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Procyanidin B2 prevents lupus nephritis development in mice by inhibiting NLRP3 inflammasome activation

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Abstract

Lupus nephritis (LN) is a multifactorial event that contributes to the long-term mortality of systemic lupus erythematosus (SLE). Activation of NLRP3 inflammasome has been known to play a role in SLE pathogenesis. We evaluated the renal protection effects of procyanidin B2 (PCB2) and the involvement of NLRP3 in a mouse model involving MRL/lpr and MRL/MpJ mice. Kidney injury was evaluated by measuring the renal clinical and pathological features, renal immune complex deposition, and serum anti-double-stranded (anti-dsDNA) Abs. ELISA and Western blotting were used to detect NLRP3 inflammasome activation and IL-1β/IL-18 production. NLRP3 gene silencing was introduced into MRL/lpr mice by short hairpin RNA, and the renal damage was compared with the treatment of PCB2. PCB2 remarkably reduced renal damage in MRL/lpr mice, reflected by the reduced proteinuria, and serum levels of blood urea nitrogen and creatinine, as well as pathological features with less renal injury. PCB2 significantly reduced renal immune complex deposition and serum anti-dsDNA levels, notably inhibited the NLRP3 inflammasome activation, and reduced the renal and serum levels of IL-1β and IL-18 in MRL/lpr mice compared with those of NLRP3 gene-silenced MRL-lpr mice. PCB2 significantly suppressed LN in MRL-lpr mice by inhibiting the activation of NLRP3 inflammasome and subsequent IL-1β and IL-18 production. This finding explores a novel mechanism by which procyanidin exerts inflammatory suppression effects and its clinical benefits in LN prevention.

Keywords

Lupus nephritis, procyanidin B2, renal damage, NLRP3 inflammasome, anti-inflammation

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Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease that mainly affects women's health and typically causes damage in multiple organs, among which the kidney is the most commonly influenced one.[1] Lupus nephritis (LN) is associated with the mortality of SLE over time, potentially leading to end-stage renal disease of patients.[2] While the 10-yr survival rate of SLE is about 70%,[3] the diverse clinical manifestations of SLE and the complicated etiology that combines genetic and environmental factors remain challenges to be further understood for the development of the effective treatment of SLE.

One of the main characterizations of SLE is the formation of auto-Abs to nuclear components, the deposition of immune complex and organ damage mediated by inflammatory cells. In the past few years, a number of studies have showed that activation of NLRP3 inflammasome, a molecular complex that activates caspase-1 and furthers the release of cytokines IL-1β and IL-18, plays important roles in promoting organ damage occurring in the SLE pathogenesis.[4]

IL-1β has been known as one of the key molecules in the inflammation progression of LN. For instance, IL-1β levels are increased in the kidneys as well as the cultured macrophages from glomeruli of mice with nephritis.[5] Experimental SLE mice deficient in IL-1β

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developed milder manifestation than normal controls, indicating the mediation of IL-1β in the pathogenesis of SLE.[6] Treatment of IL-1 receptor reduced proteinuria, the levels of auto-Abs and also improved the survival rate in established MRL/lpr mice.[7]

IL-18 is highly related to IL-1, which is activated by IL-1β-converting enzyme (caspase-1) from the inactive precursor of IL-18 (pro-IL-18).[8] In the kidney tissue of MRL/lpr mice, IL-18 is found to be overexpressed and the expression level increases with the severity of LN.[9] In comparison with healthy controls, patients with lupus presented with significantly higher IL-18 levels in serum, which reflects the severity extent of renal damage in lupus.[10,11] Recent studies have demonstrated that lpr mice administered an IL-18 DNA vaccine exhibited a significant reduction in glomerulonephritis and renal damage,[12] suggesting that inhibition of IL-18 production may be an effective therapeutic strategy for LN.

Procyanidin B2 (PCB2) is a phenolic compound that is mainly found in cocoa, apples or grapes, and studies have shown that procyanidins have protective properties against inflammation.[13] For instance, it has been shown that PCB2 significantly inhibited NLRP3 inflammasome activation, suppressed subsequent caspase-1 activation and IL-1β secretion in LPS-induced acute inflammation in human umbilical vein endothelial cells and macrophages.[14,15] Given the enhancement of NLRP3 inflammasome activation in the pathogenesis of SLE, and regulation of PCB2 on NLRP3 activation, herein we aim to test the reno-protective effects of PCB2 using MRL/lpr mice, and to explore the modes of action relevant to NLRP3 inflammasome activation.

Materials and methods

Animals

Since SLE is more common and severe in female mice, female MRL/lpr mice and age-matched female MRL/MpJ mice (Shanghai SLAC laboratory Animal Co, Ltd, Shanghai, China) were used in this study, and housed at pathogen-free facilities with *ad libitum* access to chow and water in the Experimental Animal Center at Heze Municipal Hospital. Prior to the initiation of the experiment, the mice were acclimatized to the new laboratory environment for 7 d. The experiments were approved by the Institutional Ethics Committee of Heze Municipal Hospital and performed in accordance with the National Institutes of Health Guide for Care and Use of Animals.

