Beneficial effect of magnolol on lupus nephritis in MRL/lpr mice by attenuating the NLRP3 inflammasome and NF-κB signaling pathway: A mechanistic analysis

FENG HUANG[1], RUI-YUN ZHANG[2] and LEI SONG[1]

[1]Department of Urology and [2]Headquarters of Emergency Room, Linyi People's Hospital, Linyi, Shandong 276003, P.R. China

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Abstract. Lupus nephritis (LN) is a common complication of systemic lupus erythematosus. The present study aimed to elucidate the protective effect of magnolol (MG) on the progression of LN, via inhibition of key signaling pathways. The results of the present study demonstrated that administration of MG caused inhibition of the activation of NACHT, LRR and PYD domains-containing protein 3 and interleukin-1β production. Histopathological analysis confirmed that the vehicle-treated group exhibited characteristic glomerular disease, which was observed to be suppressed following the administration of MG; a marked decrease in glomerular and vascular lesions was observed compared with the vehicle control. This decrease was further demonstrated through analysis of kidney sections. The expression level of cell surface glycoprotein F4/80 was demonstrated to be markedly decreased in the MG-treated mice compared with the vehicle control group. The MG-treated mice exhibited a marked decrease in serum and renal tumor necrosis factor-α expression levels.

Introduction

Among the chronic autoimmune diseases, lupus nephritis (LN) is characterized by complex inflammation of nephrons in the kidney, affecting primarily women of child-bearing age (1). LN is caused by systemic lupus erythematosus (SLE), a disease which results in the destruction of bodily tissues by the immune system (2). It has been estimated that, in China alone, the incidence of LN is increasing and affects ~7/10,000 individuals (3). Immune complex glomerulonephritis is considered to be an important cause of morbidity and mortality.

There are a number of treatment options for LN. These therapeutic interventions include corticosteroids, immunosuppressive agents, and drugs to prevent the formation of blood clots or to reduce blood pressure (4). Effective management of the disease may improve the survival rate of patients, although it does not exert a marked effect on life expectancy compared with the general population (5). In addition, treatment is frequently associated with limitations and side-effects which decrease clinical utility. The efficacy of anti-inflammatory agents in the management of LN has been well-established (6). Anti-inflammatory agents are able to target the activation of nuclear factor (NF)-κB, as a primary mechanism of providing relief from the associated inflammation. Dormant NF-κB exists in the bound form with inhibitor of NF-κB (IκB) molecules in the cytoplasm, and activation by numerous stimuli causes phosphorylation of IκB by IκB kinases (IKKs). Phosphorylation of IκB results in the degradation of NF-κB from the bound complex and its translocation to the nucleus, where it promotes the transcription of genes associated with inflammation (7). A number of studies have demonstrated that NF-κB signaling serves a critical role in the progression of lupus-like disease. In humans, the level of IκB kinase-α expression has been observed to be increased with activated NF-κB in the kidneys, and demonstrated to serve a role in LN (8-10).

Therefore, research is required to identify novel and selective agents that are able to modulate the important catalytic pathways of LN, in order to inhibit the progression of the disease with minimal side effects. Magnolol (MG), a bioactive compound obtained from the bark of *Magnolia officinalis* or *M. grandiflora*, has been demonstrated to be associated with numerous pharmacological properties, including anti-oxidative (11), anti-inflammatory (12), antitumor (13) and anti-microbial properties (14), in preclinical models. The present study aimed to elucidate the effect of MG on LN in MRL/lpr mice.

Materials and methods

Mice. A total of 16 female MRL/lpr mice (age, 12 weeks; weight, 30±5 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Throughout the study, MRL/lpr mice of comparable sex and age served as healthy controls. Mice were housed in polypropylene cages in a controlled environment under a 12-h light/dark cycle, at 21˚C with free access to laboratory food and water. The present

Correspondence to: Dr Lei Song, Department of Urology, Linyi People's Hospital, 27 Jiefang Road, Linyi, Shandong 276003, P.R. China
E-mail: songleislsl@hotmail.com

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study was ethically approved and performed according to the guidelines of the Institutional Animal Ethical Committee of Linyi People's Hospital (Linyi, China).

