Astragaloside IV Attenuates Experimental Autoimmune Encephalomyelitis of Mice by Counteracting Oxidative Stress at Multiple Levels

Yixin He1,2*, Min Du3, Yan Gao1, Hongshuai Liu1, Hongwei Wang1, Xiaojun Wu1*, Zhengtao Wang1*

1 Shanghai Key Laboratory of Complex Prescription, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, P.R. China, 2 Department of Pharmacognosy, China Pharmaceutical University, Nanjing, P.R. China, 3 Unit of Immune Signaling and Regulation, Institut Pasteur of Shanghai, Chinese Academy of Sciences, Shanghai, P.R. China

Abstract

Multiple sclerosis (MS) is a chronic autoimmune neuroinflammatory disease found mostly in young adults in the western world. Oxidative stress induced neuronal apoptosis plays an important role in the pathogenesis of MS. In current study, astragaloside IV (ASI), a natural saponin molecule isolated from Astragalus membranaceus, given at 20 mg/kg daily attenuated the severity of experimental autoimmune encephalomyelitis (EAE) in mice significantly. Further studies disclosed that ASI treatment inhibited the increase of ROS and pro-inflammatory cytokine levels, down-regulation of SOD and GSH-Px activities, and elevation of iNOS, p53 and phosphorylated tau in central nervous system (CNS) as well as the leakage of BBB of EAE mice. Meanwhile, the decreased ratio of Bcl-2/Bax was reversed by ASI. Moreover, ASI regulated T-cell differentiation and infiltration into CNS. In neuroblast SH-SY5Y cells, ASI dose-dependently reduced cellular ROS level and phosphorylation of tau in response to hydrogen peroxide challenge by modulation of Bcl-2/Bax ratio. ASI also inhibited activation of microglia both in vivo and in vitro. iNOS up-regulation induced by IFN-γ stimulation was abolished by ASI dose-dependently in BV-2 cells. In summary, ASI prevented the severity of EAE progression possibly by counterbalancing oxidative stress and its effects via reduction of cellular ROS level, enhancement of antioxidant defense system, increase of anti-apoptotic and anti-inflammatory pathways, as well as modulation of T-cell differentiation and infiltration into CNS. The study suggested ASI may be effective for clinical therapy/prevention of MS.

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* E-mail: xiaojunwu320@126.com (XW); wangzht@hotmail.com (ZW)

These authors contributed equally to this work.

Introduction

Multiple sclerosis (MS) is a chronic autoimmune neuroinflammatory disease found mostly in young adults in the western world [1–2]. Focal lesions of the white matter with inflammation, demyelination, infiltration of immune cells, oligodendroglial death and consequent axonal damage are the major neurophysiological deficits in patients with MS [3]. Although many approved first-line drugs such as IFNB, glatiramer acetate, mitoxantrone, and natalizumab, along with a number of treatments under investigation may curtail attacks or improve the progression of the disease [4], significant adverse effects including depression, infection, cardiotoxicity, nausea, and anemia have been found associated with the long-term therapy [3]. Moreover, as most of the available first-line drugs are either immunoregulators or immunosuppressants, so far there is no known cure for effectively halting neurodegeneration, and promoting remyelination and neuronal repair of the disease, which determine the final recovery of damaged neural system. Therefore, development of novel treatments with less adverse effects targeting not only immune system but also neuroregeneration or neuronal repair perhaps will benefit the therapy of the disease.

