Astragaloside IV inhibits experimental autoimmune encephalomyelitis by modulating the polarization of both microglia/macrophages and astrocytes

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Abstract
Astragaloside IV (AST IV), a major saponin component and active ingredient isolated from Astragalus membranaceus, has been well known to exhibit neuroprotective effects on diverse models of neurological diseases. Accumulating evidence suggests that dynamic balance of microglia/macrophages and astrocytes plays a vital role in neuroprotection and remyelination. However, dysregulation of microglia/macrophages and astrocytes orchestrate the pathogenesis of nervous system disorders. Therefore, we hypothesized that switching the transformation of microglia/macrophages and astrocytes into the neuroprotective M2 and A2 phenotypes, respectively, could be a potential target for therapeutic intervention.

In the present study, we evaluate the efficacy of AST IV intervention on the effects of microglia/macrophages and astrocytes in an experimental autoimmune encephalomyelitis (EAE) model. AST IV improved paralysis and pathology of EAE by inhibiting the neurotoxic M1 microglia/macrophage phenotype, promoting M2 phenotype, shifting astrocytes towards a neuroprotective A2 phenotype, and protecting neurons from apoptosis through inhibition of TLR4/Myd88/NF-κB signalling pathway. Our study showed that AST IV could be a potential and promising drug for multiple sclerosis treatment.

Key words: experimental autoimmune encephalomyelitis (EAE), microglial, macrophages, astrocytes.

Introduction
Multiple sclerosis (MS) is an inflammatory demyelinating disease caused by immune system disorders, genetic susceptibility, and environmental exposures [12], however the exact pathogenesis of the disease is yet unclear. The pathological changes of experimental autoimmune encephalomyelitis (EAE) are characterized by the progressive enlargement of an area of demyelination, accompanied by the infiltration of inflammatory cells, activation of glial cells, and the loss of axons [2,3]. EAE is a commonly used animal model for MS due to similarity in pathological changes.

Microglia and astrocytes play a vital role in neuronal development and neurohomeostasis by regulating the communication among neurons, and participating in the degeneration and regeneration response to injury or disease of the central nervous system (CNS).
The immune glial cells in the CNS are microglia and astrocytes. They maintain neurohomeostasis under physiological conditions by regulating an inflammatory response in a pathological condition [52]. Microglia in the brain show a similar function to macrophages in the periphery as front-line of immune defence in the CNS [14]. Microglia-mediated neuroinflammation has been implicated in neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and MS and share a common pathophysiological mechanism [48].

Microglia exhibit a dual role in neuroinflammation and neuroprotection, depending on the surrounding microenvironment [8]. Microglia have been categorized into neurotoxic M1 and neuroprotective M2 phenotypes [22]. M1 phenotype microglia release pro-inflammatory mediators such as nitric oxide (NO) [22], interleukin (IL)-1β, and tumour necrosis factor α (TNF-α), IL-6 [58], chemokines [42], matrix metalloproteinase 9 (MMP-9) [40], and CD206 [41] causing neurotoxicity and myelin damage. However, M2 phenotype microglia promote release of neurotrophic molecules and anti-inflammatory cytokines, such as insulin-like growth factor-1 (IGF-1), glial cell-derived neurotrophic factor (GDNF), and brain-derived neurotrophic factor (BDNF), CD206, arginase-1 (Arg-1), IL-10, and transforming growth factor (TGF)-β [47,55-57,66]. These molecules promote oligodendrocyte progenitor differentiation, enhanced neuroprotection, and myelin repair. Activated microglia or macrophages produce inflammatory/anti-inflammatory mediators to coordinate neuroinflammation and neuroprotection. Functional phenotypic modulators were used as potential therapies in neurodegenerative diseases due to distinct roles of M1 and M2 microglia/macrophages [49]. Astrocytes are a highly plastic cell type in the brain, adopt diverse morphologies and phenotypes, play a dual role in neurotransmission, signal gradients, and inter-synapse relationships [67,68]. Reactive astrogliosis is a universal response to the CNS injuries and diseases such as trauma, infection, neurodegeneration, and ischemia [5,35,54]. Molecular and functional transformation in subtypes of astrocytes are dependent on the type of injury and/or disease. During neuroinflammation or ischemia, astrocytes are more sensitive to damage. Reactive astrocytes exhibit neuroprotective or neurotoxic responses by releasing diverse immune and pro-/anti-inflammatory cytokines/chemokines [35]. Similar to the activation of microglia, and consistent with the functional significance of beneficial or detrimental effects, reactive astrocytes are also divided into neurotoxic (A1) or neurotrophic (A2) reactive astrocytes. A1s, induced by lipopolysaccharides, are detrimental to synapse and neuron maintenance [26], while A2s exhibit phagocytosis and are beneficial or protective to synapse formation and neuronal survival after injury. A1s secrete neurotoxins including IL-1β, TNF-α, NO, P2X7R and Lcn2 into the injured synapse by upregulating several classical complement cascade components such as complement component 1q (C1q), TNF-α, and IL-1α [5-7,28,36]. While A2s produce diverse neurotrophic factors such as BDNF, VEGF, CNTF, TNFα, and bFGF to repair synapses and protect neurons by regulating IL-1β, IL-6, NFIA, and silencing miR-21 [3,6,7]. Divergent functions of A1/A2 astrocytes offer a potential therapeutic target for the treatment of neurological diseases. Reactive glial cells have been postulated to play numerous important roles in the pathogenesis of EAE. Apoptosis of neurons and oligodendrocytes during EAE has been extensively studied [9]; however, the role of activated glial cells in EAE pathogenesis remains unclear.

