Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;50:1560-1573
DOI: 10.1159/000494654
Published online: 26 October 2018
Accepted: 18 October 2018

Original Paper

Mangiferin Attenuates Murine Lupus Nephritis by Inducing CD4$^+$Foxp3$^+$ Regulatory T Cells via Suppression of mTOR Signaling

Chun-Ling Liang[a] Weihui Lu[a] Jiu-Yao Zhou[b] Yuchao Chen[a] Qunfang Zhang[a] Huazhen Liu[a] Feifei Qiu[a] Zhenhua Dai[a]

[a]Section of Immunology and Joint Immunology Program, the Second Affiliated Hospital, Guangzhou University of Chinese Medicine, and Guangdong Provincial Academy of Chinese Medical Sciences, Guangzhou, [b]School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou, China

Key Words
Immunosuppression • Immunoregulation • Lupus nephritis • Mangiferin • And Treg

Abstract
Background/Aims: Lupus nephritis (LN) is an autoimmune glomerulonephritis that frequently develops secondary to systemic lupus erythematosus. Patients with LN require extensive treatments with global immunosuppressive agents. However, long-term treatment with conventional immunosuppressants may cause various side effects. Therefore, it's important to seek alternative drugs for treating LN. Here we aimed to investigate the immunoregulatory effects of mangiferin (MG), an ingredient that was originally extracted from natural herbs, including *Mangifera Indica Linn.* and *Rhizoma Anemarrhenae*. ***Methods:*** FasL-deficient B6/gld mice were used as a spontaneous LN model. The serum anti-dsDNA Ab and creatinine levels were analyzed via ELISA. Renal histology and immunopathology were determined using H&E and PAS staining, immunofluorescence (IgG and C3), and IHC staining (CD3 and α-SMA). Cytokine gene expression was measured by RT-PCR assays while effector T cells and Tregs were enumerated by flow analysis. Finally, the proliferation and apoptosis of T cells were measured by CFSE staining and flow analysis while their mTOR signaling was detected through Western blotting. ***Results:*** We found that administration of MG ameliorated LN in lupus-prone B6/gld mice by reducing the urinary protein and serum creatinine levels, diminishing T cell infiltration in kidneys and improving renal immunopathology. MG also significantly lowered the percentages of CD44^{high}CD62L^{low} effector T cells in B6/gld mice. Importantly, treatments with MG augmented CD4$^+$FoxP3$^+$ Treg frequencies in spleens, lymph nodes and kidneys of B6/gld mice. It also induced CD4$^+$FoxP3$^+$ Tregs from CD3$^+$ T cells *in vitro* and

C.-L. Liang and W. Lu contributed equally to this work.

Zhenhua Dai
Section of Immunology, Guangdong Provincial Academy of Chinese Medical Sciences
55 Nei Huan Xi Lu Guangzhou, Guangdong 510006 (China)
Tel. 86-20-39318479, E-Mail zdai2009@outlook.com

promoted Treg proliferation. Furthermore, it inhibited $CD3^+$ T cell proliferation *in vitro* and suppressed their phosphorylation of mTOR and its downstream P70S6K. However, MG did not promote T cell apoptosis, implying that it is not cytotoxic. Depletion of $CD4^+CD25^+FoxP3^+$ Tregs in B6/gld mice abrogated its therapeutic effects on LN. ***Conclusion:*** MG exerts a novel therapeutic effect on murine LN via upregulating $CD4^+FoxP3^+$ Tregs, downregulating mTOR/p70S6K pathway and improving renal immunopathology. It may be useful for treating LN in clinic.

Introduction

Lupus nephritis (LN) is an autoimmune glomerulonephritis that develops secondary to systemic lupus erythematosus (SLE). The mechanisms underlying LN-associated renal damage include the hyperactivity of T and B lymphocytes, aberrant production of autoantibodies, formation of immune complexes that deposit in kidney tissues, and the release of subsequent proinflammatory mediators [1, 2]. T regulatory cells (Tregs), a small subset of T cells that exert immunosuppressive activities on effector immune cells, play a crucial role in maintaining immune homeostasis and tolerance [3]. Dysregulated Treg number or function has been identified in multiple autoimmune diseases, such as SLE and LN. Most of the Treg-based therapeutic approaches devoted to increasing the number and/or function of Tregs have shown promise and potential in treating SLE [4-6].

Current strategies for treating LN are mainly use of immunosuppressive drugs, including steroids, cyclophosphamide and calcineurin inhibitors, but various side effects resulting from conventional immunosuppressants are often associated with poor outcomes. Mangiferin (MG), 1, 3,6, 7-hydroxyxanthone-C2-β-D-glucoside, mainly exists in herbs, such as *Mangifera Indica Linn*. leaf*, Rhizoma Anemarrhenae, and Rhizoma Belamcandae.* MG has been shown to exert various biological activities and therapeutic effects, including anti-inflammatory [7], anti-oxidative [8], anti-tumor [9] and anti-diabetic effects [10] as well as regulation of metabolism [11]. Recent studies have demonstrated that MG attenuates inflammation in experimental colitis [12], corrects the imbalance of Th17/Treg cells in mice with TNBS-induced colitis [13] and inhibits macrophage activation. Therefore, we hypothesized that MG may be effective in treating LN via induction of Tregs. We utilized a mouse model of LN, B6/gld mice, that spontaneously develop systemic autoimmunity, such as SLE and LN, due to the mutation of Fas ligand gene with an enlarged spleen and lymph node and increased numbers of T and B cells [14]. A majority of female B6/gld mice develop SLE and LN after the age of 24 weeks. Our results demonstrated that MG protected lupus-prone B6/gld mice from developing LN and exerted anti-inflammatory and immunoregulatory effects by inducing $CD4^+FoxP3^+$ Tregs and suppressing T cell proliferation through regulating mTOR/p70S6K pathway.

Materials and Methods

Mice and animal treatments

FasL-deficient B6/gld (B6Smn.C3-Tnfsf6gld/J) and MRL/MpJ-lpr mice were purchased from the Jackson Laboratory (Bar Arbor, ME, USA) while wild-type C57BL/6 mice were obtained from Guangdong Medical Laboratory Animal Center (Fushan, Guangdong, China). All mice were housed in a specific pathogen-free (SPF) environment at 25 ± 2°C with 65% humidity. All experiments were conformed to the regulations of the National Institute of Health of USA while the animal protocol was approved by the Institutional Animal Care and Use Committee of Guangdong Provincial Hospital of Chinese Medicine. At the age of 20 weeks, mice were randomized into five groups, and mangiferin (20 or 40 mg/kg, 98% purity, Daosifu Bio-Technique Inc, Nanjing, China.), prednisone (5 mg/kg, Sigma, Shanghai, China), or distilled water containing 0.4% CMC-Na (Sodium Carboxymethyl Cellulose) was administrated daily using an oral gavage method for 12 weeks. In

some groups, depleting anti-CD25 Ab (PC61, eBioscience) was administered at 0.2mg every other day for four times beginning at the time of initial MG treatment.

Measurement of urinary protein and serum chemistries

Urine samples were collected every four weeks by housing the mice in metabolic cages for 24 hours. All mice were free to access water but forbidden to access food during the time period of sample collection. Urinary protein was determined by a colorimetric method. After the last administration, all mice were scarified for blood samples. Serum was separated by centrifugation at 4°C at 3500 rpm for 15 min and the serum anti-dsDNA Ab and creatinine (Scr) levels were analyzed using murine ds-DNA standard enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) and creatinine assay kit (Nanjing Jiancheng Bioengineering institute, Nanjing, China), respectively.

Histopathology analysis

The renal tissues were fixed with 10% neutral formalin phosphate buffer, dehydrated through a series of graded alcohol, and embedded in paraffin. Kidney sections (3 μm) were stained for hematoxylin-eosin (H&E) or Periodic acid-Schiff (PAS). Glomerular mesangial proliferation, inflammatory cell infiltration, the pathological change in mesangial matrix and glomerular basement membrane, and renal tubular damage were evaluated under 400× and 200× light microscope fields.