Although different mechanisms may result in the demyelination and neurodegeneration in MS, growing evidence indicates that oxidative stress plays the greatest role in the pathogenesis by contributing to myelin and oligodendroglia degeneration that finally leading to neuronal apoptosis [6]. Remarkably elevated oxidants have been found [7–9] in serum or cerebrospinal fluid (CSF) of MS patients as well as that in rodents induced with experimental autoimmune encephalomyelitis (EAE), the animal model of MS [10–11]. The generation of free radicals, mostly by infiltrated monocytes and activated residential microglial cells, leads to a disruption of neuronal membrane integrity by interacting with the lipids, proteins, and nucleic acids and thus results in neuronal damage [12–14]. Additionally, weakened cellular antioxidant defense systems in the central nervous system (CNS) and enhanced vulnerability to oxidative stress effects in MS may increase damage [15]. Therefore, treatment with antioxidants might theoretically prevent against the damages and improve the survival of neuronal tissues in MS.
Astragaloside IV (ASI) is a small molecular saponin found in Astragalus membranaceus (Fisch.) Bge, which is a widely used herb in China. The herb shows anti-oxidation effects by inhibition of free radicals, reduction of lipid peroxidation and elevation of antioxidants enzymes [16]. Diverse pharmacological activities have been found to be exerted by the molecule such as anti-inflammation [17], anti-infection [18], anti-hypertension [19], anti-diabetes [20], myocardial protection and anti-heart failure [21]. In addition, the natural compound has shown anti-oxidative effect in various cells, including human umbilical endothelial cells [22], rat adrenal pheochromocytoma PC12 cells [23], and rat H9C2 myocardiac cells [24]. Moreover, neuroprotective effect of ASI has been found by promoting axonal regeneration and the reconstruction of neuronal synapses [25]. Since oxidative stress is one of the major factors accounts for the pathogenesis of MS, we speculated that ASI, the neuroprotective antioxidant, might contribute to the prevention of MS progression. To testify the hypothesis, in present study C57BL/6 mice induced with EAE were treated with ASI. The results showed that ASI prevented the aggravation of EAE by counteracting oxidative stress and its effects at multiple levels, which suggested ASI may be effective for clinical therapy/prevention of MS.

Materials and Methods

Ethics Statements

All the animal experiments were carried out according to the protocol approved by Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (Protocol # 11051). Subcutaneous injection and intracardial (i.c.) perfusion of mice were conducted after anesthetizing the animals with isoflurane and urethane, respectively, and all efforts were made to minimize suffering.

EAE Induction and ASI Treatment

EAE induction was conducted in 6-week-old female C57BL/6 mice as described previously [26]. In brief, each mouse received subcutaneous injection of 100 μl of complete Freund’s adjuvant containing 300 μg of MOG35-55 and 400 μg of heat-inactivated Mycobacterium tuberculosis H37RA. Pertussis toxin (200 ng/mouse) was given intraperitoneally (i.p.) on the day of immunization and again two days later. Clinical behavior of mice was scored daily according to the criteria used by Madusha Peiris et al [27].

Astragaloside IV treatment (20 mg/kg) was given i.p. daily from the day before MOG35-55 immunization and continued for 2 weeks. Meanwhile, methylprednisolone (MPD) served as positive control drug was administered i.p. at 20 mg/kg dosage consecutively from day 8 to day 10 after MOG35-55 immunization.

Cell Culture and Treatments

Neuroblast clonal line SH-SY5Y obtained from ATCC was maintained in DMEM/F-12 (1:1 v/v) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO2. To examine the anti-oxidative effect of ASI, hydrogen peroxide (H2O2) injured cell model was used. Briefly, cells were seeded at a density of 1×10^5/well into a 6-well plate and pre-treated with 10, 20 and 50 μM ASI for 1 hr. Thereafter, H2O2 was added to the medium to the final concentration of 100 μM. After co-treated for 24 hrs, cells were harvested and lysed for further western-blotting analysis.

Histopathology and Immunohistochemistry (IHC)

Animals were anesthetized with excessive 20% urethane and perfused intracardially with PBS followed by 4% paraformaldehyde. Coronal sections of brains or spinal cords at 20 μm thickness were obtained on a Leica 1950 cryostat. Double staining of Luxol fast blue and cresyl echt violet was used to assess demyelination of CNS. Briefly, sections were stained with Luxol fast blue, washed with 95% ethanol, and then placed in lithium carbonate. Nuclei of the neuronal cells were visualized with cresyl echt violet staining. IHC was performed as described previously [26]. Primary antibodies including anti-GFAP and anti-Iba1 were incubated with sections at 4°C overnight. After thoroughly washed by PBS, the sections were further incubated with Alexa 488 or 594 conjugated secondary antibodies. Fluorescent images were taken by using an inverted fluorescent microscope (Olympus IX 81).

