FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 60, No. 3, 2022
pp. 237–246

ORIGINAL PAPER

VIA MEDICA

Celastrol alleviates murine lupus nephritis *via* inducting $CD4^+Foxp3^+$ regulatory T cells

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Abstract

Introduction. Lupus nephritis (LN) is an autoimmune glomerulonephritis secondary to systemic lupus erythematosus. Commonly, immunosuppressive agents are required for treating LN. However, frequent use of conventional immunosuppressants may produce a variety of side effects. Hence, seeking alternative drugs for treating LN is very important. This report aims to figure out the immunoregulatory efficacy of celastrol (CLT) in LN.

Material and methods. A spontaneous *in vivo* model of LN was established in FasL-deficient B6/gld mice. ELISA was used for analyzing serum creatinine (Scr) and anti-dsDNA levels in mice. IHC staining, immunofluorescence and hematoxylin-eosin and PAS staining were applied to determine renal immunopathology and histology. Cytokine gene levels were assessed using RT-qPCR. $CD4^+Foxp3^+$ Treg frequency in murine kidneys, lymph nodes and spleens was determined using flow cytometry analysis.

Results. CLT treatment alleviated renal dysfunction and renal injury in LN-prone B6/gld mice. Moreover, CLT reduced $CD3^+$ T cell infiltration and inhibited proinflammatory cytokine expression in renal tissues of B6/gld mice. Importantly, CLT enhanced $CD4^+FoxP3^+$ Treg frequency in kidneys, lymph nodes and spleens of B6/gld mice.

Conclusions. CLT exerts therapeutic effects on murine LN by improving renal function and immunopathology and inducing $CD4^+FoxP3^+$ Tregs. *(Folia Histochemica et Cytobiologica 2022, Vol. 60, No. 3, 237–246)*

Keywords: B6/gld mice; lupus nephritis; celastrol; $CD4^+FoxP3^+$ cells; kidney damage; cytokine gene expression

Introduction

As an autoimmune disease, systemic lupus erythematosus (SLE) is featured with autoantibody production, multiple organ injury and systemic inflammation [1]. As a severe complication and a typical clinicopathological characteristic of SLE, lupus nephritis (LN) is characterized by renal inflammation and malfunction [2]. Approximately 60% of adults and 80% of children are vulnerable to LN, and up to 30% LN patients could develop into end-stage renal disease, which seriously reduces life quality [3]. LN is hallmarked by excessive proinflammatory mediator release, formation of immune complex deposits in the kidney, aberrant autoantibody production and T and B lymphocyte hyperactivity [4]. As a subgroup of T cells, T regulatory cells (Tregs) exert immunosuppressive effects on effector immune cells, which maintains immune tolerance and homeostasis [5]. Treg number abnormality or dysfunction can cause various autoimmune diseases, including LN and SLE [6]. At present, the first-line drugs for treating LN are

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Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0020
ISSN 0239-8508, e-ISSN 1897-5631

immunosuppressants, such as calcineurin inhibitors, cyclophosphamide, and steroids [7]. However, the clinical outcomes of LN remain unsatisfactory due to side effects of conventional immunosuppressive drugs [8]. Therefore, finding optimal therapeutic agents for LN treatment is of great importance.

Celastrol (CLT), also known as tripterine, is a Chinese herbal compound derived from the roots of Thunder of God Vine, a traditional medicinal plant [9]. The chemical formula of CLT is C29H38O4 [10]. As a pentacyclic triterpenoid, CLT is a member of quinonemethides family and possesses anti-cancer, antioxidant, anti-autoimmune and anti-inflammation properties [11, 12]. CLT administration decreases pro-inflammatory factors IL-23 IL-17, IFNγ levels, and increases anti-inflammation cytokine IL-10 expression [13]. Many studies about CLT have focused on its role in anti-inflammation in autoimmune disorders, including colitis, psoriasis, SLE and multiple sclerosis [14]. Moreover, the potential of CLT to alleviate renal injury has been revealed in experimental models of some kidney diseases. CLT administration significantly attenuates extracellular matrix deposition, glomerular hypercellularity, inflammation and proteinuria in anti-Thy1.1 nephritis [15]. CLT treatment decreases renal collagen type IV by repressing TGF-β level [16], and reduces total IgG and serum autoantibodies in the murine serum, subsequently mitigating glomerular injury [17]. CLT ameliorates acute kidney injury caused by ischemia-reperfusion by restricting NF-κB activation and inflammation [18]. The imbalance of regulatory T (Treg) cell/T helper 17 (Th17) cell is involved in multiple immune disorders. CLT could facilitate Treg cell generation and Foxp3 expression, while inhibiting Th17 cell induction and T cell proliferation [19]. In experimental autoimmune encephalomyelitis (EAE) murine models induced by myelin-basic protein, CLT inhibits the Th17 response [20]. CLT significantly augments $CD25^{+}Foxp3^{+}$ Tregs frequencies but decreases $IL\text{-}17^{+}ROR\gamma t^{+}$ Th17 cell frequencies, thus exerting immunosuppressive effects in the joints of arthritic rats [21]. Hence, we hypothesized that CLT may exert beneficial effects for LN treatment *via* Treg induction.

Due to Fas ligand gene mutation, B6/gld mice exhibit elevated T and B cell numbers and enlarged spleens and lymph nodes, and they can spontaneously develop various systemic autoimmune diseases, including LN [22]. In this study, a mouse model of LN was established using FasL-deficient B6/gld mice. We explored whether CLT administration can improve renal pathology and mediate Treg expansion in B6/gld mice. Our study may provide a better understanding of the pharmacology and efficacy of CLT in autoimmunity. Meanwhile, this study may lay a theoretical basis for LN treatment.

Material and methods

Animal models. Wild-type male C57BL/6 mice (n = 10) were purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China) and FasL-deficient B6/gld male mice (n = 40) were obtained from the Jackson Laboratory (Bar Arbor, ME, USA). All mice were housed in a specific pathogen-free environment at 25 ± 2°C with 65% humidity. They were housed four per cage in our animal facility on a 12-h light/dark cycle with ad libitum access to a standard diet and drinking water. Commencing at 20 weeks of age, mice were placed in individual metabolic cages (Techniplast; Beijing Head Biotechnology co, LTD, Beijing, China) for 24 h for urine collection, with free access to food and water. The experiment was conducted under approvement of the Hubei Provincial Center for Disease Control (Approval number: 202110174). At the age of 20 weeks, FasL-deficient B6/gld (n = 40) were divided into four groups (n = 10/group) of B6/gld, Prednisone, low dose of CLT (LD-CLT) and high dose of CLT (HD-CLT) groups. C57BL/6 mice (n = 10) were set as the control. The control and B6/gld groups were orally administrated with distilled water containing 0.4% Sodium Carboxymethyl Cellulose (CMC-Na; Pandei, Shanghai, China) once a day for 8 weeks. The Prednisone group received prednisone (0.5 mL; 5 mg/kg; Sigma, Shanghai, China) by daily oral gavage for 8 weeks [23]. Mice in the HD-CLT group were administrated with 3 mg/kg CLT (98% purity; Chengdu Must Biotechnology, Chengdu, China) and mice in the LD-CLT group with 1 mg/kg CLT *via* tail vein injection once a day for 8 weeks [15]. After the last administration at the 28th week, all mice were sacrificed *via* cervical dislocation. Afterward, blood samples were collected. The obtained spleens, lymph nodes, and kidneys were also collected.

