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Astragaloside IV protects neurons from microglia-mediated cell damage through promoting microglia polarization

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Abstract

Astragaloside IV (AST-IV) is a major active ingredient of astragalus, with a neuroprotective effect. The current study is aimed to investigate the impact of AST-IV on the M1/M2 microglial activation in response to lipopolysaccharide (LPS) stimulation, how AST-IV attenuated microglia-mediated neuronal damage, and the molecular mechanisms underlying AST-IV's protection of neurons against microglia-mediated neuronal damage. Our results showed that AST-IV partially protected microglia from death evoked by LPS and downregulated the release of pro-inflammatory (M1) mediators including interleukin (IL)-1β, IL-6, tumour necrosis factor α (TNF-α) and nitric oxide, as well as the expression of Toll-like receptors 4 (TLR4), MyD88, and nuclear factor κB (NF-κB) of these cells. In contrast, AST-IV elevated the production of anti-inflammatory cytokine IL-10 and expression of arginase 1, an M2 marker of microglia, whose conditioned medium promoted PC12 neurons survival. These results indicate that AST-IV exerts an anti-inflammatory effect on microglia, possibly through inhibiting TLR4/NF-κB signalling pathways, and protects neurons from microglia-mediated cell death through conversion of microglia from inflammatory M1 to an anti-inflammatory M2 phenotype.

Key words: *astragaloside IV, neuroinflammatory, neurons, microglia polarization, TLR4 intracellular pathways.*

Introduction

Microglia, the innate immune cells in the central nervous system (CNS), can remove the damaged nerves, patches, infectious substances, secrete immune regulatory factors, and protect neurons. Excessive activation of microglia, however, is the cause of neurodegenerative diseases such as Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS) [5,14,27]. Activated microglia release a lot of nerve toxicity factors such as nitric oxide (NO) and superoxide free radicals (ROS) and inflammatory factors, and lead to neurodegenerative disease [29]. Thus, it has a great significance for the regulation of activated microglia to treat various neurodegenerative diseases.

Neuroinflammation contributes to a wide variety of neurodegenerative diseases, and is strongly linked with neuronal loss or dysfunction in these diseases. The activation of microglia plays a key role in neuroinflammation and activated microglia kill neurons through a number of mechanisms, such as acute activation of the phagocyte NADPH

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oxidase (PHOX), expression of the inducible nitric oxide synthase (iNOS) and release of inflammatory factors [4]. Like peripheral macrophages, microglia activation has been characterized by a recognized number of phenotypes: namely, the surveillant/non-polarized phenotype (M0), the classic activation type (M1) and the selective activation type (M2) [7]. The M1 phenotype or classical activated microglia can be induced by lipopolysaccharide (LPS), interferon γ (IFN-γ), tumour necrosis factor α (TNF-α), hypoxia, and β-amyloid with increased production of proinflammatory cytokines, chemokines and oxidative metabolites such as TNF-α, interleukin 1β (IL-1β), IL-6, iNOS, NO, matrix metalloproteinases (MMPs) reactive oxygen and nitro active species, thereby exacerbating inflammation and contributing to neuron damage and death. In contrast, M2-like microglia can suppress inflammation and promote neural repair and regeneration by secreting anti-inflammatory cytokines, such as transforming growth factor β (TGF-β), IL-4, IL-10, and neurotrophic factors such as glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF) and platelet-derived growth factor (PDGF) [18]. Induction of M2-like microglia is, therefore, an attractive strategy for the treatment of neurological diseases with neuroinflammation based on their protective functions.

Astragaloside IV (AST-IV) is a small molecular saponin and a major active ingredient of *Astragalus membranaceus*, a widely used traditional herbal medicine. It was reported that AST-IV exerted anti-inflammatory, antivirus, anti-aging, immunomodulatory, and organ protective effects [26,38]. AST-IV can reduce the damage and apoptosis of hippocampal neurons in rats and promote the survival of primary cells in the cerebral cortex and the growth of axons [10,13]. *In vivo* experiments also proved that AST-IV can reduce CNS damage, alleviate the incidence of EAE mice, reduce the loss of dopamine neurons, and have a neuroprotective effect [12].

To better understand the anti-inflammatory capacity of AST-IV in the CNS, microglia were stimulated by LPS, the effect of AST-IV was examined and the molecular mechanism was discussed. This study will provide a potential for AST-IV application in neurodegenerative diseases.

