Complement 1q protects MRL/lpr mice against lupus nephritis via inhibiting the nuclear factor-κB pathway

JUANJUAN SUN, SHUXIA GUO, FUKUN NIU, DEFENG LIU and YAN ZHUANG

Department of Nephrology, Linyi Central Hospital, Linyi, Shandong 276400, P.R. China

Abstract. Lupus nephritis (LN) is a kidney disorder that is a critical cause of mortality in patients with systemic lupus erythematosus. The present study aimed to explore the protective role of complement component 1q (C1q) on LN and the underlying mechanism involving the nuclear factor (NF)-κB singling pathway. MRL/lpr mice served as the LN mouse model, and pcDNA-C1q was injected into LN mice to d*et*ermine the role of C1q. C1q mRNA expression was detected using reverse transcription-quantitative PCR. Urine protein and blood urea nitrogen (BUN) levels were measured, and the histological damage index was determined using H&E staining. ELISA was used to measure the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, anti-C1q and anti-double stranded DNA (dsDNA). CD68- and Ki67-positivity were detected using immunofluorescence, and NF-κB-related protein expression was examined using western blotting. C1q mRNA expression was downregulated in renal tissues of LN mice. Overexpression of C1q decreased urine protein, BUN levels and the histological damage index in LN mice. The levels of TNF-α, IL-1β, IL-6, anti-C1q and anti-dsDNA in renal tissues of LN mice were also reduced after pcDNA-C1q treatment. Additionally, overexpression of C1q decreased the CD68- and Ki67-positivity in glomeruli and attenuated the expression of NF-κB-related proteins. Phorbol 12-myristate 13-acetate, an NF-κB pathway activator, reversed the inhibitory effect of C1q on inflammation, macrophage infiltration and mesangial cell (MC) proliferation in renal tissues of LN mice. Thus, it was demonstrated that C1q ameliorated inflammation and macrophage infiltration and decreased MC proliferation in renal tissues of LN mice by inhibiting the NF-κB pathway.

Correspondence to: Dr Yan Zhuang, Department of Nephrology, Linyi Central Hospital, 17 Health Road, Yishui, Linyi, Shandong 276400, P.R. China
E-mail: zhuangyan1680@163.com

Introduction

Lupus nephritis (LN) is a chronic and complex kidney disease (1) that is a frequent complication of systemic lupus erythematosus (2) and is usually associated with inflammatory cell infiltration and immune complex deposition in renal tissues (3). LN is clinically evident in ~50% of patients with systemic lupus erythematosus (4). In LN, ICs initiate the synthesis of various proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6, resulting in cellular infiltration and renal injury (5). The involvement of LN significantly increases patient morbidity and mortality rates (6). Patients with LN have a higher standardized mortality ratio (6-6.8 vs. 2.4) and lower survival rates compared with patients with systemic lupus erythematosus who do not have LN (7). LN pathogenesis is complex, and some patients with LN may develop end-stage renal disease (8). Therefore, it is essential to explore new approaches for the treatment of LN.

Complement component 1q (C1q) is a subcomponent of the C1 complex, which participates in the classical pathway of complement activation (9). C1q has numerous functions, including recognition of ICs and activation of the complement system (10). Previous studies have examined the relationship between LN and anti-C1q. For example, a significantly negative correlation between C1q and anti-C1q was found in patients with LN (11). Anti-C1q was also associated with proteinuria and renal activity score in patients with LN, and could serve as a potential biomarker of LN (12). In addition, high anti-C1q antibody titers are present in the blood of patients with LN, and the level of anti-C1q is related with disease progression (13). Anti-C1q has adequate specificity and sensitivity for LN diagnosis and can be used to evaluate renal activity (14); however, the underlying mechanism of C1q in the regulation of LN remains poorly understood.

Nuclear factor (NF)-κB not only participates in innate and adaptive immunity (15), but also is considered to be a proinflammatory transcription factor (16). The NF-κB pathway is related to several pathological processes in the kidneys, including immune response, inflammation and mesangial cell (MC) proliferation (17-19). Deletion of NF-κB p65 was found to alleviate LN in mice (20). In LN, inhibition of the NF-κB pathway suppressed the inflammatory response (21), and blocking this pathway may decrease macrophage chemotaxis and MC proliferation (22). Nevertheless, the potential regulatory

mechanism of C1q in relation to the NF-κB pathway in LN is still unknown.

In the present study, C1q expression in LN mice was evaluated and the regulatory effects of C1q on renal injury, inflammation, macrophage infiltration and MC proliferation was explored. The function of C1q in regulating NF-κB pathway activity in LN mice was also explored. These results may suggest a potential therapeutic target for LN.