Western Blot

Brain cortices of mice were homogenized in CellLyticTM MT mammalian tissue lysis reagent (Sigma, C3228) supplemented with protease inhibitor cocktail (Sigma, P3840) and phosphatase inhibitor cocktail 2 (Sigma, P5726). Afterwards, the homogenate was centrifuged at 12,000 rpm and 4°C for 10 min. Protein concentration of the supernatants was quantified by BCA method. Thirty microgram of each samples were loaded to a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to FluoroTrans® W PVDF membranes (Pall, 20685) by an electrophoretic transfer system (Bio-Rad). The membranes were blocked with 5% skim milk in PBST for 1 hr and then incubated with respective primary antibodies at 4°C overnight. After thoroughly washed by PBST, the membranes were further incubated with horseradish peroxidase conjugated secondary antibodies and visualized with enhanced chemiluminescence and WB detection reagents (Amersham Biosciences, Piscataway, NJ).

ROS Measurements

In vivo ROS level was assessed by hydroethidine (HE), which is oxidized into dihydroethidine (DHE) by superoxide. The fluorescence of DHE can be measured by a fluorescent reader. Briefly, mice were injected i.p. with 200 μl of 1 mg/ml HE 15 min prior to sacrifice. Then brain cortices of mice were dissected and homogenized at 1:5 ratio (w/v) in CellLyticTM MT mammalian tissue lysis reagent supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail 2. The homogenate was centrifuged at 12,000 rpm and 4°C for 10 min. The fluorescent intensity of resultant supernatants was measured using a Varioskan flash spectral scanning multimode reader (Thermo, excitation 540 nm; emission 595 nm) and normalized to tissue weight.

Intracellular ROS in SH-SY5Y cells was detected by 2′,7′-dichlorodihydrofluorescin (DCFH, Sigma, D6883), which is oxidized into fluorescent dichlorofluorescein (DCF) by ROS. Cells were incubated with 10 μM DCFH for 30 min. The dye was then
removed and replaced with Hanks’ Balanced Salt Solution (HBSS) with Ca\(^{2+}\) & Mg\(^{2+}\). Fluorescence of the cells was observed under inverted fluorescent microscope or measured immediately using a Varioskan flash spectral scanning multimode reader (excitation 485 nm; emission 535 nm).

Evan’s Blue Dye Extravasation

Permeability of brain was examined by Evan’s blue (EB) dye extravasation method. Mice were i.p. injected with 400 µl of 0.8% EB in PBS 2 hr before i.c. perfusion with PBS. Hemisphere of the brains was weighed and homogenized in 1 ml of 50% TCA. After centrifugation at 12,000 rpm for 10 min, the resultant supernatants were collected and the fluorescence was measured at an excitation wavelength of 620 nm and an emission wavelength of 680 nm.

Cytokine Quantification

The concentrations of IFNγ, TNFα, IL6, IL4, and IL17A in brain homogenates or cell culture medium were measured by ELISA kits (eBiosciences, San Diego, CA). Cytokine concentrations in respective samples were determined by standard curves prepared by recombinant cytokines of known concentrations.

Biochemical Analysis

Brain cortices of mice were homogenized in PBS (1:10, w/v) on ice. After centrifuged at 4000 rpm and 4°C for 15 min, supernatants of the homogenates were immediately subjected to kits to analyze the concentration of malondialdehyde (MDA) and activities of total super oxide dismutase (T-SOD) and glutathione peroxidase (GSH-Px) in accordance to manuals of manufacturer (Jiancheng Bioengineering Institute, Nanjing, China).

Quantitative PCR

Total RNA of the hippocampus and spleen was extracted using RNazol according to the manufacturer’s manual (Takara, Dalian, China). After digestion with DNase I to eliminate trace amounts of DNA contamination, total RNA was reverse transcribed into cDNA with kit. Quantitative PCR was conducted by use of Taqman SYBR kit. Concentrations of target genes in the samples were quantified by standard curves generated with template plasmids containing fragments of the respective target genes. Afterwards, they were normalized to that of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) in the same sample. All primers used were listed in table 1.

Statistical Analysis

Statistical analyses were carried out by GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Repeated measures analysis of variance (ANOVA) was performed to determine the effects of treatments between groups in terms of EAE score. Pairwise comparisons among groups were performed by one-way ANOVA followed by Tukey’s posthoc test. All data in the graphs were presented as mean±standard error of the mean.

