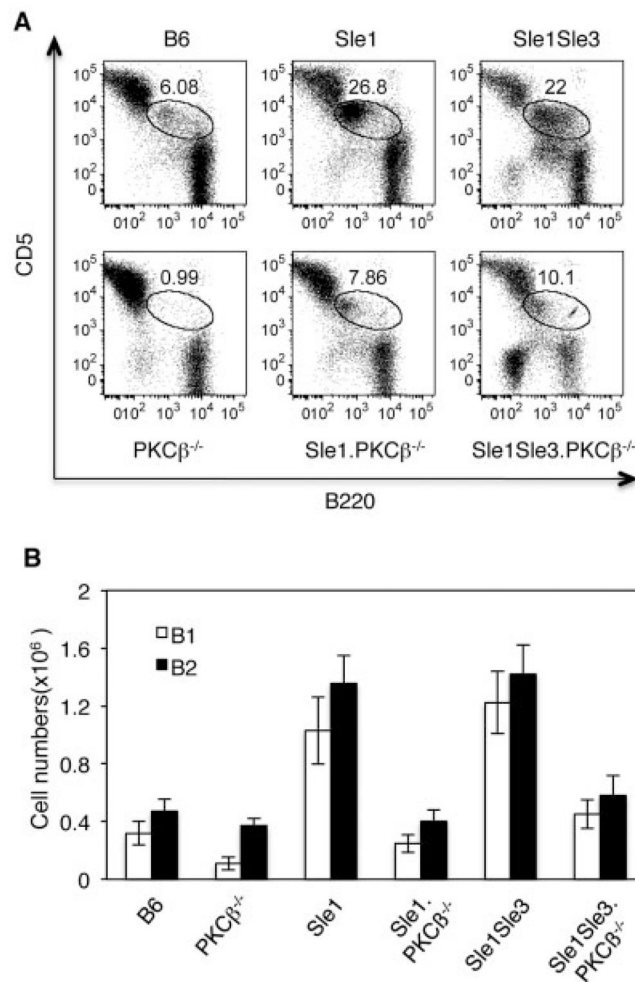
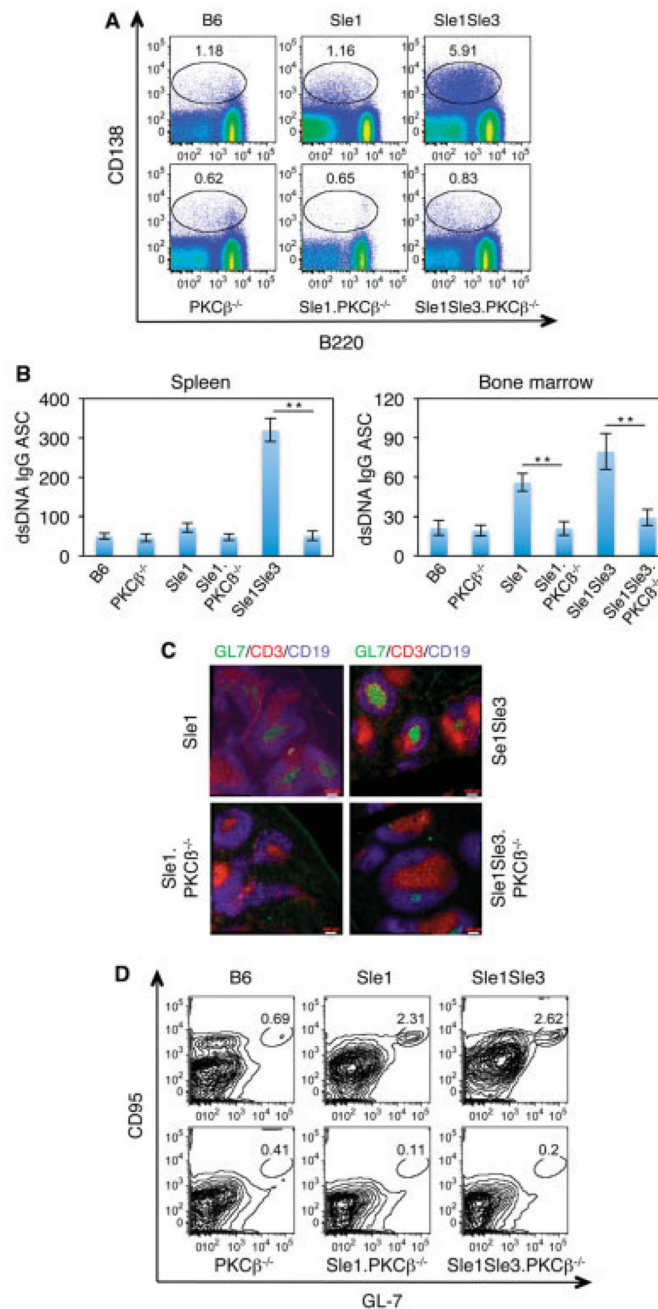