Materials and methods

Animals. In total, 60 male MRL/lpr mice (used as an LN mouse model) and 15 C57BL/6 mice (age, 3 months; body weight, 18-22 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Mice were maintained at 23-25˚C and 50-55% relative humidity, and kept under a 12 h/12 h light/dark cycle with *ad libitum* access to food and water. The present study was performed with the approval of The Animal Ethics Committee of Linyi Central Hospital (Linyi, China).

Experimental design. pcDNA-C1q and pcDNA-negative control (NC) were obtained from Sangon Biotech Co., Ltd. After one week of adjustment, LN mice were divided into pc-NC, pcDNA-C1q and Sham groups, which were injected intraperitoneally with 1 mg/kg pc-NC, 1 mg/kg pcDNA-C1q or an equivalent quantity of normal saline, respectively (15 mice in each group). C57BL/6 mice without treatment acted as the BLANK group. In addition, LN mice in the C1q + Phorbol 12-myristate 13-acetate (PMA) group were injected intraperitoneally with 1 mg/kg pcDNA-C1q and treated with PMA (25 nM; Sigma-Aldrich; Merck KGaA).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from renal tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara Bio, Inc.). The reaction mixtures were incubated at 37˚C for 60 min, 95˚C for 5 min and then held at 4˚C. miScript SYBR Green PCR kit (Qiagen, Inc.) was used to conduct qPCR. The qPCR reaction was performed on the ABI 7500HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following conditions: 95˚C for 3 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 30 sec, and a final extension step at 72˚C for 10 min. Relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (23). GAPDH was used for normalization. Primer sequences are shown in Table I.

Evaluation of renal function. The 24-h urine protein excretion was measured once every 2 weeks using Multistix 10SG reagent strips (Siemens Healthineers). After 4 weeks continuous treatment, mice were anesthetized by intraperitoneal administration of 50 mg/kg pentobarbital sodium and sacrificed by cervical dislocation. Blood samples and renal tissues were collected for future experiments. Blood urea nitrogen (BUN) levels were measured using an automatic biochemical analyzer (Hitachi, Ltd.).

H&E staining. Renal tissues were fixed in 4% paraformaldehyde for 24 h at 37˚C, embedded in paraffin, cut into 4-μm thick sections, dewaxed in xylene and rehydrated with 90% ethanol

Table I. Primer sequences used in reverse transcription-quantitative PCR.

Primer	Sequences (5'→3')
Complement 1q	F: GAAACAATGGGAACAATGGAG
	R: TGCTGAAGGTGAAGAAATACA
GAPDH	F: ACACCTTCTACAATGAGCTG
	R: CTGCTTGCTGATCCACATCT

F, forward; R, reverse.

at 37˚C. Sections were then stained with hematoxylin for 2 min and eosin for 2 min at 37˚C. Using light microscopy, the degree of histological damage in renal tissues was observed (magnification, x400). The histological damage index of glomerulus was graded on a scale of 0-3 as previously described by Muraoka *et al* (24), where 0 = normal, 1 = mild (cell proliferation and/or cell infiltration), 2 = moderate (cell proliferation and/or cell infiltration with membrane proliferation) and 3 = severe (cell proliferation and/or cell infiltration, membrane proliferation and crescent formation and/or hyalinosis).

Western blotting. Renal tissues were lysed using ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology) to obtain total protein. The concentration of total protein was detected using a bicinchoninic acid protein concentration assay kit (Cell Signaling Technology, Inc.). Total protein (60 μg/lane) was separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% separating gum and 5% concentrating gum), and subsequently transferred onto a polyvinylidene fluoride membrane. The membranes were blocked with 5% skimmed milk for 1 h at 37˚C. Then, membranes were incubated with primary antibodies overnight at 4˚C. The antibodies used were as follows: Anti-IκBα (1:1,000; cat. no. 9242), anti-phosphorylated (p)-IκBα (1:1,000; cat. no. 2859), anti-NF-κB p65 (1:1,000; cat. no. 8242) and anti-p-NF-κB p65 (1:1,000; cat. no. 8214) (all CST Biological Reagents Co., Ltd.). Next, membranes were incubated with HRP-labelled goat anti-rabbit IgG (1:2,000; cat. no. I5006MSDS) and HRP-labelled goat anti-mouse IgG secondary antibodies (1:4,000; cat. no. 12-349; both purchased from Sigma-Aldrich; Merck KGaA) for 1 h at 25˚C. Finally, protein bands were visualized using enhanced chemiluminescence exposure solution (Invitrogen; Thermo Fisher Scientific, Inc.) and semi-quantified using Quantity One 1-D software (version 4.62; Bio-Rad Laboratories, Inc.). GAPDH (1:1,000; cat. no. 100242-MM05; Sino Biological) was used as a loading control.

