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

Jingwen Yu[1], Bingtao Mu[1], Minfang Guo[1], Chunyun Liu[1], Tao Meng[1], Yuqing Yan[1], Lijuan Song[2], Jiezhong Yu[2], Gajendra Kumar[3], Cungen Ma[2]

[1]Institute of Brain Science, Shanxi Key Laboratory of Inflammatory Neurodegenerative Diseases, Shanxi Datong University, China, [2]The Key Research Laboratory of Benefiting Qi for Acting Blood Circulation Method to Treat Multiple Sclerosis of State Administration of Traditional Chinese Medicine, Research Center of Neurobiology, Shanxi University of Chinese Medicine, China, [3]Department of Neuroscience, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong

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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)

Communicating author:
Cungen Ma, The Key Research Laboratory of Benefiting Qi for Acting Blood Circulation Method to Treat Multiple Sclerosis of State Administration of Traditional Chinese Medicine, Research Center of Neurobiology, Shanxi University of Chinese Medicine, China, e-mail: macungen2001@163.com

[16,50]. 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, TM4SF1, 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 [1,38,44,64,65]. It has a distinct role with specified 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

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 MEVGWYRSPFSRVVHLYRNGK, 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 intraperitoneally 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, AST IV (200 mg/(ml·kg)) was intragastrically 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 embedded in tissue-tek OCT compound (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 fluorescence 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-Myd88 (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-TNF-α (1 : 1000, ab6671, Abcam, USA), anti-iNOS (1 : 1000, ab275330, Abcam, USA), anti-Arg-1 (1 : 1000, ab91279, Abcam, USA), anti-GFAP (1 : 1000, 12389/34001, Cell Signaling), anti-C3 (1 : 1000, ab11887, Abcam, USA), anti-S100A10 (1 : 1000, ab76472, Abcam, USA), anti-BDNF (1 : 1000, ab226843, Abcam, USA), anti-GDNF (1 : 1000, ab18956, Abcam, USA), and anti-NeuN, anti-cleave-caspase3 (c-caspase3, 1 : 500, ab44976, Abcam, USA). The following day, membranes were incubated with HRP-conjugated secondary antibodies (1 : 10,000, Earthox LLC, USA) for 2 h at room temperature followed by chemiluminescence substrate (ECL, GE Healthcare Life Sciences, USA). Immunoblots were imaged and band intensity was measured by Quantity Software (Bio-Rad, Hercules, CA, USA) to quantify relative protein abundance. GAPDH was used as the internal standard.

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-

K53, respectively; PeproTech Inc., USA). The operation of the experiment follows the manufacturer's instructions. The results were repeated at least three times in each group and cytokine concentration expressed in pg/ml.

Flow cytometry analysis

Mice was sacrificed on post-injection day 28. Spleens were harvested, placed on ice immediately and transferred in medium, to a 40 µm nylon sieve under aseptic conditions. Sterile double distilled water was added to the suspension to lyse erythrocytes osmotically and subsequently 2.7% NaCl was added to restore isotonic conditions.

Suspensions of MNCs were formed after centrifugation and resuspension, and finally adjusted to 5×10^6 cells/ml. MNCs were stained for 20 min at room temperature in 1% BSA-PBS buffer with antibodies to PE-CD16/32 (553145, BD Bioscience, USA), PE-CD206 (12-2061-80, Invitrogen, USA), PE-CD8a (557668, BD Bioscience, USA), PE-CD11c (45-0114-80, eBioscience), and PE-CD40 (12-040-81, eBioscience, USA). For intracellular marker staining, MNCs were incubated for 20 min at room temperature in 0.3% saponin in 1% BSA-PBS buffer with antibodies to PE-IL-10 (12-7101-81, Invitrogen) and PE-IL-12 (12-7123-81, Invitrogen, USA). Cells were gated using forward and sideward scatter characteristics for monocytes and at least 10,000 gated events were collected using flow cytometer (BD Biosciences, USA). Data were analysed using CellQuest software.

Statistical analyses

The data were statistically analysed with GraphPad Prism5 software. Data are presented as mean ±SEM. For clinical mean score and weight, two-way ANOVA test was performed. One-way ANOVA and *t*-test were performed for clinical mean score and body weight of each day in the three groups. One-way ANOVA was used to analyse multiple group comparison, and *t*-test was used to analyse the difference between any two groups. The NS group was used as a control group with no symptoms. Statistical differences between the NS group and the other two groups were not analysed. A statistically significant difference was assumed at $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$).

Results

AST IV delayed onset of clinical symptoms and improved the weight of EAE mouse model

The EAE model of C57BL/6 mice was established by injecting mice with the MOG_{35-55} polypeptide. The clinical score and body weight are shown in Figure 1A. 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-