Material and methods

BV-2 cell culture and treatment

The BV-2 immortalized microglial cell line was purchased from the National Infrastructure of Cell Line Resource, Beijing, China, and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, NY, USA), supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, Utah, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37°C in a humidified cell incubator with a 95%/5% (v/v) mixture of air and CO_2.

AST-IV is major active component of astragaloside that is obtained from traditional Chinese medicine Astragali radix (Sigma, St. Louis, MO, USA). After BV-2 cells had been cultivated overnight, LPS (Sigma) was added at a final concentration of 1 µg/ml and AST-IV was added at final concentrations of 1 µM/l and 5 µM/l. Wells added only with PBS served as controls. Cells in all groups were then cultured for 24 h.

PC12 cell culture

Pheochromocytoma (PC12) neurons were cultured in DMEM, supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified cell incubator with a 95%/5% (v/v) mixture of air and CO_2. Cells were subcultured 3 times a week at a density of 5×10^5/ml and incubated with BV-2-conditioned medium for 24 h.

Cell viability

Cell viability of BV-2 microglia was measured by MTT assay. Briefly, BV-2 cells (4×10^4/ml) were inoculated on 96-well plates, and cultured with AST-IV (0, 0.5, 1, 2, 5, 10, 20, 50, 100 µmol/l) for 24 h, or cultured with BV-2-conditioned medium for 24 h. Then 100 µl of 0.5 mg/ml MTT solution was added to each well, and the plates were incubated at 37°C for an additional 4 h. Plates were then centrifuged to remove the supernatants, and the crystals were dissolved in 150 µl of DMSO. Cell viability was measured by the optical density (OD) at 490 nm using a quantified microplate reader (Biotek Labsystem, Vermont, USA). All results were confirmed by replication in at least 3 independent experiments. Cell viability (%) = (OD value of the experimental well – OD value of the zero-setting well) / (OD value of the control well – OD value of the zero-setting well) × 100%.

Nitrite assay

Nitric oxide was assayed by the Griess reaction to measure the end product nitrite. Supernatants of cultured cells (100 µl) were mixed with 100 µl of Griess reagent for 10 min at RT. Absorbance was measured at 510 nm in a quantified microplate reader. Concentrations of nitrite were determined by a standard curve of sodium nitrite (Beyotime, Shanghai, China). Determinations were performed and repeated in 3 independent experiments.

Cytokine ELISA assay

Collected supernatants were measured for the concentrations of IL-1β, IL-6, TNF-α, IL-4, and IL-10 by sandwich ELISA kits (PeproTech, Rocky Hill, NJ, USA) following the manufacturer's instructions. All results were repeated in 3 independent experiments. Concentrations of cytokines were quantified by referencing to a standard curve and expressed as pg/ml.

Western blot analysis

Homogenized cells were dissolved in RIPA Lysis Buffer (Beyotime) supplemented with protease inhibitors. Protein concentration was measured by BCA (Beyotime).

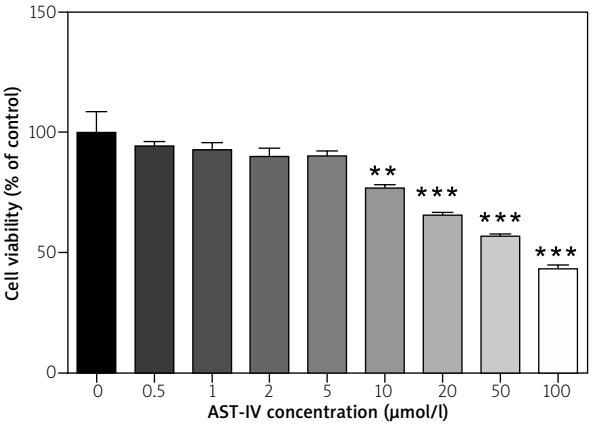

Fig. 1. The cell viability did not exhibit any statistical difference after exposure to less than or equal to 5 µmol/l and was markedly decreased after exposure to 10 µmol/l of AST-IV. The BV-2 cells were cultured in 96-well plates in the absence or presence of AST-IV (0, 0.5, 1, 2, 5, 10, 20, 50, 100 µmol/l) for 24 h. Cell viability was measured by the MTT assay. The quantitative analysis was performed based on 3 independent experiments with similar results. $^{**}p < 0.01$, $^{***}p < 0.00$ vs. 0 µmol/l.