ELISA. Renal tissue homogenate from each group was centrifuged at 3,000 x g at 4˚C for 10 min and the resulting supernatant was collected. Then, the levels of TNF-α (cat. no. ab236712), IL-1β (cat. no. ab197742), IL-6 (cat. no. ab100713) and anti-C1q (cat. no. ab170246) were measured using ELISA kits (all purchased from Abcam). The level of anti-dsDNA was measured by the automated Alegria® ELISA reader (Orgentec Diagnostika
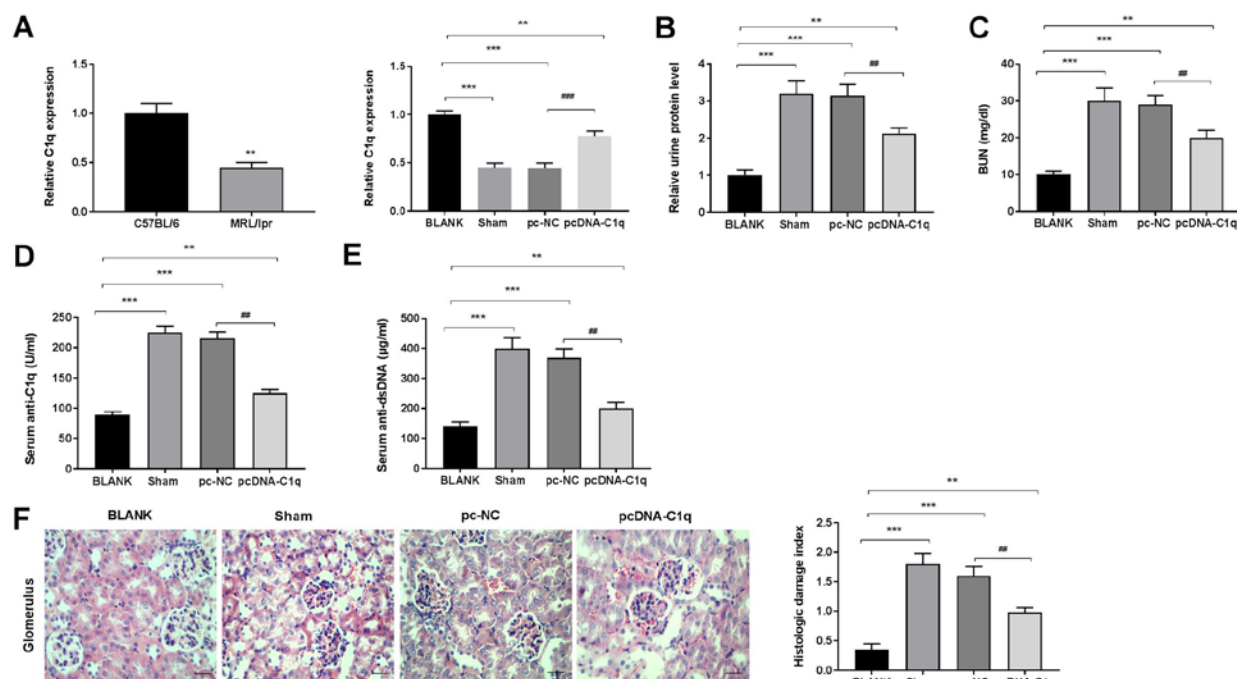
Figure 1. Overexpression of C1q alleviates renal injury in LN mice. (A) mRNA expression of C1q in renal tissues was detected using reverse transcription-quantitative PCR. (B) Relative level of urine protein at 24 h. (C) Levels of serum BUN. Levels of serum (D) anti-C1q and (E) anti-dsDNA were measured using ELISA. (F) The histological damage index of glomerulus was determined using H&E staining. There were 15 mice in each group and the experiments were repeated three times. **P<0.01, ***P<0.001 vs. C57BL/6 or BLANK; $^{##}$P<0.01, $^{###}$P<0.001 vs. pc-NC. C1q, complement component 1q; LN, lupus nephritis; BUN, blood urea nitrogen; NC, negative control; dsDNA, double stranded DNA; BLANK, C57BL/6 mice without treatment; Sham, LN mice injected intraperitoneally with normal saline; pc-NC, LN mice injected intraperitoneally with pc-NC; pcDNA-C1q, LN mice injected intraperitoneally with pcDNA-C1q.
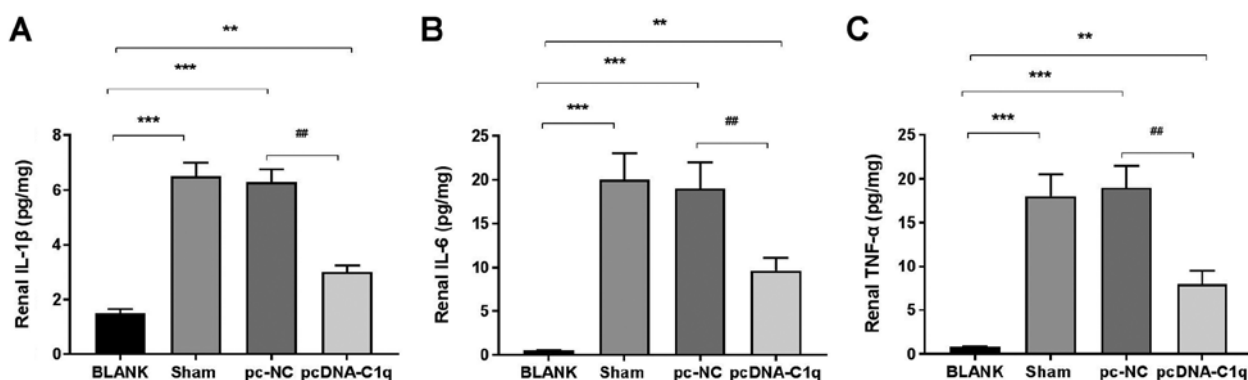

