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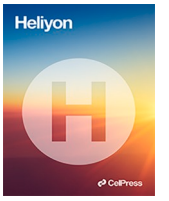

Research article

Astragaloside IV attenuates neuroinflammation and ameliorates cognitive impairment in Alzheimer's disease via inhibiting NF-κB signaling pathway

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ABSTRACT

The inflammatory process plays a significant role in the pathophysiology of Alzheimer's disease (AD). Anti-neuroinflammatory cascade is now considered an important measure for AD treatment. Astragaloside IV (AS-IV), a saponin of Astragali radix, has shown significant anti-inflammatory properties and protective effects against neurodegenerative diseases. However, the mechanisms of AS-IV in treating Alzheimer's disease (AD) have not been fully determined. The experiment research was carried out to comprehensively confirm the beneficial effects and underlying molecular mechanisms of AS-IV to AD. In this research, BV-2 cells were cultured *in vitro* and treated by AS-IV under the stimulation of LPS, qRT-PCR was adopted to analyze the mRNA expression level of inflammatory factors. Western-blot was carried out to analyze the phosphorylation level of NF-κB signaling pathway. 5xFAD mice were administrated AS-IV mixed in the diet for 3 months. Behavioral experiments were adopted to analyze learning and memory abilities. Immunohistochemical staining was adopted to observe the proliferation of microglias and the accumulation of Aβ plaques. AS-IV cut down the mRNA expression of IL-1β, COX-2, iNOS and TNF-α in LPS-stimulated BV-2 cells by suppressing the phosphorylation of IκB and p65, and inhibited the phosphorylated p65 from entering the nucleus. AS-IV increased the frequency of recognizing new objects in the novel object recognition test, shortened the escape latency, raised the number of crossing platform in the Morris water maze, inhibited the hyperplasia of microglias, and reduced the production of senile plaques in 5xFAD mice. In brief, AS-IV ameliorates learning and memory impairment by relieving the intensity of neuroinflammatory response in AD. Therefore, AS-IV is very promising to be a herbal medicine for AD treatment.

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1. Introduction

With the increase in the age of the world's population, the incidence of dementia is also increasing. Alzheimer's disease (AD), posing a heavy economical burden to elder patients, is the most common senile dementia. Up to now, only a few medicines are proven protective effects against AD. Those medicines being used in the clinic are mainly to improve symptoms directly and have low efficacy in the later period of the disease. Herbal medicines, such as Ginseng radix [1], Polygalae radix [2], and their bioactive ingredients have shown their protective effects against AD with fewer adverse effects than those currently available medicines in the clinic [3]. In Sheng Nong's Herbal Classic and Compendium of Material Medica, Astragali radix, a herbal piece of *Astragalus membranaceus* var. *mongholicus* was recorded as restorative because of its promoting metabolism and enhancing immunity of human body [4]. Recently, a growing number of academics and clinical practitioners have given importance to the research on the immunoregulatory activities of Astragali radix. Researches found that Astragali radix has a sterling clinical efficacy in anti-diabetes, anti-cancer and anti-virus [5]. The anti-cancer activity of Astragali radix is related to the effective upregulation of the level of interleukin-1β (IL-1β), IL-2, interferon-γ (IFN-γ), $CD3^+$, $CD4^+$, and the downregulation of IL-6, tumor necrosis factor-α (TNF-α) and $CD8^+$ in liver and breast cancer [6,7]. In the process of aging, the chronic inflammatory state in the brain will activate microglias. Microglias are the major phagocytes settled in the brain to clear the danger signals. However, over-activated microglias secrete TNF-α [8], IL-1β [9], IL-6 [10], reactive oxygen and nitrogen species [11], which leads to neuron death. Moreover, the phagocytosis of overactivated microglia is impaired, and the senile plaque cannot be cleaned in time. The accumulated senile plaques in turn stimulate microglias to produce inflammatory mediators, which leads to a vicious circle and accelerates the development of AD [12,13]. Multiple signaling pathways take part in the modulation of microglial activation. NF-κB is a cardinal transcription factor in regulating pro-inflammatory mediators [14]. Neuroinflammation acts as a crucial role in the progress of neurodegenerative diseases. Thus, regulating neuroinflammation would be an effective treatment to delay AD [15]. Medicines with anti-inflammatory effect are utilized in clinical practice to prevent AD. While such anti-inflammatory medicines cannot be administrated for long period due to their side effects, which holds back their application in the treatment of AD. Astragaloside IV (AS-IV), is a saponin and an active ingredient derived from Astragali radix. Researches showed that AS-IV had anti-oxidant [16] and anti-inflammatory features [17], and showed protective effect against neurodegenerative disorders, such as Parkinson's disease, cerebral ischemia [18–20] and autoimmune encephalomyelitis [21]. Recently, researchers found that AS-IV could promote the regeneration of axons and reconstruction of neuronal synapses [22], and prevent neuron apoptosis in AD mice [23,24]. However, whether AS-IV can treat AD has not quite clear. In this research, 5xFAD mice were administrated AS-IV for three months, the beneficial effect and the underlying mechanism of AS-IV to AD were verified.

2. Materials and methods

2.1. Chemicals and reagents

AS-IV (Cat.83207-58-3, and HPLC purity >98%): Tauto Biotech (Shanghai, China). LPS (*E.coli* 0111:B4) (Cat.437620), 3,3′-diaminobenzidine (DAB) (Cat.D8001), thioflavin-S (Cat.T189) and BCA protein assay reagents (Cat.BCA1): Sigma (China). Anti-Iba1 primary antibody (Cat.012-26723): Wako (Japan). Biotin-rabbit anti-mouse IgG (Cat.7074): Life Technologies (USA). Streptavidin conjugated-horseradish peroxidase (Cat.RABHRP3) and TRIzol: Invitrogen (USA). p-P65 (Ser536) (Cat.3033), p-IκBα (Ser32/36) (Cat.9246), AF488 fluorescent antibody (Cat.15008) and corresponding antibodies against total proteins: Cell Signaling Technology (Shanghai, China). Protein and nucleic acid precipitation solution (Cat.A7124) (Cat.A7943): Promega Biotech (China). Agarose (Cat. A9539): Biowest (France). Nitric oxide (NO) assay kit (Cat.S0025), RIPA and CCK8 kit: Beyotime (China). Reverse transcriptase (Cat.2690A): Takara (China). SYBR selection master mix: Life Technologies (USA). DMEM culture medium, fetal bovine serum: Gibco (USA).

2.2. Animals and drug administration

Transgenic C57BL/6J mice with 3 mutated genes of amyloid-beta (Aβ) precursor protein (APP) and 2 mutated genes of presenilin 1 (PS1) in human familial Alzheimer's disease (FAD) (also abbreviated as 5xFAD in Jackson laboratory) were purchased from Nanjing Institute of Biomedicine (Jiangsu, China). The genotype of hybrid offspring mice was identified according to gene sequences of APP and PS1 [25] (Fig. S1). Wild-type C57/BL6J mice, 8 weeks, were used as the normal control. All experimental animals were raised in a specific pathogen-free (SPF) room of animal center. The feed mixed with AS-IV was processed by Xietong Biotech (Jiangsu, China). There were 10 male mice in each group and four groups of mice showed in Table 1. The AS-IV dose in AS-IV low dose (ASL) group and AS-IV high dose (ASH) group was referenced from Li Min [20]. Mice were administered AS-IV for 3 months. The animal ethics

Table 1
Experimental grouping.