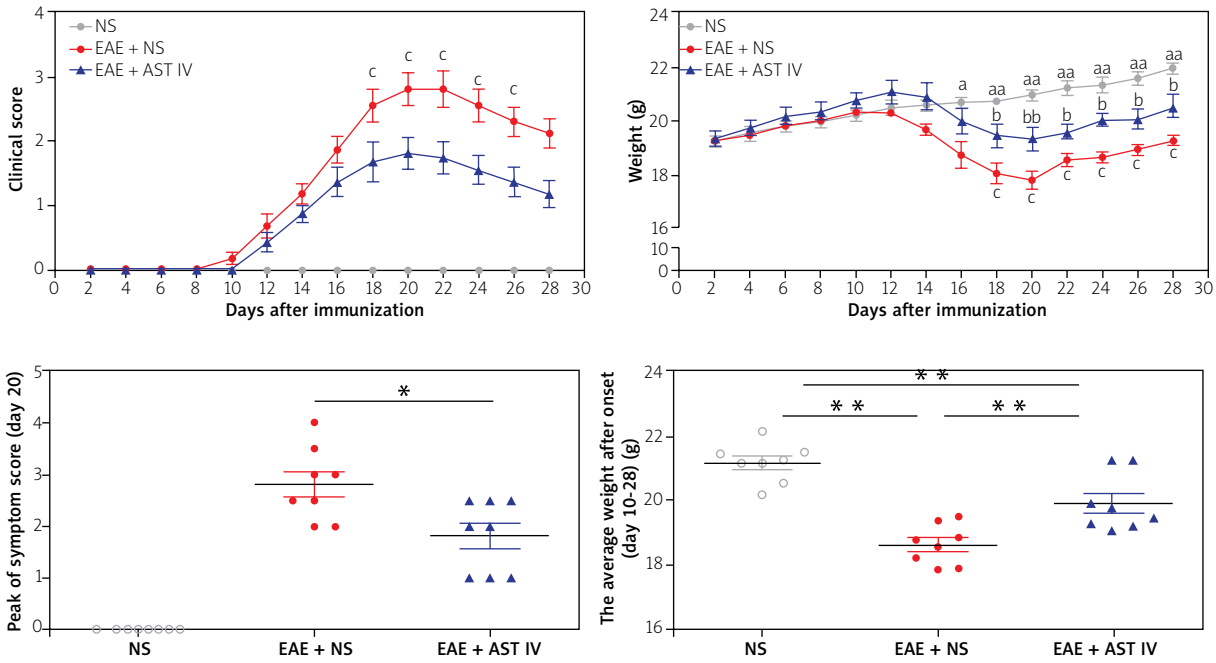

Fig. 1. Astragaloside IV inhibited the severity of experimental autoimmune encephalomyelitis (EAE). Chronic EAE immunized with MOG_{35-55} in C57BL/6 mice (18-20 g). Astragaloside IV was injected intragastrically at 800 µg/d from day 3 p.i. to day 27 p.i. as the AST IV group (EAE + AST IV, $n = 8$). Normal saline was injected to EAE mice as the model group (EAE + NS, $n = 8$) in a similar manner. Normal saline was injected to wild mice as the control group (NS, $n = 8$). **A**) Mean clinical symptom score and mean body weight. Data represent mean ±SEM ($n = 8$ each group). The NS group compared to the EAE + NS group: $^{a}p < 0.05$, $^{aa}p < 0.01$. The NS group compared to the EAE + AST IV group: $^{b}p < 0.05$, $^{bb}p < 0.01$. The EAE + NS group compared to the EAE + AST IV group: $^{c}p < 0.05$, $^{cc}p < 0.01$. Data represent mean ±SEM. **B**) The scatter diagrams about the peak of symptom score and the average weight after onset. $^{*}p < 0.05$, $^{**}p < 0.01$.

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.

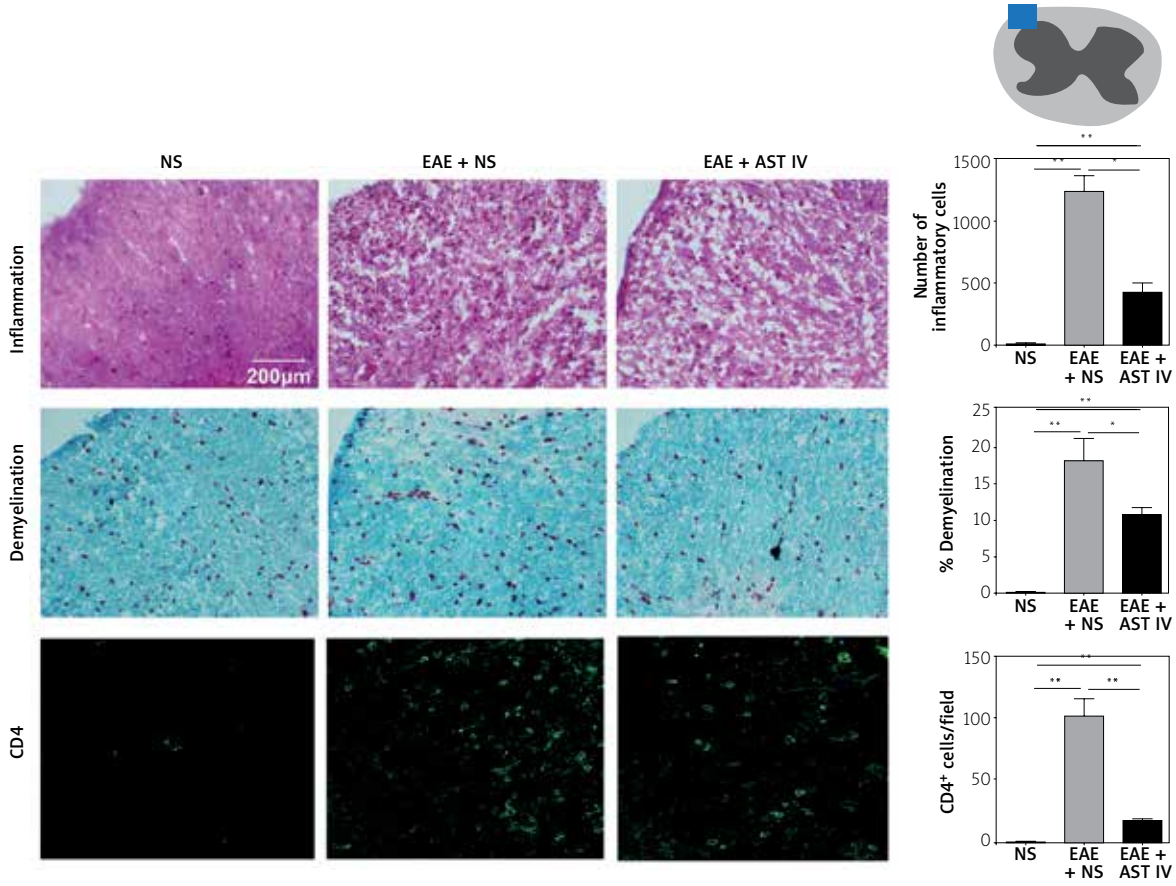

Fig. 2. Astragaloside IV reduced inflammatory cell infiltration, demyelination, and CD4$^+$ T cell activation in the CNS. Inflammation stained with H&E. H&E stain number of inflammatory foci was calculated (Fig. 2 top). Demyelination stained with luxol fast blue was quantitatively analysed on the area (%) of demyelination in total white matter (Fig. 2 middle). CD4$^+$ T cells were counted by immunohistochemistry, and the positive cells were shown, then the percentages of positive cells were analysed (Fig. 2 bottom). The above quantitative analyses were performed by Image-Pro Plus software. Data represent mean ±SEM (n = 4 each group). $^*p < 0.05$, $^{**}p < 0.01$.

