

Original Article

***Cordyceps sinensis* may inhibit Th22 cell chemotaxis to improve kidney function in IgA nephropathy**

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Abstract: IgA nephropathy is the most common form of primary glomerulonephritis and an important cause of kidney failure. *Cordyceps sinensis* (CS) is a parasitic fungus that has a long history of use in Chinese medicine for the treatment of nephritis. Interleukin (IL)-22-producing helper T cells (Th22 cells) have been reported to be involved in IgA nephropathy. Th22 cells link the immune response to tissue inflammation. To elucidate the possible efficacy and mechanisms by which CS counteracts nephritis, we established an IgA nephropathy model in 6-week-old female BALB/c mice. The mice were randomly separated into 3 groups, the normal control, IgA nephropathy and CS (5 mg/kg/d) treatment groups. The Th22 cell frequencies and the relative pathological and cytokine changes were measured with flow cytometry, whereas the serum chemokine ligand 27 (CCL27) and IL-22 concentrations were detected with ELISA. The Th22 cell frequency decreased after 1 month of CS therapy. Additionally, mesangial cell proliferation decreased. Moreover, the chemokine receptor type 10 (CCR10), CCL27 and IL-22 expression levels were significantly reduced. In conclusion, CS may modulate the chemotaxis of Th22 cells to suppress inflammatory responses in IgA nephropathy.

Keywords: *Cordyceps sinensis*, IgA nephropathy, Th22 cells, CCR10, CCL27

Introduction

IgA nephropathy (IgAN) represents the leading cause of kidney failure among East Asian populations and is the most frequent form of primary glomerulonephritis among Asians, Europeans and some regions of the USA [1-3]. Increasing evidence has shown that inflammatory immune responses play an important role in the development and progression of IgAN.

Native CD4⁺ T cells can develop into various helper T subsets with different cytokine profiles that play discriminative roles in translating antigen-specific immune responses into tissue functions or immunopathologic changes. Cells that produce IL-22 are part of a new helper T (Th) cell subgroup that may be important for skin pathology [2]. Interleukin (IL)-22 is a member of the IL-10 cytokine family and is involved in inflammatory and wound healing processes. Th22 cells are characterized by par-

ticularly high IL-22 production and specifically express the CCR10 [4]. Th22 cells are distinct from Th17 and Th1 cells and play important roles in skin homeostasis and pathological changes. Accumulating evidence suggests that IL-22 is important in epithelial cell homeostasis, infections, tissue repair and wound healing [5-7]. Th22 cells are positively correlated with the plasma IL-22 level in IgAN patients. Chemokines, which are members of a protein superfamily of structurally related, small, secreted proteins, are key mediators of leukocyte recruitment to inflammatory lesions where damage may have occurred. Chemokines have been shown to regulate trafficking of distinct leukocyte subsets into peripheral tissues [8]. CCL27 selectively attracts cutaneous lymphocyte-associated antigen (CLA)⁺ memory T cells by interacting with the CCR10 expressed by lymphocytes. The CCL27-CCR10 interaction plays a pivotal role in T cell-mediated skin inflamma-

tion [9]. Moreover, a significant positive correlation has been observed between Th22 and Th17 cells in IgAN patients. Furthermore, IgAN patients with proteinuria have a higher percentage of Th22 cells than IgAN patients without proteinuria [10]. In our previous study, we found an overrepresentation of Th22 cells in IgAN patients, which might be attributable to the actions of kidney chemokines and cytokines. Additionally, we found that Losartan and Dexamethasone might suppress inflammatory responses by inhibiting the chemotaxis of Th22 cells in IgA nephropathy patients [11, 12].