Astragalus membranaceus is a traditional Chinese medicinal material. It has been widely used in clinics for the treatment of cardiovascular, cerebrovascular diseases, liver disease, kidney disease, tumours and aging [1,38,44,65]. It has a distinct role with specific formulations or in combination with other drugs. Astragaloside (AST IV) is a monomer component of Astragalus membranaceus. In our previous studies, we have reported that AST IV acts as an anti-inflammatory agent and has a neuroprotective role in microglia and neurons in the co-culture system [63]. AST IV attenuates EAE by counteracting oxidative stress at multiple levels [53], protects blood-brain barrier integrity [24], regulates differentiation and induces apoptosis of activated CD4+ T cells [61], suppresses maturation and function of dendritic cells [60]. Till now, AST IV has not been reported in the treatment of MS. Our study will be an enhancement in the research of AST IV on improving MS treatment, which is one of the long striving goals by the scientists in this area. In the present study, we have evaluated the effect of microglia/macrophages and astrocytes in the EAE mice model, dissected the mechanism of interactions between microglia/macrophages and astrocytes and explored the therapeutic potential of AST IV intervention. Our results provide a novel treatment strategy for EAE by targeting the phenotypic modulation of microglia/macrophages and astrocytes phenotype.

Material and methods

Animals

8-10-week-old female C57BL/6 mice (18-20 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All animal procedures of this study were approved by the ethics committee of the Shanxi Datong University, Datong, China (2021024). All animal protocols were performed in accordance
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with the International Council for Laboratory Animal Science guidelines.

**EAE mice model development and symptom evaluation**

Five milligrams of myelin oligodendrocyte glycoprotein peptide$_{35-55}$ (MOG$_{35-55}$, CL, Bio-Scientific Company, Xian, China; aminophenol sequence MEVGWYRSPFS-RVVHLYRNGK, purity > 95%) were dissolved in 1 ml saline and 6 mg mycobacterium tuberculosis H37Ra (TB, 20120216, BD Difco, USA) was dissolved in 1 ml complete Freund’s adjuvant (F5881, Sigma, St. Louis, MO, USA) containing 1 mg TB. These two solutions were mixed at equal volumes into an oil-in-water suspension by a needle-tube mixer. The milky white suspension was injected subcutaneously with MOG$_{35-55}$ Freund’s complete adjuvant at a dose of 0.1 ml/mouse. Pertussis toxin (PTX, 350 ng/mouse, BML-G100, Enzo Life Sciences, Farmingdale, NY, USA) was injected intra-peritoneally after immunization and repeated after 48 hours. Mice were screened and randomly assigned into 3 groups (n = 8/group): control group (saline group, NS group), model group (EAE + NS group), and treatment group (EAE + AST IV group). In the EAE + AST IV group, the EAE + AST IV (200 mg/(ml·kg)) was intrastratically injected on day 3 and continued until day 27 post-injection. In the NS group, mice were injected with the same volume of saline. Mice were weighed on alternate day and scored for clinical symptoms. The clinical scores of mice were evaluated by using an international 5 points scoring system: 0 – healthy, 1 – tail tension disappeared or slight gait awkwardness, 2 – ataxia or paresis of hind limbs, 3 – paralysis of hind limbs or paresis of forelimbs, 4 – bilateral posterior paralysis with forelimb paralysis, 5 – on the brink of death or death. Symptoms between the two criteria were measured as ±0.5 points [2].