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).

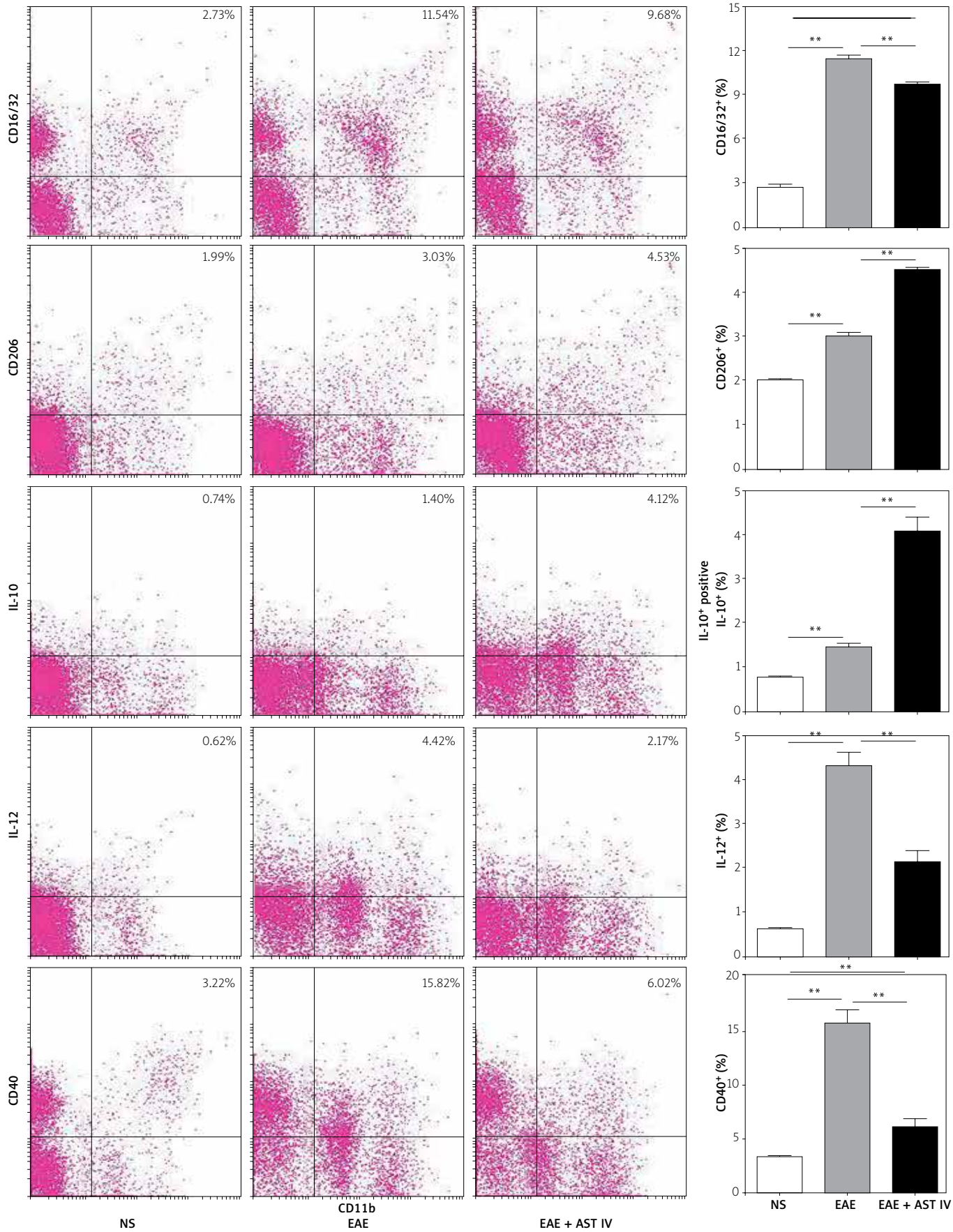

Fig. 3. Astragaloside IV shifted M1 to M2 phenotype of macrophage. Mice were sacrificed on day 28 p.i. and splenic MNCs were prepared for staining with macrophage marker CD11b and M1/M2 markers, analysed by flow cytometry. Scatterplot, histogram, quantitative results were analysed for 4 mice in each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

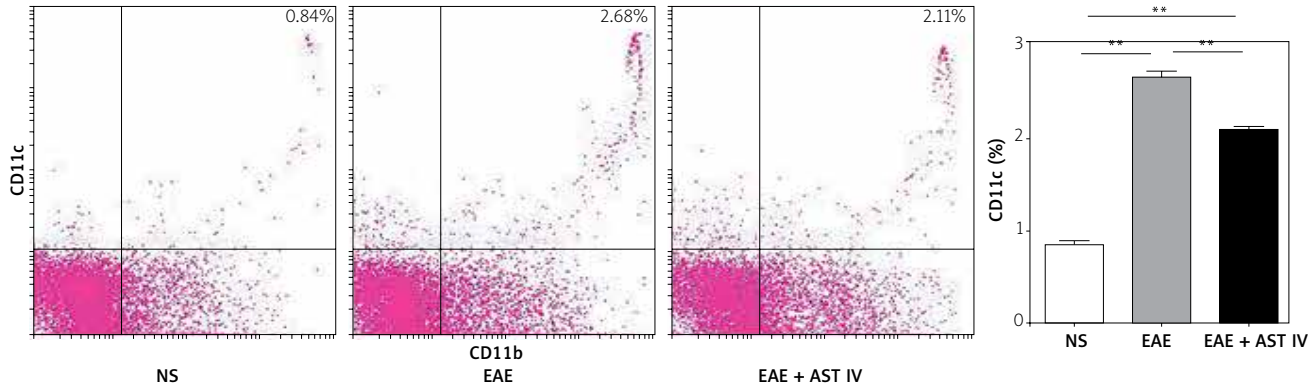

Fig. 3. Cont. Astragaloside IV shifts M1 to M2 phenotype of macrophage. Mice were sacrificed on day 28 p.i. and splenic MNCs were prepared for staining with macrophage marker CD11b and M1/M2 markers, analysed by flow cytometry. Scatterplot, histogram, quantitative results were analysed for 4 mice in each group. $^{*}p < 0.05$, $^{**}p < 0.01$.

