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Astragaloside IV improves renal function and alleviates renal damage and inflammation in rats with chronic glomerulonephritis

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Abstract: From Astragalus membranaceus (Fisch.) Bge.var. mongholicus (Bge.) Hsiao, astragaloside IV (AS-IV), a saponin can be purified and is considered traditional Chinese medicine. The purpose of this study was to evaluate the AS-IV-mediated mechanism on chronic glomerulonephritis (CGN). A cationic bovine serum albumin-induced CGN rat model was established and 10, 15, or 20 mg/kg of AS-IV was administered to measure renal function and inflammatory infiltration. Influences of AS-IV on proliferation, cell cycle, and inflammation of LPS-induced rat mesangial cells (RMCs) were determined. The results demonstrated that AS-IV alleviated renal dysfunction, renal lesions, and inflammation in CGN rats. AS-IV prolonged the G0-G1 phase, shortened the S phase, and inhibited cell proliferation and inflammation in RMCs. AS-IV can promote miR-181d-5p expression to inhibit CSF1. miR-181d-5p promotion or CSF1 suppression could further enhance the therapeutic role of AS-IV in CGN rats, while miR-181d-5p silencing or CSF1 overexpression abolished the effect of AS-IV. In conclusion, AS-IV by mediating the miR-181d-5p/CSF1 axis protects against CGN.

Key words: Astragaloside IV, miR-181d-5p, CSF1, chronic glomerulonephritis, kidney function, inflammation

1. Introduction
The prevalence and disease burden of chronic kidney disease (CKD) has been increasing for decades. Global CKD cases are estimated to exceed 697 million in 2019 (Liang et al., 2022). Belonging to CKD, chronic glomerulonephritis (CGN) is a common cause of end-stage renal disease in patients with CKD (Gao et al., 2022). In clinical practice, drug therapy for CGN has been developed (Fernandez-Juárez et al., 2006; Rovin et al., 2021), but long-term drug use has several side effects, such as muscle wasting, central obesity, hypertension, high cholesterol, and renal dysfunction (Kou et al., 2014; Gao et al., 2016). Therefore, it is requested to find new therapeutic drugs to solve the problem of clinical medication.

It has been confirmed that traditional Chinese medicine has some pharmacological effects, but few side effects (Rodrigues et al., 2016). As an active component of astragalus, astragaloside IV (AS-IV) has multiple pharmacological effects, including antioxidant (Mei et al., 2015; Li et al., 2018), antiinflammatory (Zhou et al., 2017; Zhang et al., 2019), antitumor (Li et al., 2022), and hepatoprotective functions (Zhang et al., 2020). A series of studies have demonstrated the efficacy of AS-IV for kidney diseases, such as obstructive nephropathy (Meng et al., 2011), membranous nephropathy (Zheng et al., 2012), and childhood IgA nephropathy (Liu et al., 2021). Recently, AS-IV has been described to have a renoprotective effect on CGN (Lu et al., 2020). However, current studies have not fully elucidated the underlying mechanism of AS-IV in the treatment of CGN. Therefore, an in-depth study of the regulatory mechanism of AS-IV on CGN progression is still required.

