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# PKK deficiency in B cells prevents lupus development in Sle lupus mice

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## Abstract

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of autoantibodies that can result in damage to multiple organs. It is well documented that B cells play a critical role in the development of the disease. We previously showed that protein kinase C associated kinase (PKK) is required for B1 cell development as well as for the survival of recirculating mature B cells and B- lymphoma cells. Here, we investigated the role of PKK in lupus development in a lupus mouse model. We demonstrate that the conditional deletion of PKK in B cells prevents lupus development in Sle1Sle3 mice. The loss of PKK in Sle mice resulted in the amelioration of multiple classical lupus-associated phenotypes and histologic features of lupus nephritis, including marked reduction in the levels of serum autoantibodies,

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proteinuria, spleen size, peritoneal B-1 cell population and the number of activated CD4 T cells. In addition, the abundance of autoreactive plasma cells normally seen in Sle lupus mice was also significantly decreased in the PKK-deficient Sle mice. Sle B cells deficient in PKK display defective proliferation responses to BCR and LPS stimulation. Consistently, B cell receptor-mediated NF-κB activation, which is required for the survival of activated B cells, was impaired in the PKK-deficient B cells. Taken together, our work uncovers a critical role of PKK in lupus development and suggests that targeting the PKK-mediated pathway may represent a promising therapeutic strategy for lupus treatment.

**Keywords**

Lupus; PKK; mouse model; BCR

## 1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with multiple organ pathologies and, in severe cases, kidney destruction. It has been shown that B cells play a central role in autoimmune diseases through their unique functions including auto-antibody production and antibody-independent functions such as antigen presentation to T cells and cytokine production [1–6]. The deregulation of B cell development pathways, such as alterations in B cell receptor (BCR) signaling, can lead to the development of lupus [7–11]. A crucial signaling event downstream of BCR engagement is the protein kinase C beta (PKCβ)-mediated activation of the transcription factor NF-κB, which up-regulates the expression of a large number of pro-proliferation and survival molecules [12–17].

Several mouse models, including the Sle1Sle3 mouse model, have been extensively used for the study of lupus pathogenesis [18–24]. Sle1Sle3 mice carry the bicongenic Sle1 and Sle3 loci that confer susceptability to lupus development [22]. Notably, Sle1Sle3 mice (referred as Sle mice in this report) can develop full-blown lupus nephritis when they are aged, and multiple proliferative pathways, including the NF-κB pathway in B cells, become activated as lupus evolves in these mice [20, 25]. Using the Sle mouse model, we previously showed that PKCβ is required both for lupus development in Sle mice and for the survival of human autoreactive B cells in culture [26], thus demonstrating the significance of PKCβ-mediated signaling in the pathogenesis of lupus.

The RIPK family member protein kinase C-associated kinase (PKK, also known as RIPK4), which was originally identified through its interaction with mouse protein kinase C (PKC) β, mediates NF-κB activation in both B cells and non-B cells [27–33]. Employing B cell-specific PKK conditional knockout mice, we recently demonstrated that PKK is required for the development of B1 cells and the maintenance of long-lived recirculating mature B cells through, at least in part, the regulation of BCR-mediated NF-κB activation [30]. Consistent with its role in the maintenance of active B cells, PKK was also shown to be required for the proliferation and survival of diffuse large B cell lymphoma cells in our previous studies [34]. Given the interaction of PKK with PKCβ and its function in B cell development, we hypothesized that PKK may also play a role in the development of lupus. In this study, we

investigated the effect of B-cell specific ablation of PKK on lupus development in the Sle1Sle3 lupus mouse model. Our results indicate that PKK is required for lupus development in mice, as PKK deficiency in B cells markedly ameliorated multiple pathological features in Sle mice.

## 2. Materials and Methods

### 2.1. Generation of Sle mice with conditional PKK knockout in B cells

The congenic mouse strain B6.*Sle1.Sle3* [20, 26, 35] and the B-cell specific PKK conditional knockout mice $PKK^{f/f}$CD19-Cre [30] were previously described. These mice were utilized to generate mice that carried the Sle1 and Sle3 loci with PKK deletion in B cells (Sle1.Sle3. $PKK^{f/f}$.CD19-Cre). The presence of the four alleles (*Sle1*, *Sle3*, $PKK^{f/f}$ and CD19-Cre) was confirmed by PCR using the specific primers as previously described [26, 30, 36]. Ablation of the PKK in spleen B cells was confirmed by PCR as previously described [30]. The mice described in this study were all heterozygous for CD19-Cre (CD19-$Cre^{+/-}$) so that one functional CD19 allele, which is required for B cell development, was maintained. For simplicity, the Sle1.Sle3.CD19-$Cre^{+/-}$ mice are referred as Sle mice, and the Sle1.Sle3.CD19-$Cre^{+/-}$.$PKK^{f/f}$ mice are referred as Sle-cKO mice in this report. Mice used in this study were on B6 background, and the mice with different genotypes used in each experiment were age-matched or littermates. Only female mice were used in this study, as severe lupus-associated phenotypes develop predominantly in female Sle mice [22, 37]. All animal experiments were carried out following the protocols approved by the University of Rochester Committee on Animal Resources.

### 2.2. Immunohistochemistry and Immunofluorescence analysis

Paraffin-embedded kidney tissues were sectioned (4 μm) and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff reagent [26]. Immunofluorescence analysis of frozen spleen sections was performed as previously described [26, 38]. IgG deposition was detected with fluorescein isothiocyanate–conjugated goat anti-mouse IgG, IgG2b, or IgG2c (Molecular Probes) as previously described [26, 32].

### 2.3. Assays for autoantibodies and urinary protein concentrations

Serum immunoglobulin concentrations of various isotypes were analyzed *by enzyme-linked immunosorbent assay (ELISA)* [22, 26, 38]. 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 antibodies (SouthernBiotech) and an alkaline phosphatase substrate kit (Bio-Rad). Optical density at 450 nm was read on a microplate reader (BioTek Instruments).

The IgG anti–double-stranded DNA (anti-dsDNA) antibody-secreting cells (ASCs) were assessed by *enzyme-linked immunospot (ELISpot) assay* using MultiScreen filter plates (Millipore) as previously described [26, 39]. Boiled salmon-sperm DNA (ThermoFisher Scientific)was used as the source of dsDNA.