**Immunocytochemistry and immunofluorescence staining**

Mice were sacrificed on post-injection day 28, and perfused with saline followed by 4% buffered paraformaldehyde. Spinal cords (lower thoracic lumbar) were perfused with saline followed by 4% buffered paraformaldehyde (Sakura Finetek, Zoeterwoude, Netherlands), frozen in a liquid nitrogen atmosphere, and sectioned at 10 μm thickness.

Pathological changes were detected by haematoxylin and eosin (H&E) and luxol fast blue staining. For immunofluorescence staining, sections were washed with 0.01 M phosphate-buffered saline (PBS), blocked in 1% bovine serum albumin for 1 h, permeabilized in 400 μl of 0.3% Triton X-100/1% bovine serum, and incubated overnight at 4°C with primary antibodies against anti-Iba-1 (1 : 1000, ab178846, Abcam, USA), anti-Arg-1 (1 : 1000, 2118S, Cell Signaling), anti-INOS (1 : 1000, ADI-905-431-1, Enzo Life Sciences, USA), anti-CD4 (1 : 500, ab183685, Abcam, USA), anti-GFAP (1 : 1000, ab7260, Abcam, USA), anti-C3 (1 : 1000, ab181147, Abcam, USA), anti-S100A10 (1 : 1000, ab76472, Abcam, USA), anti-BDNF (1 : 1000, ab108319, Abcam, USA), or anti-NeuN (1 : 1000, ab104224, Abcam, USA). Next day, sections were washed with PBS and incubated with a corresponding fluorescein-labelled secondary antibody for 2 h in the dark at room temperature. Sections were cover-slipped with an anti-fluorescence quench sealing solution and were counted under the fluorescent microscope at wavelengths of 450, 488 or 594 nm using Image-Pro Plus 6.0 software.

**Western blotting**

Spinal cords were homogenized with an ultrasonic processor using RIPA lysis buffer (Beyotime Institute of Biotechnology, PR China) supplemented with protease inhibitors. Protein concentration was measured by BCA (Beyotime). All protein extracts (30 μg) were separated on 8-12% SDS-polyacrylamide gels and transferred onto a PVDF membrane (Immobilon-P, Millipore). After blocking with 5% milk at room temperature for 2 h, membranes were incubated at 4°C overnight with anti-MybD88 (1 : 1000, ab2064, Lot GR203889-1, Abcam, USA), anti-TLR4 (1 : 1000, 2219S, Cell Signaling Technology, USA), anti-NF-κB (1 : 1000, 8242, Cell Signaling), anti-GAPDH (1 : 1000, 2118S, Cell Signaling), anti-IL-1β (1 : 1000, ab254360, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), anti-iNOS (1 : 1000, ab275330, Abcam, USA), anti-TNF-α (1 : 1000, ab6671, Abcam, USA), anti-IL-10 (1 : 1000, ab275330, Abcam, USA), anti-CD4 (1 : 500, ab183685, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), anti-GFAP (1 : 1000, 12389/34001, Cell Signaling), anti-C3 (1 : 1000, ab181147, Abcam, USA), anti-S100A10 (1 : 1000, ab76472, Abcam, USA), anti-BDNF (1 : 1000, ab226843, Abcam, USA), anti-GDNF (1 : 1000, ab11887, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), anti-iNOS (1 : 1000, ab275330, Abcam, USA), anti-CD4 (1 : 500, ab183685, Abcam, USA), anti-NF-κB (1 : 1000, ab6671, Abcam, USA), anti-IL-1β (1 : 1000, ab2064, Lot GR203889-1, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), anti-GFAP (1 : 1000, 12389/34001, Cell Signaling), anti-C3 (1 : 1000, ab181147, Abcam, USA), anti-S100A10 (1 : 1000, ab76472, Abcam, USA), anti-BDNF (1 : 1000, ab226843, Abcam, USA), anti-GDNF (1 : 1000, ab11887, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), and anti-NeuN (1 : 1000, ab104224, Abcam, USA). The following day, membranes were incubated with HRP-conjugated secondary antibody for 2 h in the dark at room temperature. Sections were washed with 0.1 M glycine/0.1 M Tris buffer and incubated with a corresponding fluorescein-labelled secondary antibody for 2 h in the dark at room temperature. Sections were cover-slipped with an anti-fluorescence quench sealing solution and were counted under the fluorescent microscope at wavelengths of 450, 488 or 594 nm using Image-Pro Plus 6.0 software.