Cell extracts (30 µg) were loaded onto 10% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane (Merck Millipore, Tullagreen Carrigtwohill, Cork, Ireland), blocked by 5% milk at room temperature (RT) for 2 h. Membranes were incubated at 4°C overnight with primary antibodies Toll-like receptors 4 – TLR4 (1 : 1000, 2246, Cell Signaling, Boston, USA), nuclear factor κB – NF-κB (p65) (1 : 1000, 30335, Cell Signaling), myeloid differentiation factor – MyD88 (1 : 1000, ab2064, Abcam, Cambridge, UK), IL-1β (1 : 1000, Abcam, ab200478), iNOS (1 : 1000, ADI-905-431-1, Enzo Life Sciences, NY, USA), Arg-1 (1 : 1000, 610708, BD Biosciences, NY, USA), and β-actin (1 : 10,000, 4970, Cell Signaling). Horseradish peroxidase-conjugated secondary antibody goat anti-mouse (1 : 10,000, E030110-01, Earth, San Francisco, CA, USA) and goat anti-rabbit (1 : 10,000, E030120-01, Earth) were incubated for 2 h on the next day. Immunoblots were measured by Quantity Software (Bio-Rad, Hercules, CA, USA). To compare the protein loading, β-actin was used as the optical density of internal reference.

Immunofluorescent staining

BV-2 microglia were cultured and treated based on experimental requirements in a 24-well plate with slides. BV-2 microglia were fixed with 4% paraformaldehyde for 30 min and stained with the following antibodies: TLR4 (1 : 1000), NF-κB (1 : 1000), iNOS (1 : 1000), Arg-1 (1 : 1000), and kept overnight at 4°C. The next day, Alexa Fluor 488-conjugated secondary antibodies (1 : 1000; Invitrogen, Eugene, USA) or Alexa Fluor 555-conjugated secondary antibodies (1 : 1000; Invitrogen) were added at RT for 2 h. The stained slides were examined by fluorescence microscope (Olympus, Tokyo, Japan).

Results

Effect of AST-IV on microglia viability

The viability of BV-2 microglia at different AST-IV concentrations was tested by MTT. The effect of AST-IV on the viability of BV-2 microglia viability of less than or equal to 5 µmol/l did not exhibit any statistical difference ($p > 0.05$) compared with the control group (Fig. 1). Cell viability was markedly decreased after exposure to 10 µmol/l of AST-IV, indicating that a higher dose of AST-IV is cytotoxic. The dose of AST-IV is different from that in other articles, on account of DMSO as the auxiliary solvent, which may have an effect on cell toxicity. Concentrations of AST-IV at 1 and 5 µmol/l were, therefore, used in the following experiments.