Urinary protein concentrations were assayed using Uristix strips (Siemens Diagnostics).

### 2.4. Flow cytometric analysis

Single-cell suspensions were prepared from the indicated tissues, and subsets of B cells and T cells were identified using a combination of antibodies as previously described [30, 40, 41]. All antibodies used in the experiments described here were from BD Biosciences. Flow cytometry data were collected using FACSCanto II or LSR II instruments (BD Biosciences) at the University of Rochester Flow Cytometry Core Facility and were analyzed using the FlowJo software version 8.5.3 (Tree Star).

### 2.5. In vitro viability and proliferation assays

To analyze B cell viability in vitro, B cells were purified from the spleens using CD43 microbeads (Miltenyi Biotec) as described by the manufacturer. The purified B cells were either left untreated in the medium (RPMI with 10% FBS) or stimulated with 10 μg/ml of anti-IgM $F(ab')_2$ (Jackson ImmunoResearch) or lipopolysaccharide (LPS, 5 μg/ml) for the indicated times. The viability of cells was measured by trypan blue exclusion assay (Invitrogen).

For analysis of apoptosis, splenocytes were stimulated with 10 μg/ml of anti-IgM $F(ab')_2$ (Jackson ImmunoResearch) for 30 hours. The cells were stained with anti-B220 antibody, and the apoptotic B cells ($B220^+$ gated cells) were assayed using the Annexin V apoptosis detection kit (BD Bioscience) as previously described [30, 34].

In vitro proliferation assay was performed as previously described [30, 42, 43]. Briefly, splenocytes ($5 \times 10^6$) were stained with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 2 μM for 10 minutes. The cells were washed and then treated either with medium (RPMI with 10% FBS) alone or medium plus anti-IgM $F(ab')_2$ or LPS for 30 hours. The flow cytometry analysis was carried out on $B220^+$ gated cells [26, 30].

### 2.6. Intracellular calcium measurements

The flux of $Ca^{2+}$ was measured with Fura Red (Catalog# F3021, Invitrogen) according to the manufacturer's instruction [26]. (Splenocytes ($4 \times 10^6$/ml) were loaded with 1 μM Fura Red, followed by staining with phycoerythrin-conjugated anti-B220 antibody. The Fura Red fluorescence ratios in $B220^+$ gated cells were measured using a BD FACSVantage SE system to indicate the changes in intracellular $Ca^{2+}$ levels in B cells. Cells were stimulated with 10 μg/ml anti-IgM ($F(ab')_2$) and basal readings were taken for 45 seconds prior to stimulation. Data were analyzed using FlowJo software (Tree Star) and presented as the emission intensity ratio of 610/660 nm over time. Owing to the properties of Fura Red, a greater increase in $Ca^{2+}$ flux following α-IgM stimulation produces a smaller increase in the emission intensity ratio.

### 2.7. Western blot analysis

B cells were purified from splenocytes using anti-CD19-conjugated magnetic beads (Miltenyl Biotec) and treated with either anti-mouse IgM $F(ab')_2$, μ-chain specific (10 μg/ml), or lipopolysaccharide (5 μg/ml) for the indicated times. The lysates were prepared from the isolated cells for western blot analysis [34, 44]. Antibodies specific for Bcl-xL, Bcl2, IκBα and GAPDH were from Santa Cruz Biotechnology.

To measure activation of NF-κB, the DNA-binding activity of nuclear p65 was analyzed using an enzyme-linked immunoabsorbent assay (ELISA) based method (Active Motif, Carlsbad, CA) as previously described [30, 34].

### 2.9. Statistical analysis

Two-group comparisons were analyzed using the student *t* test, assuming unequal variances between the two samples. A difference was considered significant when the p value was less than 0.05.

## 3. Results

### 3.1. B cell-specific PKK conditional knockout prevents autoantibody production and nephritis progression in Sle mice

To investigate the role of PKK in lupus development, we generated a Sle1Sle3 mouse strain with PKK conditional ablation in B lymphocytes. These mice (referred as Sle-cKO mice in this report) were produced by breeding the well-established congenic Sle (B6.Sle1.Sle3) lupus mice [21, 22] with mice carrying the B cell-specific PKK deletion mediated by CD19-cre (B6.PKK$^{f/f}$CD19-Cre) [30]. We chose the B6.Sle1Sle3 congenic Sle mouse model in our study because this model recapitulates many SLE features and has been extensively explored for the study of the pathogenesis of lupus [20, 45]. Female B6.Sle1Sle3 mice (referred here as Sle mice) develop full-blown lupus nephritis in 8 to 10 months. In addition, these mice display splenomegaly, higher CD4:CD8 T cell ratios, increased activation of B cells and T cells and have high levels of antinuclear, including anti-chromatin and anti-dsDNA, autoantibodies (ANAs) that are often associated with the severity of glomerulonephritis (GN) in lupus mice [21, 46, 47]. Moreover, *Sle1* and *Sle3* loci have been shown to exert a great impact on B cells [22, 25].

While Sle mice had high levels of IgG1, IgG2b and IgG2c anti-dsDNA and anti-histone/anti-DNA antibodies compared with the wild-type B6 mice as previous reported [21, 22, 46, 47], the levels of these antibodies were dramatically reduced in the PKK-deficient Sle-cKO mice (Figure 1A). The levels of IgM anti-dsDNA and IgM antihistone/anti-dsDNA, known to be involved in the development of lupus nephritis [48], were also significantly decreased in Sle-cKO mice (Figure 1A). In addition, total IgM levels in PKK-deficient Sle mice (mean ± SD = 191 μg/ml ± 43.5, n=4) were decreased 3-fold as compared to those (mean ± SD = 615.25 μg/ml ± 105, n = 4) in Sle mice ($p < 0.01$). These observations are consistent with our previous report that PKK deficiency resulted in a significant reduction in the peritoneal B-1 cell population [30] (also see Figure 3 below), which is a major source of serum IgM and IgG3 antibodies.