Treatment protocols. The MRL/lpr mice of 12 weeks of age were divided randomly into two groups (8 mice/group). A solution of MG (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), in 10% (v/v) dimethyl sulfoxide in PBS, was prepared for the assay. A dose of 5 mg/kg MG was administered via intraperitoneal injection 3 times/week, while the control animals received only vehicle solution. The MG was administered for 8 weeks, and the mice were sacrificed at the age of 20 weeks. For further analysis, blood and kidney samples were taken and the residual blood was removed from the kidney for preservation. The kidney sections were fixed in paraffin containing 10% neutral-buffered formalin at room temperature for 8 h. The remaining kidney tissue was preserved in liquid nitrogen for further analysis.

Estimation of renal function. Renal function was estimated through quantification of the level of blood urea nitrogen (BUN) using an auto-analyzer, at week 20. From the first week of the experiment, urine was collected every 12 h for 2 weeks. The protein content of the urine was determined using Multistix 10SG reagent strips (Bayer AG, Leverkusen, Germany), according to the manufacturer's protocol.

Evaluation of histopathology and immune complex. For the assessment of morphological changes in the kidney, sections of kidney fixed in paraffin were stained with 0.2% hematoxylin and eosin, and with periodic acid-Schiff reagents at room temperature for 8 h. The histopathology of the vascular and glomerular system was semi-quantitatively analyzed by an independent histopathologist. For the estimation of the renal immune complex, kidney sections (3 μm) frozen in liquid nitrogen were stained for 90 min at room temperature with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (Ig)G (1:120; cat no. sc-2012; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), following masking with 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA). The absorbance of the samples was measured between 250 and 600 nm using a UV-1650 PC UV-Vis spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Experiments were performed in triplicate and the mean value was calculated.

ELISA. The 96-well plates used for this assay had been previously prepared with 5 μg/ml calf thymus double-stranded (ds) DNA (Sigma-Aldrich; Merck KGaA). The reference standard curve was prepared using mouse anti-dsDNA monoclonal antibody (1:200; cat no. MAB030; EMD Millipore, Billerica, MA, USA). The absorbance was measured at 450 nm and the anti-dsDNA concentrations were estimated using a Vmax microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA). Normal mouse IgG served as a negative control.

ELISA kits obtained from R&D Systems, Inc. (Minneapolis, MN, USA) were used to analyze the levels of the pro-inflammatory cytokines interleukin (IL)-1β (cat no. RLB00) and tumor necrosis factor (TNF)-α (cat no. RTA00) in serum and kidney homogenates, according to the manufacturer's protocol.

Western blot analysis for total protein estimation. Cell lysis buffer containing 50 μM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 100 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology, Haimen, China) was used to extract total proteins from kidney tissue samples. Protein concentrations were determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Nuclear and cytosolic proteins were extracted using a nuclear extraction kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). A total of 60 μg protein was subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane (EMD Millipore). The membrane was blocked with 5% non-fat milk in TBS containing 0.1% Tween-20 at room temperature for 1 h. The membrane was then incubated with the following primary antibodies overnight at 4˚C: Anti-apoptosis-associated speck-like protein containing a CARD (Asc; 1:200; cat no. sc-514414; Santa Cruz Biotechnology, Inc.), anti-NACHT, LRR and PYD domains-containing protein 3 (NLRP3; 1:1,000; cat no. ab4207; Abcam, Cambridge, UK), anti-NF-κB-p65 (1:1,000; cat no. ab16502; Abcam), anti-caspase-1 p20 (1:10,000; cat no. sc-1218; Santa Cruz Biotechnology, Inc.), anti-cell surface glycoprotein F4/80 (F4/80; 1:100; cat no. ab6640), anti-C-C motif chemokine 2 (CCL2; 1:200; cat no. ab25124) (both from Abcam) and anti-phosphorylated-IκB (1:200; cat no. 4814; Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (1:20,000; cat no. ab205718; Abcam) at 25˚C for 1 h, following washing with PBS. Anti-GAPDH antibody (1:2,000; cat no. 2118; Cell Signaling Technology, Inc.) served as an internal control. Protein bands were visualized using enhanced chemiluminescence (EMD Millipore). Blots were semi-quantified by densitometric analysis using ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Experimental data are presented as the mean ± standard error of the mean of 3 independent experiments. The statistical significance of the differences between groups was assessed using Student's t-test, one-way analysis of variance followed by a post hoc Tukey test, or nonparametric Wilcoxon rank-sum test. $P<0.05$ was considered to indicate a statistically significant difference. GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses.