Experimental design

The mice were randomly divided into three groups. First, normal MRL/MpJ mice received distilled water by gavage for 8 wk consecutively (10 ml/kg body mass). Further, MRL/lpr mice were randomly divided into two groups. The vehicle group received distilled water by gavage for 8 wk consecutively (10 ml/kg body mass) and the PCB2 group received PCB2 (Chengdu Biopurify Phytochemicals Ltd, China) dissolved in distilled water by gavage for 8 wk consecutively (100 mg/kg);[16] $n = 10$ mice for each group.

Evaluation of renal clinical features

Starting at 12 wk, mice were placed in cages for 24-h urine collection every 2 wk. Urinary protein levels were tested using Multistix 10SG reagent strips (Bayer Healthcare, IN, USA) and scored on a scale of 0–4, where 0, negative; 1, trace; 2 (30 mg/dl); 3 (100 mg/dl); 4 (300 mg/dl); 5 (2000 mg/dl or more).[17] Further, after 8 wk of treatment, the mice were euthanized, and blood samples were collected for analysis. Blood urea nitrogen (BUN) and creatinine levels were analyzed using Beckman SynchronX3 Clinical System autoanalyzer (Beckman Coulter Inc, CA, USA). The blood and renal levels of IL-1β and IL-18 were measured by ELISA kits (eBioscience, CA, USA).

Assessment of renal histopathologic features

After euthanasia, the kidney tissues of the mice were dissected and were fixed in 10% neutral-buffered formalin and then embedded in optimal cutting temperature compound, followed by staining with hematoxylin and eosin and periodic acid-Schiff reagents. Renal pathology, glomerular proliferation, crescent formation, neutrophil infiltration, fibrinoid necrosis, and peri-glomerular inflammation were analyzed on 50 randomly selected glomeruli according to previous methods,[18] and a glomerulonephritis activity score (range 0–24) was calculated as described previously.[18]

Detection of renal immune complex deposition

In order to detect the immune complex deposition, frozen kidney sections were blocked with 10% FBS (Sigma-Aldrich), and stained with FITC-conjugated rabbit anti-mouse IgG (Santa Cruz Biotechnology, CA, USA). The mean fluorescence intensity indicating mouse IgG levels was scored 0–3, following previously described methods.[19]

Assessment of serum anti-double-stranded DNA (anti-dsDNA) Abs

Anti-dsDNA Abs in serum were assessed using an anti-mouse dsDNA ELISA kit (Biovision, CA, USA) according to the manufacturer's protocol. The absorbance at 450 nm was measured by microplate reader (Molecular Device, PA, USA).

Western blotting

Kidney tissue was homogenized in cell lysis buffer (Cell Signaling Technology, USA) and centrifuged at 9660 *g* for 5 min. Twenty μg of total proteins were loaded on 12% SDS-PAGE, and transferred onto a nitrocellulose membrane (Thermo-Fisher Scientific, MD, USA). Further, the membrane was blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween 20) for 2 h at room temperature and incubated overnight at 4 °C with the following primary Abs against NLRP3 (1:500), ASC (1:600) (Abcam, MA, USA), and Abs against procaspase-1 (1:1000) and β-actin (1:2000) (Santa Cruz Biotechnology, USA). After sufficient washing, membranes were incubated with HRP-conjugated secondary Abs. The membranes were developed with enhanced chemiluminescence (Cell Signaling Technology, CA, USA), and binding signals were visualized by Amersham Imager 600 (GE Healthcare, IL, USA).

NLRP3 gene silencing

Short hairpin RNA (shRNA) sets in pLKO.1 clones against NLRP3 were obtained from Hanyin Biotechnology (Shanghai, China). The mice were injected with NLRP3 shRNA and scrambled shRNA plasmids via the tail vein at 8 μg/g, respectively, followed by Western blotting to check the NLRP3 inflammasome activation and the expression levels of IL-1β and IL-18, in parallel with mice allocated into the normal, vehicle and PCB2 groups.

Statistical analysis

All data analysis was performed using SPSS software (version 16.0). The results are shown as the mean ± SEM. Student *t* test and one-way ANOVA test were used for data analysis. Statistical significance was defined as $P < 0.05$.