Immunofluorescence

Immunofluorescence staining was carried out on frozen sections embedded in OTC. Renal tissues were sectioned at 5 μm using freezing microtome. Then they were fixed in acetone for 10 min, washed in PBS for 3 min, stained with 1:100 diluted rabbit anti-rat IgG or C3 antibody (Proteintech Group, Wuhan, China) at 4°C overnight, washed twice in PBS, incubated with FITC or Cy™3-conjugated goat anti-rabbit IgG (Proteintech Group, Wuhan, China). These sections finally were mounted using DAPI-Fluoromount-G clear mounting agents (SouthernBiotech, Birmingham, UK) and examined under an inverted fluorescence microscope with eight micrographs per slice obtained randomly (magnification 400×). The fluorescence signal was quantified using image-processing software (Image J 1.47), with six sections per group. The data were presented as average density (per pixel) (average density = integrated density/area).

Immunohistochemistry (IHC)

Kidney tissues were fixed with 10% neutral formalin phosphate buffer, dehydrated using a series of graded alcohol, embedded in paraffin, and cut into 3-μm sections. The paraffin sections were processed for immunohistochemistry by deparaffinization. Antigen retrieval was performed using sodium citrate solution (0.01M，PH: 6.0) for antigen repair, and the tissues were then quenched in 3% hydrogen peroxide solution to block any endogenous peroxidase activity, and incubated with primary rabbit anti-CD3 or anti-α-SMA (smooth muscle actin) antibody (Abcam, Cambridge, UK) overnight at 4°C. A Non-Biotin MaxVisionTM2-HRP–Polymer anti-mouse IgG detection system (Maixin Biotech, Fuzhou, China) was used to visualize the bound primary antibody. Sections were incubated sequentially with reagent 1 (Polymer Hepler) and reagent 2 (polyperoxidase-anti-mouse/rabbit IgG) for 30 min at room temperature, washed, and counterstained with 3'-diaminobenzidine substrate.

Cytokine gene expression

Intrarenal mRNA expressions of TNF-α, IFN-γ and IL-6 were measured. Renal RNA was extracted using RNAiso Plus reagent (Takara biomedical technology, Being, China). RNA concentration was determined according to absorbance at 260 nm and purity was evaluated by A260/A280 ratios. cDNAs were synthesized using Takara Reverse transcription reagent (Takara biomedical technology, Being, China). SYBR Premix EX Taq (Takara biomedical technology, Being, China) was utilized for the Polymerase chain reaction (PCR) using ViiA 7 Sequence Detection System. The oligonucleotide primers were described as following: TNF-α (Forward 5'-ACTGATGAGAGGGAGGCCAT-3', Reverse 5'-CCGTGGGTTGGACAGATGAA-3'), IFN-γ (Forward 5'-CACGGCACAGTCATTGAAAG-3', Reverse 5'-CATCCTTTTGCCAGTTCCTC-3'), IL-6 (Forward 5'-TTCTTGGGACTGATGCTGGT-3', Reverse 5'-CCTCCGACTTGTGAAGTGGT-3'), GAPDH (Forward 5'-GGAGAGTGTTTCCTCGTCCC-3', Reverse 5'-ACTGTGCCGTTGAATTTGCC-3'). All samples were run in triplicates and the relative expression values were normalized using GADPH values.

Flow cytometry

Single-cell suspensions from spleens, draining lymph nodes and kidneys were prepared. Briefly, the spleens, lymph nodes or perfused kidneys were minced and filtered through 40 μm nylon meshes. Subsequently, erythrocytes in spleen samples were lysed with ammonium chloride (BD Biosciences, San Jose, CA). And the resulting cells were stained with fluorochrome-conjugated Abs against surface markers CD4 (PE), CD8 (PE), CD44 (PE-Cy5) and/or CD62L (FITC) (BD Biosciences, San Jose, CA). For intracellular FoxP3 staining, cells were first stained with anti-CD4-PE, permeabilized using intracellular fixation/permeabilization kit (eBioscience, San Diego, CA), and then stained with anti-FoxP3 (APC) Ab (eBioscience, San Diego, CA). Effecter T cells (CD4+CD44+CD62L^{low} or CD8+CD44+CD62L^{low}) and CD4+Foxp3+ Tregs were finally enumerated by a FACSCalibur (BD Biosciences). To measure intracellular phosphorylated mTOR (p-mTOR) via flow cytometer, cultured T cells were first stained with anti-CD4-FITC or anti-CD8-FITC and then anti-p-mTOR Ab following cell permeabilization.

T cell proliferation/apoptosis and CD4+FoxP3+ Treg generation/proliferation in vitro

Splenic cells of C57BL/6J were prepared as described above and CD3+CD25- T cells were purified by cell sorting via a FACSAria III (BD Biosciences). For CFSE staining, the purified T cells were incubated with CFSE (Invitrogen, San Diego, CA, 2 μM) dye at 37°C for 5 min and cultured in anti-CD3/anti-CD28-coated 96-well plates (2.5x10^5 cells/well) in complete RPMI-1640 media. Rapamycin (100nM, Sigma, MO, USA), recombinant TGF-β1 (5 ng/ml), MG (40 μM). Four days later, cell proliferation was analyzed via a FACS. To detect T cell apoptosis, cells were stained with Annexin V-FITC without CFSE staining before FACS analysis. To determine Treg generation, cultured CD3$^+$ T cells were first stained with anti-CD4-PE and then were permeabilized before intracellular FoxP3 staining as described under the subheading: Flow cytometer. To measure Treg proliferation, FACS-sorted CD4$^+$CD25$^+$ Tregs were also labeled with CFSE and cultured in the presence of anti-CD3/anti-CD28 Abs (2.5x10^5 cells/well) for four days. Existing Treg proliferation was measured via FACS analysis.

Western blot

Cultured T cells were harvested and lysed, and the extracted protein was quantified by bicinchoninic acid assay kit. Protein samples (30μg/each) were electrophoresed using 10% SDS-polyacrylamide gel electrophoresis, and electro-blotted onto a PVDF membrane. After blocking in TBST containing 5% skimmed milk for one hour, the membrane was incubated with rabbit anti-mTOR, anti-p-mTOR, anti-P70S6K, anti-p-P70S6K, or anti-GADPH (1:1000, all from CST, Beverly, MA) antibody at 4°C overnight. After washing, the membrane was incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (1:2000, CST, Beverly, MA) for two hours at room temperature. The signals were visualized using ECL Advance reagent (Millipore, Billerica, MA) and the specific protein bands were quantified using ImageLab software.

Statistical analysis

Comparisons of the mean were performed using ANOVA. Analyses were performed using Prism-6 software (GraphPad Software, La Jolla, CA). Data were presented as Mean ± SD. A value of $P < 0.05$ was considered statistically significant.

Results

MG ameliorates LN in lupus-prone B6/gld mice

SLE often affects the kidney, resulting in proteinuria, renal functional loss and glomerular inflammation. To assess the development of nephritis in lupus-prone B6/gld mice, proteinuria was monitored every 4 weeks. As shown in Fig. 1A, proteinuria in B6/gld mice began to increase at age of 20 weeks compared with age-matched normal C57BL/6 mice. Either MG or Prednisone treatment significantly improved the proteinuria at age of 28 or 32 weeks. Meanwhile, we detected the serum creatinine (Scr) at age of 32 weeks. In agreement with the proteinuria, B6/gld mice spontaneously developed renal dysfunction with an increase in Scr levels (Fig. 1B). In contrast, mice treated with MG or prednisone maintained lower Scr levels 12 weeks after the treatment. Furthermore, we found higher serum anti-double-stranded