Group	Mice	AS-IV administration
WT	C57/BL6J	No
AD	5xFAD	No
ASL	5xFAD	10 mg/kg/d
ASH	5xFAD	20 mg/kg/d

committee of Shanghai University of traditional Chinese medicine approved the mice experiments (PZSHUTCM210108002), and we abided by the guideline for laboratory animals.

2.3. Quantitative RT-PCR

1×10^6 BV-2 cells were cultured with 10, 25, or 50 μM AS-IV, stimulated simultaneously with LPS for 6 h, add TRIzol reagent for the lysis of cells. The extracted total RNA was reverse-transcribed into cDNA. DNA template was amplified with SYBR green fluorescent quantitative PCR kit. The specific primers of inflammatory factors were cited from Ref. [26], and synthesized by Shenggong (Shanghai, China). The reaction was performed under the condition of 94 °C, 30 s, 57 °C, 45 s, 72 °C, 30 s, 35 cycles. The relative mRNA expression of inflammatory factors was calculated by the $2^{-\Delta\Delta CT}$ method. GAPDH was used as the housekeeping gene.

2.4. Nitrite assay

2×10^6 BV-2 cells were handled in media with 10, 25, or 50 μM AS-IV for 24 h, under stimulation of LPS. NO in cell supernatant was assayed with Griess reagent [27]. Briefly, the same volume of Griess reagent was added to cell supernatant, and then reacted at RT for half an hour. The OD_{540} value of the mixture was read by ELISA machine. The concentration of NO was calculated based on sodium nitrite.

2.5. Western-blot

As described in a previous study with modifications [28], BV-2 cells were cultured with 25 μM AS-IV, stimulated simultaneously with LPS, harvested and dissolved the cells. Protein concentration of sample was determined based on BCA method. Protein band was parted by gel electrophoresis and transferred to NC membranes. After blocked, NC membranes were incubated with P65 and IκB antibodies overnight at 4 °C. Then, NC membranes were rinsed on the shaker with TBST solution 3 times, and immersed in secondary antibody for 2 h at RT. Finally, NC membranes were immersed in a chemiluminescent reagent, and read in a chemiluminescence meter. Gray value of protein band was quantified with Image J software, and analyzed relative quantification compared with GAPDH.

2.6. Cell immunofluorescent analysis

The method was the same as that described in a reference [29], 2×10^4 BV-2 cells on sterile coverslips were cultured with 25 μM AS-IV, stimulated simultaneously with LPS for 1 h. Cells were fixed for 10 min with formalin, washed with PBS, cells were immersed in 1% Triton X-100, and blocked with TBST solution with 5% BSA. The fixed cells were immersed in the Iba1 antibody solution overnight at 4 °C, and rinsed with TBST solution, then incubated for 60 min with AF488 fluorescent antibody at RT. Cells were then incubated with DAPI and washed. Finally, the coverslips with cells were dried at RT and sealed. Cell pictures of four groups were taken by confocal microscope (Zeiss, Germany).

2.7. Immunohistochemical analysis

As previously described with minor modifications [30], 40 μm brain coronal section was cut. The sections were rinsed in PBS, and immersed in solution with PBS, formaldehyde and 3% H_2O_2 in ratio of 5: 4: 1 for 30 min, then immersed in 0.1% Triton X 100 for 10 min, blocked with TBST containing 5% BSA for 1 h. Brain sections were immersed in antibody solution in an ice room, overnight. Rinsed with TBST, slides were immersed in secondary antibody coupling with streptavidin horseradish peroxidase for 2 h, rinsed, and then reacted with DAB staining solution. The brain sections were immersed in xylene and alcohol for dehydration and degreasing. Slides were sealed with neutral resin in order to save for a long time. Pictures were taken with LSM 880 microscope (Zeiss Germany).

2.8. Thioflavin S staining

As described in reference with modifications [31], brain sections were stained with 0.05% Thio-S solution avoiding light for 8 min, and then react with 80% alcohol for 10 s, washed 4 times with ddH_2O, brain sections were rinsed in PBS with a high concentration of sodium chloride at ice room for half an hour, and rinsed 4 times with ddH_2O. The stained images of Aβ plaques were read with a Zeiss microscope.

2.9. Novel object recognition task

The task was carried out based on a cited protocol with modification slightly [32]. The process of the task was recorded by a camera, and analyzed with Noldus (Beijing, China). At the beginning of the task, there were two objects (X and Y) were placed in a white box (50 × 50 × 40 cm). Mice were put in the box near the box wall, and moved freely to look for novel objects for 5 min. 1 h and 24 h later, object X was replaced with object Z. Objects X, Y and Z were the same size, but different in color and shape. The mice were put back in the box to look for novel objects again for 5 min after 1 h and 24 h of the first test. The preference index at 0 h was calculated as a ratio of time of looking for object X over time of looking for objects X and Y. Preference index at 1 h or 24 h was calculated as a ratio of time of looking for object Z over time of looking for object Y and Z.

2.10. Morris water maze task

The task, used to assess the memory of mice, was based on a cited protocol with minor modifications [33]. The movement of mice in the swimming pool was tracked by a camera. The water pool (100 cm in diameter) was apart into A, B, C and D quadrants on average. A platform hidden under 1 cm of water was put at A quadrant. The water temperature was held at 22 °C. The water maze was carried out for 6 days. Every mouse started the task from the center of A, B and C quadrants, and swam freely to look for the hidden platform within 1 min. The mouse was allowed to be familiar with the surrounding environment on the platform for 10 s. The platform was taken away at the last task, and the mice swam freely beginning at the opposite side of the D quadrant for 1 min. Mouse activities were tracked and recorded by Noldus.

2.11. Statistical analysis

The statistical difference was compared based on one-way or two-way ANOVA, and shown as *, $P < 0.05$ or **, $P < 0.01$. The analysis was carried out with GraphPad Prism 5 (CA, USA). The statistic parameters were shown in Table S1. The DOI of raw data is https://doi.org/10.6084/m9.figshare.21701402.

3. Results

3.1. AS-IV attenuates microglias activation stimulated by LPS

Cells were cultured *in vitro* and incubated with 0, 25, 50 or 100 μM AS-IV. CCK-8 assay showed that AS-IV did not exhibit cytotoxicity to BV-2 cells under the concentration of 25 μM–100 μM ($P > 0.05$) (Fig. S2). BV-2 cells cultured *in vitro* without stimulation of LPS were polygonal and protuberant. BV-2 cells cultured *in vitro* with stimulation of LPS were round, and there were fewer branches on their membranes. BV-2 cells cultured *in vitro* with stimulation of LPS and treatment of 25 μM AS-IV were triangular and there were more branches on their membranes (Fig. S3). BV-2 cells were incubated with anti-Iba1 antibody, and the expression of Iba1 molecule distinctly raised on the LPS-stimulated cells compared to that on the cells without stimulation of LPS, while dropped on the 25 μM AS-IV-treated cells compared to that on the LPS-stimulated cells ($P < 0.01$) (Fig. 1A and B). Thus, AS-IV attenuates microglias activation under stimulation of LPS.