Results

ASI Reduced Severity of EAE Mice

Generally, onset of EAE disease was 8 days after MOG\(_{35-55}\) immunization and limb defect of mice attained its peak on day 14 post-immunization in mice without treatment (Fig. 1A). Afterwards, the behavioral symptom of mice showed slightly improvement in the following days. When MPD (20 mg/kg) was administered, the developmental episode in the mice was similar to that in EAE group without treatment as the onset of disease was still around 8 days. After induction, the limb defect of animals reached its top score on day 14 post-immunization. However, the extent of severity in MPD treated group mice was remarkably alleviated. ASI treatment at 20 mg/kg dosage from the day before immunization to day 14 post-immunization altered the disease developmental pattern. The compound not only prevented the deterioration of EAE from day 12, but also reduced the average behavioral score of mice. From day 13 post-immunization, behavioral score of ASI treated mice did not deteriorate too much. And the disease in treated mice remitted again from day 20 post-immunization. In addition, the average clinical score of ASI treated mice was even lower than that of MPD treated mice during the incidence of the disease. Compared with EAE mice without treatment, both MPD- and ASI-treated mice showed significantly decreased clinical score during the incidence of disease (p<0.01 and p<0.001, respectively, shown in inset of Fig. 1A). To confirm the results of clinical score, we monitored the body weight of mice weekly. As illustrated in Fig. 1B, mice showed persistent loss of body weight with the progression of EAE if received no treatment. However, ASI treatment prevented against body weight loss of EAE mice, which indicated the preventive effect of the compound.

| Gene name | Primer sequence |
|-----------|----------------|
| GAPDH FP  | ATGGTCCGCTCGTGGATCTGA |
| RP        | ATGGCCTGTCCTCCAGCTTC |
| GFAP RP   | GCCGTCCTTGCTTAGTGATGA |
| GFAP RP   | GCACTGCGAAGAACCTGGGAATG |
| T-bet RP  | GCCGTCCTGCGTTGATGATGA |
| Foxp3 RP  | GGCAACCTGGAAGACTCCATT |

ASI Attenuated Demyelination and Neuroinflammation of EAE Mice

To examine whether ASI treatment could lessen demyelination of CNS, LFB staining was conducted on spinal cord sections. As shown in Fig. 2A–C, compared with control mice, EAE mice displayed the severest demyelination. In contrast, those mice treated with ASI exhibited significantly improved symptom demonstrated by the enhanced LFB staining. Moreover, EAE induction led to much more infiltration of monocytes
into spinal cord, especially in anterior median fissure region, which were stained by cresyl echt violet (insets in Fig. 2A–C). ASI administration inhibited the infiltration of monocytes to the same region.

To assess if ASI could attenuate neuroinflammation indicated by activation of glial cells, we conducted fluorescent IHC by using GFAP and Iba I antibodies to label astrocytes and microglia, respectively (Fig. 2D–I). In dentate gyrus of hippocampus, significantly activated astrocytes as well as microglia were found in EAE mice. Those activated glial cells showed enlarged cell bodies, retracted processes and increased GFAP or Iba I immunoreactivity. However, in ASI treated mice, those cells looked like normal ones in resting condition. Both GFAP and Iba I immunoreactivities were reduced compared with that in EAE mice without any treatment. Although a few number of activated microglia still could be found in ASI treated mice, most of the microglial cells showed ramified shape that suggested less occurrence of neuroinflammation.