Cordyceps sinensis (CS), which is a well-known traditional Chinese medicine, is a fungus that develops stroma and is found on the larvae of lepidopteran caterpillars. Numerous studies have demonstrated multiple pharmacologic actions of CS [13-15], such as reducing damage to renal tubules and protecting the Na⁺-K⁺-ATPase on cellular membranes [16]. CS can decrease chronic renal insufficiency, reduce resistance and pressure in the arteries and promote platelet formation [17]. Thus, CS may help prevent hypoxia by acting as a monoamine oxidase inhibitor [18]. In our previous study, we found that the proportion of Th22 cells was increased in IgA nephropathy [11, 19]. Because the role for CS in the regulation of Th22 cells is unknown, we aim to investigate whether CS blocks Th22 cell infiltration and to demonstrate the possible mechanisms of action. We established an IgAN mouse model to elucidate how CS modulated Th22 cells.

Materials and methods

Ethics statement

The mice were housed under controlled humidity, temperature and lighting conditions in facilities accredited by the Experimental Animal Center of Central South University (Changsha, Hunan, China). All animals had free access to standard mouse chow and drinking water. Our study was conducted in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The study protocol was approved by the Animal Experimental Ethics Committee of Hunan Province (Permit Number: 20150003). All efforts were made to minimize animal suffering.

Experimental animals

Female BALB/c mice (19±2 g, n=20, 5 mice/treatment group) were obtained from the Experimental Animal Center of Central South University (Changsha, Hunan, China) at 6 weeks of age. The IgAN model was induced by administering bovine serum albumin (BSA) (Roche, USA) in acidified water, CCL4, and castor oil combined with LPS (Sigma, USA) (50 µg) at different time points for two months after one week of adaptive feeding [20].

Drug treatment

The mice were randomly divided into 3 groups: the control mice (control), IgAN mice (IgAN) and CS-treated IgAN mice (CS-IgAN). For the CS-treated IgAN mouse group, IgAN mice were sensitized by intragastric gavage with CS (5 mg/kg/d) [21]. The control and IgAN groups received an equal amount of distilled water. At 4 weeks, all mice were anesthetized with 10% chloral hydrate, blood was collected by orbital bleeding, and the mice were sacrificed via cervical dislocation. Then, the tissues were collected.

Leukocyte isolation

The mice were exsanguinated via the retro-orbital plexus. The blood was placed in a heparin anticoagulant tube and diluted with PBS in a 1:2 ratio. The diluted blood was layered onto 3 ml of Ficoll lymphocyte separation liquid (GE, USA) in a 15-ml centrifuge tube and centrifuged for 30 min at 450 × g. Following centrifugation, the layer containing the peripheral mononuclear cells (PBMCs) was transferred to another test tube and washed twice with 10 ml of PBS at 450 × g. The PBMCs were resuspended in 1 ml of RPMI 1640 medium and counted.

Flow cytometry

The isolated murine leukocytes were equally distributed into tubes. The expression of T cell markers in the blood was determined via flow cytometry after the cells were stained for surface or intracellular markers with anti-mouse-specific antibodies conjugated to APC/Cy7, FITC, or PE. These antibodies included anti-CD3, anti-CD4 and anti-IL-22 and were purchased from BD Biosciences (Franklin Lakes,

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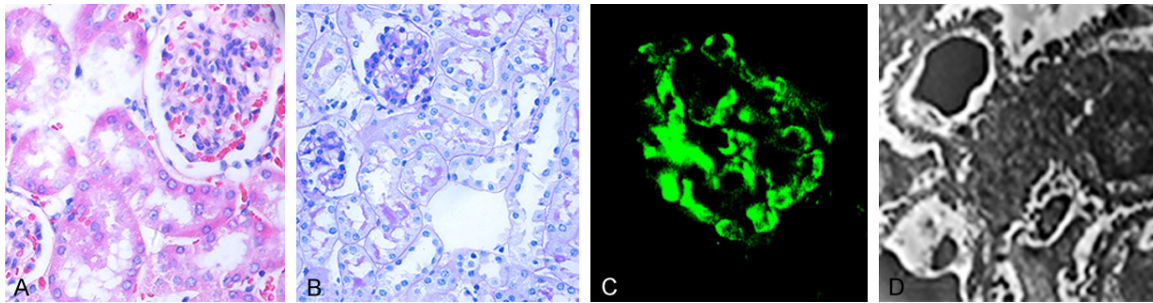


Figure 1. Basic pathological characteristics of the IgA nephropathy model mice. A: HE staining ($\times 400$); B: PAS staining ($\times 400$); C: Immunofluorescence for IgA ($\times 400$); D: Electron microscopy ($\times 2000$).