Besides the dramatic decrease in the levels of serum autoantibodies in PKK-deficient Sle mice, the immune complexes, usually seen in Sle mice, were absent in Sle-cKO mice (Figure 1B). In addition, deletion of PKK in B cells also markedly alleviated histologic features of active glomerulonephritis (GN), such as the glomerular enlargement and marked global mesangial and endocapillary proliferation that were usually observed in Sle mice (Figure 1C). While 75% of Sle lupus mice had high levels (≥100 mg/dl) of urinary proteins

at the age of 10 month, less than 10% (1 out 12) of Sle-cKO mice developed such proteinuria (Figure 1D), and the rest of Sle-cKO mice had the urinary protein concentrations in the normal range (<30 mg/dl) found in wild-type B6 mice. Taken together, our results demonstrate that lack of PKK in B cells abrogates multiple hallmarks associated with lupus in the Sle mouse model.

### 3.2. PKK deletion in B cells averts multiple SLE-associated cellular abnormalities in Sle mice

Splenomegaly is a feature of murine lupus that is associated with disease progression and severity [22]. Sle lupus mice develop considerably larger spleens than wild-type mice. In contrast, Sle-cKO mice display a spleen size comparable to that of the wild-type B6 mice (Figure 2A). Consistently, the number of splenocytes in Sle-cKO mice was greatly decreased as compared with that in Sle lupus mice (Figure 2B). Thus, PKK deletion in B cells also averts splenomegaly in Sle mice.

An increase in the B-1 cell population has been associated with lupus in both human patients and mouse models including Sle1Sle3 mice [49, 50]. One of the most noticeable phenotypes in the CD19Cre-driven PKK conditional knockout mice is the severe reduction in peritoneal B cell population [30]. Notably, the B1-a cell population in PKK-deficient Sle mice was also dramatically reduced when compared with Sle mice (Figure 3).

To examine whether PKK deletion affects other B cell compartments in Sle mice, we carried out a detailed flow cytometric analysis [30, 41, 51] on various B cell populations. While PKK deletion had no effect on early B cell development in bone marrow and spleen B cell subpopulations such as marginal zone (MZ) B cells ($IgM^{high}IgD^{lo}CD21^{hi}CD23^{lo/-}$) and mature follicular B cells ($IgM^{int}IgD^{hi}$) in Sle mice (data not shown), the frequency of $B220^{low/-}CD138^{+}$ plasma cells in the spleen decreased approximately 4-fold in Sle-cKO mice as compared with Sle mice (Figure 4A). The decrease in the frequency of ASCs (antigen-specific antibody-secreting cells) was confirmed by functional ELISpot assays (Figure 4B), which is in agreement with the above ELISA results (Figure 1A) showing the dramatic reduction in the serum levels of IgG anti-dsDNA antibodies in PKK-deficient Sle mice as compared with those in Sle mice.

Germinal center (GC) formation is associated with the overall lupus disease progression, and Sle mice develop GC spontaneously as they age [38, 52, 53]. In contrast to Sle mice, PKK-deficient Sle mice lack spontaneous GC formation (Figure 4C). In addition, the population of GC B cells ($B220^{+}GL7^{+}CD95^{+}$) was markedly reduced in Sle-cKO mice when compared to Sle mice (Figure 4D). Such a decrease in GC B cells may contribute to the reduced de novo production of plasma cells in the GCs.

The expansion of activated $CD4^{+}$ T cells is another characteristic of Sle mice [21, 47]. Interestingly, the deletion of PKK in B cells significantly reduced the population of the activated $CD4^{+}$ T cells (as defined by the high CD69 expression) in Sle mice (Figure 5), while the CD4:CD8 T cell ratio in Sle-cKO mice was similar to that of Sle lupus mice. Thus, lack of PKK reduced the expansion of activated $CD4^{+}$ T cells in Sle mice. Collectively, PKK deficiency in B cells results in the reversal of multiple lupus-associated cellular

abnormalities, including splenomegaly, expansion of B1 cells, spontaneous GC formation and the increase of activated T cells in Sle mice.

### 3.3. Defective response of the PKK-deficient Sle B cells to BCR and LPS stimulation

Elevated BCR signaling, observed in various lupus mouse models as well as in human lupus patients, has been shown to contribute to the development of lupus [7, 54–58]. BCR signaling, as well as toll like receptor 4 (TLR4) signaling, is known to promote B cell proliferation and survival. We previously showed that PKK deletion in B cells leads to defective responses to anti-IgM as well as LPS stimulation [30]. As such defective responses may contribute to the amelioration of the lupus-associated phenotypes in the PKK-deficient Sle mice, we thus examined whether the response of PKK-deficient B cells in Sle mice to these stimuli are also impaired. While the B cells from Sle mice displayed elevated viability compared to the B cells from wild-type B6 mice, PKK deficiency in Sle mice abolished such an increase in viability in response to BCR stimulation (Figure 6A–B). In fact, B cells from Sle-cKO mice exhibited slightly worse viability than that of the B6 control B cells (Figure 6A–B). In addition to affecting the survival of B cells, the lack of PKK in Sle mice led to a dramatic decrease in the proliferation rate of B cells in response to anti-IgM and LPS stimulation (Figure 6C). In accordance with the above observation, the upregulation of B cell activation markers CD69 and CD86 was also attenuated in PKK-deficient Sle mice when compared with Sle lupus mice (Figure 6D).

BCR activation increases intracellular calcium mobilization, which is essential for proper BCR signaling. Given the above-observed effects of PKK deletion on BCR signaling, we thus also investigated the effect of PKK deletion on calcium mobilization in PKK-deficient Sle B cells. Upon BCR stimulation, the PKK-deficient B cells had a much smaller increase in $Ca^{2+}$ flux than the B cells with wild-type PKK in Sle mice (Figure 6E), lending further support for the notion that PKK is required for BCR signaling in lupus B cells. Together, the above observations indicate that B cells from PKK-deficient Sle mice display defective responses to proliferation and survival stimuli, which may ultimately lead to the prevention of lupus development in Sle mice.

