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

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ORIGINAL PAPER

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

Short title: Celastrol alleviates murine lupus nephritis

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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.

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 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-17⁺RORγt⁺ 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 Harbor, 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 approval of the Institutional Ethics Review Committee of Wuhan Hospital of Traditional Chinese Medicine (Hubei, China). 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 ×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, Abcam, ab124964) 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^−ΔΔCt method [25]. Primer sequences are shown in Table 1.

Statistical analysis. All data were evaluated using SPSS 21.0 statistical software
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 20\textsuperscript{th}, 22\textsuperscript{nd}, 24\textsuperscript{th}, 26\textsuperscript{th}, and 28\textsuperscript{th} 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 26\textsuperscript{th} and the 28\textsuperscript{th} week (Fig. 1A). Additionally, B6/gld mice exhibited higher serum Scr, serum anti-dsDNA and BUN levels than C57BL/6 mice at the 28\textsuperscript{th} 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 dysfunction 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 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 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.

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 of 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 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 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


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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 < 0.001 or ####P < 0.0001 vs. control, and *P < 0.05 or **P < 0.01 or ***P < 0.001 vs. B6/gld. [where should we put B.??]
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 α-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.
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-α of 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.
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.