Results and Discussion

During the inflammatory response, the increase in leukocyte infiltration has been hypothesized to be the primary cause of tissue injury and disease progression (15-17). However, consistent with other inflammatory diseases, LN leads to increased permeability, which implicates the role of activated NF-κB in the disease (18-22). Inhibition of NF-κB activation has been demonstrated to be responsible for the suppression of renal tubulo-interstitial injury induced by proteinuria. In addition, a number of studies have demonstrated that increased expression of IKK-α, and increased NF-κB activation, are associated with the extent of renal lesions and loss of function (23-26). In the present study (Fig. 1), it was demonstrated that the

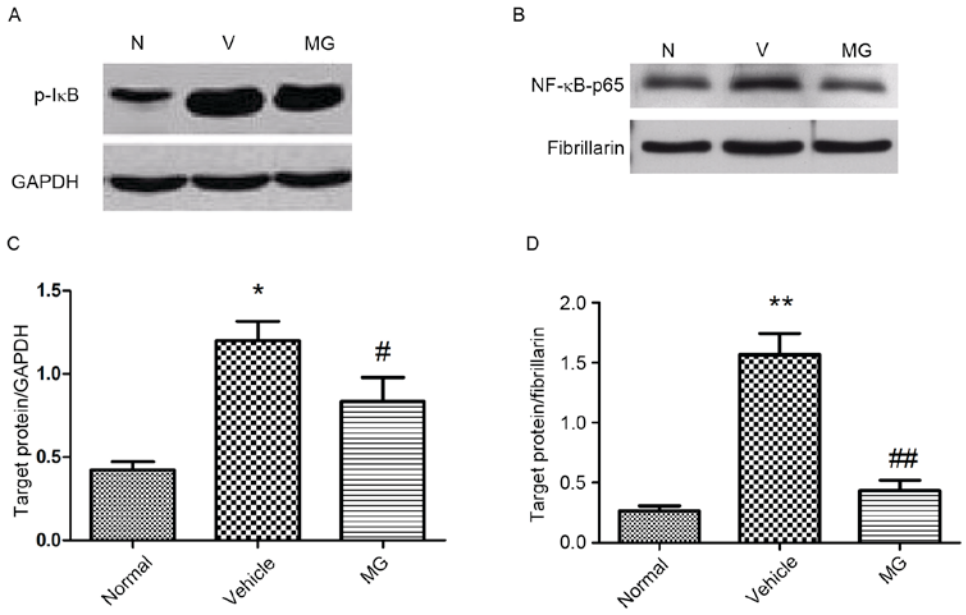

Figure 1. Effect of MG on NF-κB activation in the kidneys of MRL/lpr mice determined using western blot analysis. (A) Expression level of p-IκB. (B) Expression level of NF-κB-p65. Semi-quantitative analysis of the expression of (C) p-IκB and (D) NF-κB. Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01 vs. normal; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. vehicle. MG, magnolol; NF-κB, nuclear factor-κB; p-IκB, phosphorylated inhibitor of NF-κB; N, healthy control; V, vehicle control.