Renal function estimation. Murine urine was collected every 2 weeks. All mice were allowed to drink freely during sample collection, while they had no access to food during sample collection. Determination of urinary proteins was conducted *via* colorimetry [24]. Blood samples were prepared to separate serum by centrifugation at 3500 rpm for 15 min at 4°C. Serum creatinine (Scr) and anti-dsDNA antibodies levels were analyzed using creatinine assay kit (Jingkang, Shanghai, China) and murine ds-DNA standard ELISA kit (Unison Biotech Inc, Hsinchu, Taiwan) following the manufacturers' guidelines. The blood urea nitrogen (BUN) levels were assessed using BUN detection kit (Unison Biotech).

Histopathology. The kidney tissues were fixed with 10% neutral-buffered formalin, dehydrated in ethanol gradients, and embedded in paraffin. Renal sections (3 μm) were stained with hematoxylin and eosin (H&E) (Yulu, Nanchang, China) for 15 min. Interstitial cellular infiltration was observed using a Nikon ECLIPSE 80i microscope (Nikon, Tokyo, Japan). The cell infiltration was determined in 5 fields of each section (at

10.5603/FHC.a2022.0020
ISSN 0239-8508, e-ISSN 1897-5631

×200 magnification). The ratio of the area of inflammatory cell infiltration to total area as well as the number of infiltrating cells was determined by an average of 5 readings of each sample using 20–24 Image J software. Sections were stained with Periodic acid-Schiff (PAS) (Senbei, Nanjing, China) for 10 min based on the manufacturers' guidelines. Renal tubular damage, glomerular basement membrane, changes in mesangial matrix and glomerular mesangial proliferation were evaluated by light microscopy in Olympus BX5 microscope (Olympus, Tokyo, Japan).

Immunohistochemistry (IHC). Paraffined renal sections (3 μm) were dewaxed for immunohistochemistry. Sodium citrate buffer (0.01M, pH 6.0) solution was used for antigen retrieval at 95–100°C for 15 min. Subsequently, and the tissues were then quenched in 3% hydrogen peroxide solution to block endogenous peroxidase activities. Then, renal tissues were incubated with primary rabbit anti-CD3 (1:150, Abcam, ab16669) or anti-α-smooth muscle actin [α-SMA, 1:2500, ab124964 (Abcam, Cambridge, UK)] overnight at 4°C. The bound primary antibody was visualized using Non-Biotin MaxVisionTM2HRP–Polymer anti-mouse IgG detection system (Southern Biotech, Birmingham, UK). Sections were incubated sequentially with polyperoxidase-anti-mouse IgG and Polymer Hepler for 30 min at 37°C. 3,3-diaminobenzidine (Sigma-Aldrich, Burlington, MA, USA) was used for color development. Subsequently, hematoxylin was used to counterstain nuclei. Positive controls consisted of renal sections from mice previously known to contain target antigens. For negative controls, the primary antibody was replaced by its commercial diluent. Images of random fields were captured on a Nikon Eclipse 90i microscope with a Nikon Digital Sight DS-Fi1 camera.

Flow cytometry analysis. In brief, the perfused kidneys, lymph nodes or spleens were minced. Single-cell suspensions were generated by homogenization using a 40 μm sterile strainer on a Petri dish in 10 mL of phosphate-buffered saline (PBS) containing 1% fetal bovine serum (FBS). Afterward, erythrocytes in the spleen were lysed with 0.87% ammonium chloride solution (BD Biosciences, San Jose, CA, USA). Cells were washed with PBS +1% FBS, counted, and resuspended in PBS +1% FBS. Lymphocytes were isolated from heparinized blood using Lympholyte M Cell Separation Media (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). Then, 1×10^6 cells/mL were stained with anti-mouse CD3e (BD Pharmingen, San Diego, CA, USA), anti-mouse CD4 (BD Pharmingen), or isotype controls for 1 h at 4°C in the dark. The cells were then fixed and permeabilized with the BD CytoFix/CytoPerm Fixation/Permeabilization Kit (BD Pharmingen). Intracellular staining was performed using anti-mouse FoxP3 (eBioscience, San Diego, CA, USA). Flow cytometric data for CD4+/FoxP3+ lymphocytes were acquired on a FACS Aria III flow cytometer (BD Biosciences) and analyzed using FlowJo software.

RT-qPCR. Renal RNA was extracted using RNAiso Plus reagent (Takara, Shanghai, China). RNA concentration was determined according to absorbance at 260 nm and purity was evaluated by A260/A280 ratios. cDNAs were synthesized using PrimeScript RT reagent Kit (TaKaRa). Transcription levels of specific genes were utilized for RT-qPCR analysis using TB Green Premix Ex Taq II (TaKaRa) on CFX96™ Real-Time PCR Detection Systems (Bio-rad, Hercules, CA, USA). The conditions of qRT-PCR are as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s, with dissociation at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. GAPDH acted as internal reference and gene levels were calculated using the $2^{-\Delta\Delta Ct}$ method [25]. Primer sequences are shown in Table 1.

Table 1. Primer sequences used for real-time PCR analysis of gene expression

Gene	Primer	Oligonucleotide sequence (5′-3)
IL-6	Forward	TTCTTGGGACTGATGCTGGT
	Reverse	CCTCCGACTTGTGAAGTGGT
IFN-γ	Forward	CACGGCACAGTCATTGAAAG
	Reverse	CATCCTTTTGCCAGTTCCTC
TNF-α	Forward	ACTGATGAGAGGGAGGCCAT
	Reverse	CCGTGGGTTGGACAGATGAA
GAPDH	Forward	AGGTCGGTGTGAACGGATTTG
	Reverse	GGGGTCGTTGATGGCAACA

Statistical analysis. All data were evaluated using SPSS 21.0 statistical software (IBM, Armonk, NY, USA). The data were normally distributed which had been detected by Kolmogorov-Smirnov test and are expressed as the mean ± standard deviation (SD). Student's *t*-test was utilized for comparisons between two groups. One-way analysis of variance (ANOVA) followed by Tukey *post hoc* analysis was adopted for the discrepancy among multiple groups. $P < 0.05$ was considered statistically significant.