**Cytokine ELISA assay**

Suspensions of splenic mononuclear cells (MNCs) with MOG$_{35-55}$ at the concentration of 10 mg/ml were incubated at 37°C for 48 h. Supernatants were assayed by ELISA for IL-1β, TNF-α, IL-10 (900-K47, 900-K54, 900-
The incidence of symptoms in the EAE + NS group was 100% and onset of EAE symptoms was day 10 post-injection and average maximum score was 2.81 ±0.70 at the symptom peak on day 20. In AST IV treated mice, symptom onset was delayed by 2 days (day 12), and the mean maximum score was decreased significantly to 1.81 ±0.70 (Fig. 1A). Average body weight of each group was compared on day 10-28. Body weight was significantly increased in the EAE + AST IV group (19.91 ±0.89 g) as compared with the EAE group (18.61 ±0.62 g) (p < 0.05) (Fig. 1B). These data indicate that AST IV treatment delayed the onset of the disease, decreased the maximum clinical score, and increased animal weight suggesting the amelioration of EAE disease severity by AST IV.

AST IV inhibited inflammatory response during EAE

Pathogenesis of EAE is mainly caused by infiltration of inflammatory lymphocytes and macrophages into the CNS [11]. To evaluate the pathology of the CNS inflammation, demyelination, and CD4+ T cell activation; HE, LFB, and CD4+ T cell immunofluorescence staining were performed on spinal cord section. Increased inflammatory cell infiltration, myelin loss in spinal cords, and extensive autoimmune CD4+ T cell activation was observed in EAE mice as compared to saline controls (p < 0.01), however, AST IV treatment significantly decreased the extent of inflammation (p < 0.05), demyelination (p < 0.05), and activated CD4+ T cell (p < 0.01) (Fig. 2). These findings suggest that AST IV ameliorates EAE pathology by reducing demyelination, inhibiting inflammation, and reducing autoimmune CD4+ T activated cells in spinal cords.

AST IV shifted macrophages from M1 to M2 phenotype in spleen

Markers of M1 (CD16/32, IL-12, CD11c, CD40) [11,22,40-42,58] and M2 (i.e., CD206, IL-10) [45,47,55-57,67] were selected to assess the polarization of macrophages using flow cytometry. Significant reduction in M1 markers (CD16/32, IL-12, CD11c and CD40) (p < 0.01) and increased M2 markers (CD206 and IL-10) (p < 0.01) were observed in AST IV treated mice as compared to EAE controls (Fig. 3). Our results suggest a polarization of macrophages from M1 to M2 phenotype in EAE mice on treatment with AST IV.

AST IV shifted microglia from M1 to M2 phenotype in the spinal cord

Experimental autoimmune encephalomyelitis is characterized by demyelination with microglia-medi-
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ated neuroinflammation aggravating demyelinating lesions [60]. EAE mice showed significantly increased accumulation of Iba-1+ microglia in spinal cords as compared to the NS group (Fig. 4A), suggesting that myelin damage may trigger the migration and enrichment of microglia. In contrast, AST IV treatment significantly decreased the expression of Iba-1+ in spinal cords. Microglia are referred as a double-edged sword due to their neurotoxic and neurotrophic effects. Evaluation of microglia within brain micro-environments in EAE mice will be worth to understanding the pathophysiology [22]. TNF-α, iNOS and IL-1β are specific markers for M1, while Arg-1 is a specific marker for M2 microglia. The expression of iNOS, TNF-α and IL-1β and Arg-1 in spinal cords were detected by western blotting. AST IV treatment significantly decreased the abundance of TNF-α, IL-1β and iNOS in the spinal cord (p < 0.05) and increased the expression of Arg-1 (p < 0.05) as compared to EAE mice (Fig. 4C). Further, we observed the expression of iNOS and Arg-1 on activated Iba-1+ microglia in spinal cords by double-label immunohistochemistry. Figure 4A shows that AST IV markedly decreased Iba-1+INOS+ expression (p < 0.05), and increased Iba-1+Arg-1+ expression (Fig. 4B, p < 0.05). Taken together, these results indicate that AST IV transform M1 microglia to M2 phenotype, and these results are consistent with the results of flow cytometry analysis of splenocytes as described in Figure 3.