3.2. AS-IV inhibits the mRNA expression of IL-1β, COX-2, iNOS, TNF-α and production of NO in microglias stimulated by LPS

Over-activated microglias show neurotoxicity by secreting inflammatory factors, such as IL-1β, IL-6, COX-2, iNOS, NO, ROS and TNF-α [25]. BV-2 cells were incubated with AS-IV under the stimulation of LPS for 6 h, the mRNA expression of IL-1β, COX-2, iNOS and TNF-α were tested by qRT-PCR. We found that the mRNA expression of inflammatory factors raised in the LPS-stimulated cells compared to that in the resting cells, while the mRNA expression of IL-1β ($P < 0.01$), TNF-α ($P < 0.05$), COX-2 ($P < 0.01$) and iNOS ($P < 0.01$) dropped significantly in the 25 μM AS-IV-treated cells compared to that in the LPS-stimulated cells (Fig. 2A, B, C and D). BV-2

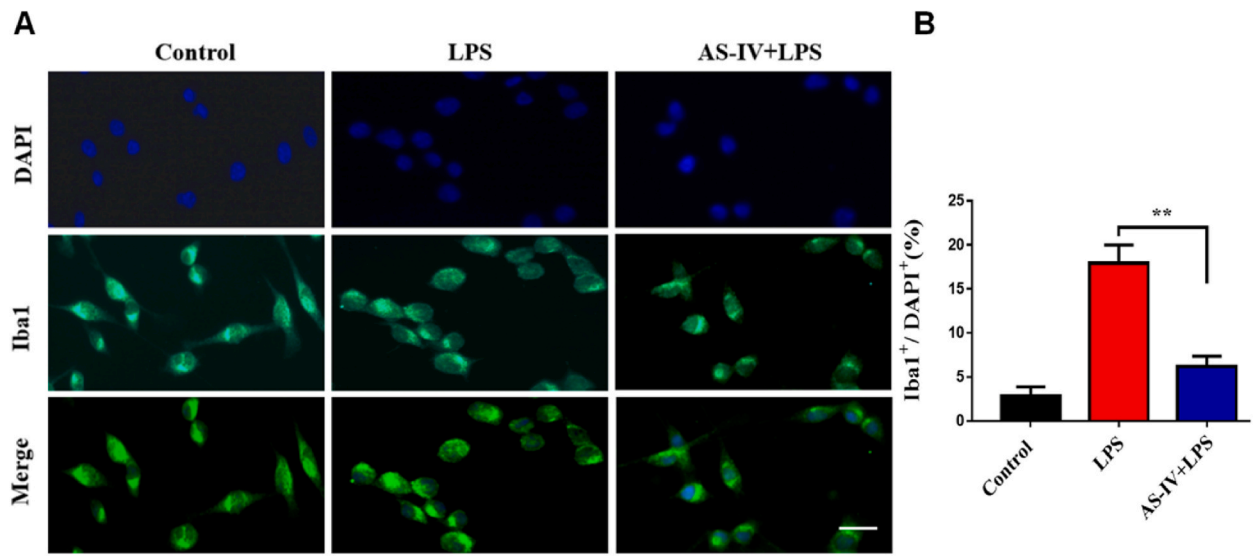

Fig. 1. AS-IV attenuates microglias activation stimulated by LPS. A: Representative cell morphology images of BV-2 in the group of control, LPS and AS-IV + LPS. Iba1: green, DAPI: blue. Scale bar: 50 μm. B: The statistical histogram of Iba1 expression on microglias (**$P < 0.01$, AS-IV + LPS group vs. LPS group).