Results

CLT relieves renal dysfunction in lupus-prone B6/gld mice

To evaluate the effects of CLT on renal function in lupus-prone B6/gld mice, the content of 24-h urinary protein, serum Scr, serum anti-dsDNA antibodies and BUN in different groups were measured. As Fig. 1A demonstrated, urinary protein was monitored at the 20th, 22nd, 24th, 26th, and 28th week. Compared to wild-type C57BL/6 mice, proteinuria in B6/gld mice increased in a time-dependent manner (Fig. 1A). Both prednisone and CLT administration significantly reduced proteinuria in B6/gld mice at the 26th and the 28th week (Fig. 1A). Additionally, B6/gld mice exhibited higher serum Scr, serum anti-dsDNA and BUN levels than C57BL/6 mice at the 28th week (Fig. 1B–D). In contrast, elevation of content of serum creatinine, serum anti-dsDNA and BUN concentrations in B6/gld mice was offset by prednisone or CLT administration (Fig. 1B–D). Overall, CLT attenuates renal dysfunc-

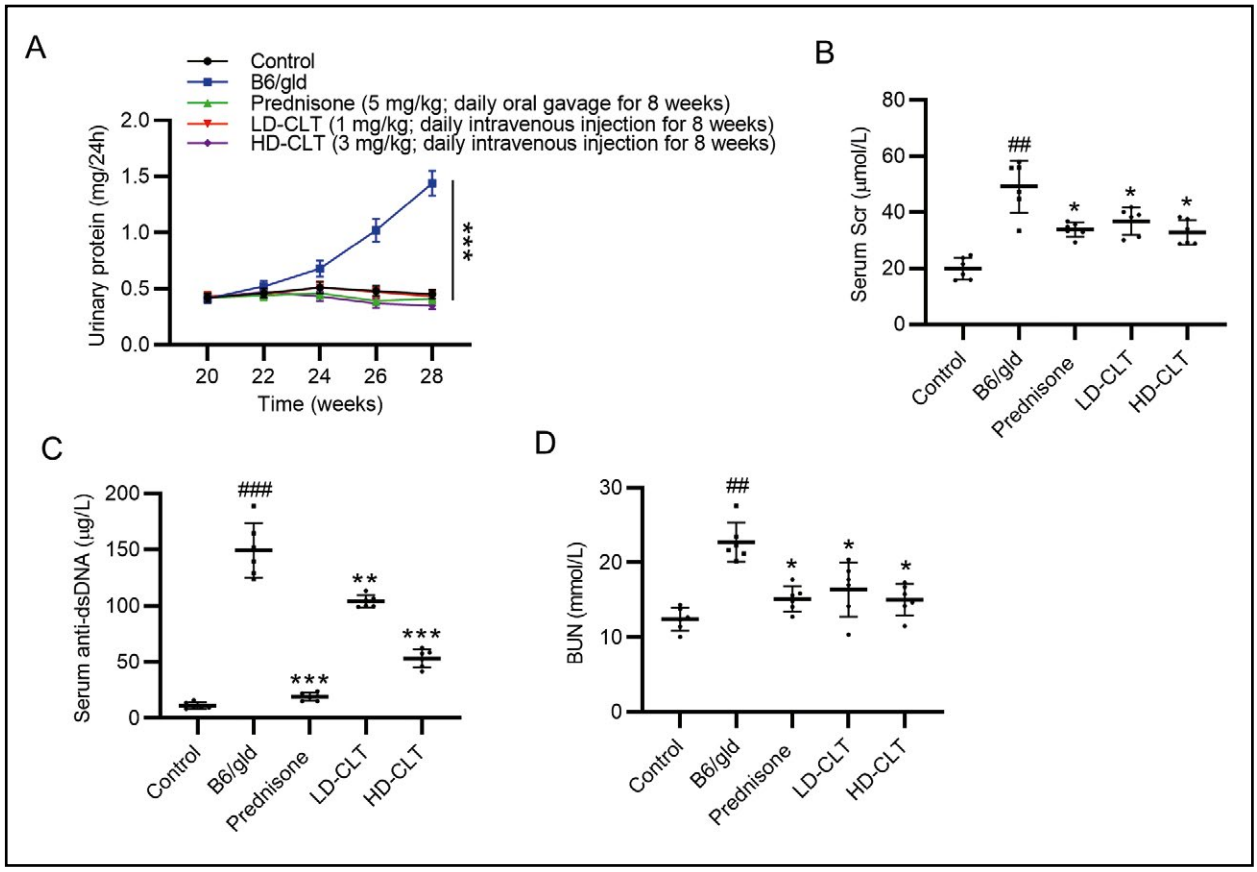

Figure 1. Celastrol (CLT) relieves renal dysfunction in lupus-prone B6/gld mice. FasL-deficient B6/gld (n = 40) were divided into four groups (n = 10/group) of B6/gld, Prednisone (5 mg/kg; daily oral gavage for 8 weeks), LD-CLT (1 mg/kg; daily intravenous injection for 8 weeks) and HD-CLT (3 mg/kg; daily intravenous injection for 8 weeks) groups. **A.** 24 h urinary protein levels of different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) was monitored at the 20th, 22nd, 24th, 26th, and 28th week. N = 10. $^{***}P < 0.001$ *vs.* B6/gld. **B.** Serum creatinine (Scr). $^{\#\#\#}P < 0.001$ *vs.* control, and $^{*}P < 0.05$ *vs.* B6/gld. **C–D.** serum anti-dsDNA and BUN levels of different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) were measured at the 28th week. N = 10. $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ *vs.* control, and $^{*}P < 0.05$ or $^{**}P < 0.01$ or $^{***}P < 0.001$ *vs.* B6/gld.

tion by decreasing urinary protein, serum Scr, serum anti-dsDNA and BUN levels in LN-prone B6/gld mice.

CLT alleviates renal injury in B6/gld mice

To figure out the expected beneficial effects of CLT against renal dysfunction, PAS and hematoxylin-eosin staining of renal tissues was carried out. The results revealed that B6/gld mice displayed abnormalities of basement membrane thickness, remarkable dilatation of Bowman's capsule and marked cellular infiltration in comparison with control C57BL/6 mice (Fig. 2A, B). However, prednisone or CLT administration mitigated glomerular lesions of B6/gld mice, which was evidenced by moderate interstitial inflammation and slightly thickening of glomerular basement membrane in the Prednisone or CLT groups (Fig. 2A, B). Moreover, the results of IHC staining demonstrated that the intensity of α-SMA staining was enhanced in renal tissues of B6/gld mice compared to that of C57BL/6 mice (Fig. 2C, D). Prednisone or CLT supplementation reduced α-SMA intensity in kidney of B6/gld mice (Fig. 2C, D), indicating that CLT can attenuate renal fibrosis of B6/gld mice. Taken together, CLT alleviates renal injury in LN mice.