### 3.4. Impaired BCR-and LPS-mediated NF-κB signaling in Sle B cells

Both BCR and TLR4-mediated NF-κB signaling play critical roles in B cell survival and proliferation. We and others have previously shown that PKK positively regulates NF-κB signaling in mammalian cells including both normal and malignant B cells [30, 31, 34]. Our finding that the lack of PKK in Sle B cells leads to defective cell survival in response to anti-IgM and LPS stimulation raises the possibility that PKK deficiency may attenuate NF-κB signaling in Sle B cells. Thus, we examined NF-κB activation in B cells induced by anti-IgM and LPS. As shown in Figure 7A, NF-κB activation induced by anti-IgM and LPS stimulation was attenuated in PKK-deficient Sle B cells, as indicated by the persistence of protein IκBα, a NF-κB inhibitor. In addition, decreased nuclear activity of the NF-κB protein p65 (RelA) was observed in Sle B cells lacking PKK (Figure 7B). Thus, our results indicate that PKK is required for the activation of NF-κB induced by BCR-and TLR in the B cells of Sle mice.

The pro-survival BCL2 family proteins, which are downstream targets of transcriptional factor NF-κB, are critical for B cell survival following BCR stimulation [12, 14, 59–61]. Consistent with the impaired NF-κB activation in PKK-deficient Sle mice, the expression of Bcl-$x_L$ and Bcl-2 proteins in response to anti-IgM stimulation and LPS was also impaired in PKK-deficient Sle B cells (Figure 7C). Thus, the impaired survival of Sle-cKO B cells may result from the failure of NF-κB-mediated up-regulation of survival factors.

## 4. Discussion

In this study, we demonstrate that PKK is required for lupus development in the Sle lupus mouse model. Ablation of PKK in Sle mouse B cells induced a dramatic reduction in SLE-related anti-DNA and anti-histone antibodies as well as anti-dsDNA ASCs. Remarkably, PKK deficiency greatly alleviated clinical and histologic manifestations of GN, which is one of major organ targets of SLE and determinants of lupus outcome. In addition, lack of PKK in B cells reversed many other lupus phenotypes such as splenomegaly, spontaneous formation of GCs, and activation of T cells. Thus, our studies uncover PKK as a potential critical player in lupus pathogenesis.

The effect of the B cell-specific PKK deficiency in preventing lupus development in Sle mice may be attributed largely to the critical role of PKK in promoting the survival of activated B cells. Our previous work demonstrated that PKK deficiency in B cells results in a defective proliferative response of mature B cells to BCR and TLR4 stimulation [30]. Consistently, PKK deficiency in Sle B cells also leads to an impaired proliferative response to BCR stimulation (Figure 6A–B). Thus, the reduction of GC B cells and plasma cells, as well as diminished de novo production of autoantibodies, may result from the defective proliferation and survival response of the mature B cells in the PKK-deficient Sle mice.

BCR signaling plays a critical role in the survival of B cells including autoreactive B cells [62–65]. Effective BCR signaling requires proper activation of NF-κB, which up-regulates the expression of the anti-apoptotic Bcl-2 family members [66, 67]. While NF-κB activation is essential for the survival of activated B cells [68, 69], exaggerated NF-κB activation and prolonged B cell survival may cause deleterious autoimmune responses [70]. Dysregulated apoptotic and anti-apoptotic genes increase B-cell lifespans and thereby promote survival of self-reactive B-cells, thus leading to autoantibodies and multiple autoimmune diseases [71, 72]. Transgenic mouse experiments showed that overexpression of Bcl-2 or Bcl-xL can allow inappropriate survival of autoreactive B cell clones, selectively alter a negative selection process, and promote survival, maintenance and differentiation of autoreactive B cells [70, 73, 74]. Similar to our previous observations in the PKK-knockout B cells [30], B cells from the PKK-deficient Sle mice display reduced NF-κB activation and expression of Bcl2 and Bcl-xL (Figure 7A–C) in response to BCR stimulation. Failure to up-regulate anti-apoptotic protein expression upon BCR stimulation, due to defective NF-κB activation in the absence of PKK, may thus likely be responsible for the impaired survival of activated B cells in PKK-deficient Sle mice, which in turn may ultimately lead to the reversal of many lupus features observed in PKK-deficient Sle mice. Consistent with this view, it was recently reported that the loss of NF-κB family members resulted in the amelioration of a SLE-like autoimmune disease in a lupus mouse model [75].

It is of interest to note that PKK deficiency reversed the large increase of Fas (CD95)-expressing B cells in Sle mice (Figure 4D), suggesting that PKK may play a role in the maintenance of B cells with high active Fas signaling. Additional pathways, such as TLR7 and TLR9 mediated signaling, have also been implicated in lupus development [76]. We observed no significant effect of PKK deficiency on the viability of B cells in response to the treatment by either ssRNA (which acts through TLR7) or CpG-containing DNA (which acts through TLR9) in our previous studies. It remains, however, to be determined in future experiments whether PKK affects TLR7 and TLR9 signaling in Sle B cells and whether such signaling plays a role in lupus development in Sle mice.

PKK deficiency in Sle mice results in marked reduction in peritoneal B-1a cells (Figure 3), similar to what we previously observed in the B-cell specific PKK knockout mice [30]. Although the role of B-1 cells in lupus development remains to be clarified, the accumulation of a large number of B-1 cells in peritoneal and, to a lesser extent in spleen cells has been observed in certain mouse lupus models. In addition, numerous studies have indicated that B-1 cells contribute to lupus manifestation through the production of autoantibodies, IL10-mediated B cell proliferation [77], and enhanced antigen-presenting capability [78]. This is in line with our results that PKK-deficient Sle mice have much lower anti-dsDNA IgM antibodies, which are known to be involved in the development of lupus nephritis. Although expansion of spleen B-1a cells has been observed in certain mouse lupus models, the spleen B-1a population was not significantly expended in the Sle model used in this study, suggesting that spleen B-1a cells may not contribute significantly to the lupus development in this Sle mouse model [22].