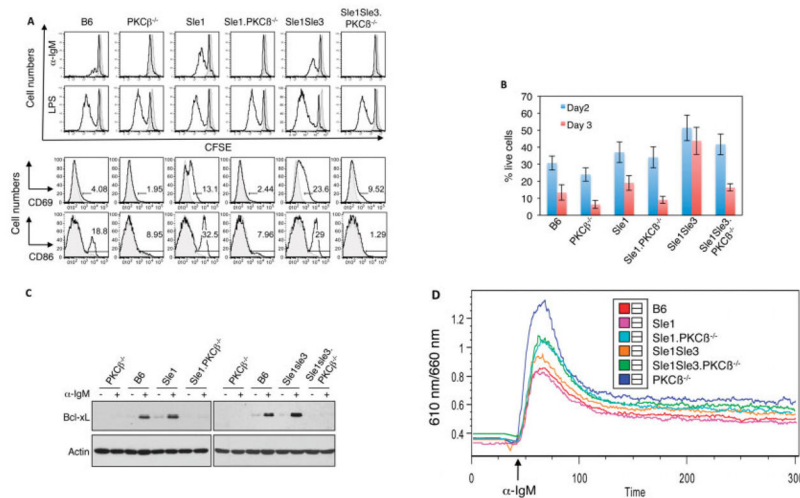
Figure 3.

Protein kinase C (PKC) deficiency decreases the number of peritoneal B-1 cells in Sle mice. **A**, Flow cytometric analysis of peritoneal B-1 cells from the indicated mouse strains (5–6 months old). The cells were stained with B220 and CD5 antibodies. The encircled areas show B-1a cells. Results are representative of 3 independent experiments. **B**, Absolute numbers of B-1a (CD5+B220^{low}) and B-2 (B220+CD23+) cells in the peritonea of the indicated mouse strains. Bars show the mean \pm SD of 3 independent experiments.

**Figure 4.**

PKC^{-/-} deficiency results in a reduction in plasma cell numbers and abrogates spontaneous germinal cell (GC) formation in Sle mice. **A**, Flow cytometric analysis of splenocytes from 5–6-month-old PKC^{-/-} deficient Sle mice. Cells were stained with the indicated antibodies. The encircled areas show spleen plasmablasts (CD138^{high}B220⁺) and plasma cells (CD138^{high}B220^{low/negative}). Results are representative of 3 independent experiments. **B**, Expression of IgG anti-dsDNA plasma cells (antibody-secreting cells [ASCs]) in 1 × 10⁶ cells from spleen or bone marrow, as determined by enzyme-linked immunospot assay. Bars show the mean ± SD. ** = *P* < 0.01. **C**, Representative immunofluorescence images of spontaneous GC (CD19⁺GL7⁺) formation in spleen sections. Spleen sections from the

indicated mouse strains were stained with GL7 (green), CD3 (red), and CD19 (blue). Original magnification $\times 20$. **D**, Representative results of flow cytometric analysis of GC cells (CD19+CD95+GL7+) in the indicated mouse strains. Results shown in **A**, **B**, and **C** were obtained from the same group of mice. See Figure 1 for other definitions.