CLT alleviates CD3$^+$ T cell infiltration and suppresses pro-inflammatory factor mRNA levels in kidneys of B6/gld mice

T cells are related to the pathology of kidney damage [26]. To visualize cellular infiltration in the kidney, IHC staining of CD3$^+$ T cells on paraffin-embedded renal tissues was conducted. As Fig. 3A revealed, no CD3$^+$ T cells were observed in kidney tissues of C57BL/6 mice. In contrast, B6/gld mice exhibited extensive CD3$^+$ T cell infiltration in the glomeruli and tubular interstice (Fig. 3A). In B6/gld mice which received prednisone or CLT, renal CD3$^+$T cell infiltration was attenuated (Fig. 3A). Moreover, proinflammatory cytokine mRNA levels in renal tissues were evaluated using RT-qPCR. Our data showed that B6/gld mice had

10.5603/FHC.a2022.0020
ISSN 0239-8508, e-ISSN 1897-5631

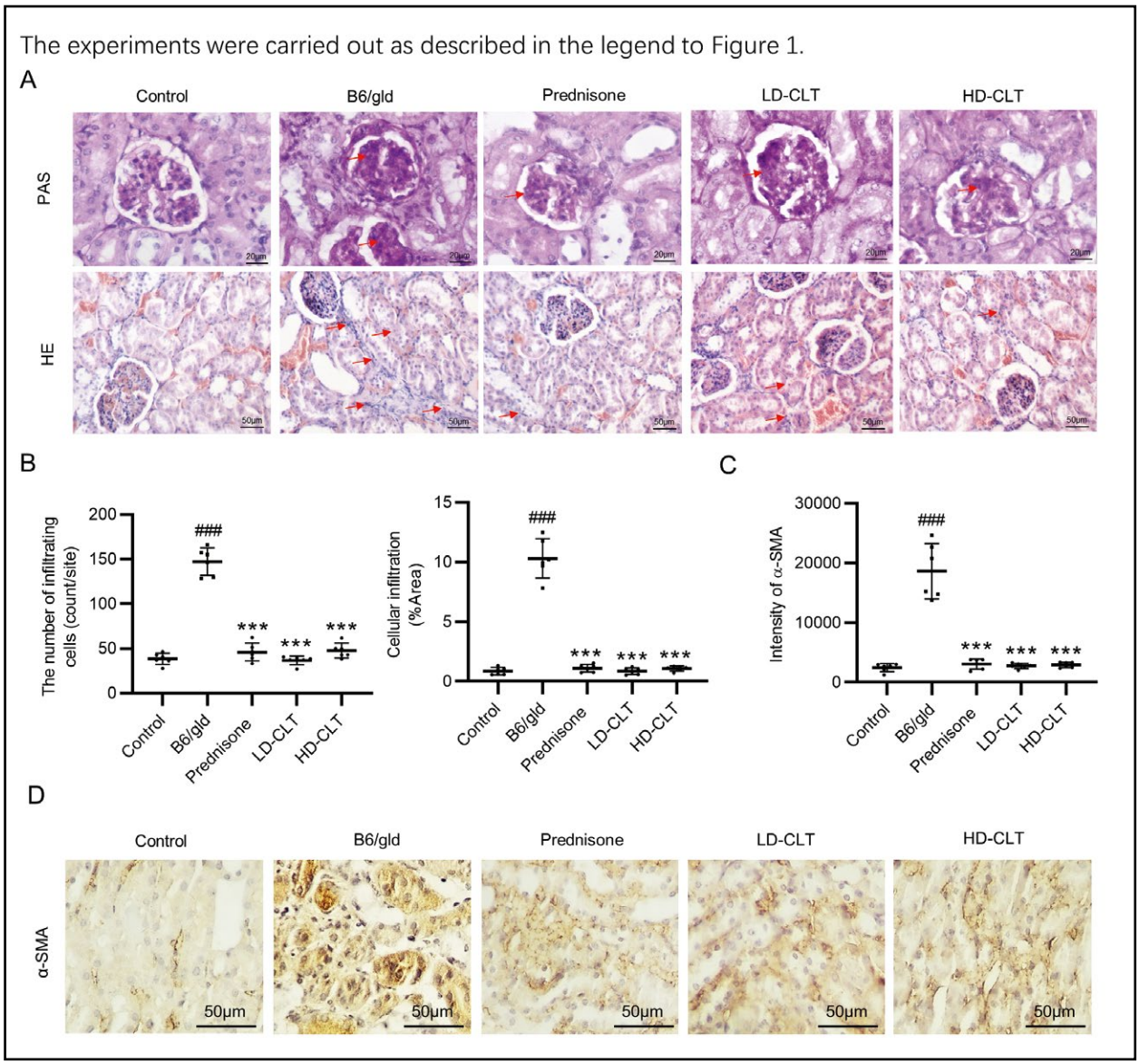

Figure 2. CLT alleviates renal injury in B6/gld mice**.** The experiments were carried out as described in the legend to Fig. 1. **A.** Renal pathological changes in different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) were evaluated by PAS staining (scale bar: 20 μm) and hematoxylin-eosin staining (scale bar: 50 μm). Red arrows indicate the glomerular basement membrane. N = 10. **B.** The percentage of the area with cell infiltration and the number of infiltrating cells in different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT). The cell infiltration was determined in 5 fields of each section (at ×200 magnification). The ratio of the area of inflammatory cell infiltration to total area as well as the number of infiltrating cells was determined by an average of 5 readings of each sample using 20–24 Image J software. N = 10. $^{###}P < 0.001$ *vs.* control, and $^{***}P < 0.001$ *vs.* B6/gld. **C–D.** IHC staining of a-SMA in kidneys of different experimental groups, and intensity of this staining. (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) (scale bar: 50 μm). N = 10. $^{###}P < 0.001$ *vs.* control, and $^{***}P < 0.001$ *vs.* B6/gld.

higher renal mRNA levels of TNF-α, IL-6 and IFN-γ genes than C57BL/6 mice, while prednisone or CLT supplementation reduced mRNA expression of these pro-inflammatory factors in the kidneys of B6/gld mice (Fig. 3B–D). To sum up, CLT ameliorates renal T cell infiltration and suppresses the expression of proinflammatory cytokines' genes in LN mice

CLT induces CD4$^+$Foxp3$^+$ T regulatory lymphocyte in LN

CD4$^+$Foxp3$^+$ Tregs play a crucial role in the maintenance of immune homeostasis and the deficiencies of CD4$^+$Foxp3$^+$ Tregs contribute to the progression of autoimmune disorders [27]. After all mice were sacrificed, permeabilized cells isolated earlier from spleen, lymph node and kidney were stained with intracellular