Abnormal B cell activation contributes to SLE pathogenesis through both antibody-dependent and independent fashions [1, 54, 79–81]. In addition to the impaired B cell survival, B cell-specific deletion of PKK also resulted in significant reduction of activated $CD4^+$ T cells in Sle mice (Figure 5), and PKK-deficient Sle B cells fail to efficiently up-regulate the critical T cell-costimulatory molecules CD80 and CD86 in response to BCR stimulation (Figure 6C). These observations raise the possibility that PKK deletion in B cells may also lead to reduced T cell-mediated autoimmunity, by compromising the antigen presentation and costimulatory function of B cells. Consistently, PKK-deficient Sle mice exhibited an impaired response to T-cell-dependent antigens (Trinitrophenyl (TNP)-ovalbumin), as the serum IgG anti-TNP level was reduced about 40% in PKK-deficient mice as compared with Sle mice 4 weeks after immunization (data not shown). It is interesting to note that human lupus B cells display a marked up-regulation of CD86 expression, and anti-CD80 antibody treatment induces a dramatic decrease in anti-dsDNA production and reduces kidney pathology in lupus mice [82].

PKK was initially identified through its interaction with PKCβ [27], and the human PKK ortholog (DIK) was shown to interact with PKCδ [28]. Similar to PKCβ, PKK mediates BCR-induced NF-κB activation in lymphoma and primary B cells, suggesting that PKK and PKCβ may function in common pathways [30, 34]. Notably, we previously showed that knockout of PKCβ or pharmacological inhibition of PKCβ activity with PKCβ-specific inhibitor Enzastaurin completely abrogated lupus-associated phenotypes in Sle mice [26]. Although the molecular mechanism underlying NF-κB activation mediated by PKK and the

significance of the interaction between PKK and PKCβ remain to be determined, PKK knockout in Sle B cells exhibits similar phenotypes as PKCβ knockout in Sle mice in the prevention of lupus development. Future studies will be needed to address the molecular mechanisms of PKK mediated lupus B cell survival and its relevance to human lupus B.

In summary, our results demonstrate that PKK plays a critical role in lupus development in a mouse model of SLE. PKK-deficiency in Sle B cells leads to a striking improvement of clinical, serological and histological lupus phenotypes, supporting the view that B cells play an important role in lupus development. Our study suggests that targeting PKK-mediated function in B cells may represent a promising strategy for the treatment of lupus.

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## Highlights

- Lupus development in the Sle mouse model is blocked by B-cell specific PKK ablation.
- Multiple lupus-associated phenotypes are eliminated in PKK conditional knockout mice.
- Elevated BCR signaling seen in lupus mice is abrogated by PKK deficiency.

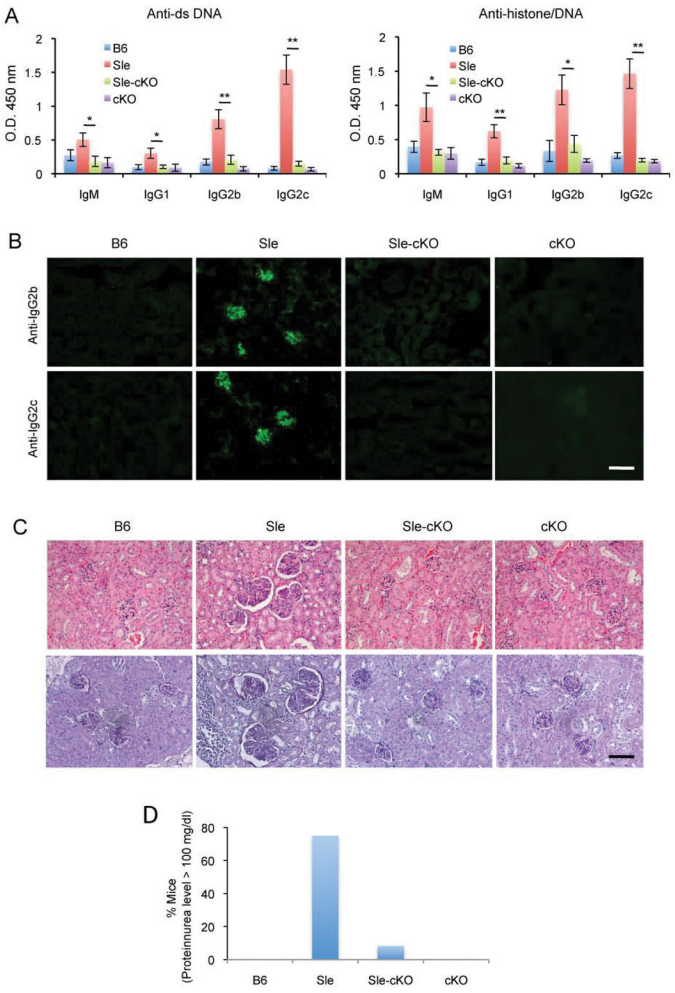


**Figure 1. B cell-specific PKK conditional knockout prevents autoantibody production and nephritis progression in Sle mice**

**A**. Levels of serum IgM and IgG anti-double-stranded DNA (anti-dsDNA) and anti-histone/anti-dsDNA autoantibodies from the indicated mouse strains at 10 months old. Antibody levels were measured by ELISA. Values are the mean ± SD (standard deviation) of 4 mice per group. *$p < 0.05$, and **$p < 0.01$. **B**. Snap-frozen kidney sections from the indicated 10-month-old mice, stained with fluorescein (FITC)–labeled anti-mouse IgG2b or anti-IgG2c antibodies. **C.** Paraffin-embedded kidney sections from the indicated 10-month-old mice, stained with H&E (top panels) and periodic acid–Schiff reagent (lower panels). Enlarged glomeruli, typical for *Sle1.Sle3* mice, were not observed in PKK-deficient Sle mice. Representative images from 2 independent experiments are shown in (B) and (C). The scale bar represents 100 μm. **D.** Proteinuria levels of the indicated mice (10 months old, n = 12 in each group).

