Astragaloside IV inhibiting rotenone-induced α-syn presentation and CD4 T-cell immune response

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Research Article

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Abstract

Background

The increased α-synuclein (α-syn)-dependent activation of CD4 T cells leads to the progressive loss of Parkinson's disease (PD) dopaminergic (DA) neurons in the substantia nigra (SN). Astragaloside IV (AS-IV) protects DA neurons against neuroinflammation. The effects of AS-IV on CD4 T-cell-mediated immune responses in PD remain to be defined.

Methods

Rotenone (ROT) injected unilaterally into the substantia nigra compact part (SNc) of rats induced PD. AS-IV (20 mg/kg) was intraperitoneally injected once a day for 14 days. The limb hanging test and rotarod test were performed to evaluate the alteration of behavior at 4 and 6 weeks. Total gastrointestinal transit tests were performed at 4 weeks. Western blotting was used to detect the expression of proinflammatory cytokine proteins. Immunofluorescence staining was conducted to test the expression and localization of major histocompatibility complex class I (MHC I), cleaved caspase-1 and α-syn in astrocytes. Flow cytometry analysis, immunohistochemistry and immunofluorescence staining were used to measure the expression of CD4 T-cell subsets in the SN.

Results

The application of AS-IV protected against the loss of DA neurons and behavioral deficits in ROT-induced PD rat models. AS-IV administration inhibited the aggregation of α-syn in DA neurons and the expression of proinflammatory cytokines such as TNF-α, IL-18 and IL-1β. AS-IV decreased the activation of CD4 T cells and three CD4 T-cell subsets: Tfh, Treg and Th1. AS-IV interrupted the ROT-induced interaction between astrocytes and CD4 T cells and the colocalization of MHC I and α-syn in astrocytes. AS-IV inhibited the expression of α-syn in astrocytes and the colocalization of α-syn and cleaved caspase-1 in astrocytes.

Conclusion

AS-IV prevents the loss of DA neurons in PD by inhibiting the activation of α-syn-specific CD4 T cells, which is regulated by MHC I-mediated antigen presentation in astrocytes.

1 Introduction

The hallmarks of Parkinson's disease (PD) are the progressive loss of dopaminergic (DA) neurons in the substantia nigra (SN) and the aggregation of α-synuclein (α-syn). Aging, oxidative stress, ferroptosis, mitochondrial dysfunction and other factors have been considered potential contributing factors in the neuropathology of PD [1]. PD was also considered a chronic neuroinflammatory disease. T cell immune response was involved in the regulation of DA neuron loss [2].
In pathogenesis of PD, misfolded α-syn was considered an important initiator of the immune response [3]. Microglia, innate immune cells, are activated by the aggregation of α-syn and participate in immune surveillance within the brain [4]. Moreover, astrocytes are active players in the innate immune response and the spread of inflammation in the CNS [5]. Previous study indicated that microglia and astrocytes have been verified as antigen-presenting cells (APCs) in the PD brain.

The CD4 T-cell-mediated adaptive immune response contributes to persistent DA neuron loss in PD [6]. Increasing evidence suggested that activation of α-syn-specific CD4 T-cell responses was a feature of preclinical and early motor of PD [7]. CD4 T cells are activated by the interaction of the T-cell receptor (TCR) with peptide complexes, which consist of major histocompatibility complex (MHC) and antigen peptides [8]. Many studies have suggested that polymorphisms in the HLA-DR locus are associated with sporadic late-onset PD [9]. MHC and MHCI are mainly expressed in microglia, astrocytes and DA neurons [10].

More evidence indicated that MHC-mediated antigen presentation of the α-syn peptide to CD4 T cells by microglia and astrocytes induced an immune response [8]. C-terminal truncation, found in Lewy bodies (LBs), modified the antigenicity of α-syn [11]. A previous study indicated that caspase-1 was increased in the LBs of PD patients. Cleaved caspase-1 causes truncation and aggregation of α-syn [12].

Astragaloside (AS-) was purified from the Chinese medicinal herb Astragalus membranaceus. AS- has been reported to exert anti-inflammatory, antioxidative and antiaging functions [13]. AS-IV application alleviated DA neuron degeneration through the inhibition of astrocyte senescence and oxidative stress in PD models [14]. However, the effects of AS- on α-syn-specific CD4 T-cell responses in the pathogenesis of PD remains to be further studied. In this study, we investigated the effect of AS-IV on the immune response of CD4 T cells.