NJ, USA) or R&D Systems (Minneapolis, MN, USA). Appropriate species-matched antibodies served as the isotype controls. After stimulation with Leukocyte Activation Cocktail (BD, USA) in an incubator for 6 h at 37°C with 5% CO_2 , the cells were stained with fluorochrome-labeled antibodies specific for CD3 (APC-Cy7; BD, USA) and CD4 (FITC; BD) for 30 min at 4°C . After membrane permeabilization and fixation, intracellular staining for IL-22 (PE, BioLegend, USA) was performed. Flow cytometry was performed using the FACS Can to II flow cytometer (BD Biosciences), and the data were analyzed using the BD FACS and FlowJo software.

Histological analysis

The renal tissues were fixed in 4% paraformaldehyde, embedded in paraffin and serially cut. Tissue sections (1.5-mm thick) were stained with a periodic acid-Schiff reagent. The stained kidney sections were examined by a renal pathologist under a light microscope. For the immunofluorescence analysis, the renal tissues were cut into frozen slices and fixed in acetone for 1 min. After fixation, 5% normal goat serum in PBS (pH 7.4) was used to block non-specific protein binding sites. IgA in renal tissues was detected with fluorescein-labeled goat anti-mouse IgA (Abcam, USA). The renal tissues were incubated at 37°C for 1 h, washed 3 times in PBS for 3 min, mounted with glycerol, and visualized under a fluorescence microscope. Then, electron microscopic examination procedures were performed. The samples were examined using the H-7700 transmission electron microscope (Hitachi, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The CCL27 and IL-22 levels in the renal tissue homogenates and sera were quantified using

ELISA kits (eBiosciences, USA) according to the manufacturer's instructions. All samples were assayed in duplicate.

Statistical analysis

The 2-tailed Mann-Whitney non-parametric U test was used to determine differences between two groups. A P -value < 0.05 was considered significant. The statistical analyses were performed using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA).

Results

The IgAN model and the basic pathological manifestations of the kidney were verified

Two BALB/c mice were randomly selected in the 9th week. Twenty-four hour urine specimens were collected in metabolic cages. The kidneys were removed for pathological examination. Microscopic hematuria was observed, and the qualitative proteinuria results were positive. Glomerular mesangial cell proliferation was demonstrated with hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) staining. Specifically, IgA was deposited in the glomeruli, and electron microscopy showed electron-dense deposits in the mesangial area (**Figure 1**).

Th22 cells were decreased after CS treatment

After we confirmed that the model was successfully generated, the lymphocytes were separated according to the treatment group and examined using flow cytometry to detect Th22 cells in the 13th week. In the first step, we analyzed the expression of Th22 cells in the normal BALB/c, IgAN and CS-treated IgAN mice. The isolated blood leukocytes were stained and