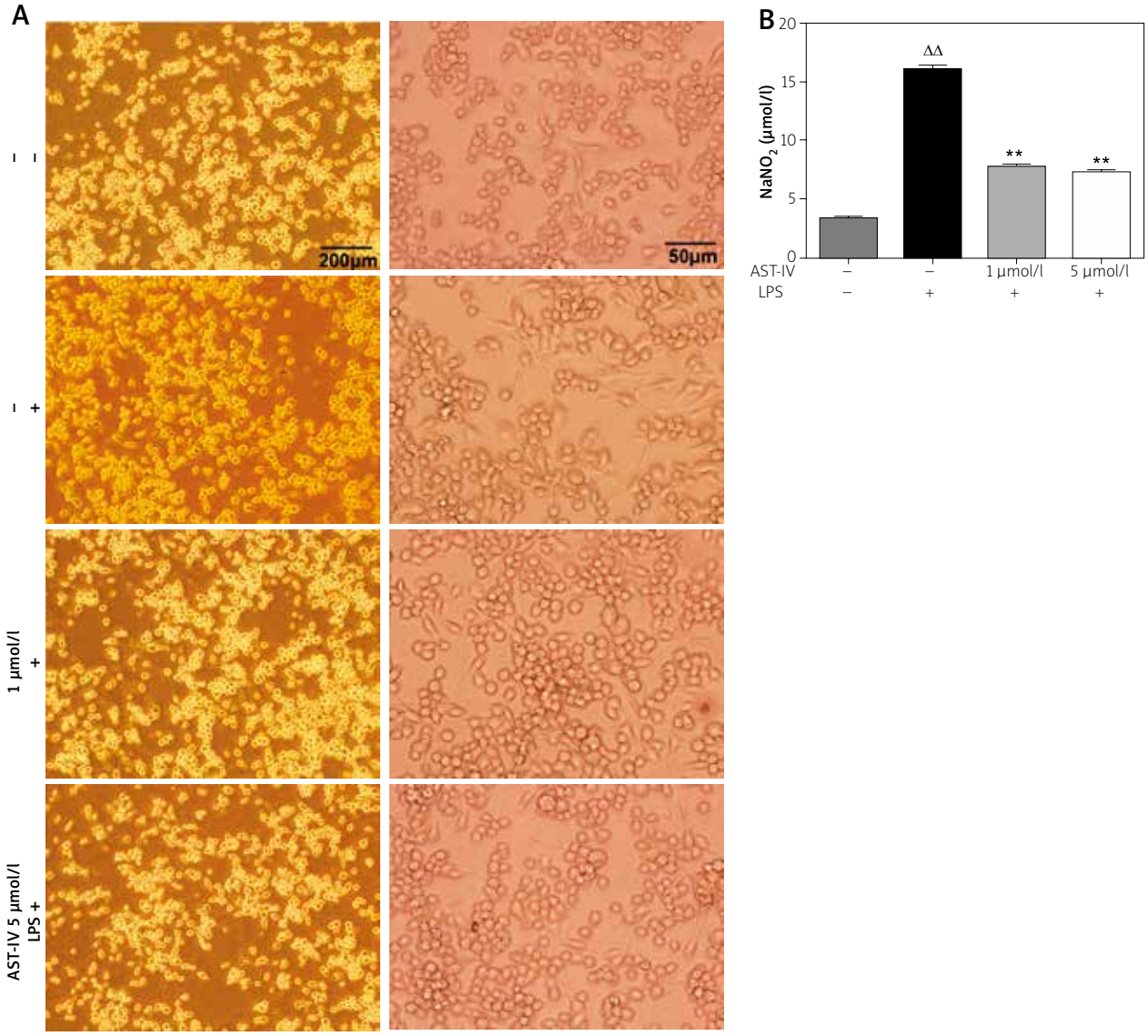

Fig. 2. AST-IV inhibited the activation of BV-2 cells that were treated with LPS and showed a resting morphology. AST-IV effectively inhibited the production of NO. The BV-2 cells were cultured in 24-well plates in the absence or presence of LPS (1 μg/ml)/AST-IV (1 μmol/l and 5 μmol/l) for 24 h. The cell morphology was obtained by bright field imaging using an inverted Olympus microscope. Representative pictures were exhibited on BV-2 cells (**A**), and release of NO was detected by Griess assay (**B**). The quantitative analysis was performed based on 3 independent experiments with similar results. $^{\Delta\Delta}p < 0.01$ vs. LPS(–) AST-IV(–), $^{**}p < 0.01$ vs. LPS(+) AST-IV(–).

Effect of AST-IV on microglia morphology and nitric oxide production

Resting BV-2 microglia is characterized by rounded and fusiformis cell bodies, clear in cell edge and bright cytoplasm, whereas activated BV-2 cellular morphology like a broken egg or a worm, swollen cell body, blur in the edge, and dim cytoplasm. Cell morphology changed after 24 h of treatment with PBS, LPS (1 μg/ml) and AST-IV (1 and 5 μmol/l). A resting morphology was observed in PBS-treated microglia, and cells treated with LPS exhibited an activated one (Fig. 2A). In contrast, microglia activation was inhibited by AST-IV, which showed a resting morphology.