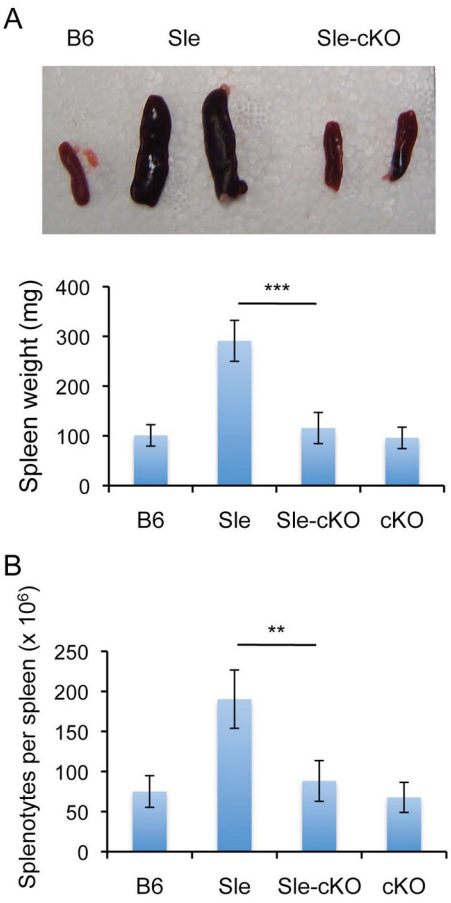


**Figure 2. PKK deficiency reverses splenomegaly**

**A.** Top panel: representative images of the spleens from the indicated 10-month old mice. Bottom panel: the average spleen weight of the indicated mice. Shown are mean ± SD (n = 4 in each group. ***$p < 0.001$). **B.** The absolute number of splenocytes per spleen in the indicated mice (10 months old, n = 4 in each group, **$p < 0.01$).

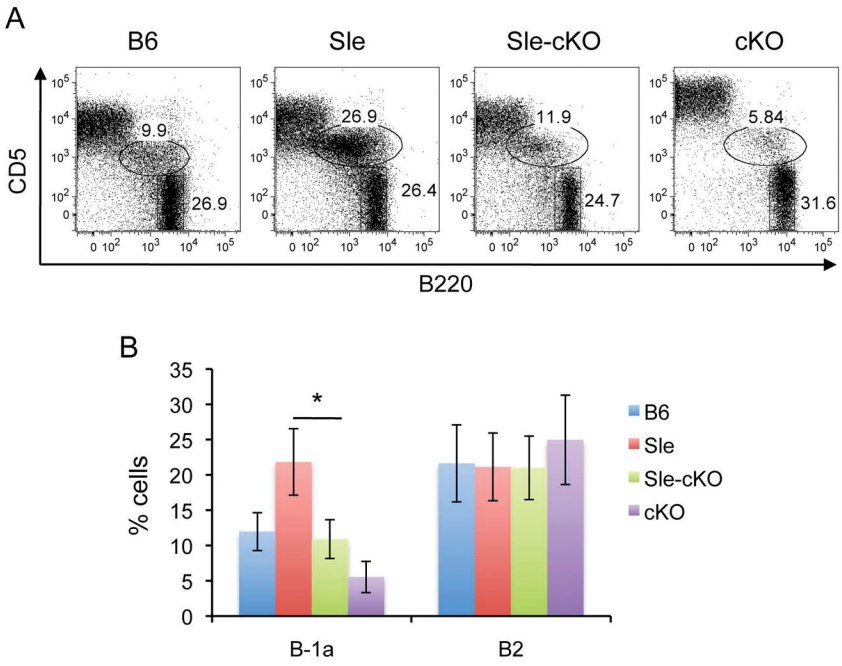


**Figure 3. PKK deficiency decreases the number of peritoneal B-1a cells in Sle mice**

**A.** Flow cytometric analysis of peritoneal B-1a cells from indicated mice (10 months old ). The cells were stained with B220 and CD5 antibodies and analyzed by flow cytometry. The peritoneal B-1a (B200+CD5+) cells are marked with ovals, and B2 cells are marked with rectangles. Shown are representative results from three independent experiments. **B**. The percentage of of B-1a ($CD5^{+}B220^{low}$) and B-2 ($B220^{+}CD23^{+}$) cells in the peritoneum of the indicated mice. The mean ± SD of three independent experiments (n =3 in each group)are depicted.

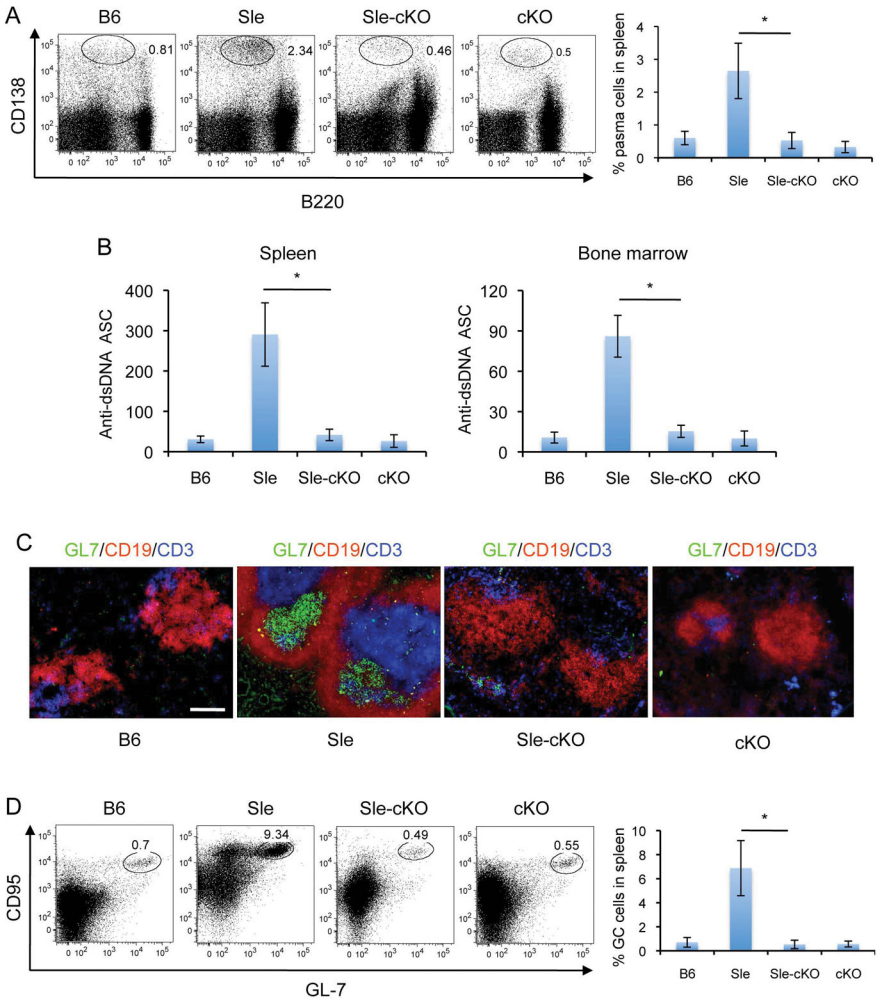


**Figure 4. PKK deficiency results in reductions in plasma cells and germinal cells as well as IgG anti-dsDNA plasma cells in spleen and bone marrow**