ASI Decreased ROS Stress in EAE Mice

As opening of BBB and oxidative stress are known to be involved in the pathogenesis of EAE [28], we firstly analyzed the BBB permeability by measuring infiltrated Evan’s blue dye. In agreement with previous reports, EAE resulted in leakage of BBB even three weeks post-immunization (p<0.05, Fig. 3A). ASI treatment reversed the increased BBB leakage. Sequentially, we estimated in vivo ROS levels in CNS of mice by measuring infiltrated DHE. Compared with the control mice, ROS level in the cortices of EAE mice was much higher (p<0.01, Fig. 3B). As a result, more MDA, the breakdown product of oxidation of...
polyunsaturated fatty acids served as a reliable oxidant marker of oxidative stress-mediated lipid peroxidation [29], were generated in EAE mice \( (p < 0.05, \text{Fig. 3F}) \) as well as iNOS \( (p < 0.001, \text{Fig. 3E}) \). Opposite to increased ROS level, MDA and iNOS production, GSH-Px and total SOD activities in EAE mice were down-regulated \( (p < 0.05, \text{Fig. 3C and D}) \). ASI administration reversed ROS and anti-oxidative enzymes levels in EAE mice as less ROS indicated by infiltrated DHE concentration \( (p < 0.05, \text{Fig. 3A}) \) were found in cortices from ASI treated mice as well as less MDA \( (p = 0.08, \text{Fig. 3B}) \) and iNOS \( (p < 0.05, \text{Fig. 3E}) \) were generated. Furthermore, GSH-Px and total SOD activities in ASI treated mice were significantly elevated compared to that in EAE mice \( (p < 0.05 \text{ and } p < 0.001, \text{respectively, Fig. 3C and D}) \). Moreover, total SOD activity in ASI treated mice was increased even higher than that in control mice in spite of no statistical significance.

ASI Affected mRNA Expressions of Hippocampal GFAP, CD11b but not Sod1 and Gpx1

In consistent with IHC pattern, mRNA expression level of GFAP in hippocampi of EAE mice was significantly elevated \( (p < 0.01, \text{Fig. 4A}) \) as well as that of CD11b, the microglial marker in CNS \( (p < 0.05, \text{Fig. 4B}) \). When treated with ASI, mRNA expression levels of both GFAP and CD11b were down-regulated remarkably \( (p < 0.05, \text{Fig. 4A-B}) \), which indicated the decline of neuroinflammation. Not surprisingly, mRNA expression of Sod1, one of the subtypes of SOD, was reduced in EAE mice \( (p < 0.01, \text{Fig. 4C}) \). But glutathione peroxidase-1 (Gpx1) mRNA was not changed \( (\text{Fig. 4D}) \). To our surprise, ASI treatment had not altered the mRNA expression levels of both Sod1 and Gpx1.

ASI Modulated mRNA Expressions of Splenic T-bet, RORγt, and Foxp3

To investigate if ASI could regulate T cell differentiation, we examined splenic RORγt, T-bet and Foxp3 mRNA expressions using qPCR approach. As illustrated in Fig. 5, EAE caused significant elevation of RORγt gene expression \( (p < 0.01) \). In contrast, T-bet and Foxp3 mRNA expressions were inhibited a little bit \( (\text{Fig. 5 A and C}) \). ASI administration decreased RORγt mRNA expression \( (p < 0.01) \). Meanwhile, ASI enhanced significant increase of T-bet and Foxp3 mRNA levels \( (p < 0.05 \text{ and } p < 0.01, \text{respectively}) \).

ASI Regulated Cytokine Profile of EAE Mice

To assess the effect of ASI on the cytokine expression profile, which was mainly secreted by inflammatory cells, cortices of mice three weeks post-immunization were homogenized and subjected to ELISA assays. To our surprise, concentration of IL17A, the cytokine secreted by infiltrated Th17 cells, was not changed in EAE mice as our predicted \( (\text{Fig. 6}) \). Similarly, IL4, one of the Th2 cytokines, showed no remarkable alteration. But Th1 cytokines, IFNγ and TNFα, were elevated \( (p < 0.05) \). Meanwhile, IL6 was also up-regulated. After ASI treatment, increased expressions of IFNγ, TNFα and IL6 were all down-regulated significantly \( (p < 0.05) \).

ASI Modulated Expression of Apoptotic Proteins in CNS

To evaluate the impact of ASI administration on neuronal damage, expression levels of proteins associated with apoptosis in cortices of mice were analyzed \( (\text{Fig. 7}) \). Not surprisingly, GFAP, the astrocytic marker, was remarkably increased in EAE mice without any further treatment \( (p < 0.01, \text{Fig. 7A and B}) \).
p53, the pro-apoptotic protein, was also robustly up-regulated in EAE mice (p<0.01, Fig. 7A and D). As a result, the phosphorylated tau indicated neuronal damage was found to be elevated significantly in those mice (p<0.05, Fig. 7A and E).