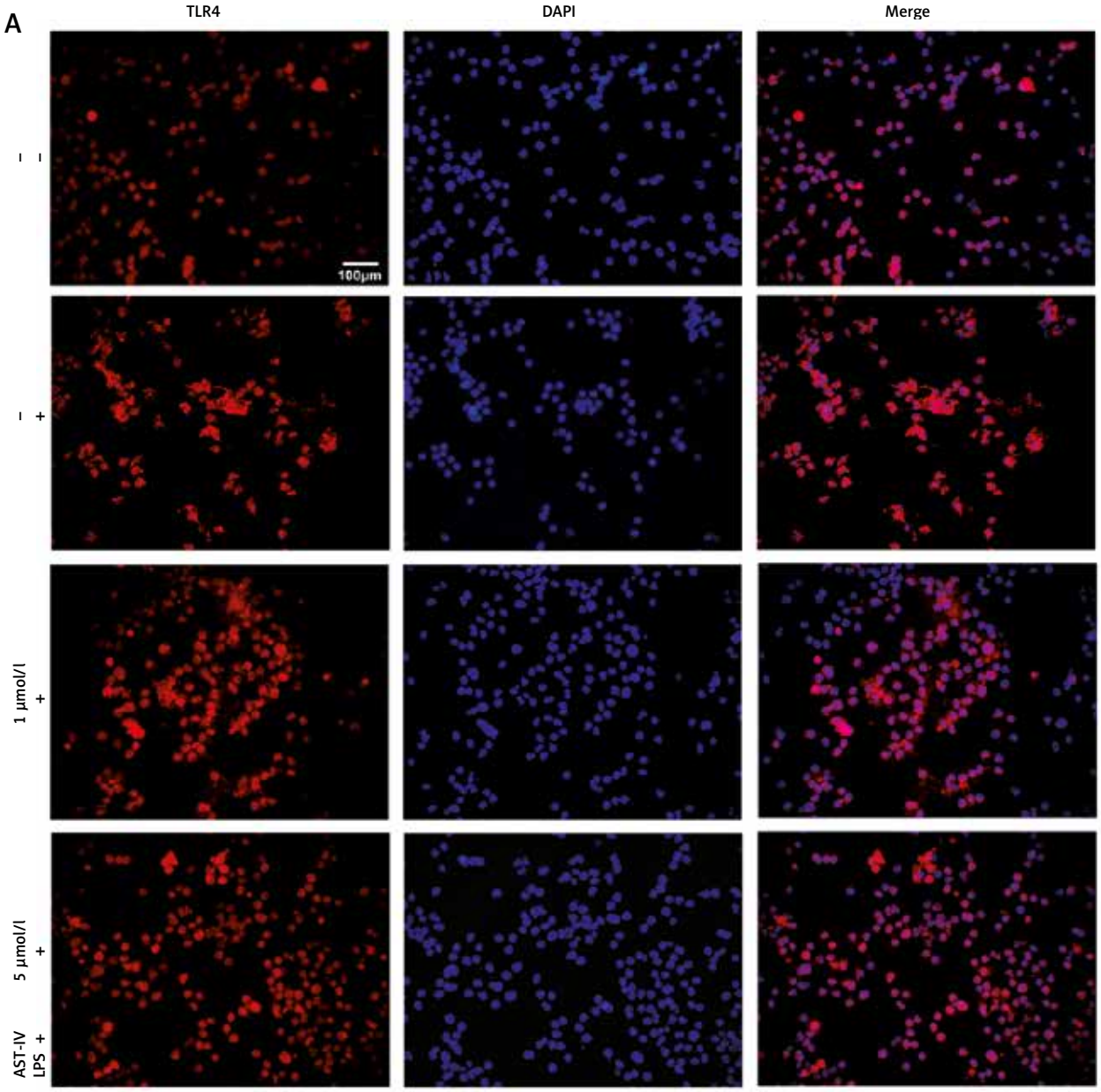

Fig. 3. AST-IV significantly inhibited the expression of Myd88, TLR4 and NF-κB in BV-2 cells. The BV-2 cells were cultured in 24-well/6-well plates in the absence or presence of LPS (1 μg/ml)/AST-IV (1 μmol/l and 5 μmol/l) for 24 h. Protein levels of TLR4 and NF-κB were measured by immunocytochemistry staining (**A** and **B**) and Western blot assay (**C**). Quantitative results are from three independent experiments with similar results. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs. LPS(–) AST-IV(–), $^{*}p < 0.05$ vs. LPS(+) AST-IV(–).

The production of nitrite, a major end product of NO metabolism, was detected in the supernatants of BV-2 cells by Griess reagent. The results showed that LPS strongly stimulated the production of nitrite (Fig. 2B; $p < 0.01$), and this production was effectively inhibited by AST-IV treatment (Fig. 2B; $p < 0.05$).