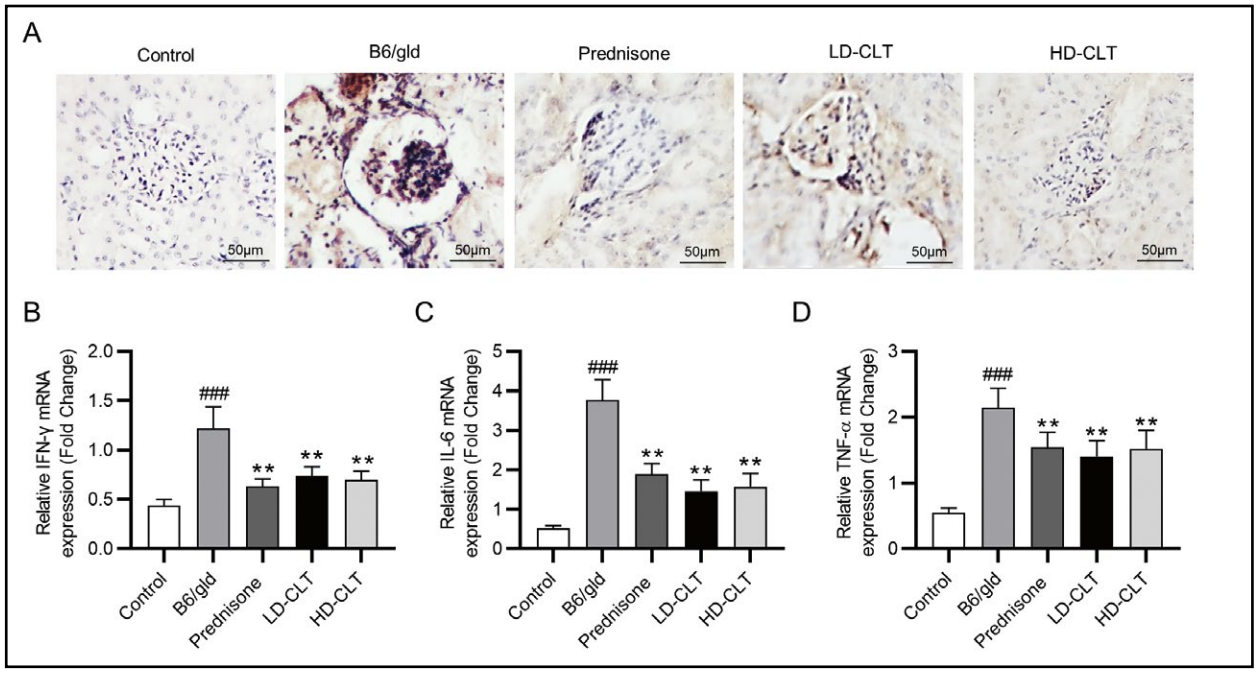

Figure 3. CLT alleviates CD3$^+$ T lymphocyte cell infiltration and suppresses proinflammatory cytokines mRNA levels in renal tissues of B6/gld mice. The experiments were carried out as described in the legend to Fig. 1. **A.** IHC staining on paraffin-embedded kidney sections was applied for detecting CD3$^+$ T cell infiltration in different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT), scale bar: 50 μm. N = 10; **B–D.** Renal mRNA levels of IFN-γ, IL-6 and TNF-αof different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) were assessed by RT-qPCR as described in Methods. N = 10. $^{###}$P < 0.001 *vs.* control, and **P < 0.01 *vs.* B6/gld.

Foxp3 and surface CD4 antibodies, and flow cytometry analysis was utilized for enumerating CD4+Foxp3+ Tregs of different groups. As Fig. 4A–D demonstrated, B6/gld mice exhibited downregulation of CD4$^+$Foxp3$^+$ Tregs in kidneys, lymph nodes and spleens compared with wild-type C57BL/6 mice. After prednisone or CLT treatment, the frequency of CD4$^+$Foxp3$^+$ Tregs in kidneys, lymph nodes and spleens of B6/gld mice was increased (Fig. 4A–D). Therefore, our results indicated that CLT upregulates CD4$^+$Foxp3$^+$ Tregs in LN-prone B6/gld mice.

Discussion

Lupus nephritis is an autoimmune glomerulonephritis [28]. Preventing end-stage renal disease and ameliorating immunopathology are the two primary goals of LN therapy [29]. Consequently, extensive immunosuppression is required for LN, which can lead to serious side effects [8]. Numerous ingredients extracted from Chinese herbs, such as taraxasterol [30], radix astragali [31] and tripterygium glycosides [32], can effectively treat diverse autoimmune diseases in animals and humans. CLT, a pentacyclic triterpene derived from Thunder of God Vine, is a potent agent with anti-tumor, anti-inflammatory, and immunosuppressive effects in animal models and clinical trial [33–36]. Previous reports demonstrated the immunomodulatory properties and effects of CLT in treating immune-based diseases. For example, CLT treatment reduces NF-κB expression, nitrites levels, and immunohistochemical expression of TLR2 and CD3+ T-lymphocytic count in a relapsing-remitting model of multiple sclerosis in rats, as well as attenuates multiple sclerosis and optic neuritis in experimental autoimmune encephalomyelitis rat models [37, 38]. CLT attenuates the severity of ongoing autoimmune arthritis in rats and suppresses the proinflammatory cytokine (*e.g.* IL-17 and IL-6). In addition, it significantly suppresses serum levels of anti-cyclic citrullinated protein/peptide (aCCP) antibodies as well as MMP-9 activity [10]. Proper immune balance between T helper cell 17 (Th17) and Tregs is necessary to avoid autoimmune-related immunopathology [39–41]. CLT has been revealed to regulate Th17/Treg ratio in the joints of rats with adjuvant arthritis [21]. Therefore, we hypothesized that CLT can exert beneficial effects against LN pathogenesis. To test our hypothesis, the effects of CLT in murine model of LN were explored in this study. Our results suggested that CLT mitigates renal dysfunction and injury *via* upregulating CD4$^+$Foxp3$^+$ Tregs. Thus, CLT has the potential to act as an immunosuppressor for LN treatment.

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0020
ISSN 0239-8508, e-ISSN 1897-5631