Figure 2. Overexpression of C1q attenuates renal inflammation in LN mice. Levels of (A) IL-1β, (B) IL-6 and (C) TNF-α in renal tissues were measured using ELISA. There were 15 mice in each group and the experiments were repeated three times. **P<0.01, ***P<0.001 vs. BLANK; $^{##}$P<0.01 vs. pc-NC. C1q, complement component 1q; LN, lupus nephritis; IL, interleukin; BLANK, C57BL/6 mice without treatment; Sham, LN mice injected intraperitoneally with normal saline; pc-NC, LN mice injected intraperitoneally with pc-NC; pcDNA-C1q, LN mice injected intraperitoneally with pcDNA-C1q.

GmbH), according to the manufacturer's instructions. The absorbance of each well was measured at 450 nm using an enzyme mark instrument (Thermo Fisher Scientific, Inc.).

Immunofluorescence staining. Frozen glomeruli sections (5 µm) were dried for 15 min at 25°C. After rinsing three times, sections were blocked in PBS containing 10% goat serum (cat. no. 16210064; Thermo Fisher Scientific, Inc.) for 1 h at 25°C. Glomeruli sections were incubated with anti-CD68 antibody (1 µg/ml; cat. no. ab201340) or anti-Ki67 antibody (1 µg/ml; cat. no. ab15580; both Abcam) overnight at 4°C, followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; cat.

no. ab150077; Abcam) for 1 h at 25°C. Glomeruli sections were counterstained with DAPI (2.5 ng/µl) at 25°C for 1 h, and the percentages of CD68- and Ki67-positivity were detected using immunofluorescence microscopy (magnification, x400; Olympus Corporation). The CellProfiler 4.0 software (www.cellprofiler.org) was used to quantify the DAPI intensity at the peri-nucleolar region of ~100 individual cells.