Bax, one of the members of Bcl-2 family, was slightly increased (Fig. 7A). However, on the contrary, Bcl-2 was decreased markedly and its ratio to Bax was down-regulated prominently (p<0.001, Fig. 7A and B). When treated with ASI, neuroinflammation indicated by up-regulated GFAP immunoreactivity was significantly reduced (p<0.05, Fig. 7A and B). Meanwhile, p53 in the CNS of ASI treated mice was decreased remarkably (p<0.01, Fig. 7A and D). Conversely, Bcl-2/Bax ratio in the cortices was elevated compared to that in EAE mice without any treatment (p<0.01, Fig. 7A and C). As a result, phosphorylation of tau in ASI treated mice was attenuated, which suggested less axonal damage (p<0.05, Fig. 7A and E).

ASI Decreased Oxidative Stress in SH-SY5Y Cells and iNOS Expression in BV-2 Cells

To examine if ASI has some direct effects on oxidative stress of neuronal cells, SH-SY5Y cells were incubated with DCFH for 30 min after pre-treated with ASI overnight. As illustrated in Fig. 8A, DCF fluorescence in ASI (50 μM) treated cells were significantly lower than that in the cells without any treatment. Moreover, ASI dose-dependently reduced DCF fluorescent intensity in normal SH-SY5Y cells (Fig. 8B). When subjected to exogenous oxidative stress challenge, for instance, 100 μM H2O2, more phosphorylated tau was found in the cells as well as elevated Bax molecules (Fig. 8C). On the contrary, Bcl-2 was shown to be reduced. After co-treated with 50 μM ASI, down-regulation of Bcl-2 was abolished without simultaneous decrease of Bax. As a result, phosphorylation of tau induced by H2O2 was alleviated. Moreover, when there’s no exogenous oxidative stress, ASI dose-
dependently (10–50 \text{M}) induced Bcl-2 expression which resulted in less phosphorylation of tau. In BV-2 cells, when stimulated with 100 ng/ml of IFN\(_c\), one of the Th1 cytokines, more iNOS proteins were induced as compared to the cells without any treatment (Fig. 8D). ASI treatment dose-dependently prevented against up-regulation of iNOS of BV-2 cells in response to IFN\(_c\) stimulation.

ASI Inhibited Secretion of Th1 and Th17 Cytokines from CD4\(^+\) Cells
Since T helper cells, especially Th1 and Th17 cells, play vital role in the pathogenesis of EAE, we investigated cytokine profile changes of CD4\(^+\) cells after ASI treatment. As shown in Fig. 9A, CD4\(^+\) cells isolated from EAE mice (21 days post-immunization) proliferated much faster than that from control mice (p<0.001). Moreover, levels of pro-inflammatory cytokines secreted by EAE CD4\(^+\) cells including IFN\(_\gamma\), TNF\(_\alpha\) and IL17 were significantly higher compared with that of the control (p<0.001, Fig. 9B-D). ASI treatment (50 \text{M}) did not prevent the proliferation of but suppressed IFN\(_\gamma\), TNF\(_\alpha\) and IL17 secretion from the CD4\(^+\) cells isolated from EAE mice (p<0.01 and p<0.001, respectively, Fig. 9B-D).

Discussion
In current study, ASI administration significantly prevented against aggravation of neuropathology of EAE mice. Further studies disclosed that ASI intervened progression of the disease perhaps via anti-oxidative stress at multi-levels. In EAE mice treated with ASI, production of ROS in CNS was decreased remarkably concurrent with robustly elevated total SOD and GSH-Px activities. Meanwhile, neuroinflammation indicated by activated astrocytes and microglia was significantly alleviated as well as cytokines secreted by the inflammatory cells. As a result, axonal damage indicated by reduction of phosphorylated tau and MDA was attenuated markedly. Further studies conducted in \textit{vivo} exposed that ASI could eliminate intracellular ROS and reduce damage to neuronal cells resulted from exogenous ROS perhaps via regulation of Bcl-2/Bax ratio, especially increase of Bcl-2 expression, and inhibition of iNOS expression in resident microglial cells.