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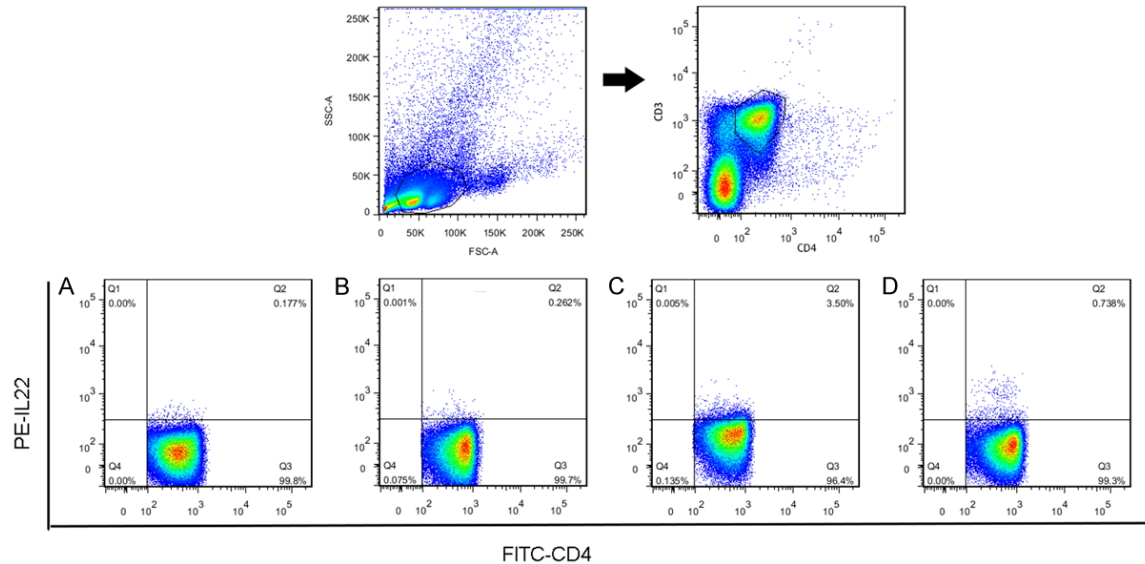


Figure 2. Representative flow chart for the percentages of Th22 cells in homotype IgG (A), normal (B), IgAN (C), and CS-treated IgAN (D). The Th22 cell population within CD4⁺ T cells was identified based on CD3⁺ and CD4⁺ expression (n=5 mice/group). The percentages of Th22 cells were determined by flow cytometry.

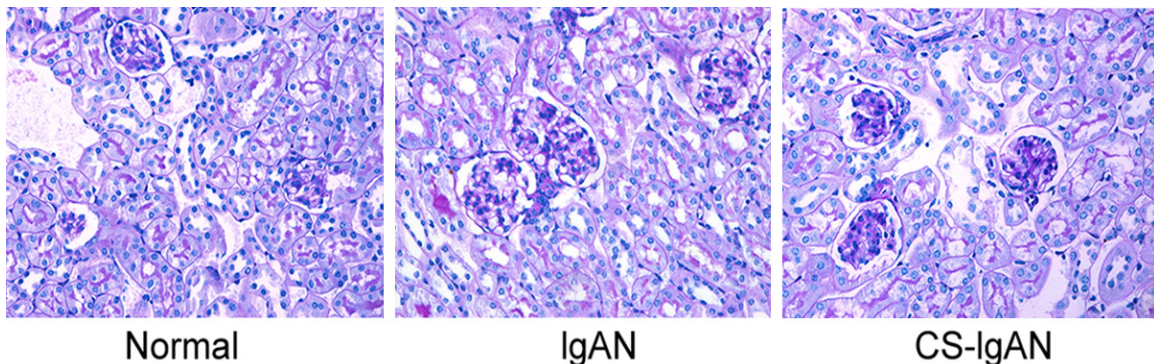


Figure 3. Representative photographs of kidney PAS staining for each group. The IgAN group demonstrates pronounced proliferation of the mesangium compared with the normal group, whereas proliferation was reduced in the CS-IgAN group.

analyzed to evaluate intracellular expression of the cytokine IL-22. Th22 cells were significantly elevated in the IgAN mice compared with the normal BABL/c mice ($0.22 \pm 0.07\%$ vs $4.71 \pm 0.56\%$, $P < 0.01$). However, the frequency of the Th22 cells was significantly reduced after CS treatment ($4.71 \pm 0.56\%$ vs $0.53 \pm 0.40\%$, $P < 0.01$) (**Figure 2**).