Statistical analysis. Statistical analysis was performed with SPSS 23.0 (IBM Corp.). Data were presented as the mean ± SD. All experiments were repeated three times. Differences among multiple groups were analyzed using
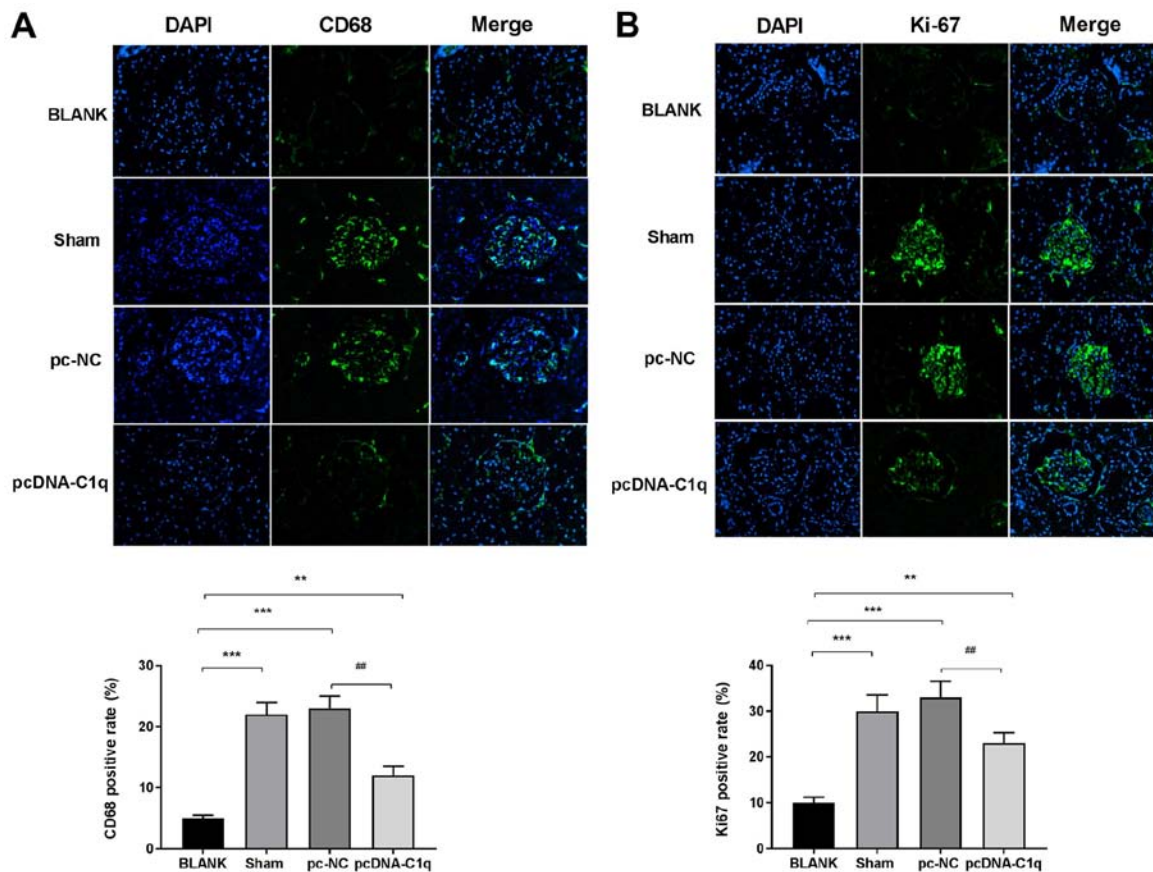
Figure 3. Overexpression of C1q inhibits macrophage infiltration and mesangial cell proliferation in renal tissues of LN mice. (A) CD68- and (B) Ki67-positivity were detected using immunofluorescence staining (magnification, x400). There were 15 mice in each group and the experiments were repeated three times. **P<0.01, ***P<0.001 vs. BLANK; ##P<0.01 vs. pc-NC. C1q, complement component 1q; LN, lupus nephritis; BLANK, C57BL/6 mice without treatment; Sham, LN mice injected intraperitoneally with normal saline; pc-NC, LN mice injected intraperitoneally with pc-NC; pcDNA-C1q, LN mice injected intraperitoneally with pcDNA-C1q.

Figure 4. Overexpression of C1q inhibits the NF-κB pathway in renal tissues of LN mice. Protein expression of p-IκBα/IκBα and p-NF-κB p65/NF-κB p65 was measured using western blot analysis. There were 15 mice in each group and the experiments were repeated three times. **P<0.01,***P<0.001 vs. BLANK; ##P<0.01 vs. pc-NC; &P<0.05 vs. pcDNA-C1q. C1q, complement component 1q; LN, lupus nephritis; p, phosphorylated; PMA, phorbol 12-myristate 13-acetate; BLANK, C57BL/6 mice without treatment; Sham, LN mice injected intraperitoneally with normal saline; pc-NC, LN mice injected intraperitoneally with pc-NC; 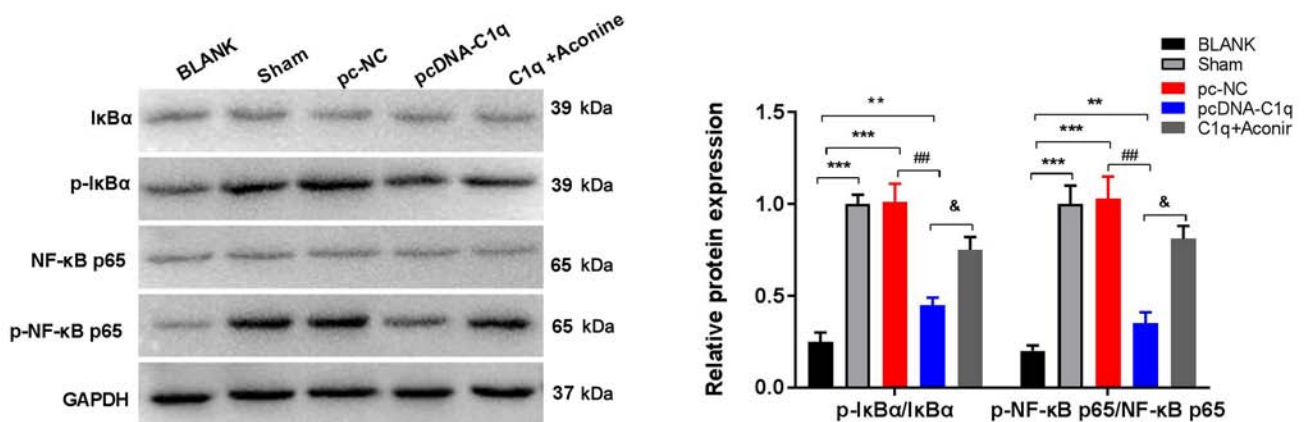pcDNA-C1q, LN mice injected intraperitoneally with pcDNA-C1q; C1q + PMA, LN mice were injected intraperitoneally with pcDNA-C1q and treated with PMA.