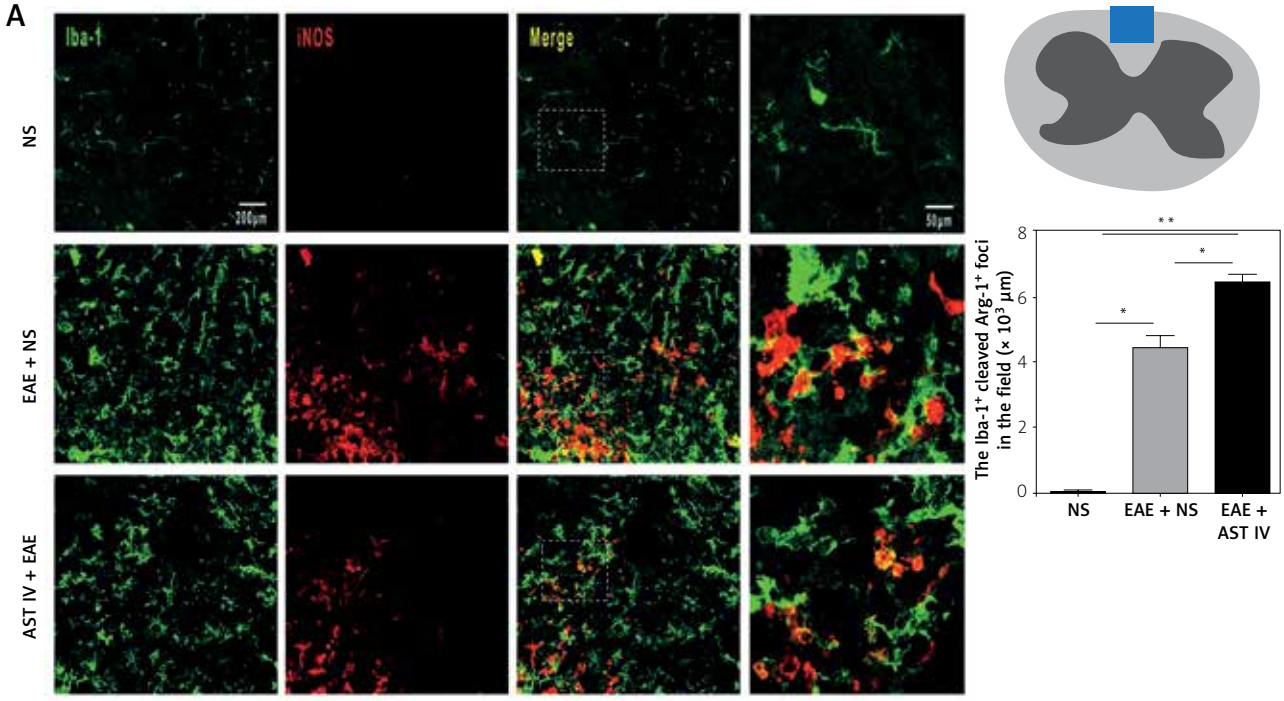

Fig. 4. Astragaloside IV shifted M1 to M2 phenotype in microglia. **A**) Spinal cords were obtained for tissue fixation and section staining with 4 mice in each group. IBa-1 (green)/iNOS (red) were detected by immunofluorescence staining and the overlay (yellow) was performed to detect IBa-1^{+}/iNOS^{+} colocalization, and quantitatively analysed by image-pro plus 6.0.

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, $p < 0.01$), 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.