**A**. Flow cytometric analysis of the spenocytes stained with anti-B220 and anti-CD138 antibodies. The circled areas show the persentage of spleen plasma cells ($CD138^{hgh}B220^{low/negative}$) from the indicated mice (Left panels). The percentage of plasma cells from the indicated mice are shown on the right (n = 3 mice for each group, *p < 0.05). **B**. The number of IgG anti-dsDNA plasma cells (antibody-secreting cells, ASC) from spleen (left panel) or bone marrow (right panel), measured by *ELISpot* with $1\times10^6$ cells. Shown are the mean ± SD (n = 4 mice for each group, *$P$ < 0.05). **C.** Representative immunofluorescence images of spontaneous GC (CD19+GL7+) formation in spleen sections of the indicated mice. Spleen sections were stained with antibodies against GL7 (green), CD3 (blue), and CD19 (red). The scale bar represents 50 μm. **D**. Representative flow cytometric analysis of spleen germinal center (GC) cells stained with antibodies specific for B220, CD95 and GL-7. Germinal center B cells (B220+CD95+GL-7+) were indicated (left panels). Shown in the right panel is the percentage of GC cells of the indicated mice (mean ± SD, n = 3, *p < 0.05). All mice used in the experiments described in this figure were 10 months old.

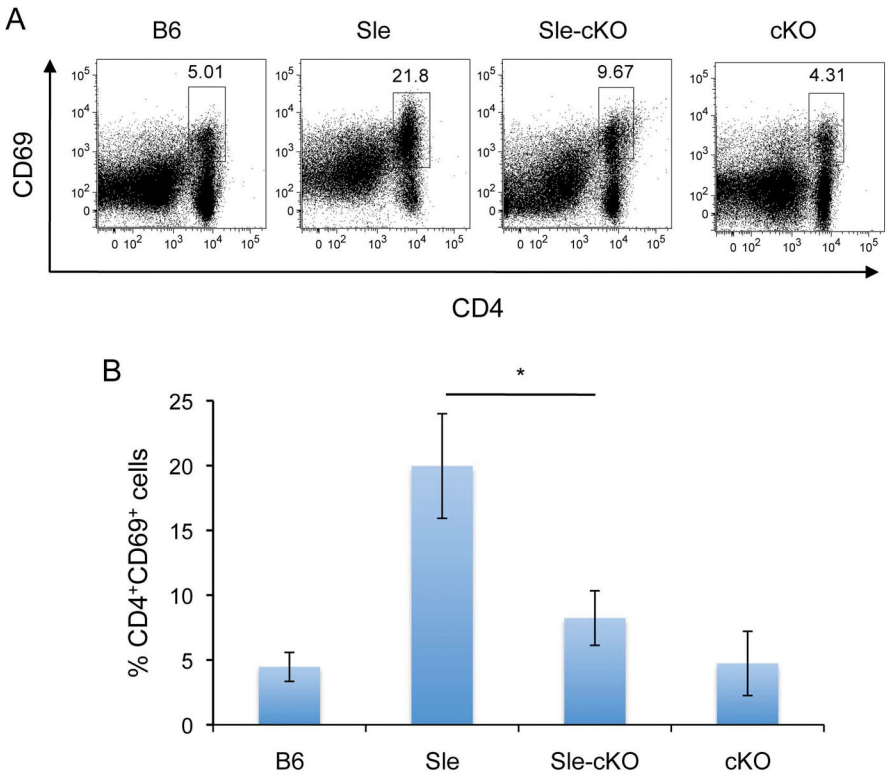


**Figure 5. PKK ablation in B cells leads to a decrease in activated CD4 T cells in Sle mice**

**A.** Flow cytometric analysis of spleen T cells from 10 month-old mice. Cells from the spleens of the indicated mice were stained with anti-CD4 and anti-CD69 antibodies and analyzed by flow cytometry. The fractions of activated T cells ($CD4^+CD69^+$) in spleen are indicated by the boxes. **B.** Percentage of the $CD69^+CD4^+$ T cells in the spleens of the indicated mice (10 months old). Shown are the mean ± SD (n = 4 in each group, *p < 0.05).