2 Materials and Methods

2.1 Reagents

The use of antibodies is shown in Table 1.

2.2 Experimental animals and treatment

Eight-week-old adult male SD rats (weight 250–320 g) were obtained from Ji’nan Pengyue Laboratory Animal Breeding Co., Ltd. Rats had free access to food and water in a room with an ambient temperature of 22°C ± 2°C and a light/dark cycle of 12:12 h. All animal procedures were approved by the guidelines of the ethical standards for laboratory animals of Binzhou Medical University.

After one week of adaptation, rats were deeply anesthetized with pentobarbital sodium and unilaterally stereotactically injected into the right substantia nigra pars compacta (SNc) (coordinates: 5.0 mm posterior to the bregma, 2.0 mm lateral to the midline, and 7.8 mm ventral to dorsal) with 1 µl rotenone (ROT) (9 µg/µl, MCE, HY-B1756, USA). The vehicle group rats were injected with 1 µl of DMSO. After
surgery, antibiotics were administered to prevent infection. After surgery, the animals were monitored until awake and then returned to new cages. Intraperitoneal injections (IP) of AS- were administered once a day for 14 days. Apomorphine- (APO-) (GIPBIO, USA)-induced rotational behavior was performed in PD rats.

### 2.3 Behavioral tests

The limb hanging test was measured over 3 consecutive days after training. The pole was 7 mm in diameter and 50 cm in length suspended 50 cm above the horizontal surface of the ground. Limb hanging time was recorded. Scoring was conducted as follows: 8 points if the rats caught the pole with four paws; 6 points if the rats caught the pole with three paws; and 0 points if they did not catch the pole with any paw.

Rotarod training for 3 days was needed to ensure that all subjects had learned the task to the same degree. Rats were placed on a rotating rod at 5–15 rpm/min. The average latencies to fall from the rotating rod during the testing periods were calculated for each mouse. Three repeated trials were investigated on the day of testing.

### 2.4 Measurement of total gastrointestinal transit time

Rats were fasted for 6 h before the test. In order to measure the total gastrointestinal transit time, carmine dye solution (2 ml) was administered to rats by oral gavage. The time was recorded until the first red-stained fecal pellet was expelled. The elapsed time from the moment when carmine dye solution was administered to rats intragastrically, to the moment when stained feces left the anus, was considered as the total gastrointestinal transit time.

### 2.5 Confocal immunofluorescence analysis

Rats were anesthetized and transcardially perfused with cold PBS and 4% paraformaldehyde (PFA) sequentially. Rat brains were maintained in 4% PFA at 4°C for 24 h and then dehydrated in 30% sucrose for 2 days. Coronal sections (40 µm) were obtained using a freezing microtome (Leica, CM1950) and stored in antifreeze solution.

For fluorescent staining, the sections were blocked with 10% normal goat serum and 0.3% Triton X-100 for 2 h at room temperature and incubated with the primary antibodies anti-TH, anti-α-syn, anti-GFAP, anti-Iba1, anti-CD4, anti-HLA-DRB3, anti-cleaved-caspase1 and anti-IL-18 in blocking buffer at 4°C overnight. The secondary antibodies were anti-Alexa Fluor 405, anti-Alexa Fluor 488, and anti-Alexa Fluor 647 in blocking buffer for 4 h at room temperature. Cultures were covered with coverslips and mounted with anti-fade fluorescence mounting medium with DAPI (ab104139, Abcam).

### 2.6 Immunohistochemistry

The frozen sections were washed in H₂O₂, quenched of endogenous peroxidases, blocked in 5% serum, and then labeled with anti-CXCR3, anti-CD25, and anti-CXCR5 antibodies overnight at 4°C. The sections
were then incubated with peroxidase-conjugated rabbit IgG (cat no. SV0002, Boster Biological Technology) for 30 min at room temperature. The immune complex was visualized with DAB staining.