The level of reactive oxygen species (ROS) is known to be enhanced in MS [30], which consequently causes increased permeability of the BBB [31–32]. Similarly, opening of the BBB and oxidative stress are known to be involved in the pathogenesis of EAE, the animal model of MS [28], [33]. In agreement with
previous studies, we observed the leakage of BBB and elevated ROS in CNS of EAE mice (Fig. 3) as well as enhanced demyelination and neuroinflammation (Fig. 2), which confirmed the association between ROS caused opening of BBB and neural damage. When treated with ASI, leakage of BBB and elevation of ROS in EAE mice were suppressed remarkably and almost resumed to normal levels (Fig. 3). As aforementioned, ASI showed anti-oxidative effect in human umbilical endothelial cells [22], whether it has similar influence on brain microvessel endothelial cells, the main component of BBB, has not been disclosed yet. Further studies to investigate if ASI has such an effect on brain microvessel endothelial cells are undergoing in our lab, which may provide a direct evidence of the protective effect of ASI on the integrity of BBB.

T cell infiltration is one of the crucial features of EAE [34], CD4+ T helper cells, including Th1 and Th17, and T regulatory (T-reg) Foxp3+ cells are key players for the EAE pathogenesis and recovery [35]. The differentiation of T cells depends on the transcription factors T-bet (Th1), RORγt (Th17) and Foxp3 (T-reg). The signature cytokines of these different subsets of T cells were modulated by the transcription factors. Th1 cytokines such as IFNγ and TNFα are regulated by T-bet [36], Th17 cytokines IL17, IL22 or IL21 by RORγt, while T-reg cytokines IL10 and TGFβ by Foxp3 [37]. Our findings indicated that ASI could regulate the splenic expression of all of the three mentioned transcription factors at mRNA levels, especially Foxp3 (Fig. 5). In agreement with our findings, ASI has been reported to antagonize the down-regulated expressions of Tregs cell phenotypes caused by high mobility group box 1 protein [38]. Consistent with the qPCR results, western blot result also showed increased CD3+ T cells infiltrated into spinal cord of EAE mice (Fig. S1, p<0.01). As been found to bind to the membranes of all mature T-cells and to be present at all stages of T-cell development, CD3 is a useful marker for T-cells in immunohistochemistry. In spinal cord of the mice treated with ASI, there was less CD3 expression (p<0.05). Moreover, in vitro experiments displayed that ASI treatment suppressed Th1 and Th17 cytokine secretion from CD4+ cells isolated from EAE mice (Fig. 9). Our unpublished data also showed that ASI inhibited Th1 and Th17 cytokine secretion by CD4+ T cell stimulated with MOG. Therefore, our findings suggested that ASI perhaps intervened the differentiation of T cells and thus prevented them from infiltrating into CNS.

As ROS was elevated in the progression of EAE, anti-oxidants such as SOD and GSH-Px were found synchronously to be gradually decreased, especially at early stages of the disease [39]. SOD catalyzes the dismutation of superoxide anion to hydrogen peroxide. For GSH-Px, it reduces lipid hydroperoxides to their corresponding alcohols and free hydrogen peroxide to water. The process is important for the relief of the oxidative damage to the organs, especially nervous system. Reduction of the enzymes causes a variety of toxic effects, including lipid peroxidation indicated by MDA. In consistence with the reports, we observed remarkably decreased SOD and GSH-Px activities in the brains of EAE mice accompanied with elevated MDA concentration. Meanwhile, the mRNA level of SOD1, the major isoenzyme of...
SOD, was up-regulated. After treatment with ASI, the down-regulated SOD and GSH-Px activities were recovered although mRNA levels of both enzymes were not enhanced synchronously, which suggests that ASI may modulate the activities of the enzymes at transcriptional or degradative levels.

The balance of pro- and anti-apoptotic proteins of Bcl-2 family plays an important role in the control of apoptotic cascade of cells. Thus, the ratio of Bcl-2 to Bax perhaps is a better determinant for cell survival than the absolute concentration of either protein alone [40–41]. The pro-apoptotic proteins of Bcl-2 family have been found to participate in neuronal death [42]. In current study, we found increased Bax in both in vivo and in vitro models (Fig. 6 and Fig. 7). When subjected to ASI treatment, the decrease of Bcl-2, the anti-apoptotic protein, was prevented although the level of it did not exceed that in normal condition. In contrast, ASI treatment did not reduce Bax expression in either EAE mice or SH-SY5Y cells challenged with H2O2. Therefore, the increased ratio of Bcl-2/Bax in ASI treated group may be due to the preventive effect of ASI on the degradation of Bcl-2 protein or the up-regulation of mRNA of Bcl-2. Our unpublished data showed that ASI could increase the phosphorylation of Bcl-2 at Ser 70 through MAPK pathway. As phosphorylation of Bcl-2 inhibited its ubiquitination and thereby conferred resistance to cellular apoptosis [43], anti-apoptotic capacity of ASI on neurons perhaps mainly via MAPK mediated Bcl-2 phosphorylation pathway.