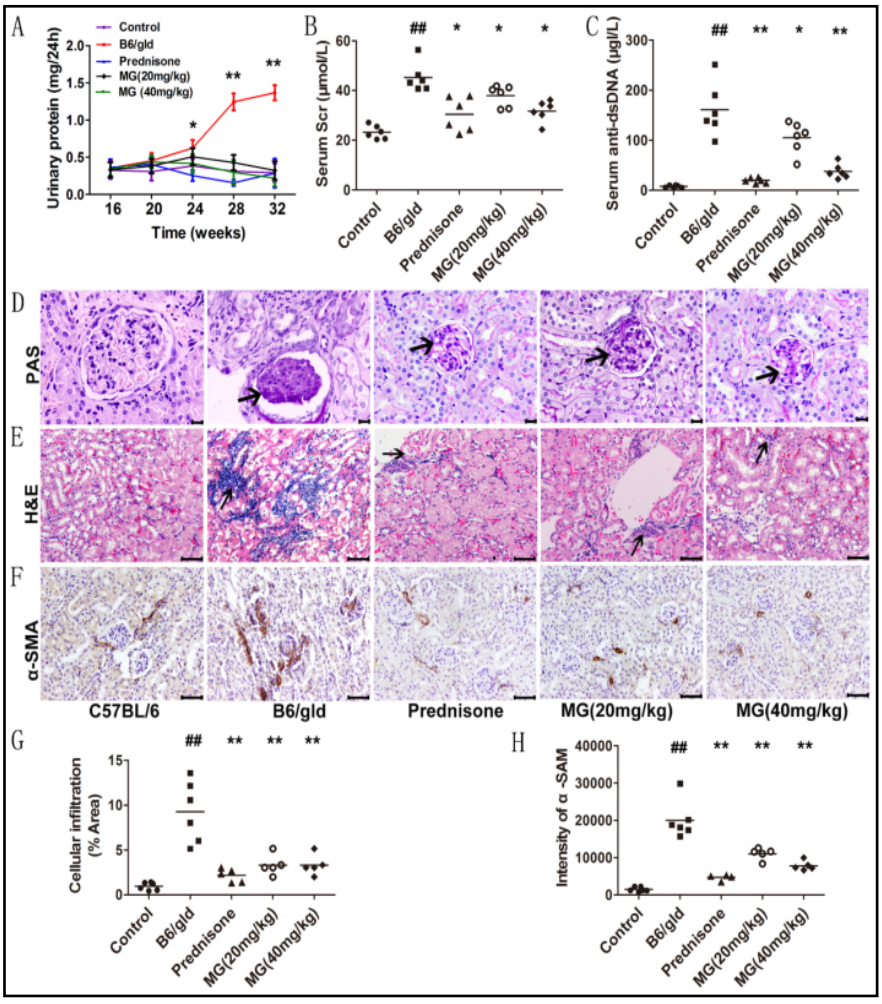

Fig. 1. Mangiferin (MG) treatment improves renal function and immunopathology and reduces serum autoantibody level in B6/gld mice. 24h-urine samples were collected every four weeks from B6/gld mice starting at the age of 16 weeks and the effects of MG on kinetics of 24h-urinary protein were observed (A). At the 32th week, all animals were sacrificed to measure serum creatinine (Scr) (B) and serum anti-dsDNA levels (C) in B6/gld mice. Data are presented as individual points with Means (n=6 mice/group). (D) PAS staining of the kidneys demonstrated the cystic glomeruli (arrowhead) with marked dilatation of Bowman's capsule and abnormal thickening of the glomerular basement membrane (arrows) in B6/gld mice (magnification: 400X). (E&G) H&E staining of the kidneys in B6/gld mice (n=5-6 mice/group) (magnification: 200X) and the percentage of the area with cellular infiltration. The percentage of the area with cellular infiltrates was measured using 20-24 Image J software. (F&H) IHC staining of α-SMA in the kidney and its intensity (n=5-6 mice/group, magnification: 200X). Shown above is one representative of three separate experiments ($^{\#\#}p<0.01$ compared with control, and $^{*}p<0.05$ or $^{**}p<0.01$ for all treated groups vs. B6/gld).

DNA Ab (anti-ds-DNA) levels in B6/gld mice than in control C57BL/6 mice at age of 32 weeks (Fig. 1 C). Treatments with MG (40 mg/kg or 20 mg/kg) or prednisone significantly lowered the level of circulating anti-ds-DNA. On the other hand, PAS and H&E staining of the kidneys demonstrated a pattern of glomerulonephritis typically found in SLE with marked dilatation of Bowman's capsule, abnormal thickening of the glomerular basement membrane (Fig. 1 D), and remarkable cellular infiltration (Fig. 1 E&G). In contrast, glomerular lesions in mice treated with MG were mild with slightly thickening of glomerular basement membrane and moderate interstitial inflammation. MG also decreased renal expression α-SMA, a protein reflecting the chronic renal fibrosis (Fig. 1 F&H). Moreover, MG also reduced proteinuria and serum creatinine levels and improved renal immunopathology in MRL/MpJ-lpr mice (data not shown). Thus, our findings demonstrated that MG improved renal function and immunopathology in murine LN.

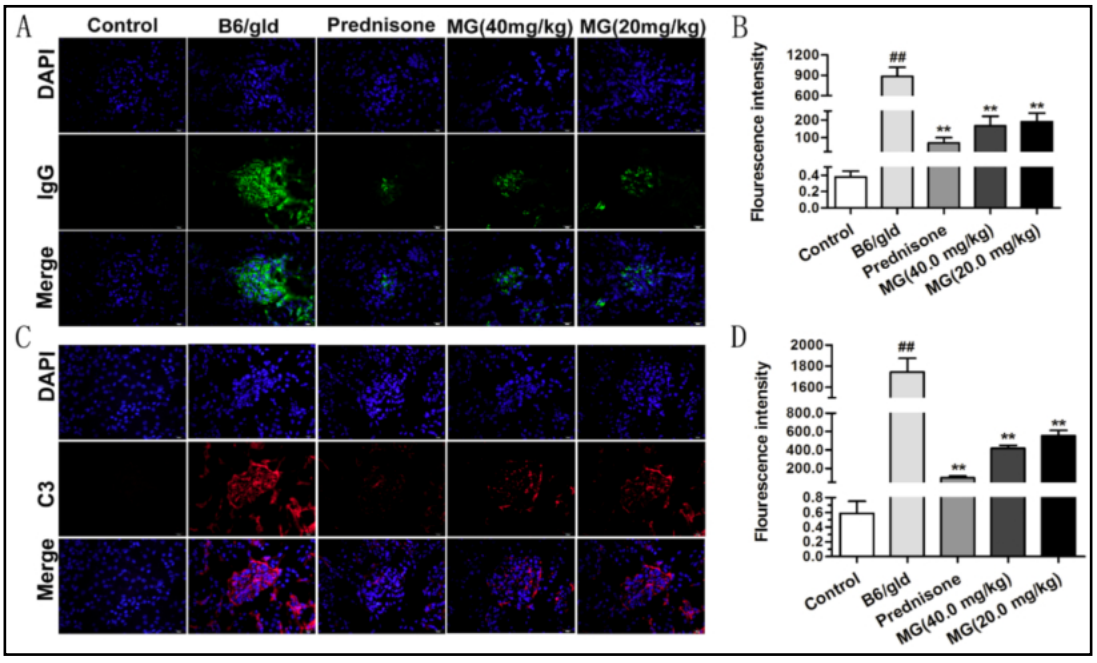

Fig. 2. MG treatment reduces the deposition of IgG and C3 in B6/gld mice. Immunofluorescence staining for total IgG (A) and complement C3 (C) in the glomeruli of B6/gld mice at age of 32 weeks were performed (n=4 mice/group). The immunological staining in C57/B6 mice is normal. In B6/gld mice, obvious granular deposition of IgG and C3 in glomerular capillary loops was observed under high magnifications. But MG or prednisone treatment significantly reduced IgG and C3 depositions. Quantitative analyses of IgG (B) or C3 (D) deposition were performed using 20-24 Image J, collected from four mice per group, Data are presented as Mean ± SD ($^{\#\#}p<0.01$ compared with control and $^{**}p<0.01$ compared with B6/gld).