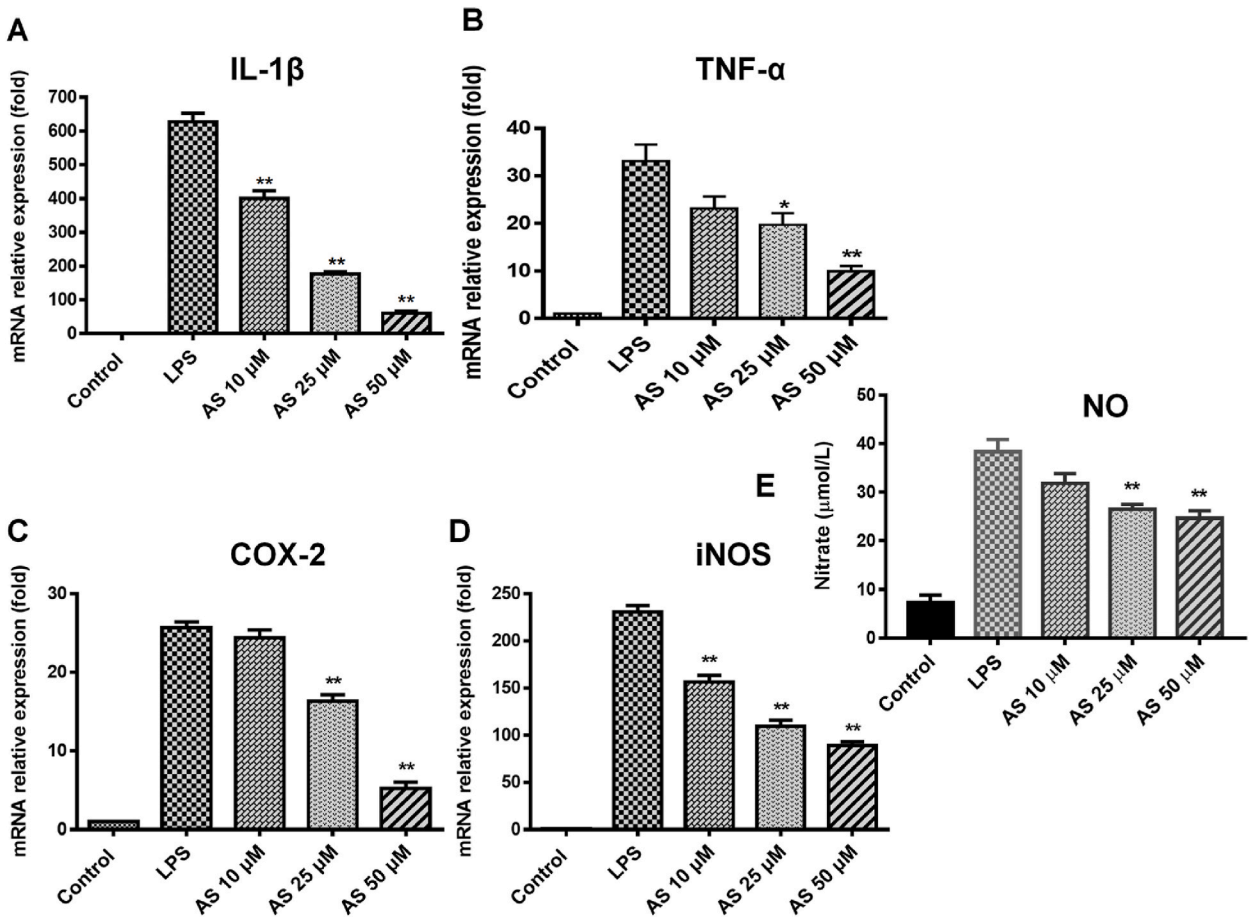

Fig. 2. AS-IV inhibits the mRNA expression of IL-1β, COX-2, iNOS, TNF-α and production of NO in microglias stimulated by LPS. A: IL-1β mRNA expression level in microglias (**$P < 0.01$, 10, 25, 50 μM AS-IV vs. LPS), B: TNF-α mRNA expression level in microglias (*$P < 0.05$, 25 μM AS-IV vs. LPS. **$P < 0.01$, 50 μM AS-IV vs. LPS), C: COX-2 mRNA expression level in microglias (**$P < 0.01$, 25, 50 μM AS-IV vs. LPS), D: iNOS mRNA expression level in microglias (**$P < 0.01$, 10, 25, 50 μM AS-IV vs. LPS), E: NO secretion level in supernatant of microglias (**$P < 0.01$, 25, 50 μM AS-IV vs. LPS). The statistical data of three independent experiments were presented as means ± SD.

cells were incubated with AS-IV for 24 h under the stimulation of LPS, NO concentration in the supernatant was checked by Griess reagent. The concentration of NO raised in the LPS-stimulated cells compared to that in the resting cells, while dropped significantly in the 25 μM AS-IV-treated cells compared to that in the LPS-stimulated cells ($P < 0.01$) (Fig. 2E). Thus, AS-IV inhibits the mRNA expression of IL-1β, COX-2, iNOS, TNF-α and the production of NO in microglias stimulated by LPS.

3.3. AS-IV inhibits the activation of NF-κB signaling pathway

To further validate the anti-inflammatory role of AS-IV, BV-2 cells were incubated with 25 μM AS-IV under the stimulation of LPS. The phosphorylated IκB and P65 were detected by Western-blot. The experimental data indicated that the phosphorylation of IκB ($P < 0.05$) and p65 ($P < 0.05$) in BV-2 cells were remarkably inhibited after AS-IV treatment (Fig. 3A, B, D and E). The results of immunofluorescent staining showed that phosphorylated p65 was inhibited from entering nucleus of BV-2 cells in 25 μM AS-IV-treated cells compared to that in cells only treated by LPS (Fig. 3C). In brief, AS-IV down-regulates the activation of NF-κB signaling pathway.

3.4. AS-IV improves cognitive impairment in 5xFAD mice

Memory, thinking and behavior of AD patients are impaired due to long period of neuroinflammation and progressive degeneration of neurons [34]. To examine whether AS-IV can improve cognitive impairment, four groups of mice were carried out novel object recognition task and Morris water maze. Before the experiments, we carried out open-field tests and analyzed the gait of mice. We found that mice did not behave anxiously or nervously (Fig. S4 A, B, C and D), move freely without abnormal gait (Fig. S5). So AS-IV is a safe candidate medicine to treat AD. At 0 h, in novel object recognition task, the preference index ($P > 0.05$) and frequency of looking for the object X ($P > 0.05$) among the four groups showed no significant difference (Fig. 4A and B). At 1 h of tests, the preference index