Results

Reduction in renal damage by PCB2 treatment in MRL/lpr mice

Female MRL/lpr mice were given vehicle or PCB2 daily by gavage for 8 wk. As shown in Figure 1a, the vehicle group showed progressively increased urine protein levels up to the end of the study. In contrast, the urine protein levels in the PCB2 group were slightly elevated at wk 2 (14-wk-old mice), and this increase lasted up to wk 4. Notably, urine protein levels started to decrease at wk 6 ($P < 0.05$) and dropped to the same level as baseline at the end of the study ($P < 0.01$), indicating the significant inhibition of PCB2 on proteinuria. The urine protein level in the normal control MRL/MpJ mice remained at the same level as baseline across the time course of the study. Similarly, serum levels of BUN (Figure 1b) and creatinine (Figure 1c) in the MRL/lpr mice were significantly higher than those for MRL/MpJ control mice, and PCB2 lowered the levels of these renal function markers significantly (both $P < 0.05$).

After 8 wk, several different pathologic features of the kidney were also compared between vehicle- and PCB2-treated MRL/lpr mice. Briefly, the vehicle-treated mice exhibited the typical features of renal disease, with enlarged hypercellular glomeruli and perivascular lesions, whereas PCB2-treated mice demonstrated significantly less hypercellular glomeruli and perivascular lesions (Figure 2a). In addition, compared to the normal MRL/MpJ mice, the percentages of glomerular proliferation, crescent formation, neutrophil infiltration, fibrinoid necrosis, peri-glomerular

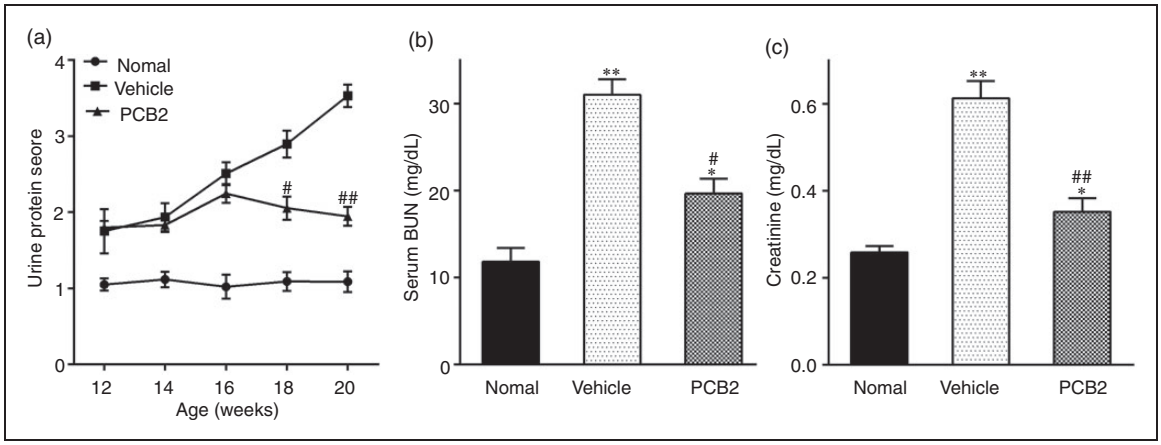

Figure 1. Effects of procyanidin B2 (PCB2) on clinical features in MRL/lpr mice. a, Urine protein time-course studies. b and (c) Serum blood urea nitrogen levels (b) and serum creatinine levels (c) at wk 20. MRL/Mpj mice were treated as the normal group and MRL/lpr mice were divided into the PCB2 group, which received 100 mg/kg per d, and the vehicle group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to vehicle group.