It has been studied that miRNAs mediate up to 30% of genes encoding human proteins (Zhao et al., 2019a). Numerous papers published recently have pointed out that miRNAs take part in the pathogenesis and progression of CKD (Lorenzen et al., 2011; Liu et al., 2018; Peters et al., 2020; Yildirim et al., 2021), including CGN (Kamyshova and Bobkova, 2017; Zhu et al., 2022). miR-181d-5p is a multifunctional miRNA that mediates inflammation by controlling key signaling pathways such as NF-κB signaling (Sun et al., 2012), as well as targets related to immune cell homeostasis (Xue et al., 2011). Recently, it is believed that miR-181d-5p suppresses inflammation and improves renal function in renal injury (Zhang et al., 2020). As previous studies have found that AS-IV has multiple targets, it can control different regulatory pathways by altering miRNAs, thereby participating in the treatment of diseases (Li et al., 2022; Wang et al., 2022a). Cytokines are small proteins that bind receptors on the surface of cell membranes and
are involved in promoting cell growth and regulating immune responses, as well as inflammatory responses. Colony-stimulating factor 1 (CSF1) is a cytokine that mainly acts on mononuclear macrophage lines to mediate cell proliferation, differentiation, and function (Hume et al., 2016). CSF1 is involved in the pathogenesis of lupus nephritis in NZBWF1 mice (Li et al., 2021). Therefore, the study hypothesized that miR-181d-5p and CSF1 were also involved in the process of AS-IV improving renal function in CGN rats. A model of C-BSA-induced CGN was established in the study, which is completely similar to human CGN and is considered to be a classical immune complex-induced model of CGN. In addition, inflammation and proliferation of RMCs were induced by LPS to explore the possible mechanism of AS-IV in abnormal proliferation and inflammation of CGN and to determine the relationship between AS-IV and miR-181d-5p/CSF1 axis. This study may provide a theoretical basis and data support for the discovery of CGN therapeutic drugs.

2. Methods

2.1. Cationic bovine serum albumin (C-BSA)-induced CGN rat model

BSA was provided by Sigma (Lot: #WXBC1232V), and C-BSA was prepared with reference to the Border method (Border et al., 1982). Adult male Sprague–Dawley rats, weighing 180–220 g, were purchased from Chengdu Dossy Experimental Animals Co., Ltd. (2020-034). All animal experimental procedures were approved by the Animal Ethics Committee of Cangzhou Central Hospital (Approval Number: Cz20180311). After 1 week of adaptive feeding, except for the rats in the sham group, the other rats were given C-BSA for 4 weeks to establish the CGN model (Wu et al., 2016). C-BSA was administered at a dose of 1.0 mg on the first 3 days, 1.5 mg on the 4th and 5th days, and 2 mg on the last 2 days; the rats were then administered 2.5 mg of C-BSA intraperitoneally daily for 3 weeks. Twenty-four-hour urinary protein was detected to ensure successful model establishment.

2.2. Lentiviral vector construction and injection

The lentiviral vector pLV-CMV containing the cloned sh-CSF1, oe-CSF1, and miR-181d-5p mimic/miR-181d-5p inhibitor were transfected into HEK293T cells with helper vectors pSPAX2 and pMD2G to produce lentivirus (3 × 10^8 TU/ml).

As shown in Table 1, the rats were randomly divided into 13 groups (6 rats in each group): sham group (no treatment), CGN group, 10 mg/kg group (intravenous injection of 10 mg/kg AS-IV in rats), 15 mg/kg group (intravenous injection of 15 mg/kg AS-IV in rats), 20 mg/kg group (intravenous injection of 20 mg/kg AS-IV in rats), AS-IV + mimic NC group (intravenous injection of 20 mg/kg AS-IV and mimic NC lentivirus in rats), AS-IV + miR-181d-5p mimic group (intravenous injection of 20 mg/kg AS-IV and miR-181d-5p mimic lentivirus in rats), AS-IV + inhibitor NC group (intravenous injection of 20 mg/kg AS-IV and inhibitor NC lentivirus in rats), AS-IV + miR-181d-5p inhibitor group (intravenous injection of 20 mg/kg AS-IV and miR-181d-5p inhibitor lentivirus in rats), AS-IV + sh-NC group (intravenous injection of 20 mg/kg AS-IV and sh-NC lentivirus in rats), AS-IV + sh-CSF1 group (intravenous injection of 20 mg/kg AS-IV and sh-CSF1 lentivirus in rats), AS-IV + oe-NC group (intravenous injection of 20 mg/kg AS-IV and oe-NC lentivirus in rats), and AS-IV + oe-CSF1 group.