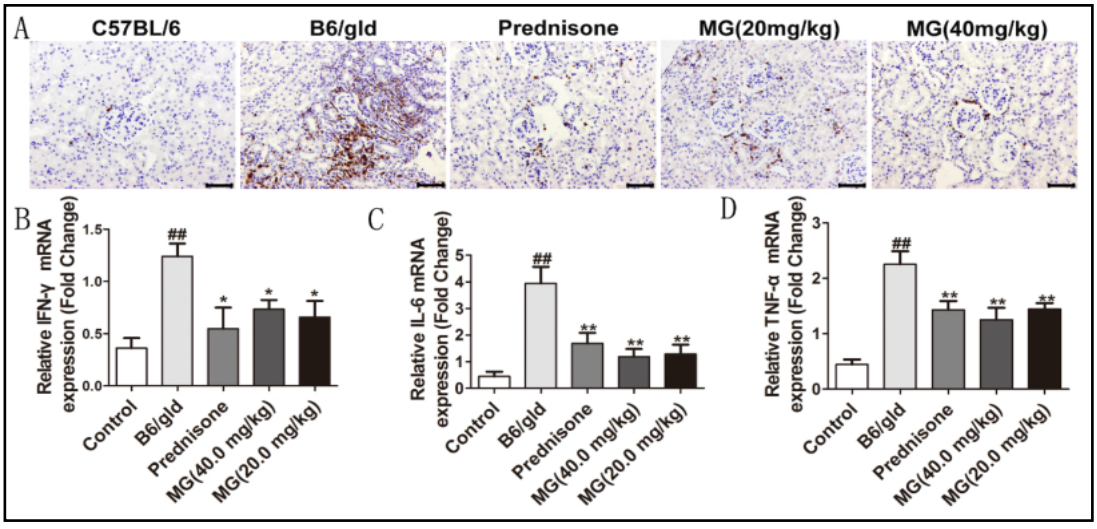

Fig. 3. MG mitigates renal T lymphocyte infiltration and inhibits gene expression of pro-inflammatory cytokines. Immunohistochemistry studies on paraffin-embedded kidney tissues were conducted to detect $CD3^+$ T lymphocyte infiltration in B6/gld mice at the 32th weeks (A). One representative staining from one mouse per group was shown (total n=4 mice/group). Further, quantitative RT-PCR was performed to evaluate the effects of MG on mRNA expression of IFN-γ (B), IL-6 (C) and TNF-α (D) in kidneys of B6/gld mice 12 weeks after the completion of treatment with MG or prednisone. MG treatment significantly inhibited the gene expression of IFN-γ, IL-6 and TNF-α in kidneys. Data are presented as Mean ± SD (n=5-6 mice/group in each of three separate experiments). One representative experiment is shown ($^{\#\#}p<0.01$ compared with control and $^{*}p<0.05$ or $^{**}p<0.01$ compared with B6/gld).

MG reduces IgG and C3 deposition in B6/gld mice

Excessive deposition of immune complexes in glomeruli plays a key role in LN pathogenesis. Here we determined if IgG and complement C3 deposition occurred in lupus-prone B6/gld mice. Immunological staining of the kidneys from B6/gld mice at age of 32 weeks showed obvious granular deposition of IgG and complement C3 in glomerular capillary loops while MG (either 40mg/kg or 20mg/kg) or prednisone treatment significantly reduced the deposition of IgG and C3 in the glomeruli of B6/gld mice (Fig. 2).

MG mitigates renal T cell infiltration and inhibits gene expression of proinflammatory cytokines

To visualize cellular infiltration in kidney tissues, we conducted IHC staining of CD3+ T cells on paraffin-embedded kidney tissues of B6/gld mice 12 weeks after MG treatment. As shown in Fig. 3A, there was almost no CD3+ T cells in normal C57/B6 kidneys. In contrast, extensive CD3+ lymphocyte infiltration was seen in the tubular interstice and glomeruli,

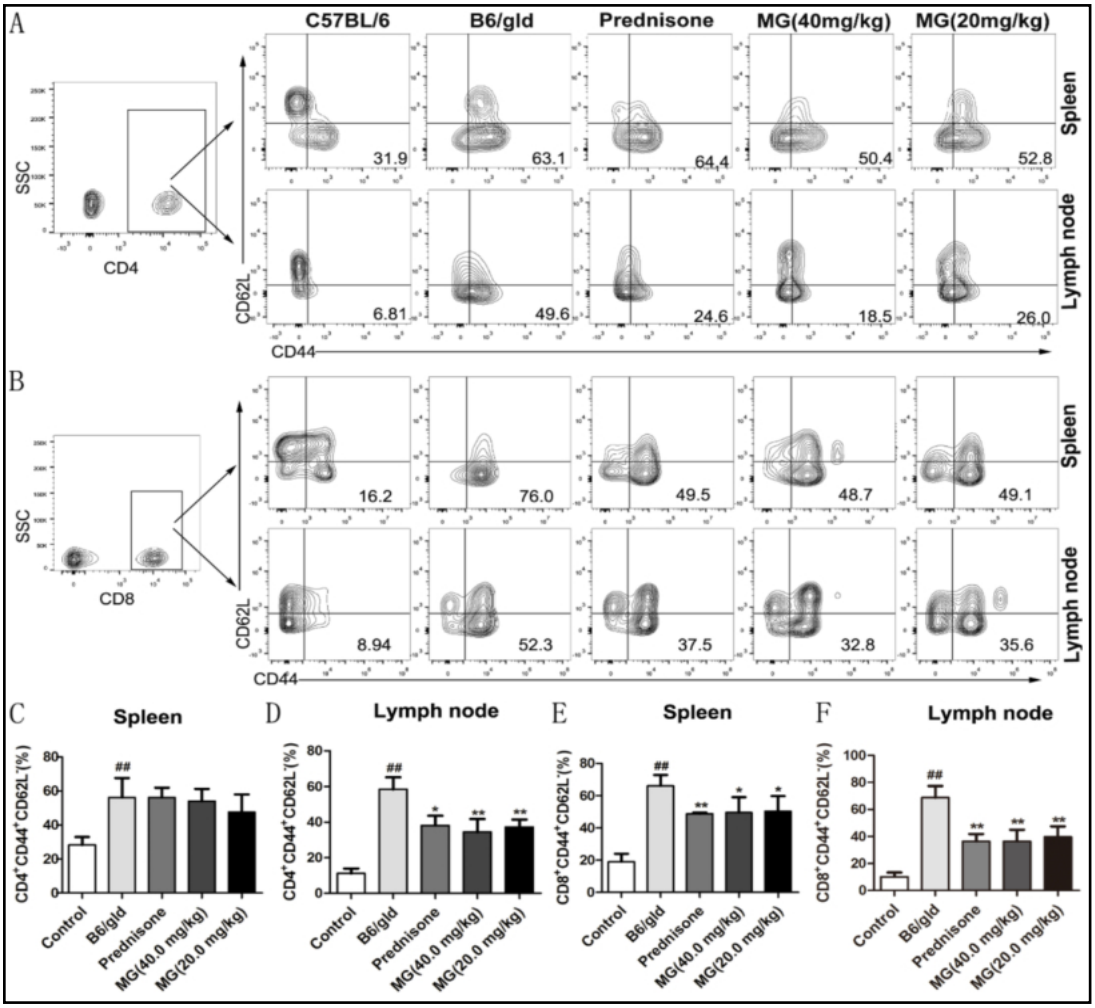

Fig. 4. MG reduces $CD4^+$ and $CD8^+$ effector T cell frequencies in B6/gld mice. Spleen and draining lymph node cells from MG-treated B6/gld mice at age of 32 weeks were stained with mAb for surface markers CD4, CD8, CD44 and CD62L, and the percentages of $CD4^+CD44^+CD62L^-$ or $CD4^+CD44^+CD62L^-$ effector T cells were determined by FACS analyses. Representative FACS dot plots stained for $CD4^+CD44^+CD62L^-$ T cells (A) and $CD8^+CD44^+CD62L^-$ T cells (B) in spleen and draining lymph node cells are shown. Histograms represent the mean percentages of $CD4^+$ T effectors in spleens (C) and lymph nodes (D), and $CD8^+$ T effectors in spleens (E) and lymph nodes (F). Data are presented as Mean ± SD (n=6 mice/group).

indicating that T cells participates in the pathology of renal damage. Only scattered or reduced tubular interstitial CD3+ lymphocyte infiltration was observed in B6/gld mice treated with MG or prednisone. Since MG was anti-inflammatory and alleviated cellular infiltration in kidneys, we asked if MG would regulate proinflammatory cytokines, such as TNF-α, IFN-γ and IL-6. 12 weeks after treatment with MG or prednisone, total mRNA in kidneys was extracted and the effects of MG on mRNA expression of IFN-γ, IL-6 and TNF-α were determined by RT-PCR assays. As shown in Fig. 3B-3D, either MG or prednisone treatment reduced mRNA expression of IFN-γ, IL-6 and TNF-α. Interestingly, there was no significant difference in their gene expression between each of the treated groups and B6/gld group when we normalized for cell numbers using infiltrated areas in Fig 1E (data not shown), suggesting that MG lowers gene expression of these proinflammatory cytokines by inhibiting cellular infiltration.