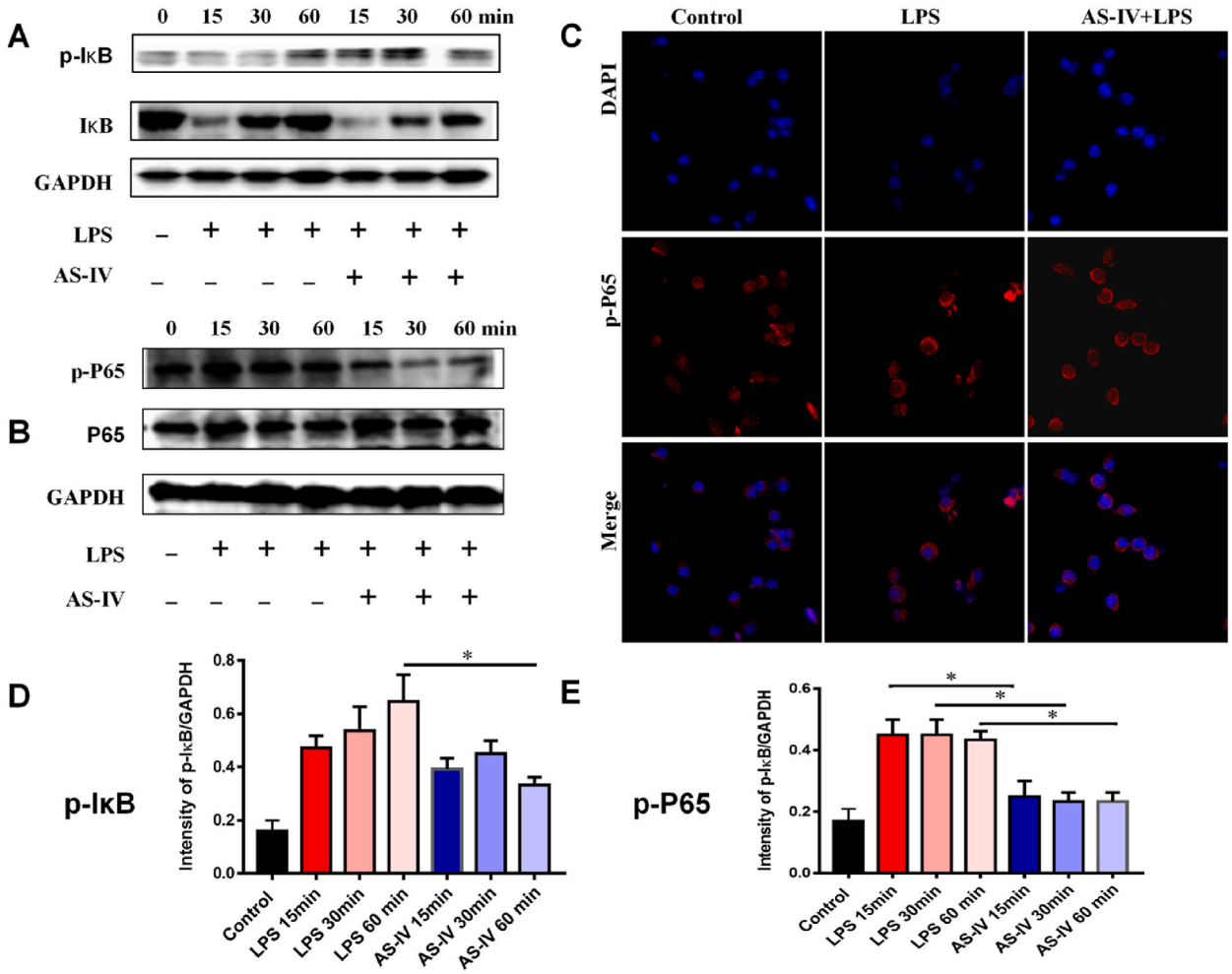

Fig. 3. AS-IV inhibits the activation of NF-κB signaling pathway. A: Representative images of Western-blot of IκB, B: Representative images of Western-blot of P65, C: Immunofluorescence staining images of AS-IV inhibition p-p65 from entering nucleus. p-P65: red, DAPI: blue. Scale bar: 50 μm, D: The statistical histogram of inhibition of AS-IV on phosphorylated IκB in microglias (*$P < 0.05$, 25 μM AS-IV 60 min vs. LPS 60 min), E: The statistical histogram of inhibition of AS-IV on phosphorylated P65 in microglias (*$P < 0.05$, 25 μM AS-IV 15 min vs. LPS 15 min, 25 μM AS-IV 30 min vs. LPS 30 min, 25 μM AS-IV 60 min vs. LPS 60 min). The statistical data of Western-blot were presented as means ± SD of three independent tests.

and frequency looking for object Z dropped apparently in AD group vs that in other three groups, while raised in ASH group vs that in AD group ($P < 0.01$) (Fig. 4C and D). At 24 h of tests, the preference index and frequency looking for object Z dropped apparently in AD group vs that in other three groups, while raised in ASH group vs that in AD group ($P < 0.01$) (Fig. 4E and F). These results indicated that the short-memory (1 h) and long-term memory (24 h) of AD group mice were impaired, and AS-IV improved the lesion in memory. In Morris water maze, the escape latency of AD group is longer than that in WT group, however, is shorter in ASH group than that in AD group ($P < 0.01$) (Fig. 5A). The swimming distance of mice in ASH group was significantly shorter than that of AD mice ($P < 0.01$) (Fig. 5B). During the period of spatial exploration, the frequency of crossing the platform dropped in AD group compared to that in WT group, while raised extremely in ASH group compared to that in AD group ($P < 0.05$) (Fig. 5C). The mice in AD group stayed shortly in D quadrant than the mice in WT group. However, the mice in ASH group stayed longer in D quadrant than the mice in AD group ($P < 0.01$) (Fig. 5D). These experimental data indicated that the 5xFAD mice were encountered impairment in learning and memory, and AS-IV reversed the cognitive impairment of AD mice. Therefore, AS-IV shows a potential protective effect to AD mice.

3.5. AS-IV inhibits neuroinflammation in 5xFAD mice

3.5.1. AS-IV reduces the production of Aβ plaques in 5xFAD mice

Senile plaques accumulate in the brain due to neuroinflammation and microglial dysfunction which is an indication of AD histopathology [13,35]. The accumulation of Aβ plaques increases the intense inflammatory response in the brain [12]. Astragali radix shows anti-inflammatory effects. The production of Aβ plaques in four groups of mice was measured by immunohistochemical staining. Tissue staining images of Aβ plaques in the brain in groups of WT, AD, ASL and ASH were exhibited in Fig. 6A. The experimental data