Table 1. Animal groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
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<tbody>
<tr>
<td>Sham</td>
<td>No treatment</td>
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<tr>
<td>CGN</td>
<td>CGN rat model was induced by C-BSA</td>
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<tr>
<td>10 mg/kg</td>
<td>Intravenous injection of 10 mg/kg AS-IV in CGN rats</td>
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<tr>
<td>15 mg/kg</td>
<td>Intravenous injection of 15 mg/kg AS-IV in CGN rats</td>
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<td>20 mg/kg</td>
<td>Intravenous injection of 20 mg/kg AS-IV in CGN rats</td>
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<tr>
<td>AS-IV + mimic NC</td>
<td>Intravenous injection of 20 mg/kg AS-IV and mimic NC lentivirus in CGN rats</td>
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<tr>
<td>AS-IV + miR-181d-5p mimic</td>
<td>Intravenous injection of 20 mg/kg AS-IV and miR-181d-5p mimic lentivirus in CGN rats</td>
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<tr>
<td>AS-IV + inhibitor NC</td>
<td>Intravenous injection of 20 mg/kg AS-IV and miR-181d-5p inhibitor NC lentivirus in CGN rats</td>
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<td>AS-IV + miR-181d-5p inhibitor</td>
<td>Intravenous injection of 20 mg/kg AS-IV and miR-181d-5p inhibitor NC lentivirus in CGN rats</td>
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<td>AS-IV + sh-NC</td>
<td>Intravenous injection of 20 mg/kg AS-IV and sh-NC lentivirus in CGN rats</td>
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<tr>
<td>AS-IV + sh-CSF1</td>
<td>Intravenous injection of 20 mg/kg AS-IV and sh-CSF1 lentivirus in CGN rats</td>
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<tr>
<td>AS-IV + oe-NC</td>
<td>Intravenous injection of 20 mg/kg AS-IV and oe-NC lentivirus in CGN rats</td>
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<tr>
<td>AS-IV + oe-CSF1</td>
<td>Intravenous injection of 20 mg/kg AS-IV and oe-CSF1 lentivirus in CGN rats</td>
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(intravenous injection of 20 mg/kg AS-IV and oe-CSF1 lentivirus in rats). AS-IV > 98% purity was provided by Chengdu Herbpurify Co., Ltd.

2.3. Blood collection and kidney tissue resection
After 4 weeks, the rats were anesthetized with isoflurane inhalation, 5 mL of blood was collected from the abdominal aorta, and serum was obtained after centrifugation at 3500 rpm. All kidney tissues were resected from euthanized rats, of which a part was fixed with 10% neutral formalin for histological staining, and the other was frozen in liquid nitrogen (Liang et al., 2014).

2.4. Detection of BUN and SCr
Serum BUN and SCr levels were determined using the Hitachi Model 7100 Automatic Analyzer.

2.5. HE staining
Kidney tissue samples were dehydrated in graded alcohol and embedded in paraffin for preparation of 5-μm-thick slides for dyeing with hematoxylin (CTS-1099, MXBio, Fuzhou, China) and 0.5% eosin (71,014,544, Sinopharm). After being fixed with neutral glue (G8590; MAIRUI, Shanghai, China), the sections were observed under a microscope (Olympus) in 3 fields of view (Wu et al., 2014).

2.6. PAS staining
Paraffin-embedded kidney tissue slides (5 μm) were stained with 10 g/L periodate solution (10,450 60-9, Nanjing Reagent, Nanjing, China), incubated with Schiff solution (DG0005, Leagene Biotech, Beijing, China), combined with hematoxylin (CTS-1099, MXBio), and observed under a microscope (Olympus) in 3 fields of view (Wang et al., 2022b).

2.7. Immunohistochemistry
Paraffin-embedded kidney tissue slides (5 μm) were dewaxed with xylene and graded alcohols. After 3% hydrogen peroxide-based inactivation of endogenous peroxidase, slides were combined with rabbit anti-CD68 antibody (1:100, Aobosen, Beijing) and then with polyperoxidase-anti-mouse/rabbit IgG. Followed by DAB development, hematoxylin counterstaining was done, and slides were imaged under a microscope (Olympus) in 3 fields of view.