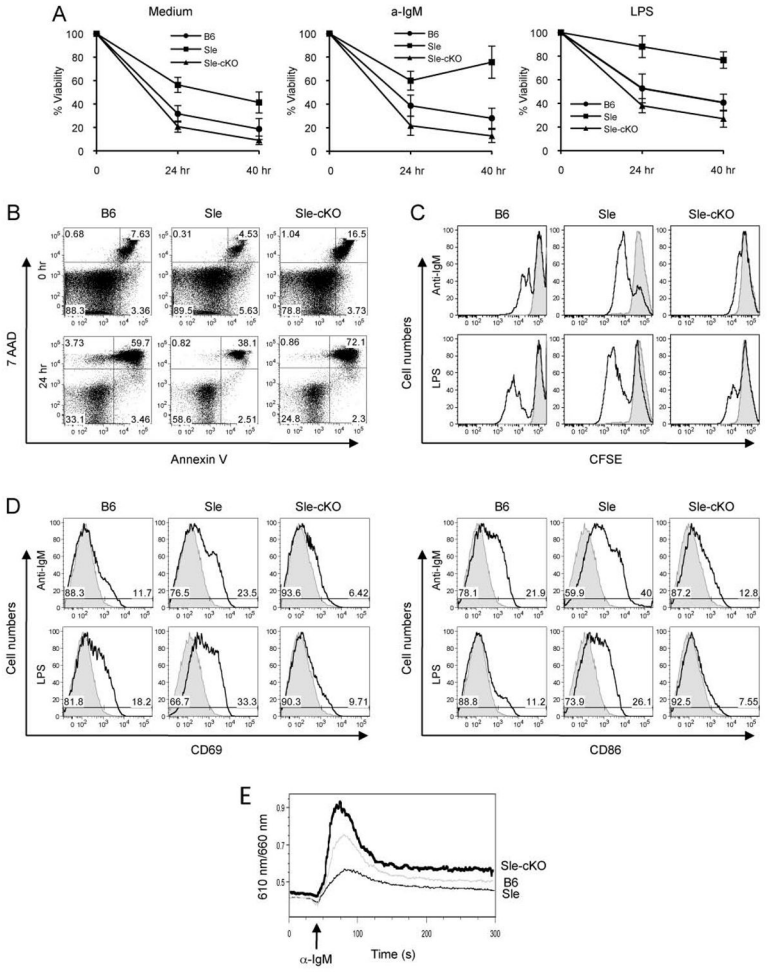


**Figure 6. Impaired proliferation and survival of PKK-deficient Sle B cells**

**A.** Decreased viability of the B cells from the PKK-deficient Sle mice in response to anti-IgM or lipopolysaccharide (LPS) stimulation. Purified spleen B cells were treated as indicated. The viable cells were determined by trypan blue exclusion assay and are depicted as the percentage of the initial input. All experiments were performed in triplicate. **B.** Increased apoptosis of PKK-deficient Sle B cells in response to anti-IgM treatment. Splenocytes isolated from each mouse strain were treated with anti-IgM antibody for 30 hours. Cells were stained with Annexin V and 7AAD, and flow cytometric analysis was performed on gated lymphoid and $B220^{+}$ cells. Shown are representative flow cytometry profiles from two independent experiments. **C.** Defective proliferation of PKK-deficient Sle B cells in response to anti-IgM and LPS stimulation. Splenocytes were labeled with CFSE and were either left in the medium without any added stimuli (shaded area) or stimulated with F(ab′) 2 anti-IgM or LPS for 30 hours (black line). Analysis was carried out on B220 gated cells. **D**. Flow cytometric analysis of CD69 (left panels) and CD86 (right panels) expression in B220 gated B cells after anti-IgM and LPS stimulation. Overlays of CD histograms (black line) on unstained controls (shaded) are shown. **E.** Decreased calcium signaling in PKK-deficient Sle B cells. Splenocytes from indicated mice strains were loaded with 1 μM Fura Red to evaluate $Ca^{2+}$ flux in response to anti-IgM stimulation. $B220^{+}$ cells

were gated for the analysis. Data in panels B–E are representative of two independent experiments. 8-month-old mice were used in the experiments described in this figure.

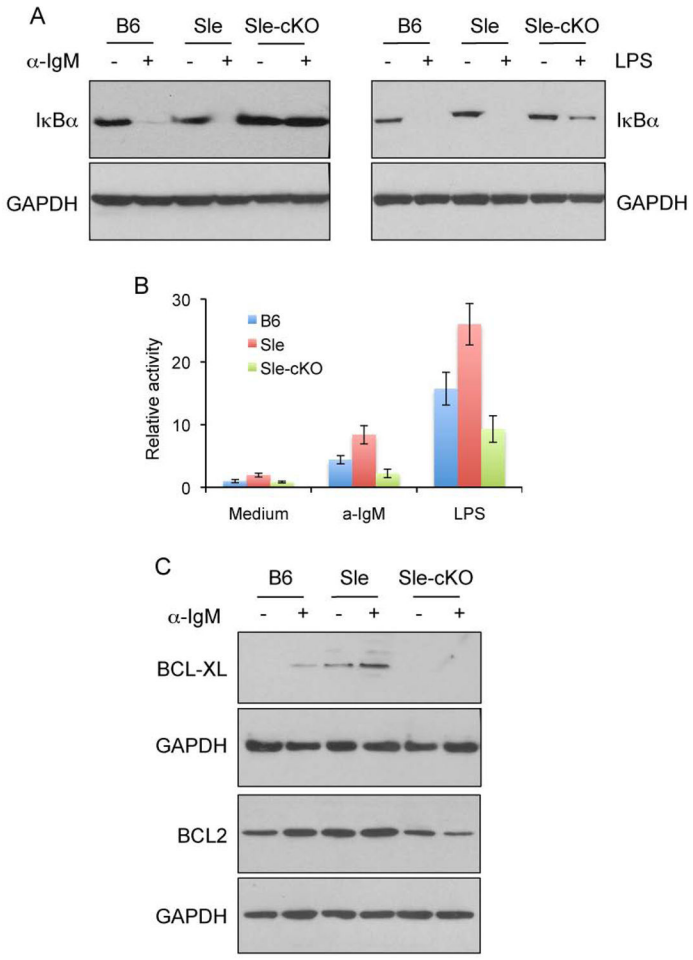


**Figure 7. Impaired NF-κB activation in PKK-deficient B cells**

**A.** Purified splenic B cells were stimulated with either anti-IgM or LPS for 1 hour. The levels of the IκBα protein in the cell lysates were analyzed by western blotting. GAPDH was used as a loading control. Shown are representative results from two independent experiments. **B.** Effect of PKK deficiency on NF-κB/p65 activation in B cells stimulated with anti-IgM or LPS. The nuclear NF-κB/p65 in the purified B cells of the indicated mice following stimulation was analyzed by an ELISA-based DNA binding assay (Active Motif) [30, 34]. Shown are relative NF-κB activation levels. The DNA binding activity from the unstimulated cells was set as 1. All tests were carried out in triplicate. **C.** Reduced expression of Bcl-xL and Bcl-2 in PKK-deficient B cells in response to anti-IgM stimulation. Splenic B cells from the PKK mutant and control mice were incubated with anti-IgM for 24 hours. The levels of Bcl-xL and Bcl-2 were analyzed by western blotting. Representative results from two independent experiments are depicted. 8-month-old mice were used in the experiments described in this figure.