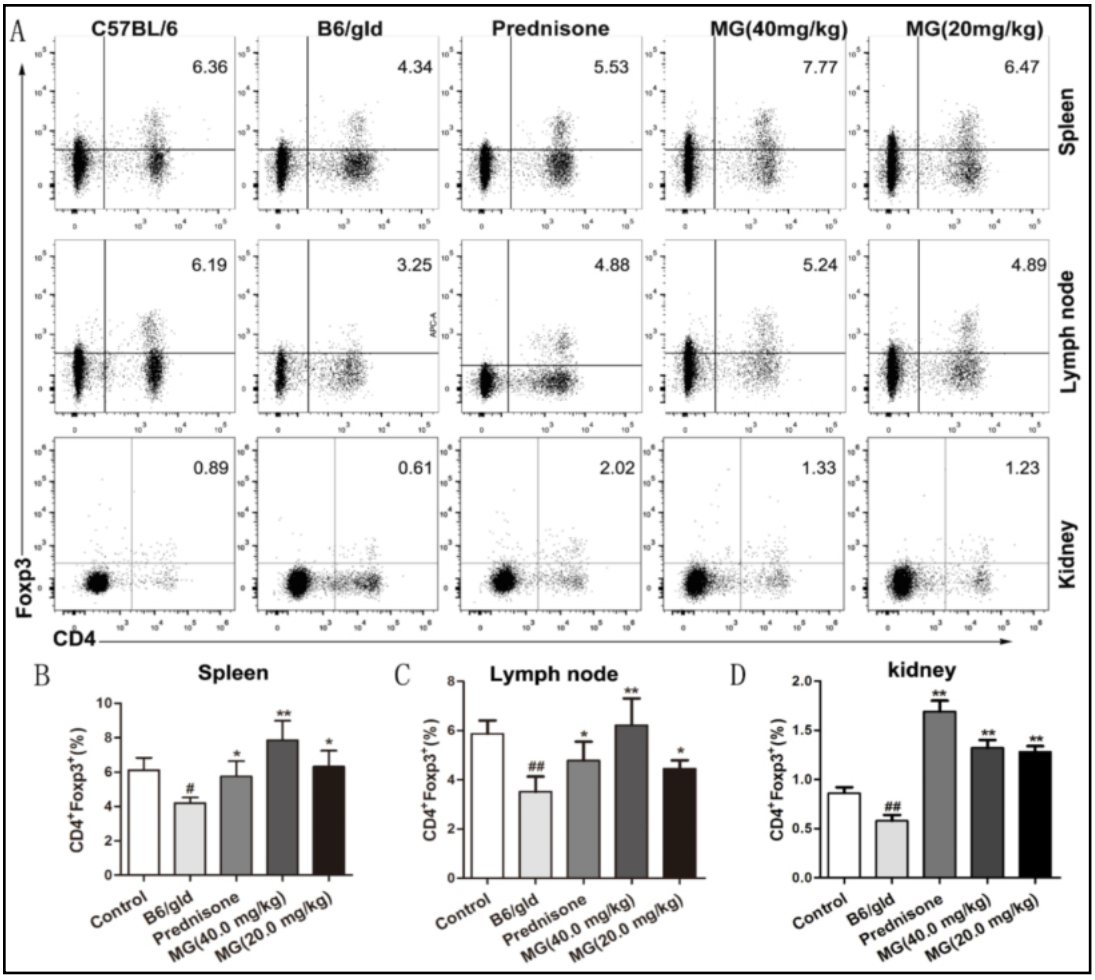

Fig. 5. MG induces CD4⁺Foxp3⁺ Tregs in B6/gld mice. Cells isolated from spleens, draining lymph nodes and kidneys of B6/gld mice were stained for surface CD4 and intracellular FoxP3 twelve weeks after administration of MG. The frequency of CD4⁺Foxp3⁺ Tregs was enumerated by FACS analyses. Representative FACS dot plots of CD4⁺Foxp3⁺ Treg populations from spleens, draining lymph nodes and kidneys are shown (A). Bar graphs represent the mean percentages of CD4+Foxp3+ Tregs in spleens (B), lymph nodes (C) and kidneys (D). Data are presented as Mean ± SD from three separate experiments (n=6-9 mice/group).

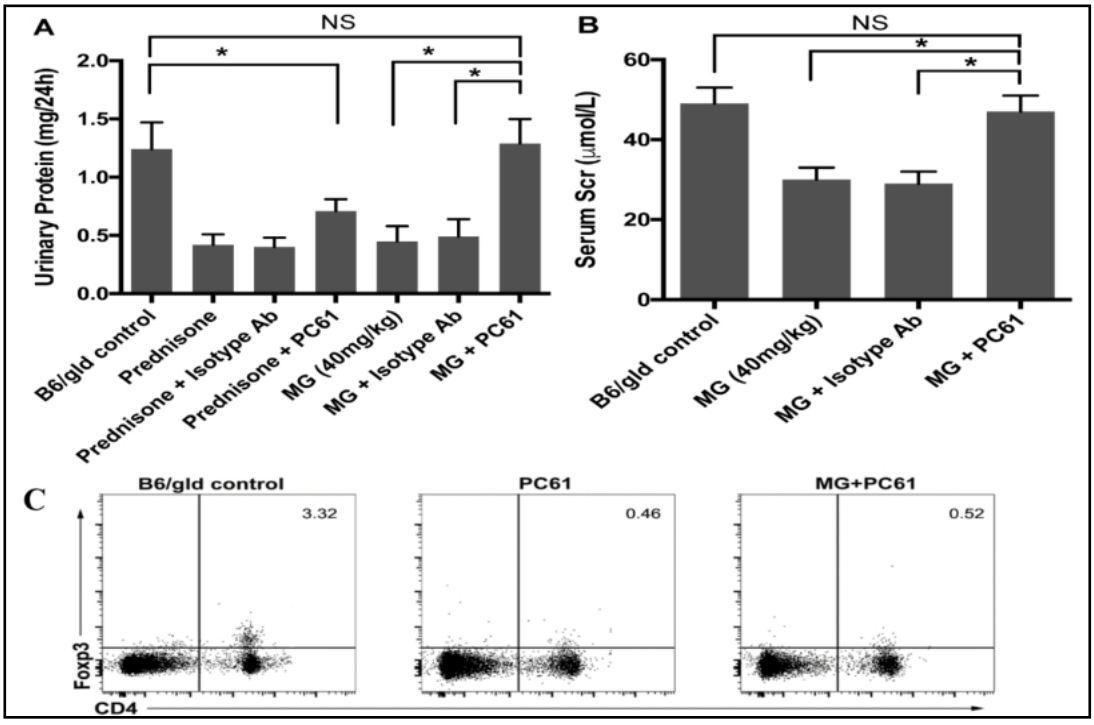

Fig. 6. Depleting $CD4^+CD25^+$Tregs in B6/gld mice abrogates therapeutic effects of MG on lupus nephritis. (A) 24h-urinary protein was measured in MG-treated B6/gld mice at the age of 32 weeks with depletion of CD25+ Tregs using depleting anti-CD25 Ab or isotype control. (B) Serum creatinine (Scr) was measured in MG-treated B6/gld mice at same age without or with depletion of CD25+ Tregs. Data are presented as Mean ± SD (n=5-6, $^*p$ <0.05 compared with B6/gld control). (C) To confirm that depleting antibody (PC61) actually deleted Tregs, splenocytes of B6/gld mice treated with PC61 were stained for CD4 and FoxP3. CD4+FoxP3+ Tregs were then quantified via FACS analysis two weeks later. One representative of two separate experiments is shown.