2.7 Western blotting

Rats were anesthetized and transcardially perfused with cold PBS and sacrificed, and the substantia nigra (SN) tissue was quickly separated. The protein of the SN was lysed in RIPA lysis buffer (Beyotime Technology, China) and centrifuged at 13000 rpm at 4°C for 10 min. The tissue protein concentrations were measured by BCA Protein Assay. Then, the protein isolates (100 µg for Western blotting) were separated on SDS–PAGE gels and electrophoretically transferred to PVDF membranes. The membranes were blocked in a solution of Tris-buffered saline with 5% dried milk with 0.1% Tween 20. The membranes were incubated with the primary antibodies anti-IL-1β, anti-IL-6, anti-IL-18, anti-IL-21, anti-Bcl-6, anti-IFN-γ, anti-CD25, anti-TNFα and anti-β-actin. After incubation with secondary antibodies, blots were developed with ECL substrate.

2.8 Flow cytometry

The midbrain was quickly removed. The midbrain tissue was shredded and digested with collagenase with DNase diluted in RPMI 1640 at 37 °C for 30 min. The midbrain tissue was filtered with a 70 µM screen. Then, lymphocytes were isolated by density gradient centrifugation. After washing three times with PBS, isolated cells were stained accordingly with fluorescent-conjugated antibodies against CD4 and CD8.

2.9 Statistical analysis

All statistical analyses were performed using GraphPad Prism 8 statistical analysis software. The results were expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA, unpaired t tests and t tests.

3 Results

3.1 AS- ameliorated motor deficits and prevented the loss of DA neurons in PD rats

As shown in Fig. 1A, PD animal models were established by stereotactically injecting ROT into the SNC. Daily intraperitoneal injection of AS- was performed continuously for 14 days. Behavior tests were used to evaluate the alteration of behavior at 2, 4 and 6 weeks. Compared with the vehicle group, the number of APO-induced contralateral rotations in the ROT group increased significantly (Fig. 1B, 312.5 ± 15.91 vs 28.91 ± 2.26, p < 0.001). There was no significant difference in APO-induced turns between the ROT and AS- groups at 2 weeks (Fig. 1C, 328.18 ± 25.44 vs 296.82 ± 19.17 per 30 min, p > 0.05). At 4 weeks, the number of rotations in the AS- group decreased apparently compared with the ROT group (Fig. 1C, 396.82 ± 14.78 vs 301.36 ± 13.18 per 30 min, p < 0.05).
Depending on the limb hanging test, in comparison with the vehicle group, ROT-treated rats exhibited a marked decrease in the hanging time to fall (Fig. 1D, 10.00 ± 0.86 s vs 18.55 ± 0.96 s, p < 0.001). After AS-treatment, the hanging time increased apparently compared with the ROT group (Fig. 1D, 14.36 ± 0.86s vs 10.00 ± 0.86, p < 0.01). The limb hanging scores of the ROT group were lower than those of the vehicle group (Fig. 1E, 2.73 ± 0.30 vs 6.18 ± 0.42, p < 0.01). AS-IV administration improved the limb hanging scores compared with the ROT group (Fig. 1E, 5.63 ± 0.53 to 2.73 ± 0.30, p < 0.05). During the rotarod test, there was a significant decline in latency time to fall in the ROT group compared with the vehicle group (Fig. 1F, 38.73 ± 2.84 s vs 88.64 ± 7.30, p < 0.001). AS- treatment obviously increased the latency time to fall compared with the ROT group (Fig. 1F, 66.82 ± 4.69 s vs 38.73 ± 2.84 s, p < 0.01).

Total gastrointestinal transit times were measured using carmine red dye delivered via oral gavage. The rats experienced no change in gastrointestinal transit time in the ROT and AS- groups (Fig. 1G, 318.2 ± 11.08 min vs 319.9 ± 11.1 min, p > 0.05).

There was a significant loss of DA neurons in the ROT group compared with the vehicle group (Fig. 1H-I, p < 0.01). Lesioned DA neurons were rescued by AS-IV application (Fig. 1H-I, p < 0.05). The aggregation of α-syn in DA neurons was reduced in the AS- group compared with the ROT group by double immunofluorescence staining with α-syn and TH antibodies (Fig. 1J-K, p < 0.05). The expression of α-syn protein increased in the SN of the ROT group, and AS- application decreased ROT-induced α-syn expression (Fig. 1L).

### 3.2 AS- inhibited the expression of proinflammatory factors in the SN

The expression of TNF-α, IL-18, IL-6, and IL-1β protein in the SN was normalized to β-actin. Compared with the vehicle group, the relative grayscale values of target proteins increased in the ROT group (Fig. 2A-B, p < 0.05). AS- treatment significantly decreased the expression of TNF-α and IL-18 compared with the ROT group (p < 0.05).