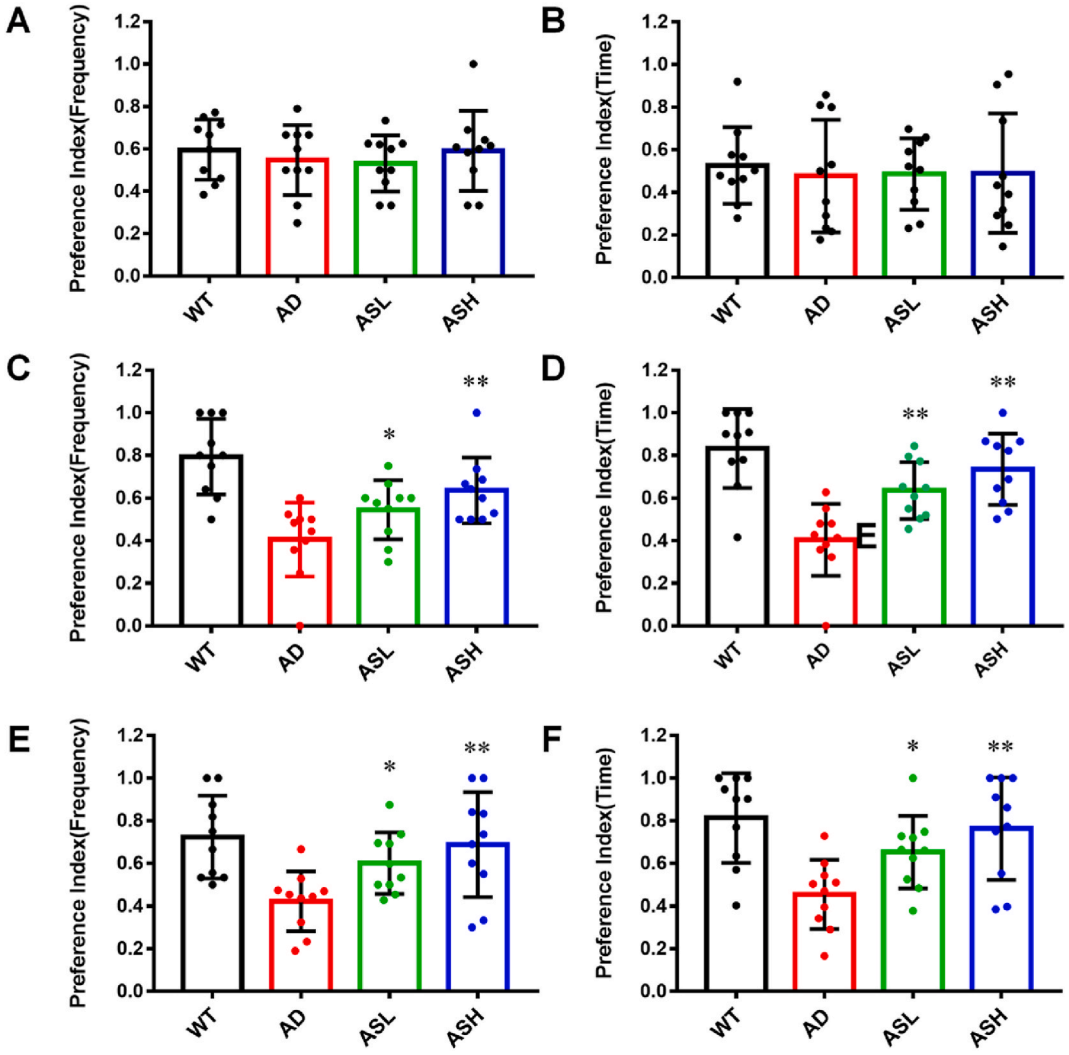

Fig. 4. AS-IV improves memory impairment in 5xFAD mice. A: At 0 h of tests, the percentage of frequency looking for object X to frequency looking for object X and Y within 5 min ($P > 0.05$, ASL vs. AD, ASH vs. AD), B: At 0 h of tests, the percentage of time looking for object X to time looking for object X and Y within 5 min ($P > 0.05$, ASL vs. AD, ASH vs. AD), C: At 1 h of tests, the percentage of frequency looking for object Z to frequency looking for object Z and Y within 5 min ($^{*}P < 0.05$, ASL vs. AD. $^{**}P < 0.01$, ASH vs. AD), D: At 1 h of tests, the percentage of time looking for object Z to time looking for object Z and Y within 5 min ($^{**}P < 0.01$, ASL, ASH vs. AD), E: At 24 h of tests, the percentage of frequency looking for object Z to frequency looking for objects Z and Y within 5 min ($^{*}P < 0.05$, ASL vs. AD. $^{**}P < 0.01$, ASH vs. AD), F: At 24 h of tests, the percentage of time looking for object Z to time looking for objects Z and Y within 5 min ($^{*}P < 0.05$, ASL vs. AD. $^{**}P < 0.01$, ASH vs. AD). The statistical data were presented as the means ± SD (n = 10).

showed that the burden of Aβ plaques raised extremely in AD group compared to that in other three groups. However, the production of Aβ plaques in region of anterior cingulate cortex (ACC) ($P < 0.05$), hippocampus dentate gyrus (DG) ($P < 0.01$) and CA1 ($P < 0.05$) dropped in ASH group compared to that in AD group (Fig. 6B). Those experimental data indicate that AS-IV greatly inhibits the production of Aβ plaques in brain of AD mice.

3.5.2. AS-IV inhibits the activation of microglias in 5xFAD mice

Microglias are the resident phagocytes and the main inflammatory cells in region of cortex and hippocampus. Excessive hyperplasia of microglias in the hippocampus is a characteristic feature and also a danger signal to AD [36]. To demonstrate the effect of AS-IV in anti-inflammation, the hyperplasia of microglias in four groups was analyzed. As shown in immunohistochemical staining images of WT, AD, ASL and ASH groups in Fig. 7A, the $Iba1^{+}$ cells piled together in AD group were much more than that in other three groups, while fewer $Iba1^{+}$ cells piled together in ASH group compared to that in AD group. As shown in Fig. 7B, the number of $Iba1^{+}$ cells raised greatly in AD group compared to that in other three groups, While dropped greatly in the hippocampus DG ($P < 0.01$), CA1($P < 0.05$) in ASH group compared to that in WT group. Those results suggested that microglias in AD mice were activated excessively and

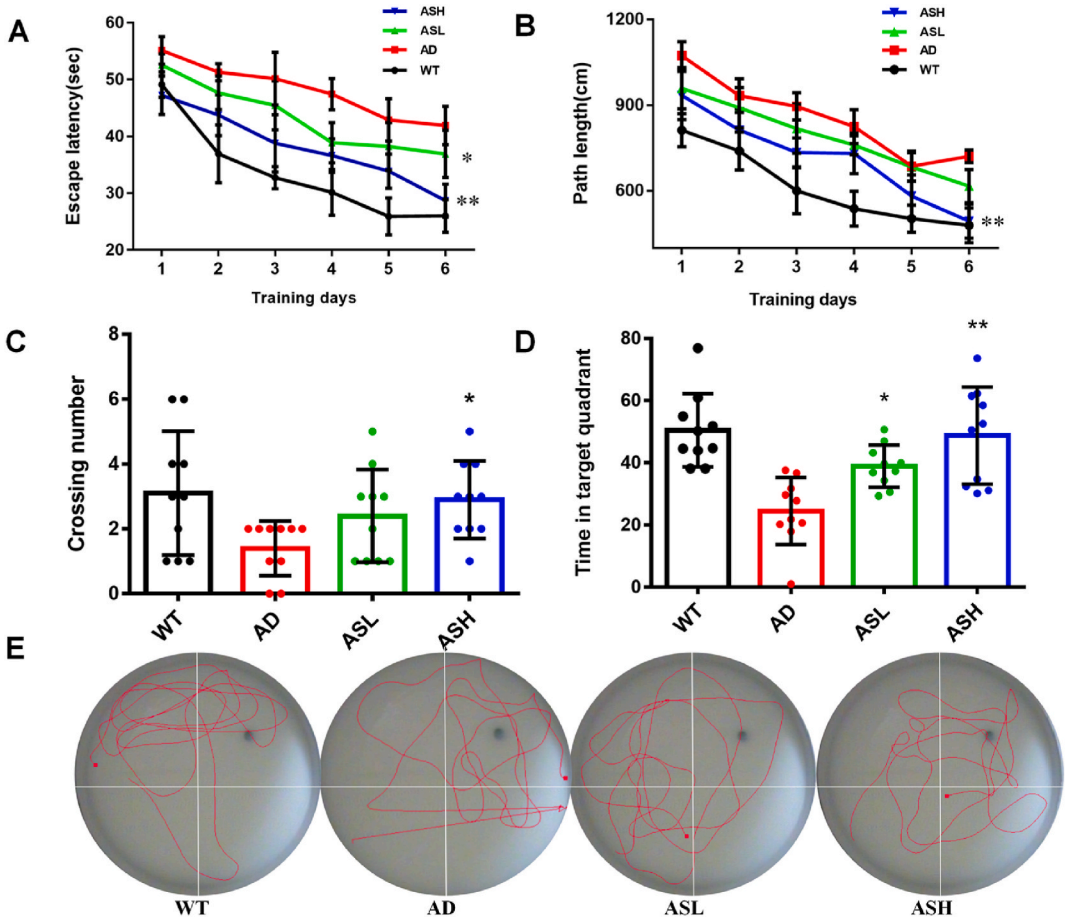

Fig. 5. AS-IV improves spatial exploration ability of 5xFAD mice. A: The escape latency of mice to look for platform in the phase of positioning navigation among four groups (*$P < 0.05$, ASL vs. AD. **$P < 0.01$, ASH vs. AD), B: Total swimming length in the phase of positioning navigation in 60 s (**$P < 0.01$, ASH vs. AD), C: Number of going through platform region in the phase of space exploration (*$P < 0.05$, ASH vs. AD), D: Time stayed in D quadrant in the phase of spatial exploration (*$P < 0.05$, ASL vs. AD. **$P < 0.01$, ASH vs. AD), E: Representative tracing of mice in the phase of spatial exploration. The statistical results were presented as the means ± SD (n = 10).

proliferated surely, and AS-IV could inhibit the situation in AD mice. Thus, AS-IV shows a protective effect on AD through anti-neuroinflammation.