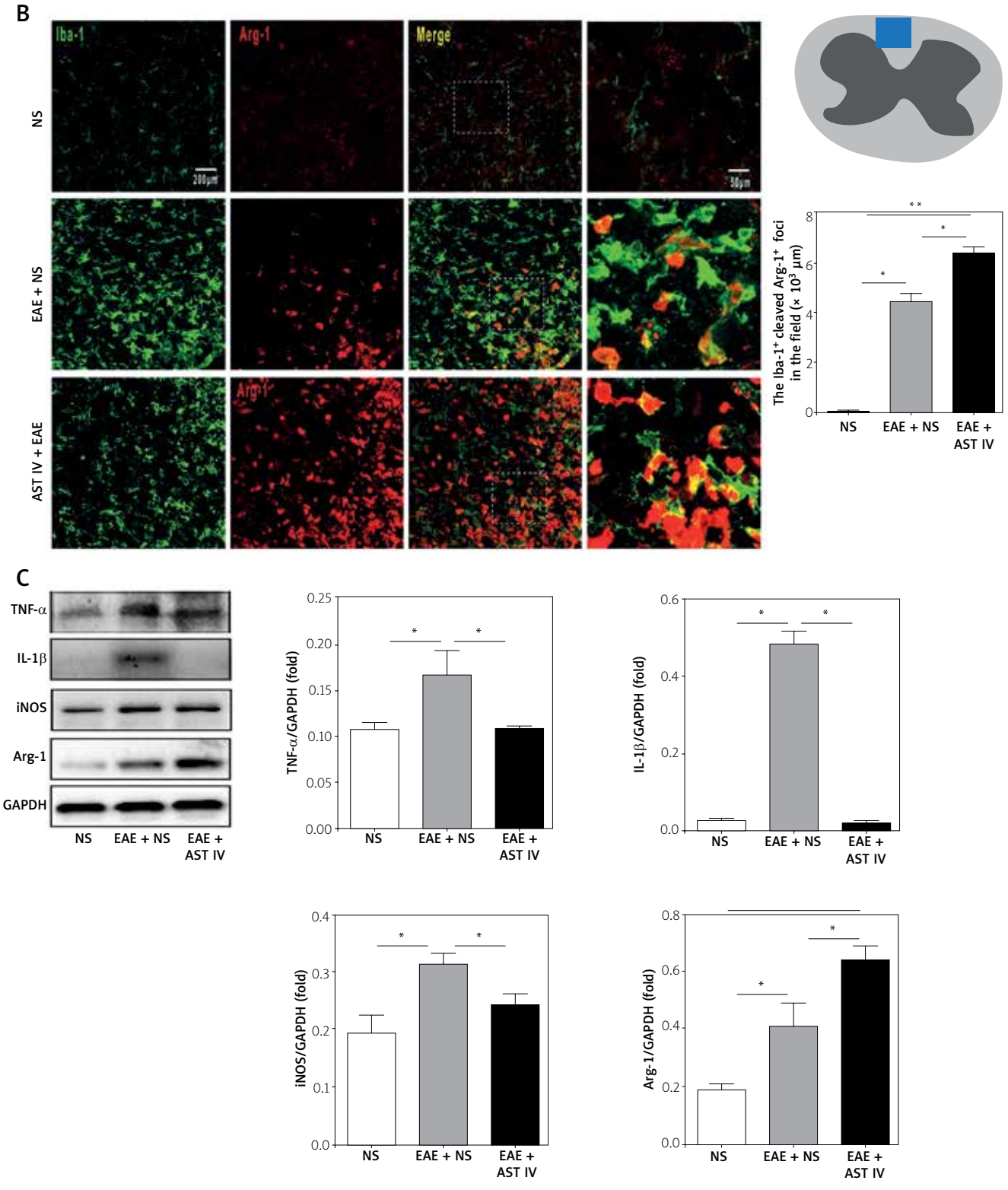

Fig. 4. Cont. **B**) IBa-1⁺ (green)/Arg-1⁺ (red). **C**) The expression of TNF-α, IL-1β, iNOS, Arg-1 was also determined by western blot and quantitatively analysed by image lab 4.0. Data represent mean ±SEM (n = 4 each group). $^{*}p < 0.05$, $^{**}p < 0.01$.

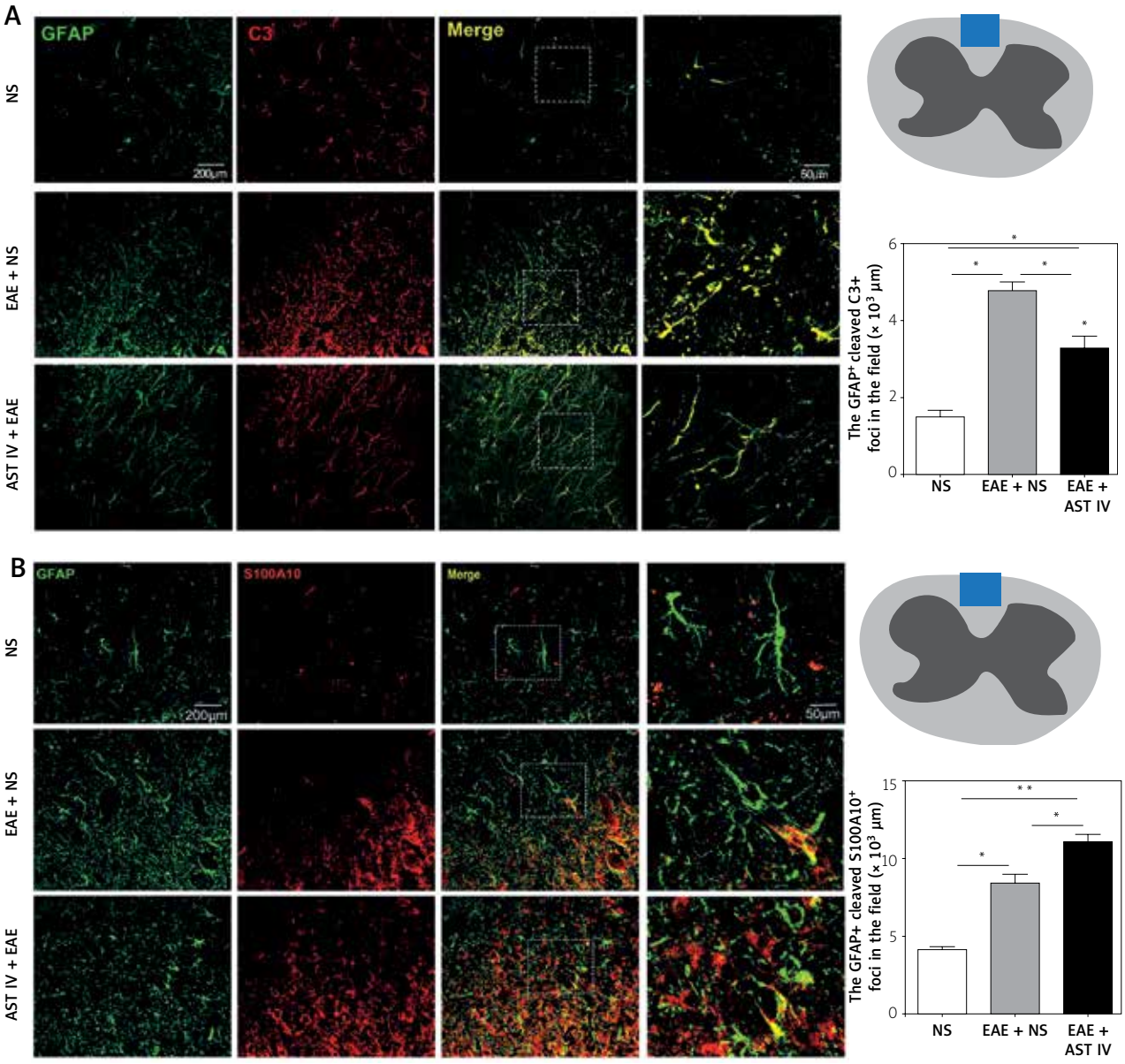

Fig. 5. Astragaloside IV shifted A1 to A2 phenotype of astrocytes. **A**) Spinal cord was harvested from mice, GFAP (green)/C3 (red) were detected by immunofluorescence staining and the overlay (yellow) was performed to detect $GFAP^+/C3^+$ colocalization, and quantitatively analysed by image-pro plus 6.0. **B**) GFAP (green)/S100A10 (red).