one-way ANOVA followed by Tukey's multiple compari-sons test. Data of two groups were assessed using unpaired Student's t-test. P<0.05 was considered to indicate a statisti-cally significant difference.

Results

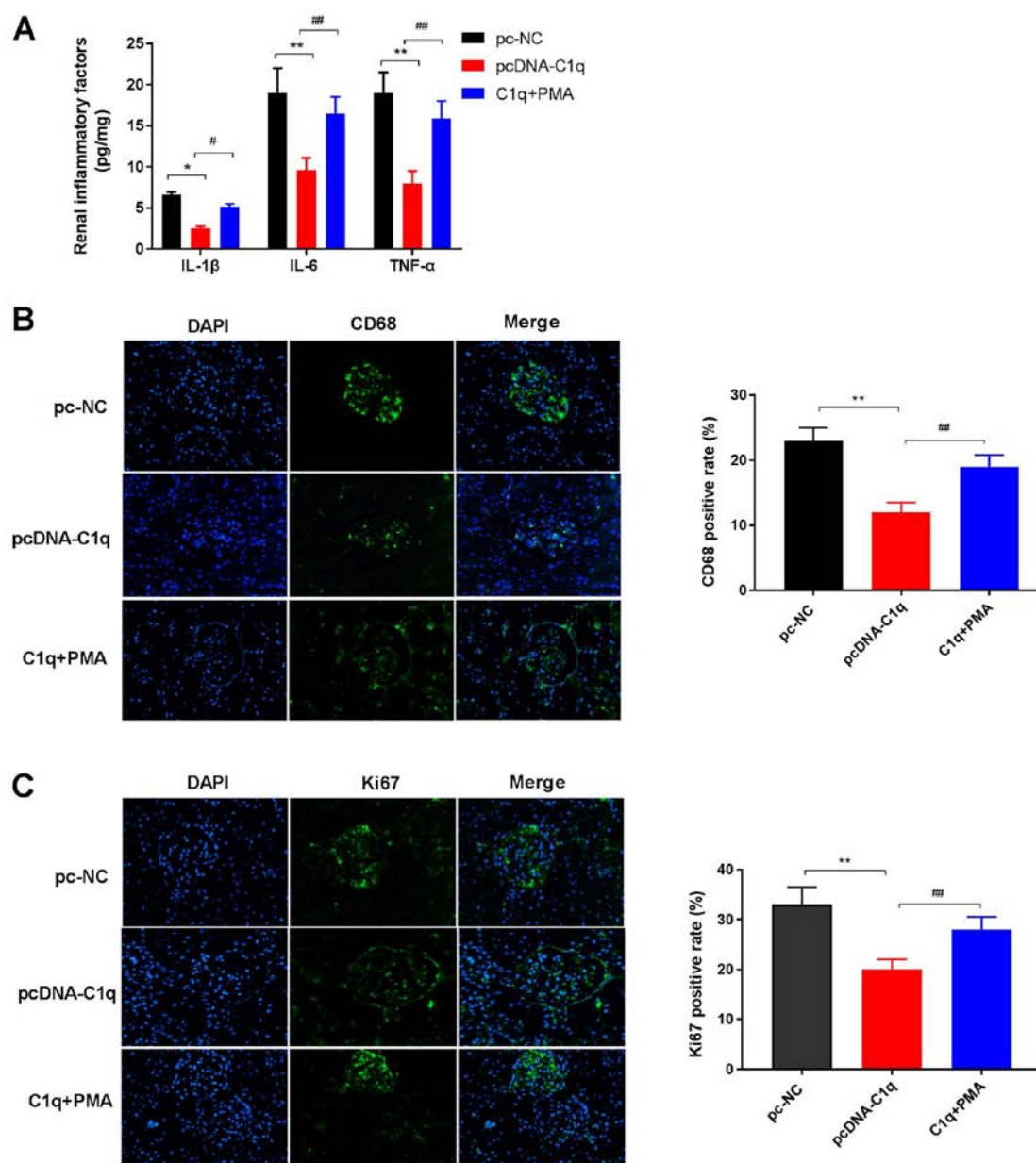*Overexpression of C1q alleviates renal injury in LN mice.* Analysis using RT-qPCR indicated that C1q mRNA