### 3.3 AS-IV inhibited the astrocyte-mediated immune response in PD rats

To further detect the innate immune responses in the SN of PD rats, the activity of astrocytes and microglia in the SN was observed. Astrocytes and microglia were mainly localized in the substantia nigra reticular part (SNr) (Fig. 3A). Compared with the vehicle group, the fluorescence intensity (the activation of astrocytes and microglia) in the SNr significantly increased in the ROT group (Fig. 3B, p < 0.05). AS-obviously alleviated the activation of astrocytes in the SNr (Fig. 3B, p < 0.05). Morphological changes in astrocytes in the SN was observed. As shown in Fig. 3C, astrocyte cell numbers were remarkably upregulated in the SN of PD rats compared with the vehicle group (Fig. 3D, p < 0.01). AS- treatment decreased the number of astrocytes (Fig. 3D, p < 0.05). Large cell bodies and increased neurite thickness were induced in the ROT group and inhibited in the AS-IV group (Fig. 3).

### 3.4 AS- inhibited the activation of CD4 T cells in PD rats
The number of CD4 T cells was detected by flow cytometry, and the expression of CD4 was examined by immunofluorescence staining in the SN. In comparison with the vehicle group, the number of CD4 T cells significantly increased in the ROT group (Fig. 4B, p < 0.001). The expression of CD4 increased in the SNr of the ROT group according to immunofluorescence analysis (Fig. 4C-D, p < 0.001). AS- application downregulated the number of CD4 T cells and the expression of CD4 in the SNr (Fig. 4B, D, p < 0.01).

The markers of CD4 T-cell subsets were detected by immunohistochemistry and western blotting. The hallmark genes of Tfh cells were CXCR5, IL-21 and Bcl-6. Compared with the vehicle group, the number of CXCR5+ cells increased in the ROT group (Fig. 4F, p < 0.001). The expression of Bcl-6 and IL-21 protein was upregulated in the ROT group compared with the vehicle group (Fig. 4G, p < 0.001). AS-IV treatment decreased the expression of CXCR5, Bcl-6 and IL-21 (Fig. 4E-G, p < 0.05).

In comparison with the vehicle group, the number of CD25 + cells (hallmark of Tregs) increased in the ROT group (Fig. 4H, p < 0.001). The expression of CD25 protein was upregulated in the ROT group compared with the vehicle group by western blotting (Fig. 4J, p < 0.05). AS-IV application downregulated the expression of CD25 compared with the ROT group (Fig. 4H-J, p < 0.05).

Th1 cells secreted CXCR3 and IFN-γ. In comparison with the vehicle group, the number of CXCR3 + cells increased in the ROT group (Fig. 4L, p < 0.001). The expression of IFN-γ and CXCR3 increased in the ROT group. AS-IV decreased IFN-γ and CXCR3 expression compared with the ROT group (Fig. 4K-M, p < 0.05).

3.5 AS- inhibited IL-18-mediated inflammation in PD rats

The expression of IL-18 was evaluated in astrocytes and microglia. The ratio of IL-18+/GFAP+ cells and IL-18+/Iba1+ cells significantly increased in the ROT group compared with the vehicle group (Fig. 5B and D, p < 0.001). AS- decreased the ratio of IL-18+/GFAP+ cells (Fig. 5B, p < 0.05). There was no significant difference in the ratio of IL-18+/Iba1+ cells between the ROT and AS- groups (Fig. 5D, p > 0.05).

The expression and localization of IL-18 and CD4 were detected by immunofluorescence staining. The number of IL-18+/CD4+ cells was upregulated in the ROT group compared with the vehicle group (Fig. 5F, p < 0.001). AS- decreased the ratio of IL-18+/CD4+ cells in the SNr (Fig. 5F, p < 0.05). In comparison with the vehicle group, the distance between IL-18+ cells and TH+ cells was shortened in the ROT group (Fig. 5H, p < 0.001). AS- treatment inhibited the ROT-induced short distance between IL-18+ cells and TH+ cells (Fig. 5H, p < 0.05).