2.8. Cell culture and AS-IV pretreatment
Rat mesangial cells (RMCs; China Center for Type Culture Collection) were maintained in DMEM (Gibco) containing 10% fetal bovine serum (Gibco) which was renewed every 3 days until RMCs were confluent. RMCs were treated with 30 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 12 h and pretreated with 10, 20, or 40 μg/mL AS-IV for 24 h (Lu et al., 2020).

2.9. Cell transfection
miR-181d-5p inhibitor, inhibitor NC, oe-CSF1, and oe-NC (40 nM) were transfected into RMCs for 7 h using Lipofectamine 2000 (Lu et al., 2020).

2.10. Cell proliferation assay
RMC proliferation was assessed according to the instructions provided by Cell Counting Kit-8 (Bioss, Beijing, China). Briefly, RMCs were collected and transferred into 96-well microplates, incubated with 100 μL of CCK8 solution for 4 h, and then the absorbance at 450 nm was recorded.

2.11. Cell cycle analysis
RMCs were seeded in 6-well plates at 1.0 × 10^5/well and incubated in fetal bovine serum-free DMEM for 12 h. RMCs were then trypsinized, suspended, and fixed in 75% ethanol, and stained with propidium iodide (50 μg/mL; Sigma, USA) and RNaseA. Cellular phases were determined on a BD flow cytometer (BD company) using Cell FIT 2.01.2 (BD company).

2.12. ELISA
ELISA kits (Beyotime) were utilized to detect IL-6 and TNF-α in renal tissue and RMCs supernatant.

2.13. RT-qPCR
Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen) and measured with NanoDrop 2000 (Thermo) according to the concentration and purity of total RNA, and reverse transcription reaction was performed to generate cDNA using the Prime Script RT Reagent Kit (Takara). PCR was performed on Step One PlusTM System (Applied Biosystems). The thermocycling conditions were as follows: Predenaturation at 95 °C for 1 min, 40 cycles at 95 °C for 20 s, and 60 °C for 1 min. The relative expression of each target gene was calculated by the 2−ΔΔCt method. Table 2 presents PCR primers.

2.14. Western blot
Based on RIPA lysis buffer (Thermo Fisher Scientific), the extraction of protein was implemented, and the products were subjected to BCA method-based quantification (Beyotime). A total of 30 μg of protein was separated on 12% SDS-PAGE, transferred to PVDF membrane (EMD Millipore), blocked with 5% nonfat milk, and then blocked with primary antibodies CSF1 (3152, 1:1000, Cell Signaling Technology) and GAPDH (ab8245, 1:1000, Abcam). Afterward, goat antirabbit IgG (ab205718; 1:2000; Abcam) was added, and ECL reagent (Cell Signaling Technology) was supplemented to develop protein bands, of which the gray values were assessed using ImageJ 5.0 (Bio-Rad Laboratories).
2.15. Dual-luciferase reporter gene assay
Wild-type plasmid CSF1-WT (containing the binding site of miR-181d-5p) and mutant plasmid CSF1-MUT (nucleotides were mutated at the binding site) were constructed. miR-181d-5p mimic or mimic NC (GenePharma) were transfected into RMCs with CSF1-WT or CSF1-MUT according to the instructions of Lipofectamine 2000 (Invitrogen). Relative luciferase activity was calculated according to a dual-luciferase assay kit (Promega).

2.16. Statistical analysis
Statistical analysis was performed using SPSS 25.0. All data are presented as mean ± standard deviation. Differences were compared using t-test or one-way analysis of variance. p < 0.05 indicates a statistically significant difference.