4. Discussion

With the increase in the age of the world's population, the incidence of AD is also increasing. AD has become the leading cause to seriously affect on people's quality of life in the aging society. It is urgent for scientists all over the world to develop effective drugs to treat AD. Up to now, most currently available medicines only offer protection against clinical symptoms, and do not stop neurons from further degeneration. Treatments directly against a single target, such as Aβ plaques, have yet to be proven effective, despite many of these medications having achieved phase 3 clinical trials [37]. Thus, the complex pathophysiology may necessitate the development of multi-target medications for AD treatment [38]. Traditional Chinese medicines were widely applied to the prevention and treatment of diseases over a long period in ancient China [39]. Recent researches highlight the protective potential of traditional Chinese medicines in neurodegenerative diseases because of their characteristics of containing multiple components and having multiple targets [40,41]. Immune regulation disorder and inflammation are important pathogenesis of AD. Therefore, medicinal plants with effects of anti-inflammation and immune regulation were often used to treat amnesia and dementia in ancient China [40].

Astragali radix is a kind of traditional Chinese medicine with anti-inflammatory and immunomodulatory effects. Researches showed that Astragali radix reduced the expression of inflammatory factors, and increased the level of TGF-β produced by T cells and macrophages, inhibited lymphocyte proliferation and promoted tissue repair [42]. Astragalus saponin, astragalus polysaccharide, astragalus flavonoid are active ingredients derived from Astragali radix. Astragalus saponin is the most content and the strongest anti-inflammatory effect in Astragali radix [43]. AS-IV, the main active saponin in Astragali radix, has been demonstrated the potential protective effect on neurodegenerative diseases [44–46]. So we want to explore whether it has protective effect on AD. In this study, we

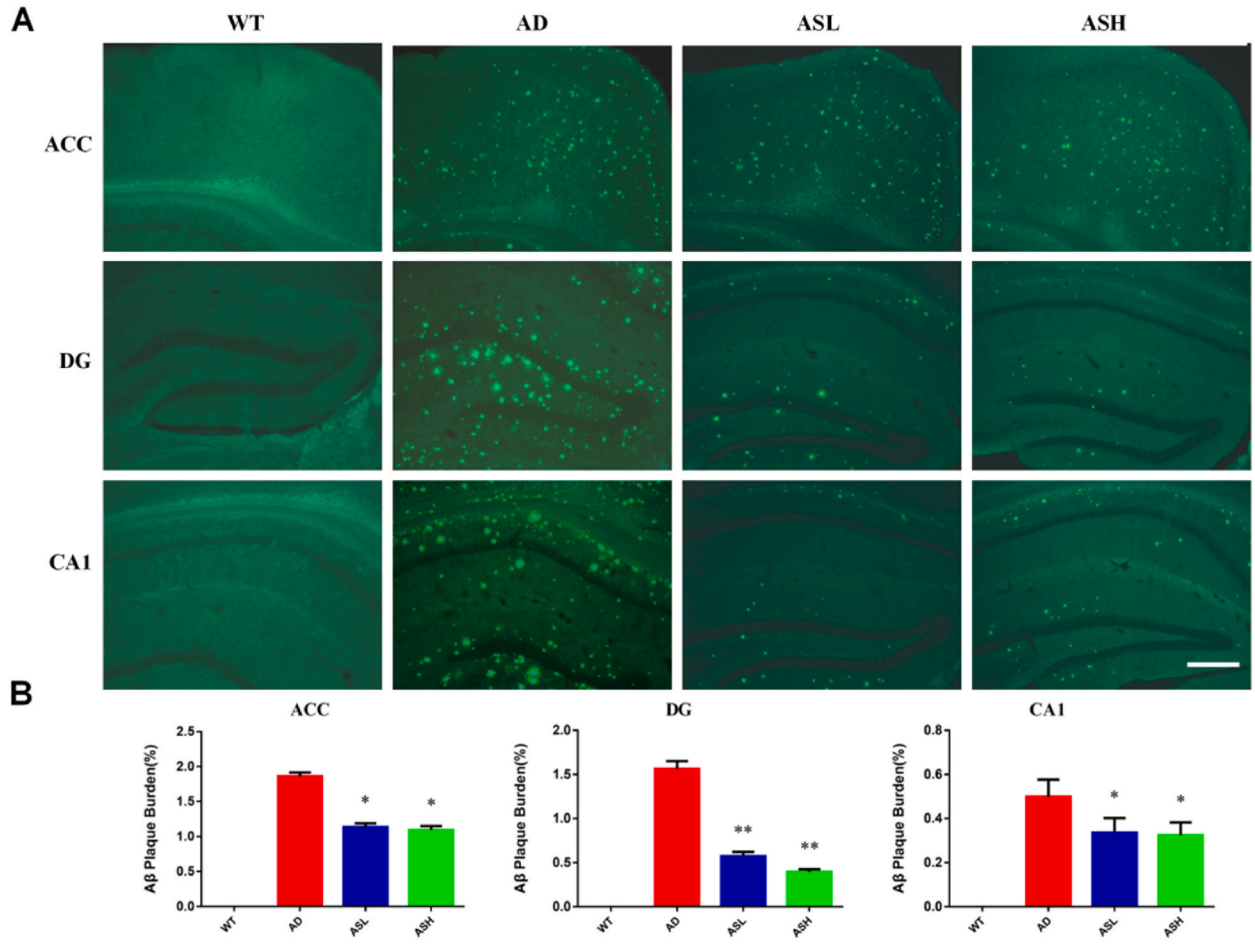

Fig. 6. AS-IV reduces the production of Aβ plaques in 5xFAD mice. A: Tissue staining images of Aβ plaques in brain in WT, AD, ASL and ASH groups. Scale bar: 200 μm, B: The statistical histogram of Aβ plaques burden in ACC (*$P < 0.05$, ASL, ASH vs. AD), hippocampus DG (**$P < 0.01$, ASL, ASH vs. AD) and CA1 (*$P < 0.05$, ASL, ASH vs. AD). The statistical data were presented as the means ± SE (n = 5). ACC: anterior cingulate cortex, DG: dentate gyrus.

found that AS-IV enhanced AD mice's memory to the new objects in the novel object recognition task, AS-IV shortened the time to find the hidden platform in the Morris water maze. In brief, AS-IV improves 5xFAD mice cognitive impairment. We further studied the protective mechanism of AS-IV on AD in vivo and *in vitro*. Numerous pieces of evidence indicate that neuroinflammation might drive the pathogenic process of AD. Anti-neuroinflammatory cascade is now considered an important measure to AD treatment [47]. Firstly, *in vitro*, we proved that AS-IV down-regulated the mRNA expression of IL-1β, TNF-α, COX-2 and iNOS, inhibited the secretion of NO in microglias via suppressing the NF-κB signaling pathway. Microglias, resident phagocytes in the brain, serve many supporting functions. Activation of microglias in AD might have different roles and effects at different stages of the disease. After exposure to the damage-related molecular pattern (DAMP), the acute reaction of microglias to damage signals aims to remove the pathological molecular, for example, Aβ fibrils, neuronal debris in AD [48]. However, with sustained exposure to DAMP, microglials are overactivated by chemokines and cytokines, which seems to be harmful for the functions of microglias especially the ability of phagocytosis to Aβ plaques [13]. Astragali radix significantly increased the phagocytosis of macrophages, increased the co-expression of major histocompatibility complex (MHC) II and CD11c on the surface of dendritic cells [49]. Therefore, we further studied the regulatory effect of AS on microglial activation in the brain. Although we did not directly detect the level of inflammatory factors in the brain, we detected the activation of microglia through brain slices immunohistochemical staining method. We found that AS-IV inhibited the activation and proliferation of microglias. Neuroinflammation is also the reason for the massive accumulation of Aβ plaques in the brain. The reduction of Aβ plaques production or accumulation in the brain can also reflect that AS has the effect of inhibiting neuroinflammation. We used immunofluorescence to stain brain slices, and found that AS-IV reduced the production and accumulation of Aβ plaques in the brain of 5xFAD mice.