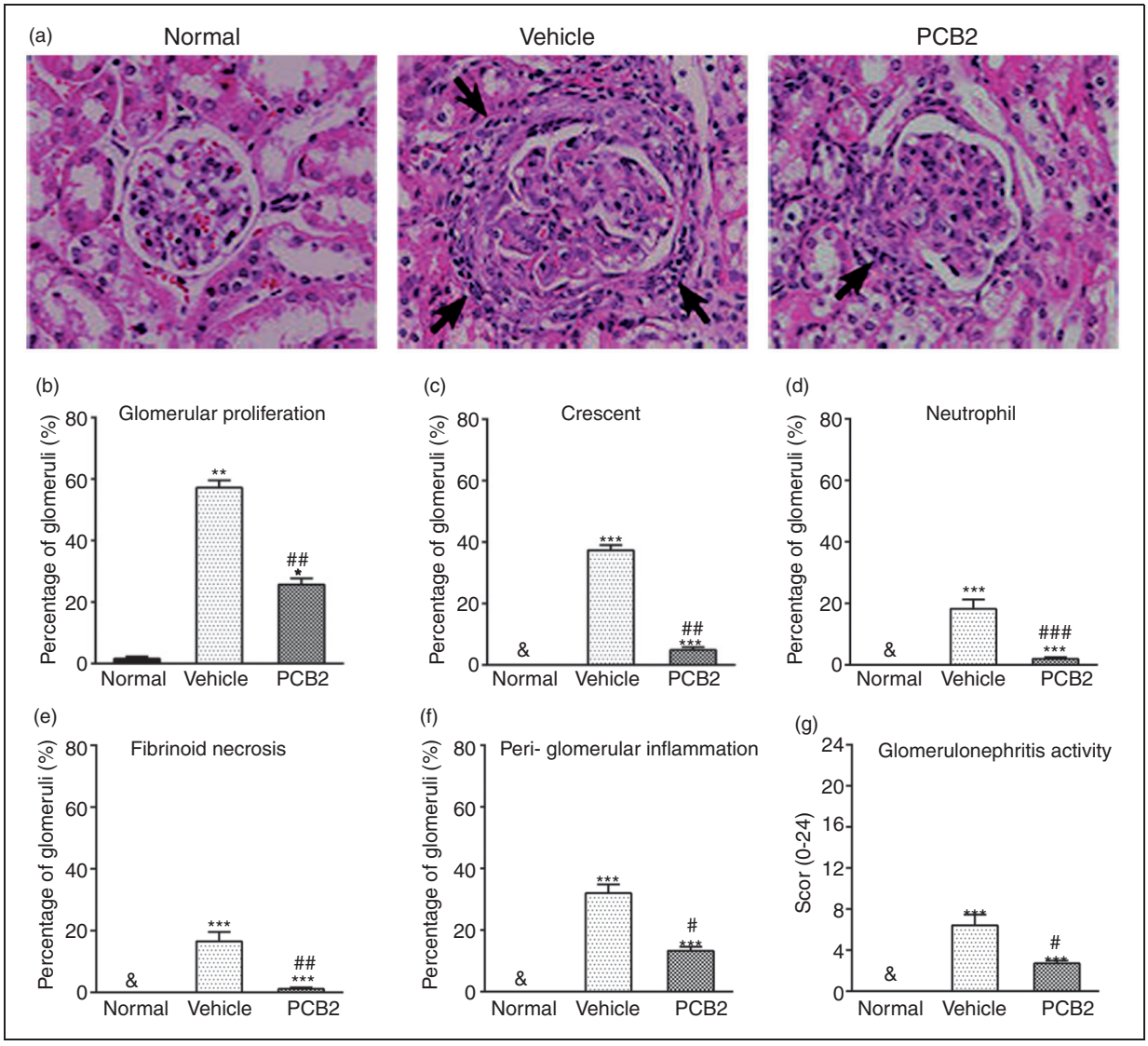

Figure 2. Effects of procyanidin B2 (PCB2) on pathological features in MRL/lpr mice. a, Renal tissue was obtained from 20-wk-old MRL/lpr mice treated with either PCB2 or vehicle and from normal control mice for histopathologic analyses. Renal sections were stained with hematoxylin and eosin to process histopathological evaluation. Original magnification ×400. The severity of renal damage was semi-quantitatively scored in each group (b–f). g, Scoring of glomerulonephritis activity. MRL/Mpj mice were treated as the normal group and MRL/lpr mice were divided into the PCB2 group, which received 100 mg/kg per d, and the vehicle group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to vehicle group. $^{\&}$Not detectable.

inflammation and glomerulonephritis activity significantly increased in the vehicle group. In contrast, PCB2-treated mice had significantly decreased glomerular hypercellularity and glomerulonephritis than the control group (Figures 2b–g).

Decrease in renal immune complex deposition and serum anti-dsDNA production by PCB2 treatment in MRL/lpr mice

To evaluate whether PCB2 mediates the immune complex deposition in the kidneys, renal sections were snap-frozen and stained for FITC-IgG. In the glomeruli of vehicle-treated mice, intensive deposition of IgG was observed (Figure 3a). Further, using a semi-quantitative scoring system, MRL/lpr mice had significantly higher IgG deposition than normal MRL/MpJ mice ($P < 0.001$), while PCB2 treatment demonstrated a significant decrease in glomerular deposition of IgG ($P < 0.01$) (Figure 3b).

Furthermore, the circulating anti-dsDNA Ab levels were determined. Our data showed that the serum anti-dsDNA Ab level in the normal control mice was at the minimum. In contrast, we saw a significant difference between the vehicle-treated control group and the PCB2-treated group at wk 8 ($P < 0.01$) (Figure 3c). Specifically, PCB2 treatment inhibited the production of anti-dsDNA Abs in MRL/lpr mice.