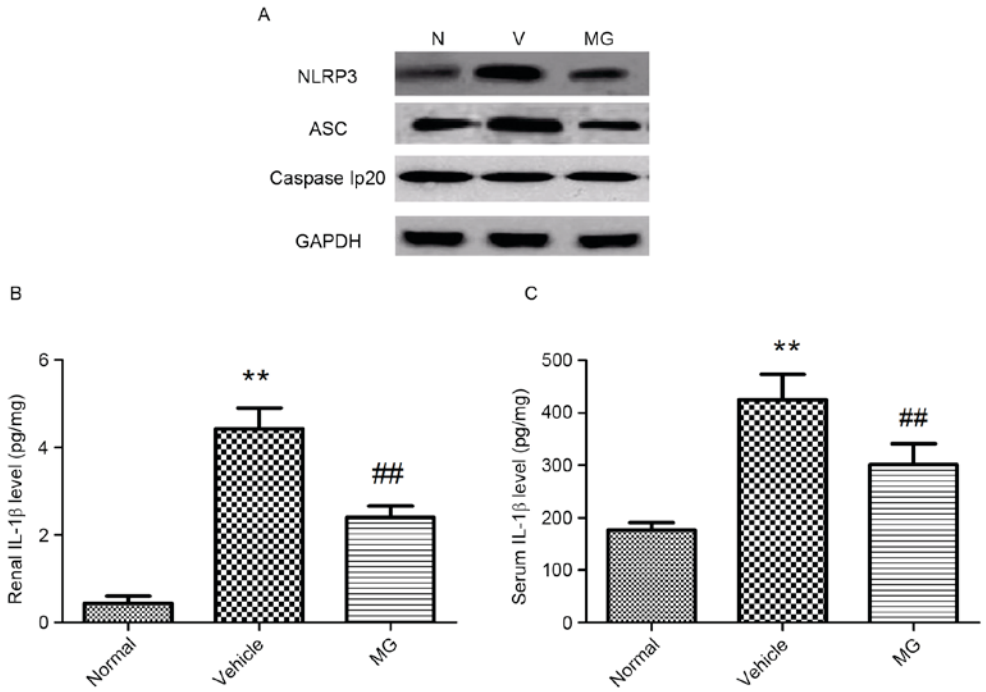

Figure 2. Effect of treatment with MG on NLRP3 inflammasome activation in the kidneys of MRL/lpr mice. (A) Western blot analysis of NLPR3 inflammasome activation. (B) Renal and (C) serum IL-1β levels. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. normal; $^{\#\#}$P<0.01 vs. vehicle. MG, magnolol; NLRP3, NACHT, LRR and PYD domains-containing protein 3; IL-1β, interleukin-1β; ASC, anti-apoptosis-associated speck-like protein containing a CARD; N, healthy control; V, vehicle control.

phosphorylation of IκB increased in the kidneys of the treated mice compared with the healthy controls (Fig. 1A and C). As presented in Fig. 1B and D, the level of NF-κB-p65 was additionally increased compared with the normal control. Therefore, the results of the present study demonstrated that administration of MG at the tested dose led to a decrease in NF-κB expression.

The role of the NLRP3 inflammasome/IL-1β signaling pathway in the inflammatory and immune response has been demonstrated in the kidneys of NZB/WF1 lupus-prone mice (27). The pathway has been demonstrated to be overexpressed in patients with SLE, suggesting its clinical significance as an important biomarker of the disease (28,29). Numerous studies have suggested that the U1-small nuclear