3.6 AS-IV inhibited antigen presentation of astrocytes to CD4 T cells.

To investigate antigen presentation in astrocytes or microglia in the SN, double immunofluorescence staining of GFAP/MHC and Iba1/MHC was used. Compared with the vehicle group, the ratio of GFAP+/MHC+ cells increased in the ROT group (Fig. 6B, p < 0.001). AS- markedly decreased the number of GFAP+/MHC+ cells (Fig. 6B, p < 0.001). There was no significant difference in the ratio of Iba1+/MHC+ cells between the ROT and AS- groups (p > 0.05).
Compared with the vehicle group, the ratio of GFAP+/CD4+ cells was enhanced in the ROT group (Fig. 6C-D, p < 0.001). AS- decreased the number of GFAP+/CD4+ cells (Fig. 6D, p < 0.01). By staining with CD4 antibody, CD4+ cells exhibited altered cell morphology in the ROT group (Fig. 6E).

3.7 AS-IV decreased antigen presentation of α-syn to CD4 T cells and the expression of cleaved caspase-1

The expression and cellular location of GFAP, MHC and α-syn were detected by triple immunofluorescence staining. In comparison with the vehicle group, the ratio of α-syn+/MHC + cells, GFAP+/MHC + cells, and α-syn+/MHC +/GFAP + cells (white arrow) in the SN of the ROT group increased (Fig. 7B, p < 0.01). AS- treatment decreased the ratio of α-syn+/MHC +/GFAP + cells (Fig. 7B, p < 0.05). Compared with the vehicle group, the proportion of α-syn+/CD4 + cells increased in the ROT group, as shown by immunofluorescence analysis (Fig. 7C-D, p < 0.01). AS- administration decreased the proportion of α-syn+/CD4 + cells (Fig. 7C-D, p < 0.05).

Immunofluorescence triple staining with α-syn, cleaved caspase-1 and GFAP showed that the proportions of α-syn+/GFAP + cells, cleaved caspase-1+/GFAP + cells, and α-syn+/cleaved caspase-1+/GFAP + cells were increased in the ROT group compared with the vehicle group (Fig. 7E-F, p < 0.05). AS- treatment decreased the proportion of α-syn+/cleaved caspase-1+/GFAP + cells in astrocytes (Fig. 7E-F, p < 0.05).

4 Discussion

In this study, AS-IV treatment alleviated the motor dysfunction and the loss of DA neurons induced by ROT. AS-IV decreased the expression of proinflammatory factors and MHC -mediated antigen presentation in astrocytes by the SN in ROT-induced PD rats. AS-IV administration inhibited the activation of α-syn-specific CD4 T cells in the SN (Fig. 8).

The most critical finding presented here was that AS-IV inhibited antigen presentation by astrocytes in ROT-induced PD rats. The latest research reported that astrocyte acted as APCs in PD [15]. Jinar Rostami et al. demonstrated that astrocytes expressed MHC and enhanced the activation of CD4 T cells in the brain parenchyma [15]. In addition, studies of multiple sclerosis (MS) showed that astrocytes were able to activate Th1 and Th17 T cells [16; 17]. In this study, we observed that astrocytes presented α-syn to CD4 T cells in the ROT-induced PD rats and induced T cells immune response. AS-IV administration reduced the presentation of α-syn to CD4 T cells.

Microglia were often suggested to be the main APCs in the brain [18]. However, early studies on antigen presentation by microglia revealed that microglia were inefficient activators of CD4 T cells [19; 20]. In this study, MHC expression was not observed in microglia, which was required for CD4 T-cell activation in ROT-induced PD rats in the SN. Therefore, in ROT-induced neuroinflammation, astrocyte might be the main APCs involved in CD4 T-cell activation.

Growing evidence indicated that CD4 T cells were critical in pathogenesis of PD. The activation of α-syn-specific CD4 T-cell predominates in neuroinflammation in early PD [8]. Two antigenic regions of human α-
syn elicited specific T-cell responses in PD patients [21]. T-cell infiltration and activation was observed in the midbrain of PD patients and animal models [22]. In our study, α-syn-specific CD4 T-cell activation was induced in the SN in ROT-induced PD rats with increased expression of proinflammatory cytokines.

The activation of different subsets of CD4 T cells was determined by a combination of cell surface markers and secreted cytokines [8]. CD4 T helper cells were mainly divided into four subgroups: Th1, Th2, Th17, and regulatory T cells (Tregs), which were activated through the MHC pathway [23]. Th1/Th17 cells produced IFN-γ via stimulation and expressed CXCR3 and CCR5. Tregs expressed CD25 at high levels [23]. In addition, follicular T helper (Tfh) cells were the specialized subset of T cells [24]. To investigate the effects of Tfh in PD, we also detected the cell markers of Tfh in ROT-induced PD rats. The results showed that the number of Tfh cells in PD increased. AS- treatment downregulated these changes in PD rats. The contribution of Tfh cells to the pathogenesis of PD requires further research.