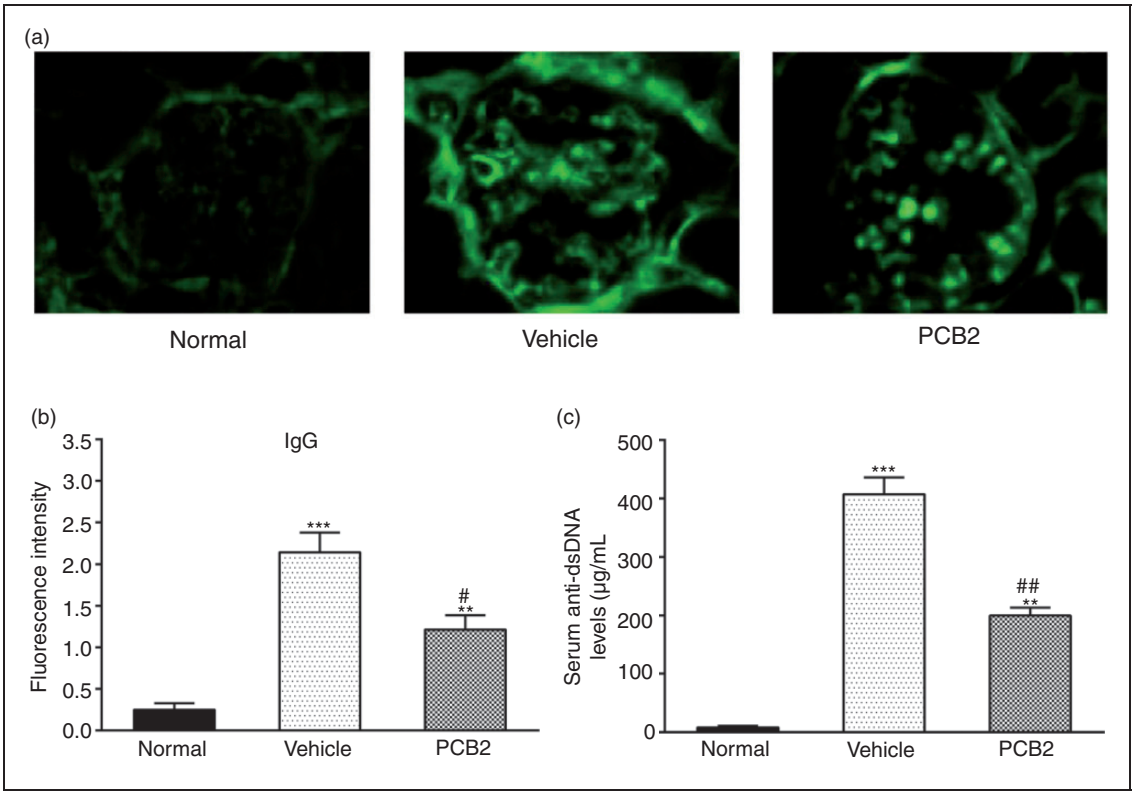

Figure 3. Effects of procyanidin B2 (PCB2) on pathological features in MRL/lpr mice. a, Renal tissue was obtained from 20-wk-old MRL/lpr mice treated with either PCB2 or vehicle and from normal control mice for histopathologic analyses. Renal sections were snap-frozen and embedded in optimal cutting temperature compound. The tissue was then analyzed by immunofluorescence microscopy to assess the deposition of IgG (representative images shown) and the intensity of green fluorescence (b). Original magnification ×400. c, Serum anti-double-stranded DNA Ab levels at wk 20. MRL/Mpj mice were treated as the normal group and MRL/lpr mice were divided into the PCB2 group, which received 100 mg/kg per d, and the vehicle group. Data are presented as mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to vehicle group.

PCB2 treatment inhibited NLRP3 inflammasome activation and reduced IL-1β/IL18 production in the kidneys of MRL/lpr mice

The effects of PCB2 treatment on NLRP3 inflammasome activation in MRL/lpr mice were examined to explore the underlying mechanisms by which PCB2 attenuates murine LN. The up-regulation of NLRP3, ASC, and procaspase-1 was significantly reduced by PCB2 treatment in the kidneys of MRL/lpr mice (Figure 4a and 4b). ELISA was used to analyze the production of IL-1β and IL-18 in different treated groups. As shown in Figure 4c–f, PCB2 treatment resulted in a significant reduction in renal and serum levels of IL-1β and IL-18 in PCB2-treated MRL/lpr mice ($P < 0.05$), suggesting the reduction of inflammatory response in MRL/lpr mice.

PCB2 treatment exhibits similar protection on MRL/lpr mice as NLRP3 knockdown

We investigated the protein levels of caspase-1 p20, and levels of IL-1β and IL-18 in retinal tissues and found that these factors were significantly more abundant in the vehicle-treated MRL/lpr mice group than in the normal MRL/MpJ mice (Figure 5a–c). Consistent with the ELISA results determined earlier, Western blotting results showed that the protein levels of IL-1β and IL-18 were significantly lower in the PCB2-treated MRL/lpr mice in comparison with the vehicle control group. Remarkably, the administration of PCB2 resulted in expression levels of IL-1β and IL-18 similar to the NLRP3 knockdown group, which both were significantly lower than the vehicle group ($P < 0.01$).