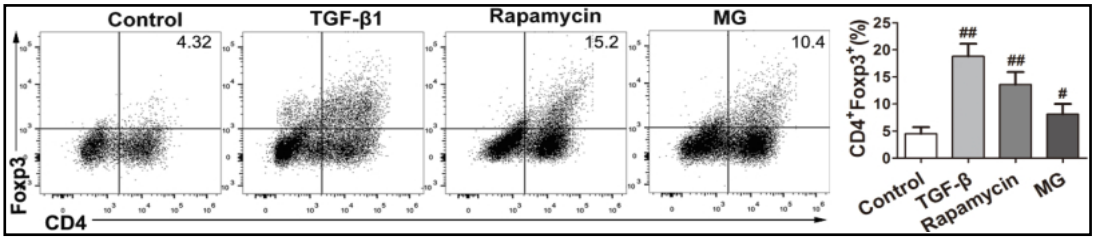

Fig. 7. MG induces $CD4^+FoxP3^+$ Tregs in vitro. FACS-sorted CD3+CD25- T cells from naïve mice were cultured in anti-CD3/CD28-coated 96-well plates in complete RPMI-1640 media in the presence of MG, TGF-β1 or rapamycin. The frequencies of CD4+FoxP3+ Tregs were detected by FACS analyses four days after the cell culture (A). Data are presented as Mean ± SD (B). One representative of three separate FACS experiments is shown.

MG decreases CD4+ and CD8+ effector T cell frequencies in B6/gld mice

Given that MG reduced renal T cell infiltration in B6/gld mice, we assumed that MG would regulate effector T cell differentiation. To test this hypothesis, spleen and lymph node cells isolated from B6/gld mice that were treated with MG or prednisone were stained with anti-CD4, anti-CD8, anti-CD44, and/or anti-CD62L Abs. And the percentage of CD44+CD62L- cells within CD4+ or CD8+ population was determined by FASC analyses. As shown in Fig. 4, MG treatment significantly reduced the frequency of CD4+CD44+CD62L- effector T cells in lymph nodes but not in spleens. In contrast, percentage of CD8+CD44+CD62L- effector T cells was decreased by MG treatment in both lymph nodes and spleens.

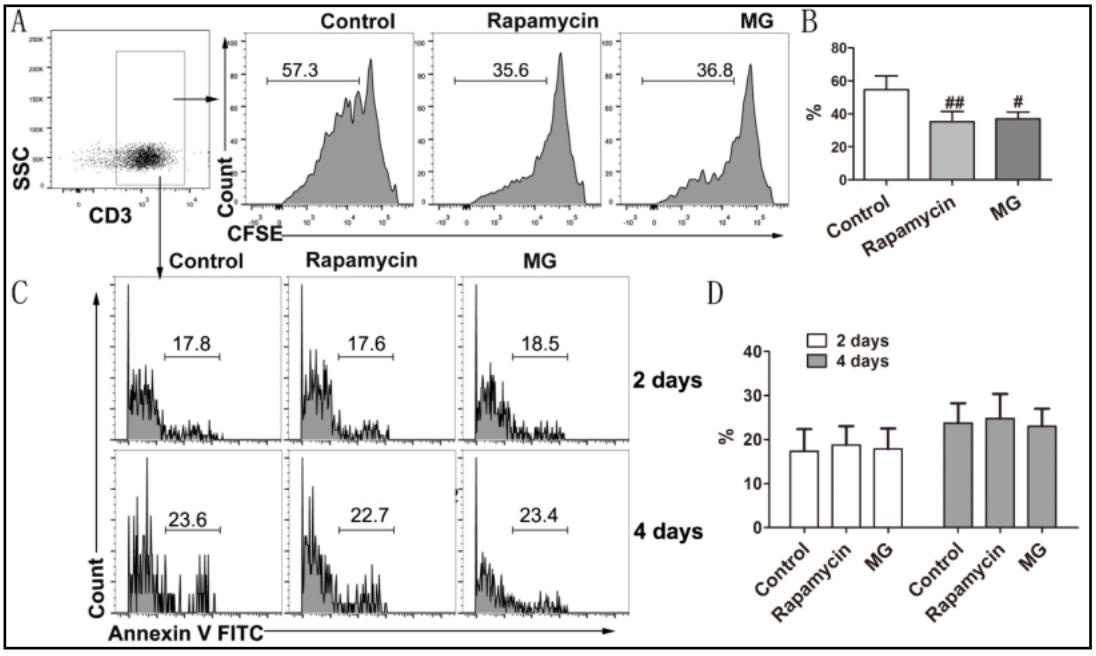

Fig. 8. MG suppresses T cell proliferation in vitro. CSFE-labeled CD3+CD25- T cells were similarly stimulated in the presence of MG or rapamycin. Four days later, T cell proliferation was determined via FACS analysis (A). To detect T cell apoptosis, similarly cultured T cells were stained with annexin V and analyzed via FACS 2 and 4 days after the culture (B). Data are presented as Mean ± SD (n=4). One representative of three separate FACS experiments is shown.

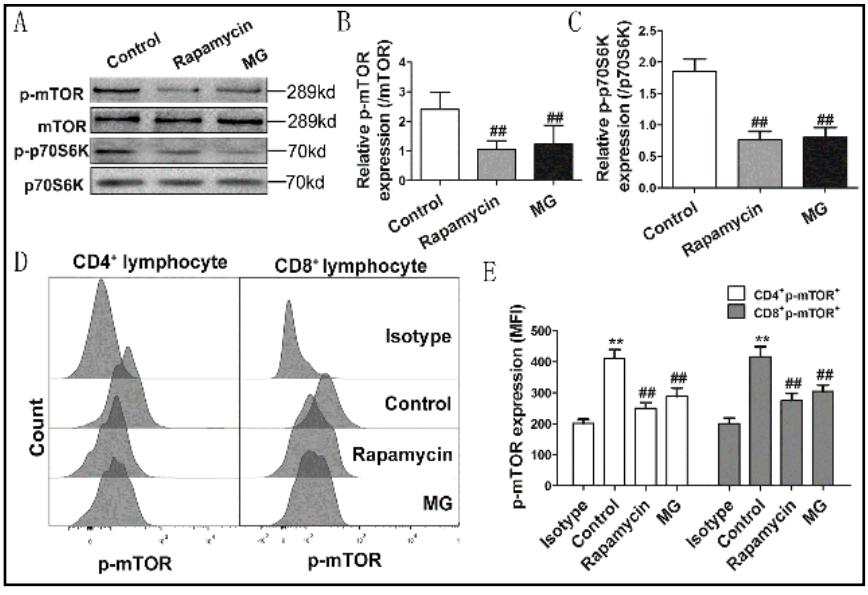

Fig. 9. MG downregulates mTOR/p70S6K pathway in T cells. FACS-sorted CD3+CD25- T cells were cultured in anti-CD3/CD28-coated 96-well plates in complete RPMI-1640 media in the absence or presence of MG/rapamycin. Phosphorylated mTOR (p-mTOR) and p-P70S6K protein expressions in CD3+ T cells were detected via Western blot analyses (A-C) and flow cytometry (D) two days after the culture. Expression of p-mTOR protein determined by FACS was presented as mean fluorescence intensity (MFI) (E). One of the four blotting images and one of three flow data are shown ($^{\#\#}p<0.01$ compared with control; $^{**}p<0.01$ compared with isotype).