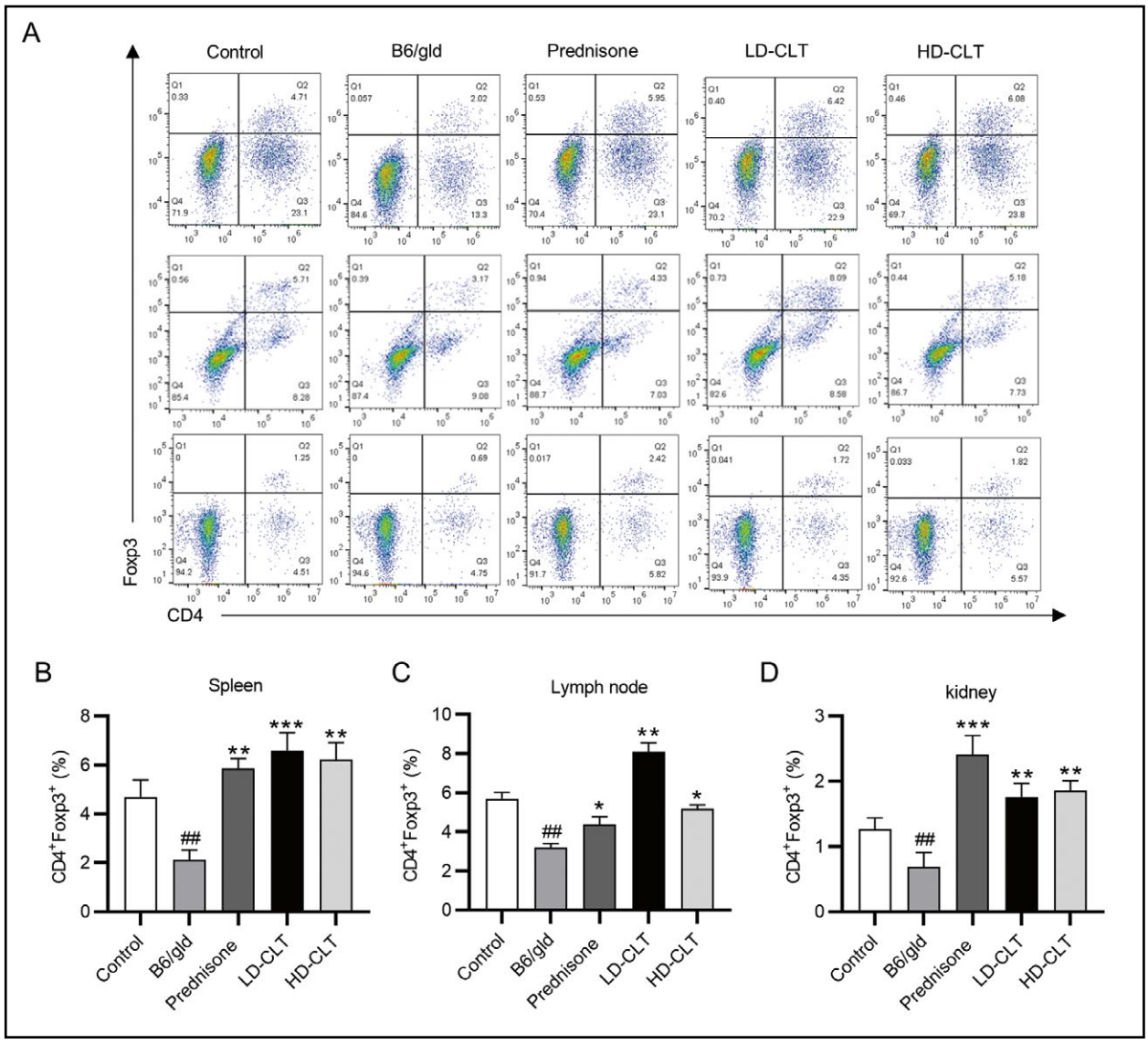

Figure 4. CLT elevates $CD4^+Foxp3^+$ Tregs in B6/gld mice. The experiments were carried out as described in the legend to Fig. 1. After CLT administration to mice for 8 weeks, cells separated from kidneys, lymph nodes and spleens of B6/gld mice were stained with antibodies against surface CD4 and intracellular Foxp3. **A–D.** CD4+Foxp3+ Treg frequency in kidneys, lymph nodes and spleens of different groups (Control, B6/gld, Prednisone, LD-CLT, and HD-CLT) was tested by flow cytometry analysis. N = 10. $^{\#\#}P < 0.01$ *vs.* control, and $^{*}P < 0.05$ or $^{**}P < 0.01$ or $^{***}P < 0.001$ *vs.* B6/gld.

In this study, a spontaneous LN model was established with B6/gld mice to investigate the effects of CLT on immunopathology and the dysfunction of kidneys. We found that CLT ameliorated renal function by reducing serum Scr, BUN and urinary protein in LN-prone B6/gld mice. Moreover, CLT inhibited interstitial inflammation and glomerular basement membrane thickening and repressed renal fibrosis by reducing α-SMA immunoreactivity (a protein that reflects chronic renal fibrosis) to attenuate kidney injury of B6/gld mice. CLT efficacy that protects against renal dysfunction was also demonstrated in other kidney injury models, such as diabetic nephropathy [42] and acute renal injury [43] in rat models, suggesting that CLT ameliorates renal dysfunction through various mechanisms. Especially, CLT protects kidney from glomerular injury with a concomitant reduction of serum autoantibodies and total immunoglobulin G (IgG) in systemic lupus erythematosus induced by active chromatin in BALB/c mice [17]. Since diverse autoimmune disorders are characterized by the presence of noxious autoantibodies, whether CLT can regulates autoimmune responses in LN *in vivo* needs further investigation. Our findings showed that

Folia Histochem Cytobiol. 2022
10.5603/FHC.a2022.0020
ISSN 0239-8508, e-ISSN 1897-5631

CLT significantly reduced anti-ds-DNA levels in the serum of B6/gld mice, meaning that CLT inhibits humoral autoimmunity. Furthermore, CLT suppressed proinflammatory cytokine levels and attenuated $CD3^+$ T cell infiltration in murine renal tissues. All in all, the outcomes indicate that CLT exerts an anti-inflammation impact by modulating both humoral and cellular autoimmune responses in lupus-prone B6/gld mice.

Tregs are a small subgroup of T cells that are indispensable to immune tolerance and immune homeostasis [44]. Deficiencies in the function or number of Tregs result in graft rejection and diverse autoimmune disorders, including rheumatoid arthritis, graft-versus-host disease and SLE [45]. Previously, $CD4^+Foxp3^+$ Tregs upregulation was reported to ameliorate LN in NZB/W F1 mice [46]. Moreover, a Brazilian patient with LN presented $CD4^+Foxp3^+$ Treg deficiency [47], and $Foxp3^+/CD3^+$ cell ratio is markedly decreased in renal biopsies of LN patients [48]. Herein, CLT administration elevated $CD4^+FoxP3^+$ Treg production in kidneys, lymph nodes and spleens of B6/gld mice, suggesting that CLT may exert pharmacological effects against LN pathogenesis *via* inducing $CD4^+FoxP3^+$ Tregs. As previously reported, mangiferin attenuates murine LN by inducing $CD4^+FoxP3^+$ Tregs by suppression of mTOR signaling [23]. Paeoniflorin inhibits effector T cell development by augmenting the frequency of CD4+Foxp3+ Treg cells in lupus-prone B6/gld mice [49]. Therefore, the induction or expansion of $CD4^+Foxp3^+$ Tregs can be a method for the LN treatment.

In conclusion, CLT ameliorates renal dysfunction and attenuates kidney injury by inhibiting renal T cell infiltration and upregulating $CD4^+FoxP3^+$ Tregs in LN-prone B6/gld mice. CLT exerts immunoregulatory and anti-inflammatory effects in LN development, which suggests that CLT may be an available drug for the treatment of LN or other autoimmune disorders in clinical application. Since our study only investigates the function of CLT in B6/gld mice, more research about the underlying molecular mechanisms of CLT action in LN should be carried out in the future.

Conflicts of interest

The authors declare that no competing interests is involved in this study.