Discussion

LN is the most known organ-threatening manifestation of SLE and is a major cause of morbidity and mortality of it.[20] In spite of the improvements in the treatment and management of lupus, the incidence of end-stage renal disease remains a big threat for SLE patients.[21] Current therapeutic options include glucocorticoids and immunosuppressive agents including cyclophosphamide, azathioprine, and mycophenolate mofetil.[22]

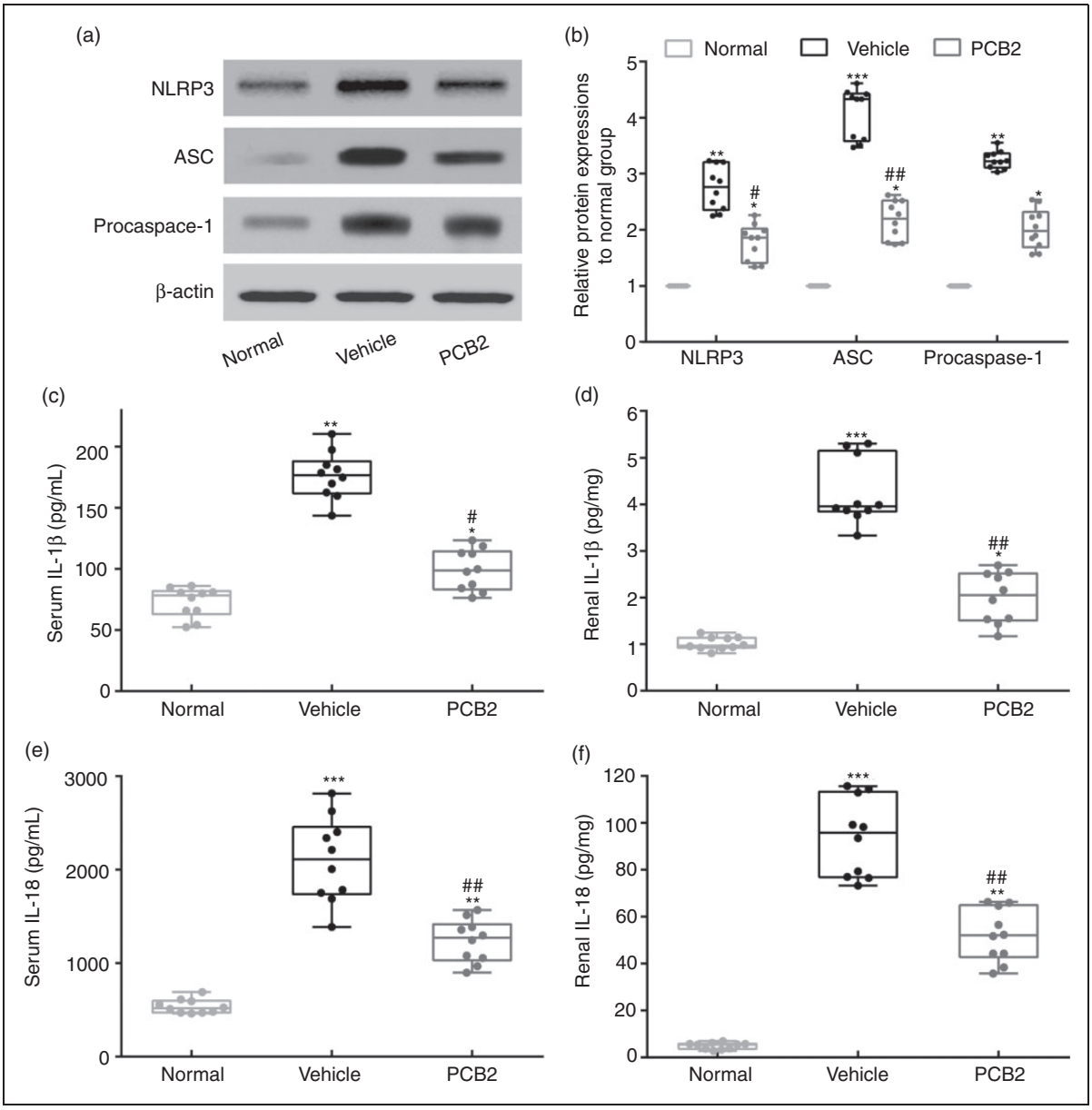

Figure 4. NLRP3 inflammasome activation is involved in LN and procyanidin B2 (PCB2) treatment. a, Western blotting (total cell lysates) was used to assay the protein expressions including NLRP3, ASC, and procaspase-1. β-Actin was used as a loading control and relative expressions from the Western blotting (b). c to f, Serum levels of IL-1β (c) and IL-18 (e) and renal levels of IL-1β (d) and IL-18 (f) at wk 20 measured by ELISA. MRL/Mpj mice were treated as the normal group and MRL/lpr mice were divided into the PCB2 group, which received 100 mg/kg per d, and the vehicle group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to vehicle group.