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, TLR4 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

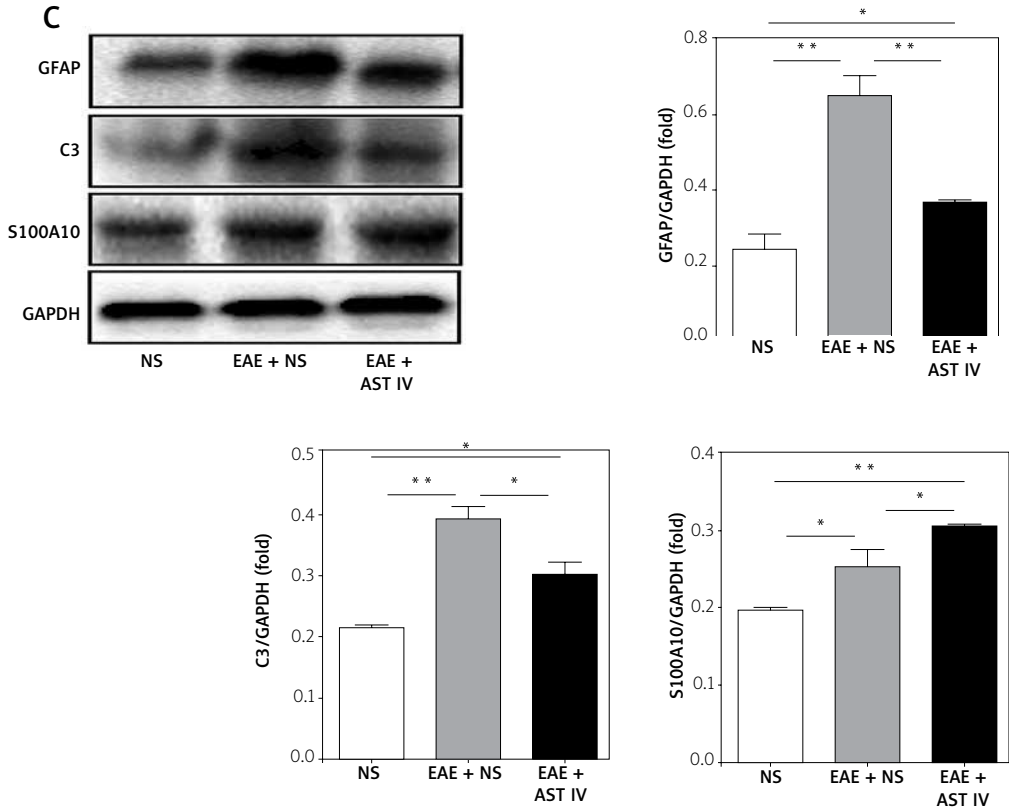

Fig. 5. Cont. **C**) The expression of GFAP, C3, S100A10 was also determined by western blot and quantitatively analysed by image lab 4.0. Data represent mean ±SEM (n = 4 each group). $^{*}p < 0.05$, $^{**}p < 0.01$.

mice (Fig. 8B, $p < 0.01$ for IL-1β; $p < 0.05$ for TNF-α). However, AST IV dramatically increased the abundance of anti-inflammatory cytokines (IL-10) (Fig. 8B, $p < 0.05$) as compared to EAE mice. These data indicate that AST IV suppresses inflammation and enhances anti-inflammatory cytokines.

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 MOG_{33-55} 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