3. Results
3.1. AS-IV can improve renal function and alleviate pathological changes in CGN rats
The chemical structure of AS-IV is shown in Figure 1A. To explore the potential regulatory mechanism of AS-IV in CGN, a CGN rat model was established and given 10, 15, or 20 mg/kg of AS-IV. The results revealed that CGN rats exhibited high levels of SCr and BUN, while AS-IV decreased SCr and BUN levels dose-dependently (Figures 1B and 1C). In addition, 24-h urinary protein increased in CGN rats, while AS-IV decreased the 24-h urinary protein in a dose-dependent manner (Figure S1A). The pathological changes of renal tissue of CGN rats were detected by HE staining and PAS staining, showing that the glomeruli and renal tubules of normal rats had a transparent capsule, while the CGN rats had severe pathological damage, mainly manifested as obvious inflammatory cell infiltration, granular degeneration, glomerulus swelling, and marked hyperplasia of the mesangial matrix. Pathological changes were alleviated after AS-IV treatment, and the therapeutic effect of AS-IV was dose-dependent (Figures 1D and 1E). To measure the inflammatory changes in CGN rats, immunohistochemistry was performed to detect CD68 and ELISA to determine inflammatory factors. Immunohistochemical results found CD68 mainly in the cytoplasm of glomeruli and tubulointerstitium, and that the infection of macrophages in the kidney tissue of CGN rats was increased, while AS-IV was a dose-dependent way to reduce the inflammatory response (Figure 1F). ELISA results manifested that IL-6 and TNF-α contents were augmented in CGN rats, while AS-IV decreased IL-6 and TNF-α contents dose-dependently (Figures 1G and 1H). miR-181d-5p was decreased in CGN rats, while AS-IV upregulated miR-181d-5p dose-dependently (Figure 1I). In conclusion, AS-IV can improve renal function and alleviate pathological changes in CGN rats, and 20 mg/kg AS-IV has the best therapeutic effect. Therefore, 20 mg/kg of AS-IV was selected for subsequent experiments.

3.2. miR-181d-5p has a healing effect on CGN rats
CGN rats were given 20 mg/kg AS-IV and injected with lentivirus that interfered with miR-181d-5p. RT-qPCR results confirmed that miR-181d-5p expression intervention was successful (Figure 2A). SCr and BUN levels were further reduced after overexpressing miR-181d-5p, while inhibiting miR-181d-5p could mitigate AS-IV-mediated SCr and BUN levels (Figures 2B and 2C). Twenty-four–hour urinary protein was further decreased after upregulating miR-181d-5p, while downregulating miR-181d-5p could reverse the effect of AS-IV on 24-h urinary protein (Figure S1A). miR-181d-5p could further alleviate renal tissue pathological damage and inflammatory response, while miR-181d-5p inhibition blocked the improvement effect of AS-IV on renal tissue pathological damage and inflammatory response (Figures 2D–2H).

3.3. miR-181d-5p inhibits CSF1 expression
By starbase database (https://starbase.sysu.edu.cn/), CSF1 might be a target gene of miR-181d-5p (Figure 3A). The
detection results of luciferase activity showed that miR-181d-5p mimic decreased firefly luciferase activity of CSF1-WT (Figure 3B). In further studies, it was found that CSF1 was upregulated in CGN rats, while it was downregulated by AS-IV dose-dependently (Figure 3C). In addition, CSF1 expression was further decreased after overexpressing miR-181d-5p, while it was increased after suppressing miR-181d-5p (Figure 3D).
Figure 2. miR-181d-5p has a healing effect on CGN rats. A: RT-qPCR to detect miR-181d-5p expression; B-C: After regulating miR-181d-5p, Automatic biochemical analyzer to determine serum BUN and Scr levels; D-E: After regulating miR-181d-5p, HE staining and PAS staining to observe renal histopathologic changes, with black arrows indicating inflammatory infiltration and matrix expansion; F: After regulating miR-181d-5p, Immunohistochemistry to measure CD68; G-H: After regulating miR-181d-5p, ELISA to analyze IL-6 and TNF-α contents; values were expressed as mean ± standard deviation (n = 6). * p < 0.05 vs. AS-IV + mimic NC; # p < 0.05 vs. AS-IV + inhibitor NC.
3.4. CSF1 inhibition protects against CGN in rats
With treatment with 20 mg/kg of AS-IV, CGN rats were injected with lentivirus that interfered with CSF1 expression. RT-qPCR and Western blot showed that CSF1 expression was successfully intervened (Figure 4A). CSF1 low expression further reduced SCr, BUN, and 24-h urinary protein, alleviated the pathological damage of renal tissue, and attenuated inflammatory response based on AS-IV treatment, while CSF1 promotion worsened the performance of AS-IV on CGN rats (Figures 4B–4H, Figure S1C).