AST IV shifted astrocytes from A1 to A2 phenotype in the spinal cord

Reactive astrocytes are known to promote EAE progression [18]. EAE mice exhibit migration and accumulation of astrocytes in spinal cords and decreased by AST IV treatment as compared to saline control mice (Fig. 5A). These results showed that EAE induced the migration and activation of astrocytes, while AST IV treatment effectively inhibits astrocyte activation. Reactive astrocytes participate in neuroinflammation by releasing immune inflammatory/anti-inflammatory mediators and perform either a neurotoxic (A1 type) or a neuroprotective (A2 type) role within the CNS.
Complement component 3 (C3) is a specific marker for A1, while S100 calcium-binding protein A10 (S100A10) is a specific marker for A2 astrocytes. The expression of C3 and S100A10 in spinal cord extracts were detected by western blot. Compared to control EAE mice, the expression of GFAP and C3 was significantly decreased in AST IV mice (Fig. 5C, \( p < 0.01 \)). In contrast, expression of the A2 marker S100A10 was increased in AST IV-treated mice (Fig. 5C, \( p < 0.05 \)). We counted the expression of C3 and S100A10 on activated astrocytes in spinal cords by double-label immunohistochemistry. The results showed that AST IV treatment decreased the double positive GFAP+C3+ population (Fig. 5A, \( p < 0.05 \)), and increased the GFAP+S100A10+ population (Fig. 5B, \( p < 0.05 \)), indicating that AST IV promotes a shift of astrocytes from the A1 to the A2 phenotype.

AST IV enhanced the secretion of neurotrophic factors by astrocytes

Studies suggest that astrocytes release neurotrophic factors to promote myelin repair [6]. Nutrient factors promote nerve cell development, prevent the death of adult neurons after injury, promote the repair of neurons and axonal regeneration, and regulate synaptic plasticity. To determine whether AST IV treatment induces astrocytes to produce nutrient factors, double-label immunohistochemistry was used to detect the expression of BDNF in GFAP+ astrocytes. We observed that AST IV treatment effectively stimulated astrocytes to up-regulate the expression of BDNF in GFAP+ spinal cord astrocytes as compared to EAE mice (Fig. 6A, \( p < 0.05 \)). Similarly, compared to EAE mice, the abundance of both BDNF and GDNF was significantly \( (p < 0.01) \) increased in spinal cord extracts as shown by western blot (Fig. 6B).
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CD16/32+ (%)  CD206+ (%)  IL-10+ (%)  IL-12+ (%)  CD40+ (%)
15  12  9  6  3  0  5  4  3  2  1  0

CD40+ positive

NS  EAE  EAE + AST IV
20%  15%  10%  5%  1%  0%

Fig. 3. Astragaloside IV shifted M1 to M2 phenotype of macrophage. Mice were sacrificed on day 28 p.i. and splenic MNCs were prepared for staining with macrophage marker CD11b and M1/M2 markers, analysed by flow cytometry. Scatterplot, histogram, quantitative results were analysed for 4 mice in each group. *p < 0.05, **p < 0.01.
We conclude that AST IV enhances the secretion of neurotrophic factors BDNF and GDNF by astrocytes.

**AST IV exhibited a neuroprotective effect on neurons**

To investigate whether AST IV treatment protects neurons from damage, we examined the neuron marker NeuN by immunohistochemistry and western blot. Our results showed a significantly decreased expression of NeuN in EAE mice (Fig. 7B, \( p < 0.05 \)), whereas AST IV treatment increased the expression of NeuN (Fig. 7B, \( p < 0.05 \)) as compared to control mice. Further, AST IV decreased the expression of c-caspase3 in western blot (Fig. 7B, \( p < 0.05 \)), suggesting that AST IV protects neurons from apoptosis.
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**Fig. 4.** Cont. B) Iba-1+ (green)/Arg-1+ (red). C) The expression of TNF-α, IL-1β, iNOS, Arg-1 was also determined by western blot and quantitatively analysed by image lab 4.0. Data represent mean ±SEM (n = 4 each group). *p < 0.05, **p < 0.01.
AST IV suppresses TLR4/Myd88/NF-κB signalling pathway