IL-18 was the product that activated the inflammasome, which secreted active IL-18 after cleavage with caspase-1 or other caspases [25]. Astrocytes and microglia induced functional inflammasomes and secreted proinflammatory cytokines such as IL-18 [26]. IL-18 stimulates innate and acquires immune responses. In the adaptive immune system, IL-18 induces IFN-γ production and promotes CD4 T-cell activation and differentiation [27]. Moreover, IL-18-null mice suppressed DA neuron loss following acute 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) treatment [28]. Therefore, IL-18 was a main cytokine involved in the activation of CD4 T cells and the loss of DA neurons. In our study, the expression of IL-18 in astrocytes and microglia increased in ROT-induced PD rats. In addition, an increase in IL-18 was accompanied by a decrease in CD4 T cells and DA neurons.

Truncated α-syn promoted the development of LBs and consequential cellular toxicity [11]. Caspase-1 was observed in LBs and cleaved α-syn to produce a C-terminal truncated α-syn121, which would be one of the sources of the antigens that triggered adaptive immune responses in PD [29]. Upregulated cleaved caspase-1 interacted with endogenous α-syn121 in cells and exacerbated endogenous phosphorylated α-syn inclusions [12]. In our study, cleaved caspase-1 and α-syn colocalized in astrocytes. The above evidence showed that in astrocytes, truncated α-syn was presented to CD4 T cells. AS-IV administration inhibited the α-syn-specific CD4 T-cell-mediated immune response.

AS- exhibited neuroprotective and anti-inflammatory effects in ROT-induced PD rats by inhibiting MHC-mediated antigen presentation of α-syn to CD4 T cells. Thus, AS-IV might be a promising therapeutic drug for the treatment of PD.

**Declarations**

Ethical approval and competing interests

No applicable.

Competing interests
The authors declare no conflicts of interest.

Authors’ contributions

Mengdi Wang and Hongcai Wang conceived and designed research. Mengdi Wang, Fengjiao Sun, Xiaofeng Han, Nan Wang, Yalan Liu, Jinfeng Cai, Shanshan Tong and Rui Wang performed the experiments. Mengdi Wang, Yalan Liu, Jinfeng Cai and Shanshan Tong analyzed and interpreted the data. Mengdi Wang and Hongcai Wang wrote the manuscript, which was read, edited, and approved by all the authors. Mengdi Wang, Fengjiao Sun, Nan Wang and Hongcai Wang contributed reagents, materials, and analysis tools. All authors read and approved the final manuscript.

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Data Availability

The data used during the current study are available from the corresponding author on reasonable request.

Ethics approval

All animal procedures were approved by the guidelines of the ethical standards for laboratory animals of Binzhou Medical University.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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References


Table 1
Table1 The antibodies were used in this study.

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<td>1:400</td>
<td>Goat</td>
<td>Abcam, ab150115</td>
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Figures
Figure 1
AS-IV ameliorated motor deficits and inhibited the loss of DA neurons in PD rats.

(A) Schematic diagram of the experimental process.

(B-C) APO-induced rotation test was examined at weeks 2 and 4. n=11 each group. *p<0.05, #p>0.05.
(D) Time in limb hanging test was investigated. n=11 each group. **p<0.01, ***p<0.001.

(E) Scores in limb hanging test was tested. n=11 each group. *p<0.05, **p<0.01.

(F) Time on the rod in rotarod test was evaluated. n=11 each group. *p<0.05, #p>0.05.

(G) Gastrointestinal motility test was monitored at 4 weeks. n=11 each group. **p<0.01, ***p<0.001.

(H-I) The representative immunofluorescence staining of TH in the SN. n=5 each group. Scale bar=20μm.

(J-K) The representative immunofluorescence staining of TH and α-syn in the SN. n=5 each group. Scale bar=20μm.

(L) The representative western blot bands and statistical graph of α-syn protein expression. n=3 per group. The protein level was normalized to β-actin. Data were presented as mean ± SEM.

![Figure 2](image)

AS- inhibited the expression of pro-inflammatory factors in PD rats.

(A) The representative western blot bands of TNF-a, IL-18, IL-6 and IL-1β.