MG induces CD4⁺Foxp3⁺ Tregs in lupus-prone B6/gld mice

CD4⁺FoxP3⁺ Tregs are critical for immune tolerance to self-antigens and their deficiency leads to development of autoimmunity. Here we asked whether MG would ameliorate LN by upregulating CD4⁺Foxp3⁺ Tregs. Cells were isolated from spleens, lymph nodes and kidneys of B6/gld mice treated with MG for 12 consecutive weeks, and CD4⁺Foxp3⁺ Tregs were enumerated by FASC analysis. As shown in Fig. 5, MG treatment significantly increased the frequency of CD4⁺Foxp3⁺ Tregs in spleens and lymph nodes as well as kidneys of B6/gld mice, suggesting that MG indeed induces CD4⁺Foxp3⁺ Tregs. Similar findings also were seen six weeks following MG treatment (data not shown). Moreover, a reduction in urinary protein and serum Scr by MG treatment was totally reversed when B6/gld mice were depleted of Tregs with depleting mAb, PC61 (Fig. 6).

MG promotes CD4⁺FoxP3⁺ Treg generation in vitro

We also determined the impact of MG on Treg generation *in vitro.* To generate CD4⁺Foxp3⁺ Tregs, FACS-sorted CD3⁺CD25⁻ T cells from naïve mice were stimulated with anti-CD3 plus anti-CD28 Abs for 96 hours. As shown in Fig. 7, addition of MG, TGF-β1 or rapamycin to the cell culture significantly increased the percentage of CD4⁺Foxp3⁺ Tregs compared with control group.

MG suppresses T cell proliferation in vitro and regulates mTOR/p70S6K pathway

FACS-sorted CD3⁺CD25⁻ T cells were labeled with CFSE and stimulated with anti-CD3 plus anti-CD28 Abs in the presence or absence of MG for 96 hours. We found that MG or rapamycin significantly suppressed the T cell proliferation (Fig. 8A). However, MG did not alter T cell apoptosis (Fig. 8B), indicating that MG is not cytotoxic. Moreover, Western blot analyses demonstrated that either MG or rapamycin markedly inhibited their phosphorylation of mTOR and p70S6K (Fig. 9A-C). FACS analyses also showed that MG suppressed the mTOR phosphorylation (Fig. 9D-E), suggesting that MG regulates T cell differentiation via mTOR/P70S6K pathway.

Discussion

Lupus nephritis (LN) is a major risk factor for morbidity and mortality among SLE patients, resulting in kidney damage in about 50% of SLE patients, and some LN patients eventually develop end-stage renal dysfunction (ESRD) [1, 15]. The overarching goal of LN treatment is to correct immunopathology and to prevent ESRD. Therefore, LN usually requires extensive global immunosuppression, which may cause severe side effects. Many herbal extracts or small molecular ingredients derived from Chinese herbs, such as artemisinin [16], tripterygium glycosides [17] and total glucosides of paeony [18], have been effective in treating autoimmune diseases. Mangiferin, a natural polyphenol, reportedly exerted a number of beneficial effects on reducing inflammation, oxidative injury, and metabolic disorders [19]. Previous studies have also confirmed that MG exhibits immunomodulatory properties and is effective in treating immune-based diseases in several animal models. For instance, MG ameliorated allergic asthma via regulating Th1/Th2 cytokine balance [20], corrected the Th17/Treg imbalance in TNBS-induced colitis [13], and demonstrated a potential for clinical application for the treatment of rheumatoid arthritis [21]. Given that the proper immunological balance between Tregs and effector T cells (Th1/Th17) is an essential process to avoid autoimmunity-associated immunopathology and that MG can regulate this critical balance in other autoimmune diseases, we hypothesized that MG could also inhibit the development of LN. In this study, we not only defined the therapeutic effects of MG on murine LN, but also elucidated the cellular and molecular mechanisms associated with its action. We found that MG alleviated the kidney injury and dysfunction in a LN model of B6/gld mice by inducing CD4⁺Foxp3⁺ Tregs. We further demonstrated that MG suppressed T cell proliferation, which was associated with downregulation of the mTOR/p70S6K pathway.

Thus, these results suggest that MG is a new immunosuppressant that acts as an mTOR-signaling inhibitor.

We utilized B6/gld mice as a spontaneous LN model to study the protective effects of MG on renal dysfunction and immunopathology. We found that MG attenuated proteinuria and renal pathological damage, including deposition of IgG and C3, and improved renal function as well. The beneficial effects of MG on renal function have also been reported in multiple kidney injury models, such as chronic nephritis, acute renal injury [22, 23], experimental hyperuricemia [24] and diabetic nephropathy [25, 26], indicating that MG preserves renal function through a variety of mechanisms. Since many autoimmune diseases, including LN, are accompanied with a significant increase in serum autoantibodies, we determined whether MG would regulate autoantibody responses in B6/gld mice. We found that MG dramatically decreased anti-ds-DNA Ab in the serum, indicating that MG generally suppresses humoral autoimmunity. Further, MG attenuated renal $CD3^+$ T cell infiltration and suppressed renal gene expression of TNF-α, INF-γ and IL-6. Overall, these data suggest that MG exerts anti-inflammatory effects via regulating both cellular and humoral autoimmune responses in lupus-prone B6/gld mice.

Regulatory T cells (Tregs) represent a small subset of T cells that are essential for maintaining immune homeostasis and tolerance. Defects in the number or function of Tregs led to various autoimmune diseases and graft rejection, including SLE, rheumatoid Arthritis (RA) and graft-versus-host disease (GVHD) [4, 6, 27, 28]. It was reported that patients with a defect in CD4+FoxP3+ Tregs developed lupus nephritis [29] and that the ratio of $FoxP3^+$/$CD3^+$ cells was significantly reduced in kidney biopsies of patients with lupus nephritis [30]. Moreover, expansion of $CD4^+Foxp3^+$ Tregs ameliorated LN in NZB/W F1 mice [31]. Therefore, $CD4^+Foxp3^+$ Treg expansion or induction has the potential to treat SLE or LN. Tregs exert their immunosuppression via multiple mechanisms. It has been known that Tregs secret anti-inflammatory/immunosuppressive cytokines, perforin and granzyme B, promote tolerogenic APC generation, and consume IL-2 at the site of inflammation, thus affecting local cell functions [32]. In this study, we demonstrated that MG induced $CD4^+Foxp3^+$ Tregs in both lymphoid organs and kidneys of the lupus-prone mice and alleviated LN. Increased Treg frequency *in vivo* by MG could be due to enhanced Treg survival, conversion of conventional T cells to Tregs, and/or augmented proliferation of the Tregs. It remains unclear whether MG increases Treg frequency mainly by enhancing conversion of conventional T cells to Tregs or promoting survival/proliferation of pre-existing Tregs. We did demonstrate that depletion of $CD4^+CD25^+$ Tregs abrogated the protective effects of MG on murine LN, suggesting that its beneficial effects may be dependent on Tregs. One could argue that depletion of Tregs likely makes any autoimmune disease harder to control and that neither MG nor another agent may control autoimmunity in the absence of Tregs. Thus, MG may not necessarily work directly by inducing Tregs. However, our data demonstrated that prednisone, but not MG, was effective even when Foxp3+ Tregs were depleted with PC61, arguing that MG, but not prednisone, acts through Tregs. Taken together, Tregs may be an important mechanism underlying MG action.

We also studied the molecular mechanisms underlying the effects of MG on $CD4^+Foxp3^+$ Treg induction and T cell proliferation and found that MG regulated the mTOR/p70S6K pathway in T cells. We demonstrated that MG treatment significantly reduced their phosphorylation of mTOR and p70S6K. It has been known that rapamycin, an mTOR inhibitor, induces $CD4^+Foxp3^+$ Tregs and suppresses T cell proliferation [33-35]. Our data revealed that MG is nearly as effective as rapamycin in suppression of T cell proliferation and regulation of mTOR/p70S6K pathway. Therefore, MG is a novel mTOR-signaling inhibitor that may be useful for treating LN or other autoimmune diseases in clinic.

Acknowledgements

This work was supported by National Natural Science Foundation of China (81803821), a PhD Start-up fund of Natural Science Foundation of Guangdong Province (2017A030310127), a joint grant from Guangdong Provincial Department of Science & Technology and Guangdong Provincial Academy of Chinese Medical Sciences (2014A020221053), and Innovative and Enhancement Research Program of Guangzhou University of Chinese Medicine (E1-KFD015181K36 and E1-KFD015181K37).