TLR4/Myd88/NF-κB signalling pathway is a classical inflammatory signalling pathway [39]. The expression of TLR4, Myd88, NF-κB in the spinal cords of our experimental groups were detected by western blotting. As shown in Figure 8, mice with EAE exhibited a significantly increased expression of Myd88, TLR4, NF-κB as compared to the NS group (Fig. 8A, p < 0.01, p < 0.05, p < 0.01, respectively), while AST IV treatment significantly reduced the expression of Myd88, TLR-4 and NF-κB (Fig. 8A, p < 0.01, p < 0.05, p < 0.05, respectively).

The levels of pro-inflammatory cytokines in the media from cultured splenocytes were measured by ELISA. Compared to EAE mice, the levels of IL-1β and TNF-α were significantly decreased in AST IV-treated
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Fig. 5. Cont. C) The expression of GFAP, C3, S100A10 was also determined by western blot and quantitatively analysed by image lab 4.0. Data represent mean ±SEM (n = 4 each group). * p < 0.05, ** p < 0.01.

Discussion

Astragalus membranaceus is a traditional Chinese medicinal herb, with a wide range of clinical applications for various diseases. The major ingredient of Astragalus membranaceus are calycosin, 3-hydroxy-9-dimethoxy silane rosewood and astragalus saponins. AST IV is one of the active saponins. Recent studies have shown the neuroprotective effect of AST IV in various neurological disorders including cerebral ischemia, Parkinson’s disease, Alzheimer’s disease, and autoimmune encephalomyelitis. It improves motor deficits and/or neurochemical activity, especially antioxidant systems, by reducing inflammation and oxidative stress [4]. AST IV has multifaceted functions in the CNS such as protection against dopaminergic neurons [66], maintenance of the blood-brain barrier [24], suppression of inflammatory response, reduction of oxidative stress [53]. In our previous study, we reported the beneficial effect of AST IV treatment on regulation of M1/M2 phenotype in BV2 cells and inflammatory microenvironment and protection of neurons in vitro [63]. Nevertheless, the effect of AST IV on microglia/macrophage-astrocyte crosstalk and astrocytic polarization in EAE mice remains poorly understood.

In MS/EAE pathogenesis, activated CD4+ T cells in the peripheral immune system cross the blood-brain barrier, infiltrate into the CNS [13,19], and produce pro-inflammatory mediators, trigger immunologic cascades, activate glial cells, amplify the inflammatory response, resulting in oligodendrocyte and neuronal death either directly, or indirectly [37,43]. In our study, AST IV treatment in MOG33-55 induced EAE improved clinical symptoms and body weight gain suggests inhibition of activated CD4+ T cells, suppression of inflammatory cell infiltration into the CNS, reduction of demyelination of the area, and alleviated inflammatory response.

Macrophages/microglia have diverse and complex phenotypes in response to immune regulation, cytotoxicity, and injury repair in the CNS. These phenotypic
changes are dependent on the type of microenvironments. iNOS, a hallmark of M1 is expressed in macrophages/microglia, and increased expression results in M1 polarization of microglia that activate a proinflammatory response. This is accomplished by producing a large amount of ROS and RNS (using arginine to produce NO), as well as releasing cytokines IL-1β and TNF-α, leading to an inflammatory cascade and exacerbation of EAE/MS progression [28,69]. Microglia/macrophages act as antigen presenting cells (APC), trigger an antigen-specific T cell response in the periphery and CNS and orchestrate the effector cells to mediate immune responses in EAE [28]. Microglia/macrophages play a dual role during the disease process, specifically, M1 phenotype microglia/macrophages induce inflammatory cascades and promote disease progression through release of IL-1β, IL-6 and TNF-α, promoting immune-derived signalling in the brain and transmit neuroinflammatory signals to peripheral immune cells by inducing chemokine production [11]. M2 phenotype blocks inflammatory responses and promotes tissue regeneration through production of Arg-1. Further M2 macrophages express CD206 and IL-10 which promotes neurogenesis, axonal remodelling, angiogenesis, oligodendrogenesis, and remyelination [11,21,45,50,57].