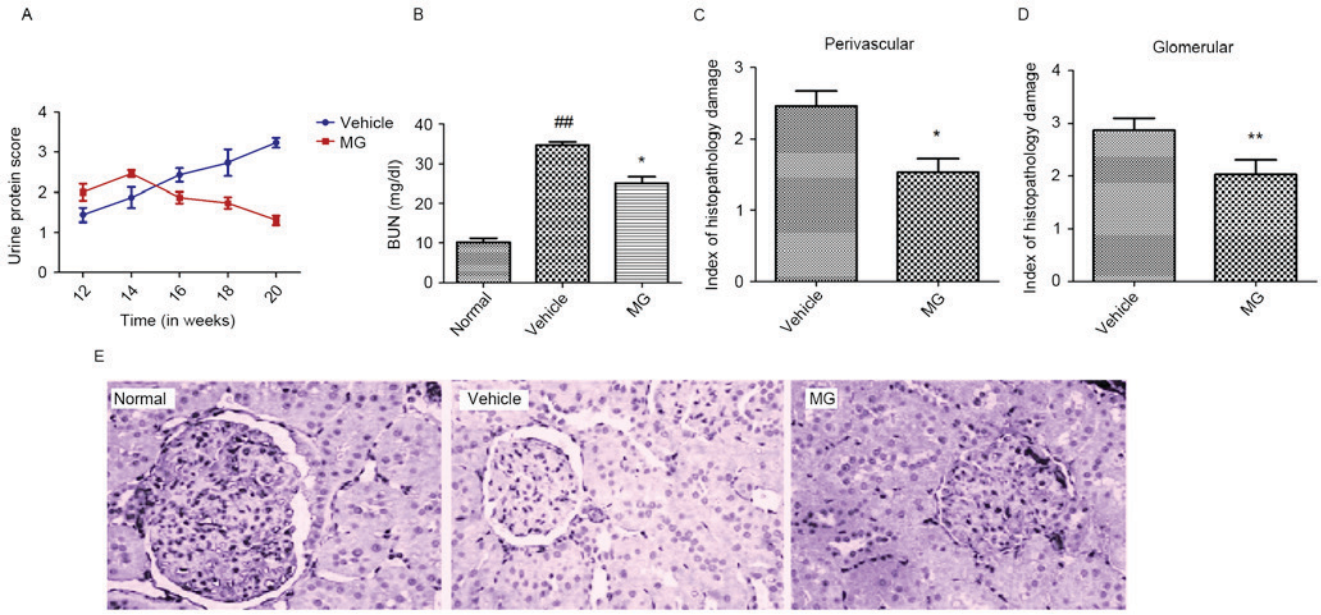

Figure 3. Effect of treatment with MG on renal lesions in MRL/lpr mice. (A) Urine protein excretion analysis. (B) BUN analysis. Histological damage index of (C) perivascular and (D) glomerular kidney tissue in MRL/lpr mice. (E) Decrease in inflammation and proliferation in kidney sections following treatment with MG (magnification, x200). Data are presented as the mean ± standard error of the mean. $^{\#\#}$P<0.01 vs. normal; *P<0.05, **P<0.01 vs. vehicle. MG, magnolol; BUN, blood urea nitrogen.

ribonucleo protein (snRNP) stimulates the NLRP3 inflammasome in human cluster of differentiation 14+ monocytes, which are particularly dependent on anti-U1-snRNP antibody. U1-snRNP leads to overproduction of IL-1β, which has been observed to be significantly elevated in LN. This overproduction has additionally been promoted by dsDNA from human monocytes following innervation of the NLRP3 inflammasome in the presence of anti-dsDNA antibodies (30-34). The results of the present study were consistent with previous reports, demonstrating that NLRP3 inflammasome components were elevated in MRL/lpr mice compared with healthy controls and the vehicle treated group (Fig. 2). The expression of caspase-1p20 and IL-1β, in addition to the level of NLRP3 activation, was observed to be decreased in the kidney homogenates of MG-treated mice compared with the healthy control group. The results additionally demonstrated that the administration of MG caused inhibition of the activation of NLRP3, and IL-1β production. Therefore, it may be hypothesized that the tested dose of MG suppressed LN by inhibiting NLRP3 activation and the NLRP3 inflammasome complex.