(B) Statistical graph. The protein levels were normalized to β-actin. n=3 per group. Data were presented as mean ± SEM. *p 0.05, **p 0.01, ***p 0.001, #p>0.05
Figure 3

AS-IV inhibited astrocyte-mediated immune response in PD rats.

(A) The cell number of astrocyte and microglia was analyzed in the SNc and SNr by immunofluorescence staining with GFAP and Iba-1 antibody. n=5 per group. Scale bars=20 μm.

(B) Statistically analysis of fluorescence was performed in vehicle, ROT and AS-IV group.

(C-D) The cell number and cell morphology were altered apparently in ROT and AS-IV group in the SN. n=5 per group. Scale bars=20 μm. Data were presented as mean ± SEM. *p 0.05, **p 0.01, ***p 0.001, #p>0.05.
Figure 4

AS-IV inhibited the activation of CD4 T cell in SN in PD rats.

(A-B) The scatter plot of the flow cytometry of CD4 and CD8. n=3 each group. ***p<0.001.
(C-D) The representative immunofluorescence staining of CD4 in the SN and statistical graph. n=5 each group. Scale bar=20μm. **p<0.01, ***p<0.001.

(E-G) Tfh cell hallmarkers were investigated in vehicle, ROT and AS-IV groups. (E-F) The representative immunohistochemistry staining of CXCR5 in the SN and statistical graph. n=5 per group. Scale bar=20μm. *p<0.05, ***p<0.001. (G) The representative western blot bands for IL-21 and Bcl-6 and statistical graph. n=3 each group. ***p<0.001.

(H-J) Treg cell hallmarkers were evaluated in vehicle, ROT and AS- groups. (H-I) The representative immunohistochemistry staining of CD25 in the SN and statistical graph. n=5 per group. Scale bar=20μm. ***p<0.001. (J) The representative western blot bands for CD25 and statistical graph. n=3 per group. ***p<0.001.

(K-M) Th1 cell hallmarkers were tested in vehicle, ROT and AS- groups. (K-L) The representative immunohistochemistry staining of CXCR3 in the SN and statistical graph. n=5 per group. Scale bar=20μm. ***p<0.001. (M) The representative western blot bands for IFN-γ and statistical graph. n=3 per group. *p<0.05, ***p<0.001. The protein levels were normalized to β-actin. Data were presented as mean ± SEM.
Figure 5

AS- inhibited IL-18-mediated inflammation in PD rats.

(A-B) The representative immunofluorescence staining of GFAP and IL-18 and statistical graph. n=5 per group. Scale bar=20 μm.
(C-D) The representative immunofluorescence staining of Iba1 and IL-18 and statistical graph. n=5 per group. Scale bar=20μm.

(E-F) The representative immunofluorescence staining of CD4 and IL-18 and statistical graph. n=5 per group. Scale bar=20μm.

(G-H) The representative immunofluorescence staining of TH and IL-18 and statistical graph. n=5 per group. Scale bar=20μm. Data were presented as mean ± SEM. *p<0.05, **p 0.01, ***p 0.001, #p > 0.05.
Figure 6

AS- inhibited the presentation of α-syn in PD rats.

(A-B) Immunofluorescence staining of GFAP/MHC and Iba1/MHC in the SN and statistical graph. n=5 per group. Scale bar=20μm.
(C-D) Immunofluorescence staining of CD4 and GFAP in the SN and statistical graph. n=5 per group. Scale bar=20μm.

(E) Immunofluorescence staining of CD4 in the SN Scale bar=100μm. Data were presented as mean ± SEM. **p  0.01, ***p  0.001, #p>0.05.
AS-IV decreased antigen presentation of α-syn to CD4 T cells and the expression of cleaved caspase-1.

**(A-B)** Immunofluorescence staining of GFAP, MHC and α-syn in the SN and statistical graph. n=5 per group. Scale bar=20μm.

**(C-D)** Immunofluorescence staining of CD4 and α-syn in the SN and statistical graph. n=5 per group. Scale bar=20μm.

**(E-F)** Immunofluorescence staining of α-syn, cleaved-caspase-1 and GFAP in the SN and statistical graph. n=5 per group. Scale bar=20μm. Data were presented as mean ± SEM. *p  0.05, **p  0.01, ***p  0.001.
Figure 8

Mechanisms that AS-IV protected DA neurons against CD4 T cell response.