In our study, flow cytometry indicated results showed that AST IV treatment shifted M1 into M2 macrophages within the peripheral immune system, and transformed

**Fig. 6.** AST IV promoted the production of neurotrophic factors GDNF and BDNF. Spinal cord was harvested from mice, examined for GFAP/BDNF/GDNF expression. A) GFAP (green)/BDNF (red) were detected by immunofluorescence staining and then quantitatively analysed. The overlay (yellow) was performed to detect GFAP+/BDNF+ colocalization, and quantitatively analysed by image-pro plus 6.0. B) Expression of neurotrophic factors BDNF and GDNF was also determined by western blot and quantitatively analysed. mean ±SEM (n = 4 each group). *p < 0.05, **p < 0.01.
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Astrocytes are involved in immune and inflammatory responses and contribute to neurological diseases. During astrocyte activation, their morphology swells and the expression of GFAP increases. Activated astrocytes with the polarized A1/A2 phenotype play a neuroinflammatory or neurodegenerative role. A1 subtype astrocytes release ROS, IL-1β, TNF-α [31], IL-17A, IL-22, MIP-1, C3, and other inflammatory cytokines [23]. These inflammatory cytokines damage neurons and oligodendrocytes [27]. A2 subtype astrocytes release growth factors such as NGF, BDNF, VEGF, and anti-inflammatory cytokines to protect neurons, increase neuronal survival, growth, and synaptogenesis [34].

In MS and EAE, activated astrocytes with elevated GFAP expression are observed throughout the CNS. Activation of reactive astrocytes and loss of A1/A2 homeostasis are important indicators of disease progression [3]. In our study, we observed activation of both microglia and astrocytes. EAE mice showed an increased number of Iba-1+ and GFAP+ cells, and an overall increase in Iba-1 and GFAP levels. These events are key to the entry of peripheral immune cells into the CNS. AST IV inhibited the activation of microglia and astrocytes, and the recruitment of immune cells from the periphery into the CNS, thereby suppressing inflammation and reducing cytokines IL-1β and TNF-α while increasing IL-10. The expression of A1 astrocytes identified by C3 was upregulated, while the expression of A2 astrocytes identified by S100A10 was downregulated in double-label immunohistochemistry and western blotting in EAE mice. AST IV treatment inhibited the increased A1 markers and promoted the expression of A2 markers.

**Fig. 7.** Astragaloside IV inhibited the apoptosis of neurons and protected neurons. A) Spinal cord was harvested from mice, NeuN expression (red) was detected by immunofluorescence staining. B) Expression of NeuN and c-caspase3 was also determined by western blot and quantitatively analysed by image lab 4.0. Data represent mean ±SEM (n = 4 each group). *p < 0.05, **p < 0.01.
of A2 astrocytes, indicating that AST IV triggered the transformation of astrocytes from A1 to A2.

Crosstalk between microglia and astrocytes is well known, where microglia modulate astrocyte phenotype and function [20]. A1 astrocytes are observed in close association with CD68+ activated microglia/macrophages, which are believed to be potent inducers of A1 astroglial phenotype [29]. On the other hand, activated astrocytes produce mediators to regulate microglial response for immunity [31]. Microglia and astrocytes are both contributors to neurological disease in response to the peripheral immune challenge. The imbalance of M1/M2 macrophages/microglia or A1/A2 astrocytes is a key factor in inflammation severity and predominantly observed in neurological diseases [15,62]. M1 macrophages/microglia or A1 astrocytes promote ongoing severe EAE, amplified immune response within lesions, and promote neurotoxicity and loss of neurotrophic functions. Thus, induction of M2-polarized microglia/macrophages polarization and transformation to neuroprotective A2 phenotype astrocytes provides a novel treatment strategy for MS [9,17]. A2 astrocytes secrete the neurotrophic factors BDNF and GDNF to protect neurons and promote the formation and differentiation of oligodendrocytes in demyelinated regions [27,34].

Our results indicate that AST IV transformed astrocytes to A2 phenotype, which secreted neurotrophic factors BDNF and GDNF to inhibit apoptosis of neurons and promote remyelination.