References

1. Kiriakidou M, Ching CL. Systemic lupus erythematosus. Ann Intern Med. 2020; 172(11): ITC81–ITC96, doi: 10.7326/AITC202006020, indexed in Pubmed: 32479157.
2. Chang A, Clark MR, Ko K. Cellular aspects of the pathogenesis of lupus nephritis. Curr Opin Rheumatol. 2021; 33(2): 197–204, doi: 10.1097/BOR.0000000000000777, indexed in Pubmed: 33394604.
3. Aljaberi N, Bennett M, Brunner HI, et al. Proteomic profiling of urine: implications for lupus nephritis. Expert Rev Proteomics. 2019; 16(4): 303–313, doi: 10.1080/14789450.2019.1592681, indexed in Pubmed: 30855196.
4. Ma HY, Chen S, Cao WD, et al. Diagnostic value of TWEAK for predicting active lupus nephritis in patients with systemic lupus erythematosus: a systematic review and meta-analysis. Ren Fail. 2021; 43(1): 20–31, doi: 10.1080/0886022X.2020.1853568, indexed in Pubmed: 33307926.
5. Zhao Y, Hu W, Chen P, et al. Immunosuppressive and metabolic agents that influence allo- and xenograft survival by in vivo expansion of T regulatory cells. Xenotransplantation. 2020; 27(6): e12640, doi: 10.1111/xen.12640, indexed in Pubmed: 32892428.
6. Xia Y, Fang X, Dai X, et al. Iguratimod ameliorates nephritis by modulating the Th17/Treg paradigm in pristane-induced lupus. Int Immunopharmacol. 2021; 96: 107563, doi: 10.1016/j.intimp.2021.107563, indexed in Pubmed: 33812258.
7. Zhao M, Shao Y, Xu J, et al. LINC00466 impacts cell proliferation, metastasis and sensitivity to temozolomide of glioma by sponging miR-137 to regulate PPP1R14B expression. Onco Targets Ther. 2021; 14: 1147–1159, doi: 10.2147/OTT.S273264, indexed in Pubmed: 33642868.
8. Aziz F, Chaudhary K. Lupus nephritis: a treatment update. Curr Clin Pharmacol. 2018; 13(1): 4–13, doi: 10.2174/1574884713666180403150359, indexed in Pubmed: 29611488.
9. Allison AC, Cacabelos R, Lombardi VR, et al. Celastrol, a potent antioxidant and anti-inflammatory drug, as a possible treatment for Alzheimer's disease. Prog Neuropsychopharmacol Biol Psychiatry. 2001; 25(7): 1341–1357, doi: 10.1016/s0278-5846(01)00192-0, indexed in Pubmed: 11513350.
10. Venkatesha SH, Yu H, Rajaiah R, et al. Celastrus-derived celastrol suppresses autoimmune arthritis by modulating antigen-induced cellular and humoral effector responses. J Biol Chem. 2011; 286(17): 15138–15146, doi: 10.1074/jbc.M111.226365, indexed in Pubmed: 21402700.
11. Lee JH, Choi KJ, Seo WD, et al. Enhancement of radiation sensitivity in lung cancer cells by celastrol is mediated by inhibition of Hsp90. Int J Mol Med. 2011; 27(3): 441–446, doi: 10.3892/ijmm.2011.601, indexed in Pubmed: 21249311.
12. Zhang X, Yang J, Chen M, et al. Metabolomics profiles delineate uridine deficiency contributes to mitochondria-mediated apoptosis induced by celastrol in human acute promyelocytic leukemia cells. Oncotarget. 2016; 7(29): 46557–46572, doi: 10.18632/oncotarget.10286, indexed in Pubmed: 27374097.
13. Shaker ME, Ashamallah SA, Houssen ME. Celastrol ameliorates murine colitis via modulating oxidative stress, inflammatory cytokines and intestinal homeostasis. Chem Biol Interact. 2014; 210: 26–33, doi: 10.1016/j.cbi.2013.12.007, indexed in Pubmed: 24384223.
14. Venkatesha SH, Dudics S, Astry B, et al. Control of autoimmune inflammation by celastrol, a natural triterpenoid. Pathog Dis. 2016; 74(6), doi: 10.1093/femspd/ftw059, indexed in Pubmed: 27405485.
15. Guo L, Luo S, Du Z, et al. Targeted delivery of celastrol to mesangial cells is effective against mesangioproliferative glomerulonephritis. Nat Commun. 2017; 8(1): 878, doi: 10.1038/s41467-017-00834-8, indexed in Pubmed: 29026082.
16. Xu X, Zhong J, Wu Z, et al. Effects of tripterine on mRNA expression of TGF-beta1 and collagen IV expression in BW F1 mice. Cell Biochem Funct. 2007; 25(5): 501–507, doi: 10.1002/cbf.1338, indexed in Pubmed: 16929507.
17. Li H, Zhang Yy, Huang XY, et al. Beneficial effect of tripterine on systemic lupus erythematosus induced by active chro-