Disclosure Statement

The authors declare no competing interests.

References

1 Hoover PJ, Costenbader KH: Insights into the epidemiology and management of lupus nephritis from the US rheumatologist's perspective. Kidney Int 2016;90:487-492.
2 Almaani S, Meara A, Rovin BH: Update on Lupus Nephritis. Clin J Am Soc Nephrol 2017;12:825-835.
3 Kasper IR, Apostolidis SA, Sharabi A, Tsokos GC: Empowering Regulatory T Cells in Autoimmunity. Trends Mol Med 2016;22:784-797.
4 Arellano B, Graber DJ, Sentman CL: Regulatory T cell-based therapies for autoimmunity. Discov Med 2016;22:73-80.
5 Hu M, Wang YM, Wang Y, Zhang GY, Zheng G, Yi S, O'Connell PJ, Harris DC, Alexander SI: Regulatory T cells in kidney disease and transplantation. Kidney Int 2016;90:502-514.
6 Giang S, La Cava A: Regulatory T Cells in SLE: Biology and Use in Treatment. Curr Rheumatol Rep 2016;18:67.
7 Saha S, Sadhukhan P, Sil PC: Mangiferin: A xanthonoid with multipotent anti-inflammatory potential. Biofactors 2016;42:459-474.
8 Saha S, Rashid K, Sadhukhan P, Agarwal N, Sil PC: Attenuative role of mangiferin in oxidative stress-mediated liver dysfunction in arsenic-intoxicated murines. Biofactors 2016;42:515-532.
9 Khurana RK, Kaur R, Lohan S, Singh KK, Singh B: Mangiferin: a promising anticancer bioactive. Pharm Pat Anal 2016;5:169-181.
10 Saleh S, El-Maraghy N, Reda E, Barakat W: Modulation of diabetes and dyslipidemia in diabetic insulin-resistant rats by mangiferin: role of adiponectin and TNF-alpha. An Acad Bras Cienc 2014;86:1935-1948.
11 Fomenko EV, Chi Y: Mangiferin modulation of metabolism and metabolic syndrome. Biofactors 2016;42:492-503.
12 Szandruk M, Merwid-Lad A, Szelag A: The impact of mangiferin from Belamcanda chinensis on experimental colitis in rats. Inflammopharmacology 2018;26:571-581.
13 Lim SM, Jeong JJ, Choi HS, Chang HB, Kim DH: Mangiferin corrects the imbalance of Th17/Treg cells in mice with TNBS-induced colitis. Int Immunopharmacol 2016;34:220-228.
14 Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S: Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 1994;76:969-976.
15 Lewis MJ, Jawad AS: The effect of ethnicity and genetic ancestry on the epidemiology, clinical features and outcome of systemic lupus erythematosus. Rheumatology (Oxford) 2017;56:i67-i77.
16 Lin ZM, Yang XQ, Zhu FH, He SJ, Tang W, Zuo JP: Artemisinin analogue SM934 attenuate collagen-induced arthritis by suppressing T follicular helper cells and T helper 17 cells. Sci Rep 2016;6:38115.
17 Xu X, Li QJ, Xia S, Wang MM, Ji W: Tripterygium Glycosides for Treating Late-onset Rheumatoid Arthritis: A Systematic Review and Meta-analysis. Altern Ther Health Med 2016;22:32-39.
18 Xu XX, Qi XM, Zhang W, Zhang CQ, Wu XX, Wu YG, Wang K, Shen JJ: Effects of total glucosides of paeony on immune regulatory toll-like receptors TLR2 and 4 in the kidney from diabetic rats. Phytomedicine 2014;21:815-823.

19 Jyotshna, Khare P, Shanker K: Mangiferin: A review of sources and interventions for biological activities. Biofactors 2016;42:504-514.
20 Guo HW, Yun CX, Hou GH, Du J, Huang X, Lu Y, Keller ET, Zhang J, Deng JG: Mangiferin attenuates TH1/TH2 cytokine imbalance in an ovalbumin-induced asthmatic mouse model. PLoS One 2014;9:e100394.
21 Song J, Li J, Hou F, Wang X, Liu B: Mangiferin inhibits endoplasmic reticulum stress-associated thioredoxin-interacting protein/NLRP3 inflammasome activation with regulation of AMPK in endothelial cells. Metabolism 2015;64:428-437.
22 He L, Peng X, Zhu J, Chen X, Liu H, Tang C, Dong Z, Liu F, Peng Y: Mangiferin attenuate sepsis-induced acute kidney injury via antioxidant and anti-inflammatory effects. Am J Nephrol 2014;40:441-450.
23 Wang B, Wan J, Gong X, Kuang G, Cheng X, Min S: Mangiferin attenuates renal ischemia-reperfusion injury by inhibiting inflammation and inducing adenosine production. Int Immunopharmacol 2015;25:148-154.
24 Yang H, Gao L, Niu Y, Zhou Y, Lin H, Jiang J, Kong X, Liu X, Li L: Mangiferin Inhibits Renal Urate Reabsorption by Modulating Urate Transporters in Experimental Hyperuricemia. Biol Pharm Bull 2015;38:1591-1598.
25 Pal PB, Sinha K, Sil PC: Mangiferin attenuates diabetic nephropathy by inhibiting oxidative stress mediated signaling cascade, TNFalpha related and mitochondrial dependent apoptotic pathways in streptozotocin-induced diabetic rats. PLoS One 2014;9:e107220.
26 Zhu X, Cheng YQ, Du L, Li Y, Zhang F, Guo H, Liu YW, Yin XX: Mangiferin attenuates renal fibrosis through down-regulation of osteopontin in diabetic rats. Phytother Res 2015;29:295-302.
27 Tao JH, Cheng M, Tang JP, Liu Q, Pan F, Li XP: Foxp3, Regulatory T Cell, and Autoimmune Diseases. Inflammation 2017;40:328-339.
28 Miyara M, Ito Y, Sakaguchi S: TREG-cell therapies for autoimmune rheumatic diseases. Nat Rev Rheumatol 2014;10:543-551.
29 Matta MC, Soares DC, Kerstenetzky MS, Freitas AC, Kim CA, Torres LC: CD4+CD25 high Foxp3+ Treg deficiency in a Brazilian patient with Gaucher disease and lupus nephritis. Hum Immunol 2016;77:196-200.
30 Afeltra A, Gigante A, Margiotta DP, Taffon C, Cianci R, Barbano B, Liberatori M, Amoroso A, Rossi Fanelli F: The involvement of T regulatory lymphocytes in a cohort of lupus nephritis patients: a pilot study. Intern Emerg Med 2015;10:677-683.
31 Yan JJ, Lee JG, Jang JY, Koo TY, Ahn C, Yang J: IL-2/anti-IL-2 complexes ameliorate lupus nephritis by expansion of CD4+CD25+Foxp3+ regulatory T cells. Kidney Int 2017;91:603-615.
32 Miyara M, Sakaguchi S: Natural regulatory T cells: mechanisms of suppression. Trends Mol Med 2007;13:108-116.
33 Strauss L, Czystowska M, Szajnik M, Mandapathil M, Whiteside TL: Differential responses of human regulatory T cells (Treg) and effector T cells to rapamycin. PloS One 2009;4:e5994.
34 Dong M, Wang X, Liu J, Zhao YX, Chen XL, Li KQ, Li G: Rapamycin Combined with Immature Dendritic Cells Attenuates Obliterative Bronchiolitis in Trachea Allograft Rats by Regulating the Balance of Regulatory and Effector T Cells. Int Arch Allergy Immunol 2015;167:177-185.
35 Bestard O, Cassis L, Cruzado JM, Torras J, Franquesa M, Gil-Vernet S, Lucia M, Grinyo JM: Costimulatory blockade with mTor inhibition abrogates effector T-cell responses allowing regulatory T-cell survival in renal transplantation. Transpl Int 2011;24:451-460.