Figure 5. Overexpression of C1q ameliorates renal injury in LN mice via inhibiting the NF-κB pathway. (A) Levels of TNF-α, IL-6 and IL-1β in renal tissues were measured using ELISA. (B) CD68- (C) Ki67-positivity were detected using immunofluorescence staining (magnification, x400). There were 15 mice in each group and the experiments were repeated three times. *P<0.05, **P<0.01 vs. pc-NC; #P<0.05, ##P<0.01 vs. pcDNA-C1q. C1q, complement component 1q; LN, lupus nephritis; IL, interleukin; PMA, phorbol 12-myristate 13-acetate; pc-NC, LN mice injected intraperitoneally with pc-NC; pcDNA-C1q, LN mice injected intraperitoneally with pcDNA-C1q; C1q + PMA, LN mice were injected intraperitoneally with pcDNA-C1q and treated with PMA.

expression was decreased in MRL/lpr mice (LN mice) compared with C57BL/6 mice. C1q-overexpression was induced by transfecting pcDNA-C1q (Fig. 1A), and the urine protein and BUN levels were examined. Compared with the BLANK group, urine protein and BUN levels were significantly increased in the Sham group, whereas transfection of pcDNA-C1q significantly decreased these levels (P<0.01; Fig. 1B and C). ELISA analysis showed that the levels of serum anti-C1q and anti-dsDNA were significantly elevated in the Sham group compared with the BLANK group. Overexpression of C1q reduced the levels of serum anti-C1q and anti-dsDNA compared with the pc-NC group (Fig. 1D and E). Compared with the BLANK group, H&E staining showed a higher histological damage index

of glomeruli in the Sham group. Additionally, the histological damage index was lower in the pcDNA-C1q group compared with that in the pc-NC group (Fig. 1F). These results suggested that C1q overexpression may attenuate renal injury in LN mice.

Overexpression of C1q attenuates renal inflammation in LN mice. ELISA analysis revealed that the levels of TNF-α, IL-6 and IL-1β in renal tissues in the Sham group were markedly increased compared with those in the BLANK group. Overexpression of C1q significantly reduced the levels of TNF-α, IL-6 and IL-1β in renal tissues in the pcDNA-C1q compared with the pc-NC group (Fig. 2A-C). Overall, these results indicated that C1q overexpression may alleviate the renal inflammation in LN mice.

Overexpression of C1q reduces macrophage infiltration and MC proliferation in renal tissues of LN mice. To investigate the effect of C1q on macrophage infiltration and the proliferation of MCs in the renal tissues of LN mice, the percentages of CD68- and Ki67-positivity were measured using immunofluorescence staining. The percentage of CD68-positivity in Sham group renal tissues was higher compared with that of the BLANK group. Furthermore, compared with the Sham group, this percentage was decreased in the pcDNA-C1q group (Fig. 3A). In addition, with the BLANK group, the percentage of Ki67-positivity in renal tissues was increased in the Sham group and was decreased by C1q-overexpression (Fig. 3B). Taken together, C1q overexpression may decrease macrophage infiltration and MC proliferation in renal tissues of LN mice.

Overexpression of C1q inhibits the NF-κB pathway in the renal tissues of LN mice. To evaluate the effect of C1q-overexpression on the NF-κB pathway in LN mouse renal tissues, the expression of NF-κB-related proteins was measured via western blotting. Compared with the BLANK group, the protein expression of p-IκBα/IκBα and p-NF-κB p65/NF-κB p65 in renal tissues was significantly increased in the Sham group. These levels in renal tissues were significantly decreased in the pcDNA-C1q group compared with the Sham group. PMA, an activator of the NF-κB pathway (25), was injected into the pcDNA-C1q-treated LN mice. Consequently, the inhibitory effect of C1q on the NF-κB pathway was attenuated by PMA compared with the pcDNA-C1q group (Fig. 4). These results demonstrated that C1q overexpression may inhibit the NF-κB pathway in the renal tissues of LN mice.

Overexpression of C1q ameliorates renal injury in LN mice via inhibiting the NF-κB pathway. PMA was administered to LN mice, and the levels of renal inflammatory factors were measured. The results showed that overexpression of C1q significantly decreased the levels of TNF-α, IL-1β and IL-6 in the renal tissues of LN mice (Fig. 5A). In addition, immunofluorescence staining demonstrated that the percentages of CD68- and Ki67-positivity in renal tissues were decreased in the pcDNA-C1q group compared with the pc-NC group (Fig. 5B and C). Moreover, PMA attenuated the effects of C1q on inflammatory factors and percentages of CD68- and Ki67-positivity in LN mice renal tissues (Fig. 5A-C). Overall this indicated that C1q overexpression may ameliorate renal injury by inhibiting the NF-κB pathway in LN mice.