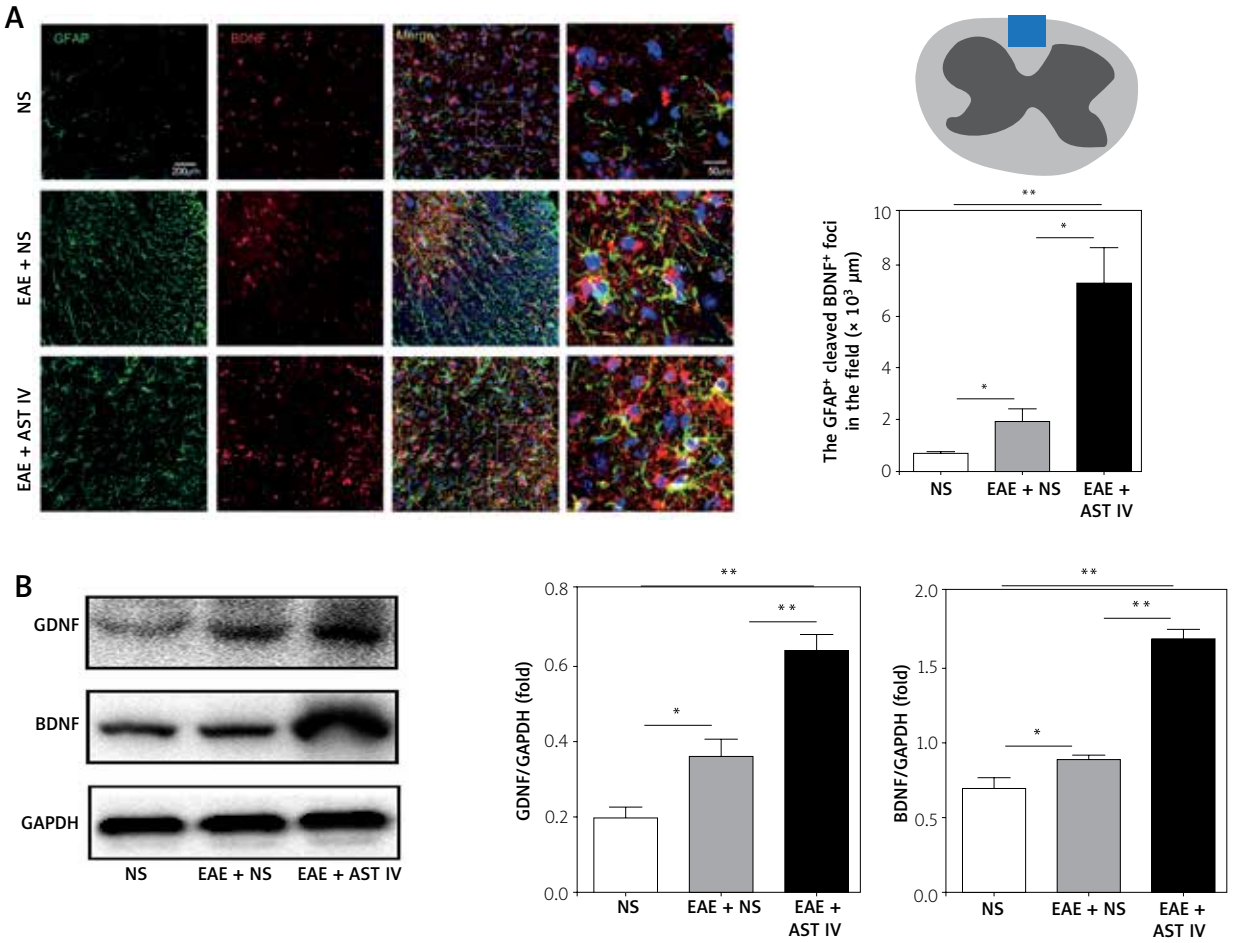

Fig. 6. AST IV promoted the production of neurotrophic factors GDNF and BDNF. Spinal cord was harvested from mice, examined for GFAP/BDNF/GDNF expression. **A**) GFAP (green)/BDNF (red) were detected by immunofluorescence staining and then quantitatively analysed. The overlay (yellow) was performed to 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). $^*p < 0.05$, $^{**}p < 0.01$.

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

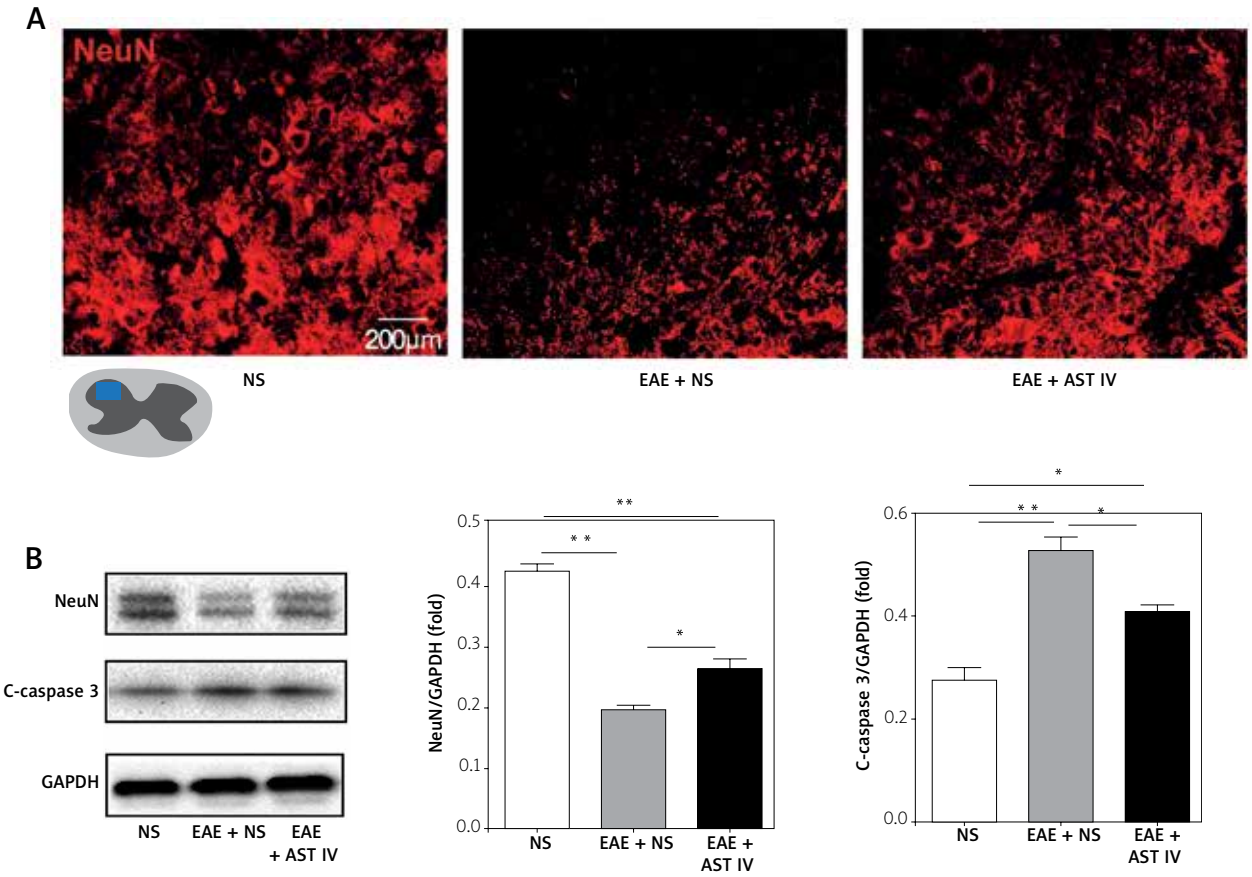

Fig. 7. Astragaloside IV inhibited the apoptosis of neurons and protected neurons. **A**) Spinal cord was harvested from mice, NeuN expression (red) was detected by immunofluorescence staining. **B**) Expression of NeuN and c-caspase3 was also determined by western blot and quantitatively analysed by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

the morphology/phenotype, as verified by immunofluorescent staining and western blot in the CNS. Presence of M1 macrophage/microglia was decreased, while M2 was increased by AST IV intervention, implying that AST IV triggered the polarization of macrophages/microglia from M1 to M2.