In order to investigate the effect of MG on renal function, the protein urea level was determined. Increased protein urea levels have been observed to be associated with loss of renal function (35). In the present study, it was observed that the two groups of MRL/lpr mice exhibited an increase in proteinuria at12 weeks of age (Fig. 3A). By contrast, between 14 and 16 weeks of age, the MG-treated mice exhibited a significantly improved condition compared with the vehicle-treated group (P<0.05). In addition, the level of BUN was decreased in the MG-treated group compared with the vehicle-treated group (Fig. 3B). In the histopathological examination, the vehicle-treated group exhibited characteristic glomerular disease. As presented in Fig. 3C and D, the administration of MG led to a marked decrease in glomerular and vascular lesions compared with the vehicle control group. The results of the present study were further demonstrated through histopathological analysis of kidney sections (Fig. 3E).

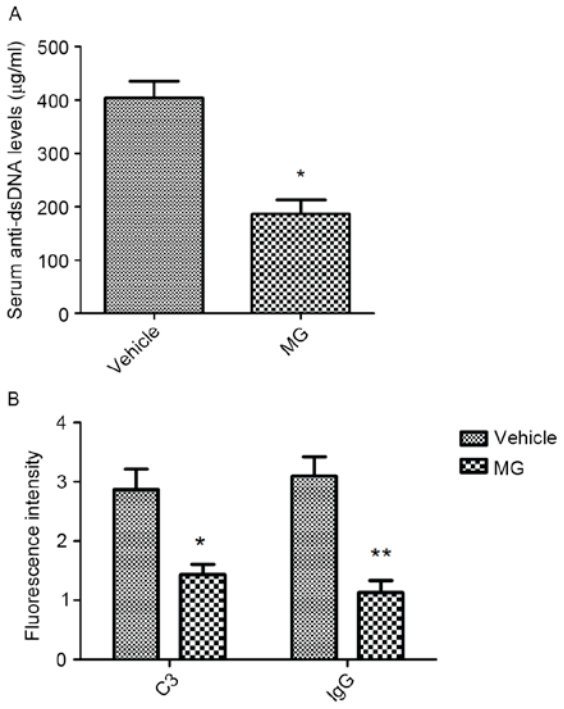

Figure 4. Effect of treatment with MG on serum anti-dsDNA antibody and renal deposition of immune complexes in MRL/lpr mice. (A) A decrease was observed in serum anti-dsDNA antibody in MG-treated mice. (B) A decrease was observed in the fluorescence intensity of C3 and IgG in the MG-treated group. Data are presented as the mean ± standard error of the mean. *P<0.05, **P<0.01 vs. vehicle. MG, magnolol; ds, double-stranded; C3, complement C3; IgG, immunoglobulin G.