matin in BALB/c mice. Eur J Pharmacol. 2005; 512(2-3): 231–237, doi: 10.1016/j.ejphar.2005.02.030, indexed in Pubmed: 15840409.
18. Chu C, He W, Kuang Y, et al. Celastrol protects kidney against ischemia-reperfusion-induced injury in rats. J Surg Res. 2014; 186(1): 398–407, doi: 10.1016/j.jss.2013.07.048, indexed in Pubmed: 23981709.
19. Zhang J, Shan J, Chen X, et al. Celastrol mediates Th17 and Treg cell generation via metabolic signaling. Biochem Biophys Res Commun. 2018; 497(3): 883–889, doi: 10.1016/j.bbrc.2018.02.163, indexed in Pubmed: 29476742.
20. Wang Y, Cao Lu, Xu LM, et al. Celastrol ameliorates EAE induction by suppressing pathogenic T cell responses in the peripheral and central nervous systems. J Neuroimmune Pharmacol. 2015; 10(3): 506–516, doi: 10.1007/s11481-015-9598-9, indexed in Pubmed: 25773257.
21. Astry B, Venkatesha SH, Laurence A, et al. Celastrol, a Chinese herbal compound, controls autoimmune inflammation by altering the balance of pathogenic and regulatory T cells in the target organ. Clin Immunol. 2015; 157(2): 228–238, doi: 10.1016/j.clim.2015.01.011, indexed in Pubmed: 25660987.
22. Takahashi T, Tanaka M, Brannan CI, et al. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell. 1994; 76(6): 969–976, doi: 10.1016/0092-8674(94)90375-1, indexed in Pubmed: 7511063.
23. Liang CL, Lu W, Zhou JY, et al. Mangiferin attenuates murine lupus nephritis by inducing CD4+Foxp3+ regulatory T cells via suppression of mTOR signaling. Cell Physiol Biochem. 2018; 50(4): 1560–1573, doi: 10.1159/000494654, indexed in Pubmed: 30359968.
24. Costa-Bauza A, Grases F, Gomila I, et al. A simple and rapid colorimetric method for determination of phytate in urine. Urol Res. 2012; 40(6): 663–669, doi: 10.1007/s00240-012-0473-3, indexed in Pubmed: 22476541.
25. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4): 402–408, doi: 10.1006/meth.2001.1262, indexed in Pubmed: 11846609.
26. Martin-Moreno PL, Tripathi S, Chandraker A. Regulatory T cells and kidney transplantation. Clin J Am Soc Nephrol. 2018; 13(11): 1760–1764, doi: 10.2215/CJN.01750218, indexed in Pubmed: 29789350.
27. Hou X, Song J, Su J, et al. CD4(+)Foxp3(+) Tregs protect against innate immune cell-mediated fulminant hepatitis in mice. Mol Immunol. 2015; 63(2): 420–427, doi: 10.1016/j.molimm.2014.09.015, indexed in Pubmed: 25315497.
28. Kronbichler A, Brezina B, Gauckler P, et al. Refractory lupus nephritis: When, why and how to treat. Autoimmun Rev. 2019; 18(5): 510–518, doi: 10.1016/j.autrev.2019.03.004, indexed in Pubmed: 30844548.
29. Wang S, Chen D, Zuo Ke, et al. Long-term renal outcomes of mesangial proliferative lupus nephritis in Chinese patients. Clin Rheumatol. 2022; 41(2): 429–436, doi: 10.1007/s10067-021-05909-y, indexed in Pubmed: 34549340.
30. Chen J, Wu W, Zhang M, et al. Taraxasterol suppresses inflammation in IL-1β-induced rheumatoid arthritis fibroblast-like synoviocytes and rheumatoid arthritis progression in mice. Int Immunopharmacol. 2019; 70: 274–283, doi: 10.1016/j.intimp.2019.02.029, indexed in Pubmed: 30851708.
31. Zhao P, Su G, Xiao X, et al. Chinese medicinal herb Radix Astragali suppresses cardiac contractile dysfunction and inflammation in a rat model of autoimmune myocarditis. Toxicol Lett. 2008; 182(1-3): 29–35, doi: 10.1016/j.toxlet.2008.08.002, indexed in Pubmed: 18782607.
32. Luo Y, Zhang Y, Kuai Le, et al. Efficacy and safety of Tripterygium glycosides in Sjögren's syndrome treatment: evidence from 12 randomized controlled trials. Ann Palliat Med. 2021; 10(7): 8215–8231, doi: 10.21037/apm-21-256, indexed in Pubmed: 34263629.
33. An L, Li Z, Shi L, et al. Inflammation-targeted celastrol nanodrug attenuates collagen-induced arthritis through nf-κb and notch1 pathways. Nano Lett. 2020; 20(10): 7728–7736, doi: 10.1021/acs.nanolett.0c03279, indexed in Pubmed: 32965124.
34. Liu Y, Xiao N, Du H, et al. Celastrol ameliorates autoimmune disorders in Trex1-deficient mice. Biochem Pharmacol. 2020; 178: 114090, doi: 10.1016/j.bcp.2020.114090, indexed in Pubmed: 32565148.
35. Lu Y, Liu Y, Zhou J, et al. Biosynthesis, total synthesis, structural modifications, bioactivity, and mechanism of action of the quinone-methide triterpenoid celastrol. Med Res Rev. 2021; 41(2): 1022–1060, doi: 10.1002/med.21751, indexed in Pubmed: 33174200.
36. Lagoa R, Silva J, Rodrigues JR, et al. Advances in phytochemical delivery systems for improved anticancer activity. Biotechnol Adv. 2020; 38: 107382, doi: 10.1016/j.biotechadv.2019.04.004, indexed in Pubmed: 30978386.
37. Abdin AA, Hasby EA. Modulatory effect of celastrol on Th1/Th2 cytokines profile, TLR2 and CD3+ T-lymphocyte expression in a relapsing-remitting model of multiple sclerosis in rats. Eur J Pharmacol. 2014; 742: 102–112, doi: 10.1016/j.ejphar.2014.09.001, indexed in Pubmed: 25218987.
38. Yang H, Liu C, Jiang J, et al. Celastrol attenuates multiple sclerosis and optic neuritis in an experimental autoimmune encephalomyelitis model. Front Pharmacol. 2017; 8: 44, doi: 10.3389/fphar.2017.00044, indexed in Pubmed: 28239352.
39. Qi WH, Zhang YY, Xing K, et al. 2', 4'-Dihydroxy-2,3-dimethoxychalcone: A pharmacological inverse agonist of RORγt ameliorating Th17-driven inflammatory diseases by regulating Th17/Treg. Int Immunopharmacol. 2022; 108: 108769, doi: 10.1016/j.intimp.2022.108769, indexed in Pubmed: 35453073.
40. Honda M, Segawa T, Ishikawa K, et al. Nephronectin influences EAE development by regulating the Th17/Treg balance via reactive oxygen species. Am J Physiol Cell Physiol. 2022; 322(4): C699–C711, doi: 10.1152/ajpcell.00376.2021, indexed in Pubmed: 35235429.
41. Zhou F, Wang X, Wang L, et al. Genetics, epigenetics, cellular immunology, and gut microbiota: emerging links with graves' disease. Front Cell Dev Biol. 2021; 9: 794912, doi: 10.3389/fcell.2021.794912, indexed in Pubmed: 35059400.
42. Nie Y, Fu C, Zhang H, et al. Celastrol slows the progression of early diabetic nephropathy in rats via the PI3K/AKT pathway. BMC Complement Med Ther. 2020; 20(1): 321, doi: 10.1186/s12906-020-03050-y, indexed in Pubmed: 33097050.
43. Hu X, Jia M, Fu Yu, et al. Novel low-toxic derivative of celastrol maintains protective effect against acute renal injury. ACS Omega. 2018; 3(3): 2652–2660, doi: 10.1021/acsomega.7b01890, indexed in Pubmed: 30023844.
44. Alahyari S, Rajaeinejad M, Jalaeikhoo H, et al. Regulatory T cells in immunopathogenesis and severity of COVID-19: a systematic review. Arch Iran Med. 2022; 25(2): 127–132, doi: 10.34172/aim.2022.22, indexed in Pubmed: 35429952.
45. Esensten JH, Muller YD, Bluestone JA, et al. Regulatory T-cell therapy for autoimmune and autoinflammatory diseases: the next frontier. J Allergy Clin Immunol. 2018; 142(6): 1710–1718, doi: 10.1016/j.jaci.2018.10.015, indexed in Pubmed: 30367909.
46. Yan JJ, Lee JG, Jang JY, et al. IL-2/anti-IL-2 complexes ameliorate lupus nephritis by expansion of CD4CD25Foxp3 regu-

latory T cells. Kidney Int. 2017; 91(3): 603–615, doi: 10.1016/j.kint.2016.09.022, indexed in Pubmed: 27914701.
47. Matta MC, Soares DC, Kerstenetzky MS, et al. CD4+CD25 high foxp3+ treg deficiency in a Brazilian patient with gaucher disease and lupus nephritis. Hum Immunol. 2016; 77(2): 196–200, doi: 10.1016/j.humimm.2015.11.018, indexed in Pubmed: 26673565.
48. Afeltra A, Gigante A, Margiotta DP, et al. The involvement of T regulatory lymphocytes in a cohort of lupus nephritis patients: a pilot study. Intern Emerg Med. 2015; 10(6): 677–683, doi: 10.1007/s11739-015-1212-x, indexed in Pubmed: 25720575.
49. Liang CL, Lu W, Qiu F, et al. Paeoniflorin ameliorates murine lupus nephritis by increasing CD4+Foxp3+ Treg cells via enhancing mTNFα-TNFR2 pathway. Biochem Pharmacol. 2021; 185: 114434, doi: 10.1016/j.bcp.2021.114434, indexed in Pubmed: 33513343.

Submitted: 6 December, 2021
Accepted after reviews: 22 June, 2022
Available as AoP: 6 July, 2022