Mesangial cell proliferation decreased after CS treatment

Next, we determined whether changes occurred in renal pathological features that would contribute to the decrease in Th22 cells. We

analyzed the PAS-stained images for each group and compared them to the normal group. Mesangial cells proliferated in the IgAN group. After 1 month of CS treatment, mesangial cell proliferation decreased significantly. These results suggest that the onset of IgAN in mice may be due to a decrease in effector Th22 cells during disease progression, as shown in **Figure 3**.

Reduced CCR10 and CCL27 expression in the CS-treated groups

We tested the possible mechanism by which CS decreased the ratio of Th22 cells and investi-

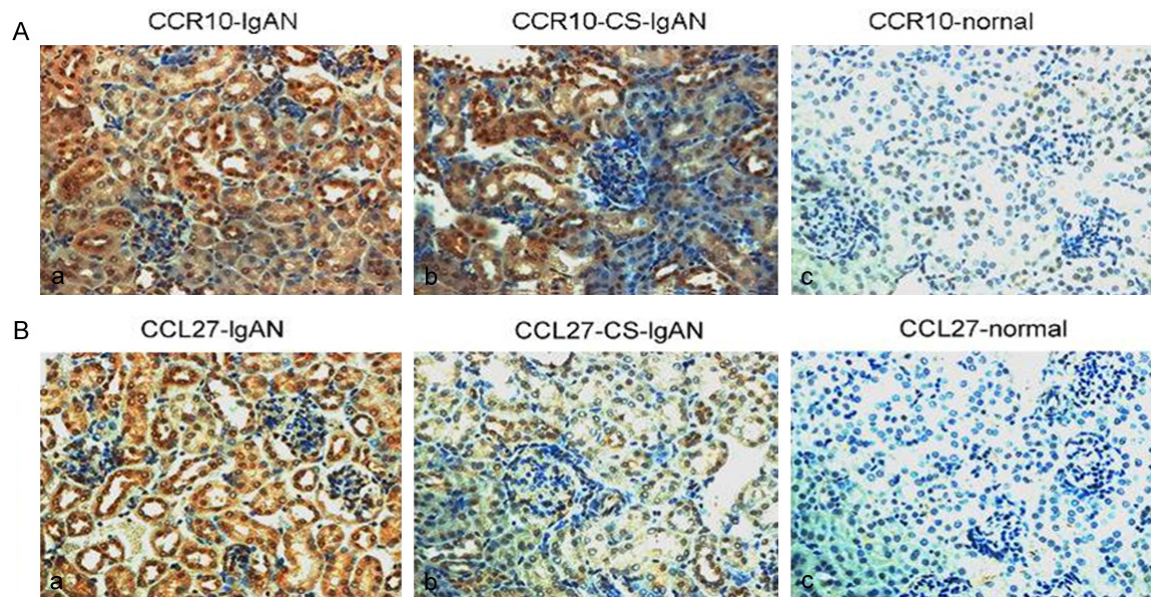


Figure 4. CCR10 and CCL27 expression in the biopsies of the control group, the IgAN group and the CS-IgAN group after one month of CS treatment. Immunohistochemistry was performed on renal biopsies from BALB/c mice ($\times 400$). A prominent accumulation of CCR10-positive or CCL27-positive cells was found around the glomerular and tubular membranes.

gated whether inhibition of chemotaxis was associated with CS treatment. We investigated the role of the chemokine CCL27 and its receptor CCR10. We examined whether CS inhibited the chemotaxis mediated by CCR10 and CCL27. The CCR10 and CCL27 expression levels were examined using immunohistochemistry. Based on the immunohistochemistry analysis, the normal BALB/c mice did not express CCR10 or CCL27, whereas the expression of these molecules was significantly increased in the IgAN mice. However, after treatment with CS for one month, both CCL27 and CCR10 expression was reduced significantly (**Figure 4**).