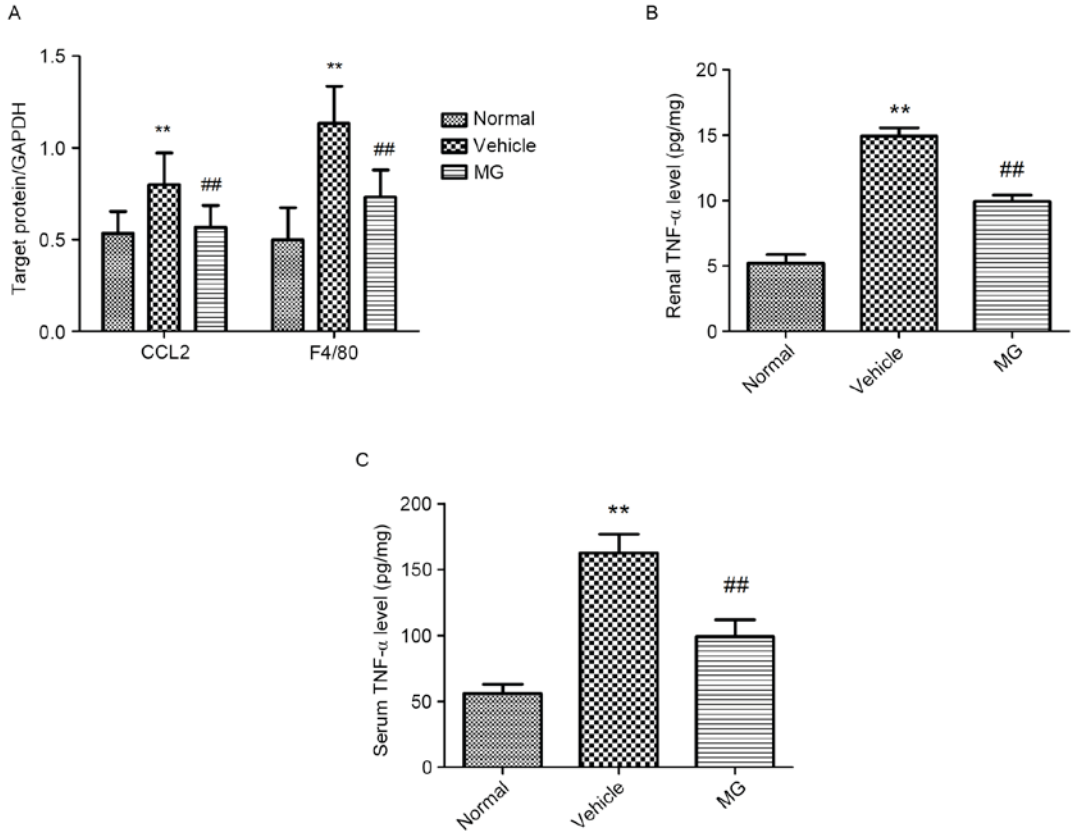

Figure 5. Effect of treatment with MG on the level of macrophage infiltration, CCL2 and TNF-α in MRL/lpr mice. (A) A decrease was observed in F4/80 and CCL2, in MG-treated mice. A decrease in (B) renal and (C) serum TNF-α levels was observed in MG-treated mice. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. normal; $^{\#\#}$P<0.01 vs. vehicle. MG, magnolol; CCL2, C-C motif chemokine 2; TNF-α, tumor necrosis factor-α; F4/80, cell surface glycoprotein F4/80.

As LN progresses, the levels of serum anti-dsDNA increase (36). In the MRL/lpr mice in the present study, the level of serum anti-dsDNA antibody increased, while the control mice exhibited no detectable anti-dsDNA. Following treatment with MG, the mice exhibited a significant decrease in the concentration of anti-dsDNA (Fig. 4A). As presented in Fig. 4B, the levels of IgG were observed to be markedly decreased in the kidneys of the MG-treated mice compared with non-treated mice. The results of the present study demonstrated that MG exerts an effect on the suppression of the production of auto antibodies and immune complex deposition. The effect of MG was additionally investigated on the infiltration of macrophages and the expression of CCL2 and TNF-α (Fig. 5). The activation of macrophages was measured by quantifying F4/80, a specific biomarker. The results of the present study demonstrated that the level of F4/80 was decreased in the MG-treated mice compared with the vehicle control group. Previous studies have reported that the levels of TNF-α and CCL2 were aberrantly activated during inflammation (37,38). As presented in Fig. 5, the MG-treated mice exhibited a significant reduction in serum and renal TNF-α levels.

In conclusion, treatment with MG may provide a protective effect against the progression of LN via the inhibition of key signaling pathways. MG was able to inhibit the NLRP3 inflammasome and NF-κB pathways, which consequently affects macrophage infiltration. The results of the present study demonstrated the potential benefit of MG in the treatment of LN. However, further studies are required to elucidate the detailed molecular mechanism.

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