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

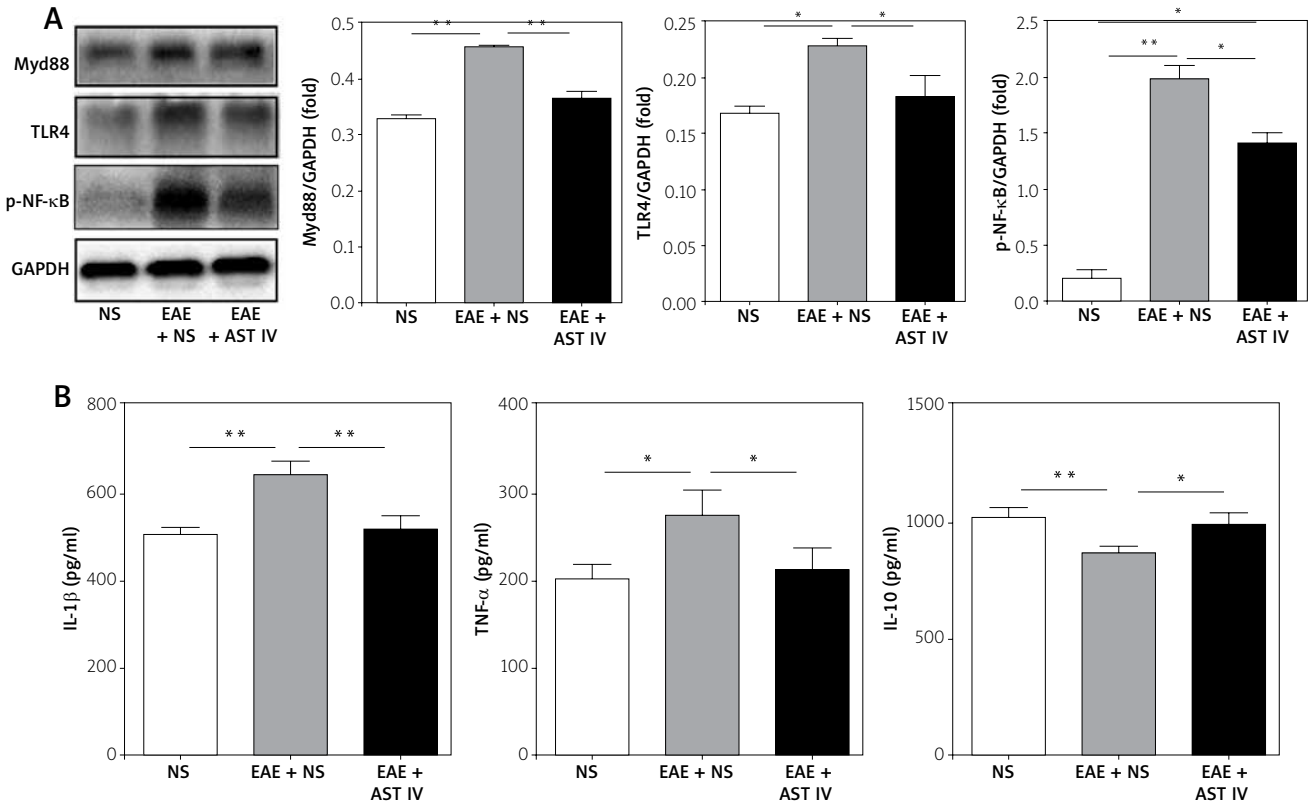

Fig. 8. Astragaloside IV inhibited the inflammatory signalling pathway in the spinal cord and regulated the secretion of cytokines in peripheral immune cells. **A**) The expression of Myd88, TLR4 and NF-κB was performed by western blot. The mice were sacrificed on day 28 p.i., parts of spinal cord were obtained for detecting protein expression. **B**) The levels of IL-1β, TNF-α, and IL-10 in spleen were measured by ELISA kits. Splenic MNCs were incubated for 48 h at 37°C in the presence of MOG_{35-55} (10 mg/ml). The supernatants were collected for ELISA. The results were represented as pg/ml. Quantitative results are mean ±SEM and analysed for 4 mice in 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

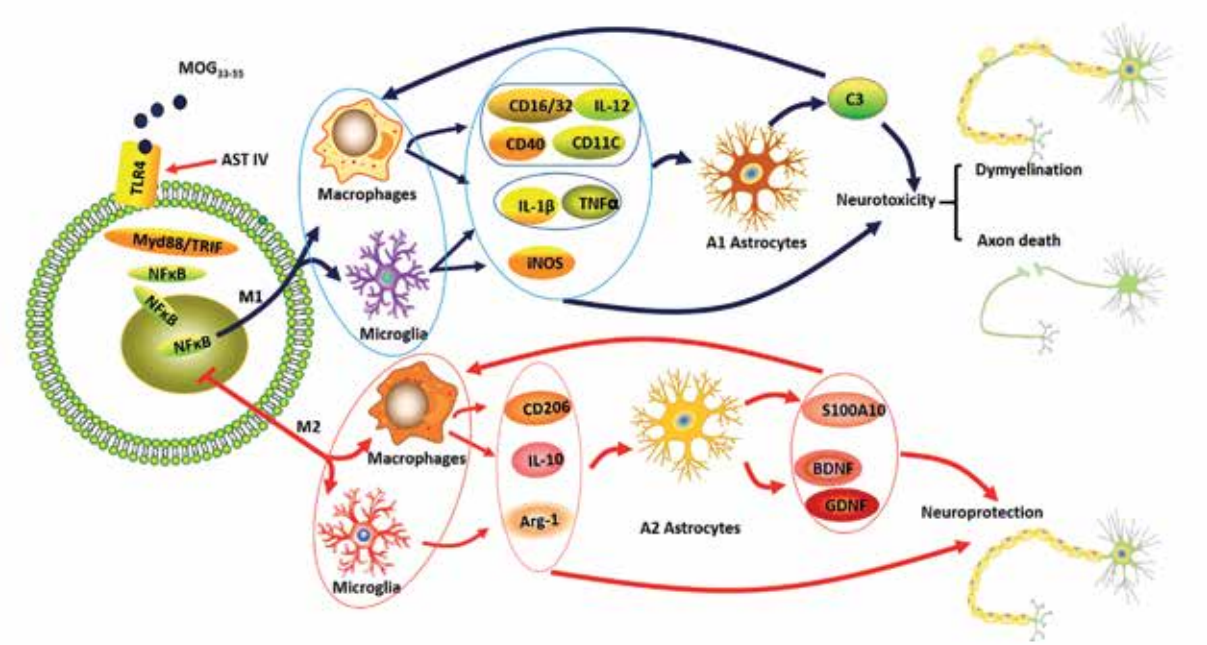

Fig. 9. Schematic diagram of the hypothesized signalling pathways summarizing the therapeutic effects of AST IV against experimental autoimmune encephalomyelitis (EAE). AST IV exhibited strong beneficial effects which may be mainly due to effectively suppressing macrophage/microglia/astrocytes inflammatory response *via* the TLR4/Myd88/NF-κB signalling pathway, and subsequently increases neurotrophic factors derived from astrocytes to protect neurons against apoptotic cell death.

form heterodimers. 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 MOG_{33-55} 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.

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Disclosure

The authors report no conflict of interest.

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