However, these drugs are incompletely effective and are associated with substantial toxicity.[23] Therefore, discovery of new strategies of LN treatment are imperative. In this study, we found that PCB2, which is one of the most important components of grape-seed procyanidin extract, significantly attenuated LN in lupus-prone MRL/lpr mice. Specifically, PCB2 remarkably reduced renal damage in MRL/lpr mice, supported by the reduced urine protein level, and serum levels of BUN and creatinine. Pathological features of the kidney showed less severity of renal injury in the PCB2-treated mice. In addition, PCB2 significantly reduced serum anti-dsDNA levels and renal immune complex deposition. Further, PCB2 notably inhibited NLRP3 inflammasome activation and reduced the renal and serum levels of IL-1β and IL-18 in MRL/lpr mice.

MRL/lpr mice develop a systemic autoimmune syndrome that shares many common characteristics of human SLE, such as severe glomerulonephritis, production of auto-Abs, splenomegaly, lymphadenopathy, arthritis, and vasculitis.[24] For this experimental animal

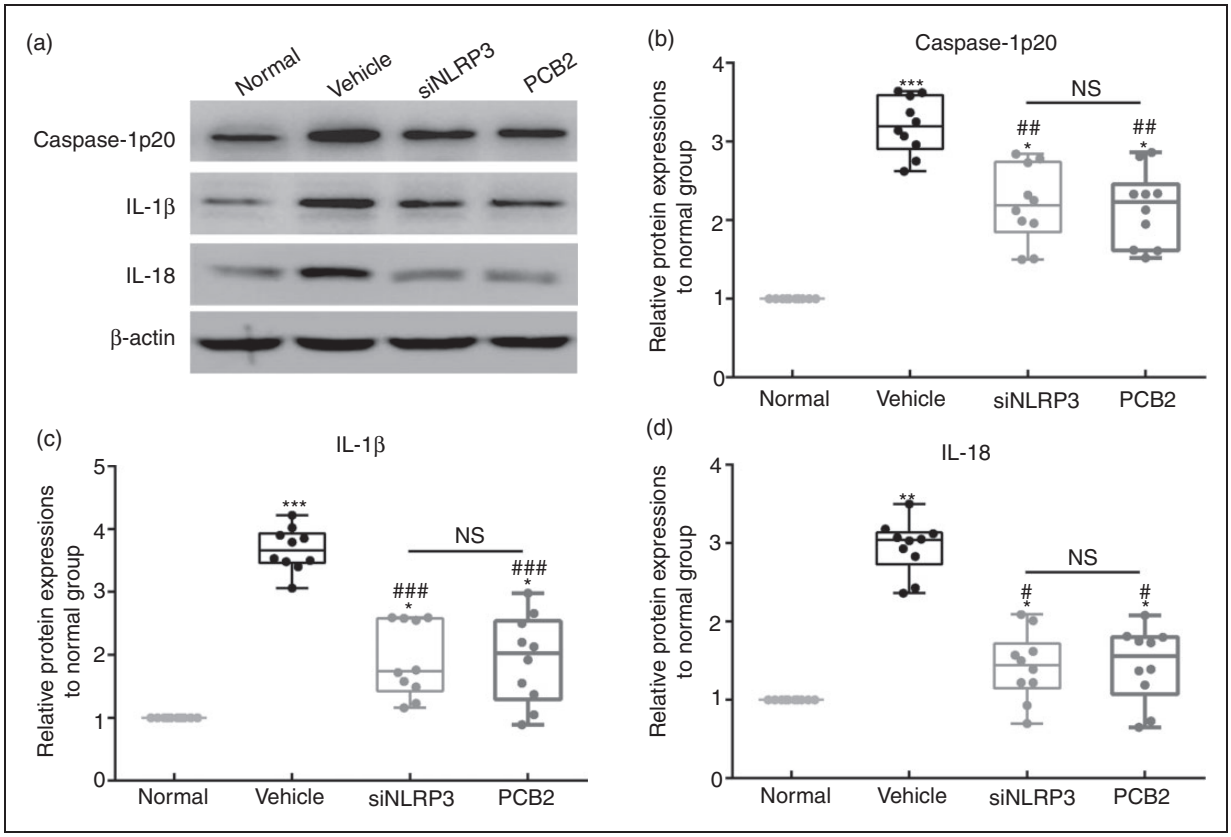

Figure 5. Procyanidin B2 (PCB2) prevents LN development in mice by inhibiting NLRP3 inflammasome activation. a, Western blotting (total cell lysates) was used to assay the protein expressions including caspase-1 p20, IL-1β and IL-18. β-Actin was used as a loading control and relative expressions from Western blotting (b–d). MRL/Mpj mice were treated as the normal group and MRL/lpr mice were divided into the PCB2 group, which received 100 mg/kg per d, and the vehicle group. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to normal group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to vehicle group. NS means no significant difference between the siNLRP3 group and PCB2 group.

model, kidney inflammation is evident at 3 mo of age, and is rapidly progressive or even fatal by 5–8 mo of age.[9] That was the reason why the administration of PCB2 were performed at wk 12, which is the time point to expect kidney damage features. Likewise, according to the regression schedule of the disease model, our study was ended before the death of the mice after 8 wk. In future studies, we could wait for longer duration of the experiment to evaluate the clinical features and survival rates of the mice.