3.5. AS-IV can inhibit RMCs proliferation and inflammation
To further validate the therapeutic effect of AS-IV on CGN, RMCs were pretreated with AS-IV at 10, 20, and 40 μg/mL, and then cellular inflammation was induced by LPS. CCK-8 results showed that AS-IV inhibited LPS-induced proliferation of RMCs dose-dependently (Figure 5A). Cell cycle detection by flow cytometry implicated that AS-IV prolonged the G0-G1 phase and shortened the S phase dose-dependently in LPS-induced RMCs (Figure 5B). ELISA finding revealed that AS-IV decreased IL-6 and TNF-α in LPS-induced RMCs dose-dependently (Figures 5C and 5D).

3.6. miR-181d-5p deficiency or CSF1 induction can aggrandize the performance of AS-IV
AS-IV promoted miR-181d-5p expression and inhibited CSF1 expression dose-dependently (Figure 6A). RMCs pretreated with 40 μg/mL of AS-IV were transfecting miR-181d-5p inhibitor or oe-CSF1 to mediate gene expression (Figure 6B). Cellular experiments suggested that miR-181d-5p suppression or CSF1 overexpression reduced AS-
IV-mediated performance on RMCs proliferation (Figure 6C), cell cycle (Figure 6D), and inflammatory factor production (Figures 6E and 6F).

4. Discussion

CGN is associated with immune-mediated inflammatory disease, frequently occurs during end-stage renal disease, and severely affects patient survival (Ding et al., 2013; Gao et al., 2016). Traditional Chinese medicine has advantages in the treatment of complex diseases (Zhao et al., 2019b), and particularly AS-IV is of clinical significance to treat various renal diseases (Meng et al., 2011; Zheng et al., 2012; Lu et al., 2020; Liu et al., 2021).

The establishment of appropriate models is the basis for simulating diseases. The C-BSA-induced CGN rat model was chosen for this study because it has previously been shown to be similar to human CGN progression (Wu et al., 2016). Studies have reported that immune-mediated inflammation and glomerular injury in the tubulointerstitial compartment are the keys to CGN progression (Zou et al., 2012; Schwalm et al., 2014; Velciov et al., 2016). The present study found that CGN rats showed significant deterioration of renal function, accompanied by massive 24-h urinary protein and abnormal serum BUN and SCr levels. CGN rats showed obvious pathological injury and inflammatory symptoms.

Figure 4. CSF1 inhibition protects against CGN in rats. A: RT-qPCR and Western blot to analyze CSF1 expression; B-C: After regulating CSF1, Automatic biochemical analyzer to determine serum BUN and SCr levels; D-E: After regulating CSF1, HE staining and PAS staining to observe renal histopathologic changes, with black arrows indicating inflammatory infiltration and matrix expansion; F: After regulating CSF1, Immunohistochemistry to measure CD68; G-H: After regulating CSF1, ELASA to analyze IL-6 and TNF-α contents; values were expressed as mean ± standard deviation (n = 6). * p < 0.05 vs. AS-IV + sh-NC; # p < 0.05 vs. AS-IV + oe-NC.
Interestingly, AS-IV treatment could improve the biochemical indicators of blood renal function in C-BSA-induced CGN rats, alleviate renal pathological damage, and inhibit the inflammatory response. Common pathologies of proliferative and inflammatory glomerular diseases in humans and experimental animals include mesangial cell proliferation (Cao et al., 2015; Singh et al., 2016; Kurihara and Sakai 2017), which may lead to excessive deposition of extracellular matrix, glomerulosclerosis, and loss of renal function. LPS is considered to be one of the strong stimulators of RMCs, and it can be used as an inducer of glomerular cell viability (Gao et al., 2018). Therefore, LPS was employed to mimic CGN in cells. The present study found that AS-IV could inhibit RMC proliferation and inflammation, which is consistent with previous findings (Wu et al., 2018).