p53, the tumor suppressor molecule, is important in inducing DNA repair, cell cycle arrest or apoptosis after genotoxic stress [44]. Endogenous factors, such as NO and its metabolites, can induce p53-dependent neuronal cell apoptosis [45–46], in part by regulation of Bcl-2 and Bax [47–48]. In our work, we observed increased p53 as well as iNOS activity in EAE mice brain (Fig. 6). Meanwhile, Bcl-2 was decreased accompanied with elevated Bax. ASI treatment significantly reversed the process in brain cortices of EAE mice. Therefore, we speculated ASI might also regulate p53 via Bcl-2/Bax pathway, which is still lack of evidence currently.

Tau protein binds to and stabilizes microtubules in a phosphorylation-dependent pattern [49]. Study shows tau is engaged in modulation of anterograde axonal transport by influencing the attachment/detachment rate of molecular motors along microtubules [50]. Hyperphosphorylation of tau results in detachment and accumulated unbound protein initiates its aggregation into toxic paired helical filaments [51]. Abnormally increased phosphorylated tau has been found to be associated with neuronal and axonal loss in EAE and MS [51–52]. In our study,

Figure 7. Effects of astragaloside IV on apoptotic proteins. A, western blots of GFAP, Bcl-2, Bax, p53, p-tau in cortices of EAE mice treated with astragaloside IV. B-E, gray intensity analysis of GFAP, Bcl-2, Bax, p53 and p-tau. All data are presented as mean±standard error of the mean and compared with EAE group. n = 5 for each group. *, p<0.05; **, p<0.01; ***, p<0.001.

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Figure 8. Astragaloside IV reduced oxidative stress in SH-SY5Y cells and decreased iNOS expression in BV-2 cells upon IFNγ stimulation. A, SH-SY5Y treated with ASI (50 μM) for 24 hr reduced cellular DCF fluorescence. B, SH-SY5Y treated with different doses of ASI for 24 hr inhibited cellular DCF fluorescent intensity (n = 5). C, SH-SH5Y cells were pretreated with ASI for 1 hr followed by challenge of H2O2 (100 μM) for 24 hr. D, BV-2 cells were pretreated with ASI for 1 hr followed by stimulation of IFNγ (100 ng/ml) for 24 hr. ASI, astragaloside IV.

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Figure 9. Effects of astragaloside IV on proliferation and cytokine secretion of CD4+ T cells. A, ASI did not prevent proliferation of CD4+ T cells isolated from EAE mice. B, ASI inhibited TNFα secretion from CD4+ T cells isolated from EAE mice. C, ASI reduced IFNγ production of CD4+ T cells isolated from EAE mice. D, ASI decreased IL17 secretion from CD4+ T cells isolated from EAE mice. Control CD4+ T cells were isolated from spleens of normal C57 BL/6 mice. EAE mice used for CD4+ T cell isolation were induced with MOG35–55 for 21 days. ASI, astragaloside IV. All data are presented as mean±standard error of the mean and compared with EAE group. n = 5 for each group. *, p<0.05; ***, p<0.001.

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regulated. In BV-2 cells, ASI dose-dependently reduced IFN-γ treatment, levels of iNOS activity and those cytokines were down-regulated both XW. Wrote the paper: XW ZW. Contributed reagents/materials/analysis tools: YH MD YG HL HW XW. Analyzed the data: MD XW.

**Author Contributions**

Conceived and designed the experiments: YH MD XW ZW. Performed the experiments: YH MD YG HL HW XW. Analyzed the data: MD XW ZW. Contributed reagents/materials/analysis tools: YH MD YG HL HW XW. Wrote the paper: XW ZW.

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