In summary, AS-IV can inhibit the activation and proliferation of microglias, decrease the secretion of inflammatory factors, reduce the formation and accumulation of Aβ plaques, and attenuate cognitive impairments in AD mice. Astragali radix has been used to treat diseases for thousands of years in ancient China. We have also confirmed that AS-IV has no cytotoxicity at the concentration of clinical

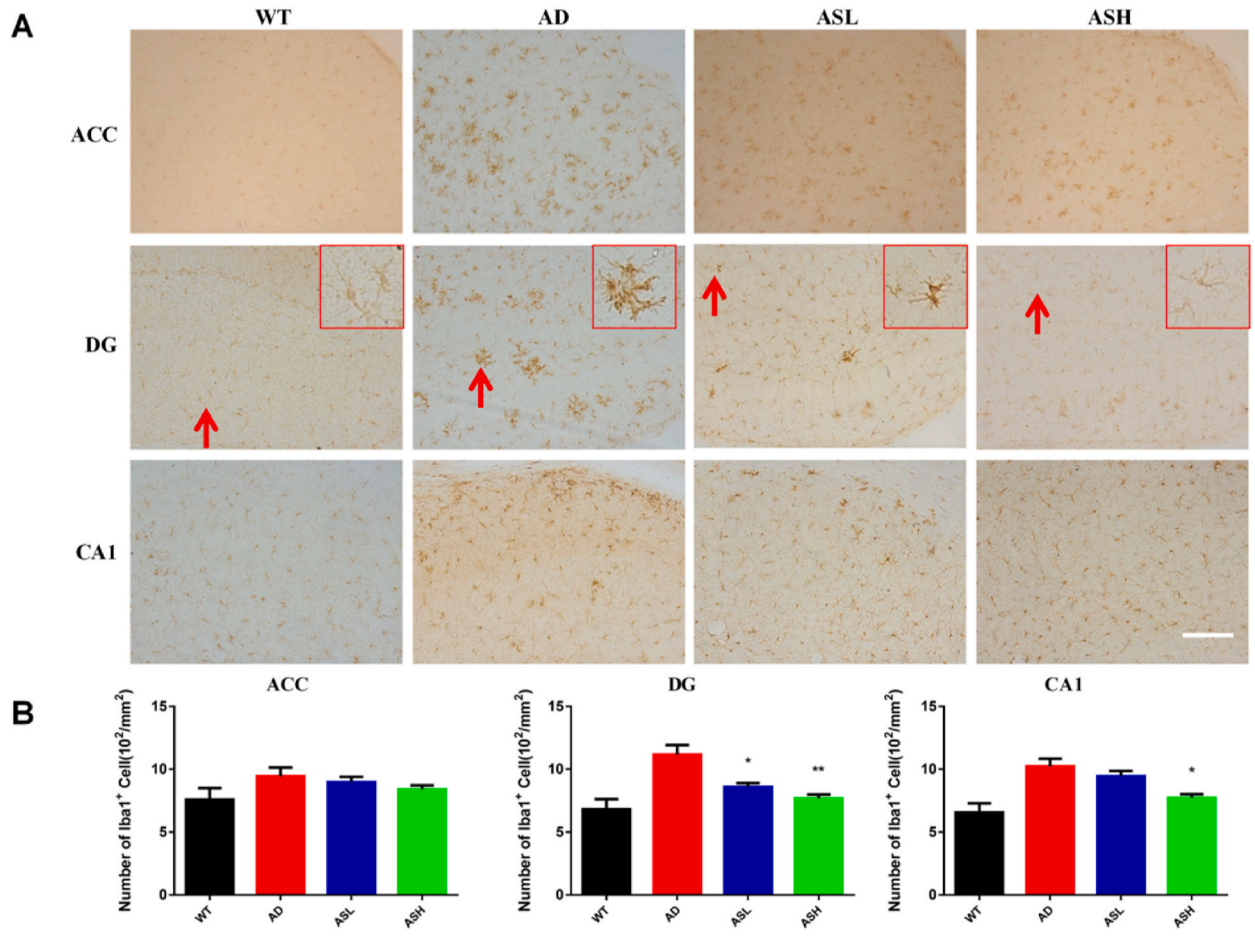

Fig. 7. AS-IV inhibits the hyperplasia of microglias in 5xFAD mice. A: Tissue staining images of Iba1^{+} cells in mice brain of four groups. Scale bar: 100 μm, B: The statistical histogram of microglias in ACC ($P > 0.05$, ASL, ASH vs. AD), hippocampus DG (*$P < 0.05$, ASL vs. AD. **$P < 0.01$, ASH vs. AD) and CA1 (*$P < 0.05$, ASH vs. AD). The statistical data were presented as the means ± SE (n = 5). ACC: anterior cingulate cortex, DG: dentate gyrus.

dose. As a botanical medicine, AS-IV has no side effects even if long-term administration. Thus, AS-IV is very promising to be a candidate therapeutic drug for AD treatment. Combined use of AS-IV and other medications provides a new choice for the clinical treatment of AD.

Production notes

Author contribution statement

Li He: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jinxia Sun: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Zhulei Miao: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Shengmin Chen: Contributed reagents, materials, analysis tools or data.
Guizhen Yang: Conceived and designed the experiments; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interest's statement

The authors declare no competing interests.

Abbreviation

AD	Alzheimer's disease
Aβ	amyloid-β
AS-IV	Astragaloside IV
ASL	AS-IV low dose
ASH	AS-IV high dose
DAB	3,3′-Diaminobenzidine
DAMP	damage related molecular pattern
DMEM	dulbecco's modified Eagle's medium
FBS	fetal bovine serum
FAD	familial Alzheimer's disease
IL-1β	interleukin-1β
iNOS	induced nitric oxide synthase
PFA	paraformaldehyde
NO	nitric oxide
NSAIDs	Non-steroid anti-inflammatory drugs
SD	standard deviation
SE	standard error
SHP	streptavidin conjugating horseradish peroxidase
SPF	specific pathogen-free
TCM	traditional Chinese medicine
TNF-α	tumor necrosis factor-α
WT	wild type

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e13411.

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