The concentration of urea in serum is one of the most frequently clinical indices for renal function estimation, and increased BUN is associated with kidney disease or failure. Creatinine, a breakdown product of creatine phosphate in muscle, is also commonly used as an index of kidney function.[25] DNA-containing nucleosomes are released abundantly into the circulation system of patients with SLE, leading to the formation of immune complex with anti-dsDNA auto-Abs.[26] Once deposited in the kidney, immune complex can trigger a series of events that result in kidney inflammation leading to complement activation, chemotactic factor production, cytokine/growth factor generation, and reactive oxygen species generation.[27,28] This study showed that PCB2 significantly reduced the renal damage of lupus-prone MRL/lpr mice as indicated by decreased proteinuria, BUN and creatinine levels, serum anti-dsDNA production, and renal immune complex deposition. We further investigated which mechanisms were behind these pathological changes. Among the inflammatory cascades in SLE, the NLRP3 inflammasome/IL-1β signaling pathway plays key roles in inflammation and autoimmunity, thus attracting increasing attention, and a recent study showed that the P2X7 signaling pathway promotes murine LN by activating the NLRP3/ASC/caspase 1 inflammasome, leading to increased IL-1β production in MLR/lpr mice.[29] Using the NZB/W F1 lupus-prone mice model, an experimental study revealed that epigallocatechin-3-gallate, one of the major bioactive polyphenols from green tea, prevents LN development by activating the Nrf2 antioxidant signaling pathway and inhibiting NLRP3 inflammasome activation in the kidney.[30] Isoflurane administration remarkably reduced LN by abrogating renal NLRP3 inflammasome formation and activation and subsequently

reducing inflammatory responses in MRL/lpr mice.[31] PCB2 demonstrated anti-inflammatory effects involving various mediators of inflammation, such as eicosanoids, cytokines, and NO via the activation of NF-κB and MAPK pathways, as well as inflammasome activation.[15]

In our study, we found that NLRP3 inflammasome components (NLRP3/ASC/caspase-1) were activated in the kidneys of MRL/lpr mice, which is consistent with previous reports.[29,31] Intriguingly, a negative correlation of NLRP3 inflammasome activation with SLE disease activity has been reported as well.[32,33] To this point, the correlation of NLRP3 inflammasome with SLE disease activity has been controversial. In our results, we did see the up-regulation of NLRP3 in the SLE mice, which was in favor of the positive role of NLRP3 in the progression of SLE. However, in the PCB2 group, the dose of 100 mg/kg lowered the NLRP3 level to only approximately 70% of the vehicle group but showed significant protection of SLE. The result further supports that NLRP3 plays a pivotal role in SLE pathogenesis, in spite of the dynamic regulation.

PCB2 administration resulted in inhibition of NLRP3 activation and suppression of IL-1β and IL-18 production, indicating that PCB2 attenuated LN partly by inhibiting NLRP3 activation. To further investigate whether NLRP3 inflammasome was a specific target for the protection of PCB2 on LN, the NLRP3 gene was silenced in the MRL/lpr mice. Our results showed that NLRP3 shRNA reduced the activation of NLRP3 inflammasome, as well as the production of subsequent IL-1β/IL-18. Notably, the expression levels of IL-1β and IL-18 in the PCB2-treated group were the same as the ones in the NLRP3 knockdown group, indicating the abrogation of NLRP3 inflammasome of PCB2 at the current dose (100 mg/kg).

SLE patients suffer from the potential side effects of the long-term use of current therapeutic drugs, and the risk of relapse, the efficacy and safety of the treatment strategy are pivotal in the development of new drugs for the maintenance of long-term kidney health in LN patients. Our study confirmed the participation of the NLRP3 inflammasome in the pathogenesis of LN, providing scientific ground for the development of NLRP3 inflammasome-targeted treatment. Dietary procyanidins have been shown to be health protective agents in the human immune system,[34] which is also supported by epidemiological studies that have uncovered lower incidences of inflammatory disease in populations that consume procyanidin-rich foods.[35] Therefore, PCB2 is relatively safe to be developed as a protective agent on the renal damage caused by autoimmune diseases.

In summary, we demonstrated for the first time that PCB2 significantly attenuated the renal damage in the LN development in MRL/lpr mice, mainly through inhibiting the activation of NLRP3 inflammasome in the pathogenesis of SLE. This finding revealed a novel potential therapeutic strategy for LN and broadens the clinical benefits of PCB2.

Conclusion

PCB2 significantly suppressed LN in MRL/lpr mice by inhibiting the activation of NLRP3 inflammasome and subsequent IL-1β and IL-18 production. This finding explored a novel mechanism by which procyanidin exerts inflammatory suppression effects and its clinical benefits in LN prevention.

Declaration of conflicting interests

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