**Figure 5.**

Protein kinase C (PKC) deficiency impairs the B cell receptor response and survival of B cells from Sle mice. **A**, In vitro responses of B cells to anti-IgM or lipopolysaccharide (LPS) stimulation. Splenocytes from 6-month-old mice were labeled with 5,6-carboxyfluorescein succinimidyl ester (CFSE) (shaded area) and stimulated with anti-IgM or LPS or were left in the medium without any added stimuli for 48 hours (black lines). Top, PKC deletion reduced the proliferative response of B cells to anti-IgM stimulation but not LPS stimulation. Bottom, PKC deficiency reduced up-regulation of CD69 and CD86 expression, as determined by fluorescence-activated cell sorting analysis. **B**, Effect of PKC deficiency on cell viability. Purified spleen B cells from the indicated mouse strains were incubated in RPMI 1640 medium. Cell viability was assayed by flow cytometry, using an annexin V detection kit. Bars show the mean \pm SD. **C**, Expression of Bcl-x_L in anti-IgM-stimulated splenocytes from the indicated mouse strains, as analyzed by Western blotting. Actin was used as a loading control. **D**, Effect of PKC deletion on calcium signaling in B cells from Sle mice. Splenocytes from the indicated mouse strains were loaded with 1 μ M Fura Red to evaluate their ability to flux Ca²⁺ in response to anti-IgM. B220⁺ cells were gated for the analysis. All data shown are representative of at least 2 independent experiments.

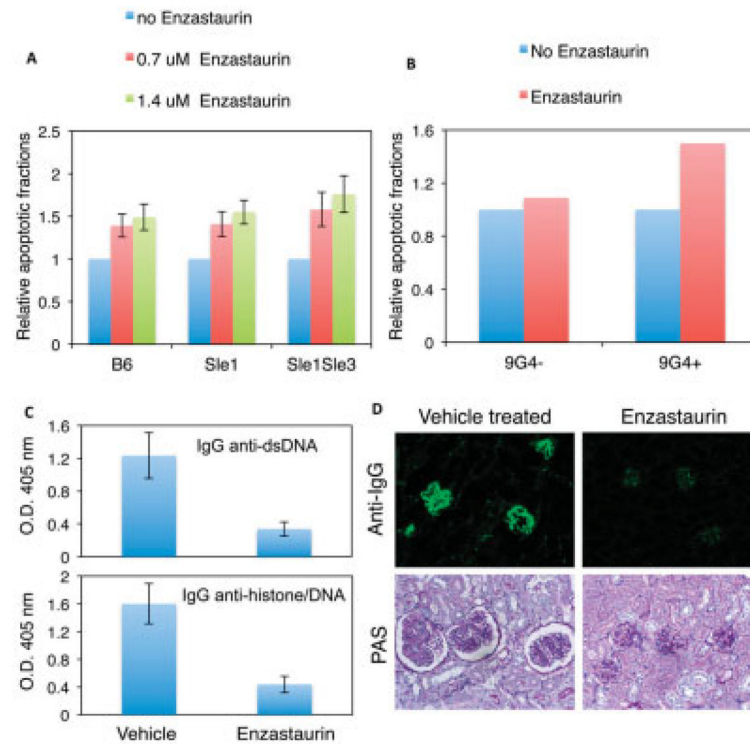


Figure 6.

The protein kinase C (PKC)-specific inhibitor enzastaurin induces apoptosis of lupus B cells and prevents lupus development in *Sle* mice. **A**, Effect of enzastaurin on apoptosis of lupus B cells. Purified splenic B cells were treated with anti-IgM antibody in the presence or absence (control) of enzastaurin for 48 hours and analyzed with an annexin V detection kit. The fractions of annexin V-positive (apoptotic) cells in the samples treated with only anti-IgM (control) are set at 1. **B**, Sensitivity of human 9G4-positive and 9G4-negative B cells to PKC inhibition. Purified splenic B cells were treated with enzastaurin for 24 hours. The apoptotic fractions from untreated samples are set at 1. Results are representative of 2 independent experiments. **C**, Levels of serum IgG anti-double-stranded DNA (anti-dsDNA) and antihistone/anti-dsDNA autoantibodies from vehicle-treated control mice and enzastaurin-treated mice, as analyzed by enzyme-linked immunosorbent assay. Bars in **A–C** show the mean \pm SD of 3 independent experiments. **D**, Representative immunofluorescent images of IgG deposition (top) and glomeruli (bottom) in kidney sections from *Sle1.Sle3* mice treated with vehicle or enzastaurin. Original magnification $\times 20$ (top); $\times 40$ (bottom). PAS = periodic acid-Schiff.