Inhibition of chemotaxis reduced Th22 cell infiltration

Recruitment from peripheral blood could contribute to the increased number of Th22 cells in the kidney. Lymphocyte migration is tightly regulated by chemokine/CCR interactions. The plasma IL-22 and CCL27 concentrations were significantly higher in the IgAN group than in the normal group. After the CS treatments, the concentrations of both IL-22 (154.0 ± 12.0 pg/ml, 634.2 ± 29.8 pg/ml and 383.5 ± 7.9 pg/ml, $P < 0.01$) and CCL27 (327.9 ± 7.5 pg/ml, 465.0 ± 6.9 pg/ml and 373.0 ± 11.5 pg/ml, $P < 0.01$) were significantly decreased, which was consis-

tent with the immunohistochemistry results (**Figure 5**).

Discussion

To the best of our knowledge, the mechanism described herein by which CS may prevent the kidney damage induced by Th22 cells is novel. In this study, we compared the Th22 cell frequencies and the relative changes in chemokines, chemokine receptors and renal pathology before and after drug intervention. The Th22 cell frequency was increased in the IgAN model compared to the normal controls but decreased after CS treatment. This finding was consistent with the renal pathological changes and ELISA results.

IgAN has been recognized as an autoimmune disease characterized by deposition of IgA in the mesangial area. Up to 40% of patients with IgAN progress to end-stage renal disease [22]. Therefore, preventing, halting and decreasing the rate of progression of IgAN is a very worthwhile goal [23]. Disease progression is determined mainly by the balance between the microorganism and the host defense system [24, 25]. IL-22 is a T cell mediator that directly promotes innate, non-specific immunity in tissues [5, 24], which may be produced by skin-