Discussion

The MRL/lpr mouse model is frequently recognized as a suitable model of human LN (26,27). In the present study, C1q expression was decreased in LN mice. The 24-h urine protein and BUN levels are reliable measures of renal function in patients with LN (28,29), and, in the present study, these levels were decreased by C1q-overexpression in LN mice. Anti-C1q and anti-dsDNA are valuable biological markers for the prediction of human LN (30,31). It was shown in the present study that transfection of pcDNA-C1q significantly reduced the levels of serum anti-C1q and anti-dsDNA in LN mice. In addition, overexpression of C1q also decreased the histological damage index of glomeruli in LN mice. Taken together, these

results suggested that C1q alleviates renal injury in LN mice through improving renal function and attenuating histological damage.

Proinflammatory cytokines play critical roles in the occurrence and developmental process of LN (32,33). For example, nucleotide-binding oligomerization domain-containing protein 2 was found to participate in LN pathogenesis through promoting the release of proinflammatory cytokines (34). Upregulation of microRNA-146a alleviated LN in patients via suppressing the gene expression of TNF-α, IL-1β and IL-6 (35). In the present study, overexpression of C1q significantly decreased the levels of proinflammatory cytokines in the renal tissues of LN mice. CD68-positivity is a macrophage marker in renal diseases (36). Macrophage infiltration in renal tissues induced by T cells is related to podocyte injury in patients with LN (37). Ki67 is a nuclear protein associated with the cell cycle and is used as a marker for MC proliferation in glomeruli (38). Normal MC proliferation is involved in maintaining glomerular function and structure (39). The results of the present study demonstrated that C1q-overexpression significantly reduced macrophage infiltration and MC proliferation in the renal tissues of LN mice. Together, these data indicated that C1q protected against LN by decreasing inflammation, macrophage infiltration and MC proliferation in renal tissues.

Previous research found that the NF-κB pathway participates in LN progression (40,41). In the present study, the expression of NF-κB-related proteins in the renal tissues of LN mice was significantly decreased by treatment with pcDNA-C1q, indicating that C1q inhibited the NF-κB pathway in these tissues. The NF-κB pathway was found to play a role in LN inflammation, and was ameliorated by demethylzeylasteral through attenuating the NF-κB pathway (42). NF-κB-related proteins are also related to the macrophage infiltration and MC proliferation in renal diseases. Curcumin alleviated macrophage infiltration of diabetic nephropathy by inhibiting NF-κB activation (43). Resveratrol reduced renal MC proliferation in diabetic nephropathy via downregulating the expression of NF-κB-related proteins (44). In the present study, it was found that overexpression of C1q decreased inflammation, macrophage infiltration and MC proliferation through inhibiting the NF-κB pathway in the renal tissues of LN mice. To further confirm this result, LN mice were treated with PMA, an NF-κB pathway activator (45). The results showed that PMA effectively reversed the inhibitory effect of C1q on inflammation, macrophage infiltration and MC proliferation in the renal tissues of LN mice. Taken together, it was demonstrated that C1q may protect against LN through inhibiting the NF-κB pathway.

The present study has some limitations. Firstly, only glomerular nephritis in mice was analyzed. Interstitial nephritis, another type of renal inflammation in LN mice (46), may more comprehensively reflect the histopathological changes in LN. Secondly, IFN-γ, a key regulator in renal tissues, was not detected. IFN-γ plays a role in the perpetuation of local inflammatory processes in the kidney by the activation of monocytes, macrophages or renal resident cells. The expression of IFN-γ in renal tissues may better reflect the inflammatory changes in LN. Finally, the detailed mechanism of action of C1q in LN remains to be studied. Further *in vitro*

experiments are needed to identify the regulatory mechanism of C1q in the development of LN.

In summary, C1q expression was decreased in the renal tissues of LN mice. Overexpression of C1q alleviated inflammation and macrophage infiltration and inhibited MC proliferation in these tissues. The protective effect of C1q on LN was closely associated with the suppression of the NF-κB pathway. Therefore, C1q may be a promising therapeutic target for LN.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JS conceived and designed the study, performed the data analyses and wrote the manuscript. SG, FN and DL contributed to the conception of the study. JS, SG, FN and DL performed the experiments. YZ contributed to analysis and manuscript preparation. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Linyi Central Hospital (approval no. 2020013; Linyi, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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