miR-181d-5p is a multifunctional miRNA that mediates inflammation and immune cell homeostasis (Sun et al., 2012) and is a great mediator for inflammation and improves renal function in renal injury (Xue et al., 2011; Zhang et al., 2020). Interestingly, miR-181d-5p was decreased in CGN rats, whereas AS-IV upregulated miR-181d-5p dose-dependently. Given that, it was speculated that AS-IV may improve renal function and alleviate pathological changes in CGN rats by upregulating miR-181d-5p. To further test our hypothesis, CGN rats were injected with 20 mg/kg of AS-IV simultaneously with lentivirus that interfered with miR-181d-5p. As the findings indicate, promoting miR-181d-5p expression could further enhance the therapeutic effect of AS-IV in CGN rats, while declining miR-181d-5p expression could attenuate the therapeutic effect of AS-IV in CGN rats.

CSF1 is a critical modifier of macrophage production, differentiation, and function (Zhang et al., 2021). CSF1 is upregulated in renal tubular epithelial cells in response to renal injury stimuli (Perry and Okusa, 2015) and could mediate inflammatory damage and apoptosis in human mesangial cells in lupus nephritis (Liao et al., 2022). Here, it was measured that CSF1 expression was upregulated in CGN rats, whereas it was downregulated by AS-IV and further decreased after upregulation of mir-181d-5p. Based on this, further functional rescue experiments were carried out, demonstrating that CSF1 inhibition could further enhance the therapeutic effect of AS-IV on CGN rats, while CSF1 did the opposite. Furthermore, in vitro cell experiments further support that AS-IV improves renal function in rats with CGN by regulating the miR-181d-5p/CSF1 axis, and attenuates renal damage and inflammation.

However, a limitation is that our findings are based on cell culture investigations and experimental animal studies without clinical practice. Furthermore, the NF-κB signaling pathway is related to the inflammatory pathogenesis of CGN (Chen et al., 2022), while AS-IV can activate different functional pathways (Zhang et al., 2022). Therefore, the downstream pathways of AS-IV can be further elucidated in the future to enrich our study.

Figure 5. AS-IV can inhibit RMCs proliferation and inflammation. A: CCK-8 to measure RMCs proliferation; B: Flow cytometry to determine RMCs cell cycle; C-D: ELASA to analyze IL-6 and TNF-α contents in RMCs supernatant; values were expressed as mean ± standard deviation (N = 3). * p < 0.05 vs. control; # p < 0.05 vs. LPS.
5. Conclusion
AS-IV improves renal function in CGN rats by mediating the miR-181d-5p/CSF1 axis and alleviates renal lesions and inflammation. In vitro, it prolongs the G0-G1 phase and shortens the S phase in RMCs, and inhibits cell proliferation and inflammation. AS-IV has the potential to become a clinical drug for the treatment of CGN. AS-IV therapy in the clinic warrants further investigation as an alternative treatment strategy to manage the progression of CGN and end-stage renal disease.

Conflict of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All animal experiments were conducted in compliance with the ARRIVE guidelines and performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of Cangzhou Central Hospital.
References


Supplementary

Figure S1. Detection of 24-h urinary protein in CGN rats. A-C: 24 h urinary protein of rats; Values were expressed as mean ± standard deviation (n = 6). *p < 0.05 vs. sham; # p < 0.05 vs. CGN; & p < 0.05 vs. AS-IV + mimic NC; $ p < 0.05 vs. AS-IV + inhibitor NC; ^ p < 0.05 vs. AS-IV + sh-NC; ^ p < 0.05 vs. AS-IV + oe-NC.