In the compromised immune system or nervous system tissue damaged, guard cells such as microglia and astrocytes, produce a wide variety of pro-inflammatory cytokines and chemokines by activation of several signalling pathways. TLR immune responses have been found in astrocytes, microglia and other brain cells, and TLRs can specifically bind to multiligand to
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**Fig. 9.** Schematic diagram of the hypothesized signalling pathways summarizing the therapeutic effects of AST IV against experimental autoimmune encephalomyelitis (EAE). AST IV exhibited strong beneficial effects which may be mainly due to effectively suppressing macrophage/microglia/astrocytes inflammatory response via the TLR4/Myd88/NF-κB signalling pathway, subsequently increases neurotrophic factors derived from astrocytes to protect neurons against apoptotic cell death.

TLR4 predominantly binds lipopolysaccharides (LPS) from gram negative bacteria. TLRs can recognize both endogenous and exogenous ligands. These ligands are isolated from macromolecular precursors in the extracellular matrix during tissue injury and/or proteolysis, and released fragments bind to TLR receptors to initiate intracellular signal transduction cascades [32,46].

Activated TLRs will recruit junction proteins containing TIR domains, inducing MyD88, TIRAP, TRIF, and TRAM [33]. Upon receptor activation together with MyD88, one or more TIR containing adaptor proteins are recruited along with IRAK kinases [30]. The IRAK kinases become activated/phosphorylated, dissociated from the adaptor protein, and interact with TRAF6. TRAF6 activate TAK1 which together with TAB adaptor proteins activates two downstream signalling pathways such as NF-κB (NF-κB kinases IKKα, β) and MAPK (ERK, JNK, p38 kinases) [10,49,51,59]. TLR4/MyD88/NF-κB signalling pathway is majorly affected due to immune inflammatory response. Activated TLRs recruit and transduce MyD88 protein to activate NF-κB. These signalling pathways play critical roles in neuroinflammation and EAE pathogenesis. Production of pro-inflammatory cytokines by microglia/macrophage and astrocytes releases inflammatory mediators. TLRs are chiefly expressed in microglia and are expressed to a lesser extent in astrocytes and neurons [25]. However, activation and overexpression of TLR transform astrocytes into the inflammatory A1 phenotype [25]. It has been reported that expression of A1 markers in astrocytes, and TLR4/MyD88 co-expression occurs in the injured but not a normal spinal cord. Antagonizing MyD88 in astrocytes significantly protects M1 microglia/macrophages from death in vitro, suggesting that reactive astrocytes undergo M1 microglia/macrophages-induced necroptosis, partially through TLR/MyD88 signalling [9]. Our results also indicated that AST IV suppressed the TLR4/Myd88/NF-κB signalling pathway, modulated the overexpression of inflammatory mediators, regulated the dynamic balance of macrophages/microglia and astrocytes, as shown in Figure 9.

In summary, AST IV ameliorated MOG33-55 induced inflammatory injury by modulating microglia/macrophage and astrocyte polarization towards the M2 and A2 phenotype, respectively, via the TLR4/Myd88/NF-κB signalling pathway. It protected neurons from apoptosis and promoted remyelination. Results of EAE mice show that the traditional Chinese medicine monomer AST IV could be a potential therapeutic candidate for MS/EAE through the following mechanisms of action: (i) inhibition of macrophage/microglia inflammatory response, induction of macrophages/microglia anti-inflammatory polarization, and prevention of excessive neuroinflammation; (ii) transforming neurotoxic A1 astrocytes into...
the neuroprotective A2 phenotype via TLR4/Myd88/NF-κB signalling pathway and (iii) increase in neurotrophic factors derived from astrocytes to protect neurons against apoptotic cell death.

**Funding**

This study was supported by the National Natural Science Foundation of China (81473577, 82004028), Basic Research Program of Shanxi Province (20210302123337, 20210302123476, 20210302123478), Shanxi Scholarship Council of China (HGKY2019089), Shanxi Province Collaborative Innovation Research Center of astra galus resources industrialization and internationalization (HGXTCXZX2016-022), Applied Basic Research Project of Datong (2020145), China Postdoctoral Science Foundation (2020M680912), International Key Research & Development Cooperation Plan of Datong (2019123), Young Scientists Cultivation Project of Shanxi University of Chinese Medicine (2021PYQN-09), and Leading Team of Medical Science and Technology, Shanxi Province (2020TD05).

**Disclosure**

The authors report no conflict of interest.

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