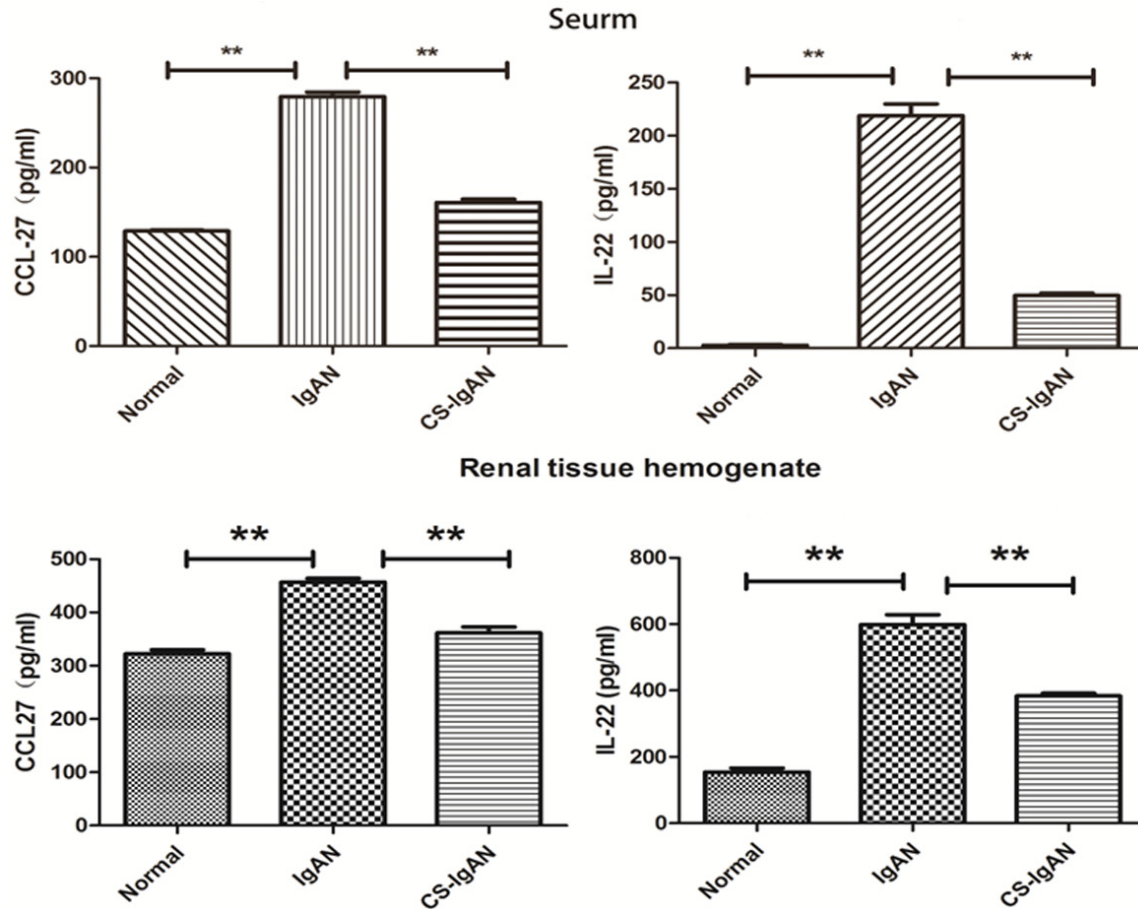


Figure 5. Plasma concentrations of the chemokines CCL27 and IL-22. Th22 cells produce IL-22, The CCL27 and IL-22 concentrations were measured using ELISA. The bars indicate the means; the data are reported as the means \pm SEMs from 5 independent experiments. Differences between groups were compared using paired-sample T tests. **P < 0.01 compared with the control.

infiltrating lymphocytes that are potentially involved in the initiation and/or maintenance of the pathogenesis of psoriasis [6]. The possible mechanisms by which Th22 cells infiltrate the kidney have been elucidated in our early stage research.

CS is a precious herbal medicine that has been used to treat diseases for many years in China. Its application areas include the nervous system, respiratory system and renal system, and its extracts have been suggested to have a protective effect in renal tubular cells [26-28]. Many bioactive components have been extracted from CS, including nucleosides, polysaccharides, sterols, proteins, amino acids and polypeptides. These constituents correspond to demonstrated pharmacologic actions, such as anti-inflammatory, antioxidant, anti-tumor, anti-apoptotic, and immunomodulatory actions [29-

33]. CS ameliorated albumin-induced EMT of HK2 cells by decreasing NADPH oxidase activity and inhibiting ROS production [34]. Additionally, CS prevented the recurrence of lupus nephritis, protected kidney function [35] and inhibited mesangial proliferation [36]. Inhibition of activated human mesangial cell proliferation by the natural product of CS may have implications for the treatment of IgA mesangial nephropathy [37]. Furthermore, CS has a mitogenic effect on spleen lymphocytes and is capable of increasing IL-2 production by splenocytes from CRF rats [31, 38]. The IL-2 absorbency of splenocytes is promoted by CS and exhibits therapeutic effects on CRF rats, such as decreasing the BUN and serum creatinine levels and increasing the hemoglobin level. These results indicate that CS has a regulatory effect on cellular immunity in CRF rats [39].

CS can modulate the Treg-to-Th17 cell ratio *in vivo* and thus contributes to the inhibition of diabetes [40], possibly through chemokine activity. In contrast, cordycepin may induce CCL-27 chemokine expression to counter inflammation. CS may contribute to the prevention of mesangial cell proliferation. These features have been further validated by our study.

CS has been proven to be a powerful immunomodulatory compound [28, 41]. An important immunomodulatory finding from our study is the new immune effect of CS. Our study showed that Th22 cells were increased significantly in IgAN mice, whereas CS reduced the number of Th22 cells. After CS treatment, mesangial proliferation was also decreased. The kidney immunohistochemistry analysis showed that CCL27 and CCR10 expression was also decreased. We speculated that CS might inhibit the chemotaxis action of Th22 cells, resulting in improved kidney function. We confirmed a significant increase in the frequency of Th22 cells, which was inhibited by CS. In light of this new evidence for the use of CS in IgAN, our study calls for further investigation of CS and future clinical studies.

In conclusion, this study shed light on the mechanism by which CS may improve kidney functions by inhibiting the chemotaxis of Th22 cells in IgAN.

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Disclosure of conflict of interest

None.

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