Effect of AST-IV on LPS-induced TLR4/MyD88/NF-κB signalling in BV-2 cells

Toll-like receptors (TLRs) are a key component of the innate immune system that defences against pathogens and the development of adaptive immunity, releases cytokines, increases costimulatory molecule expression,

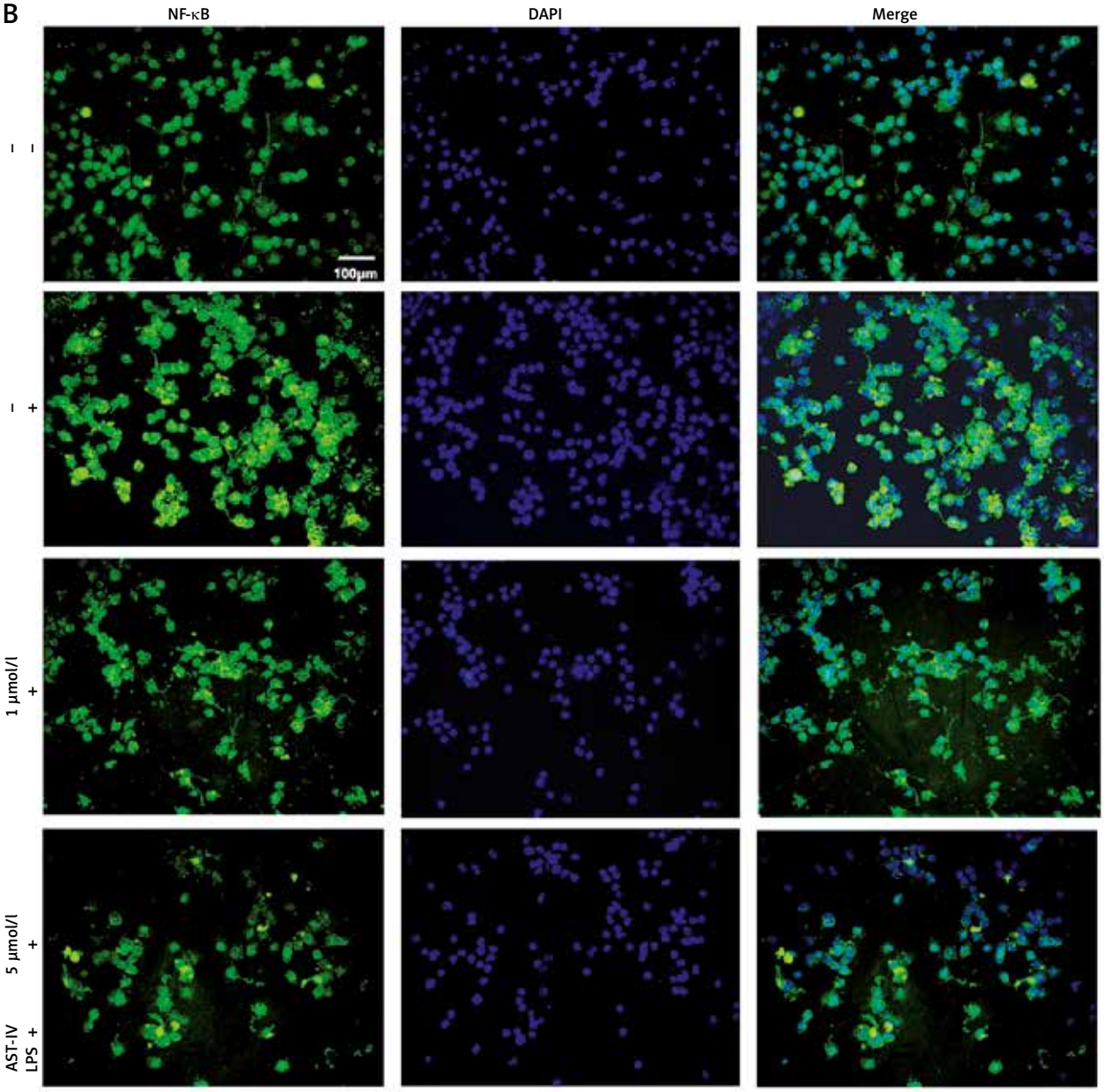

Fig. 3. Cont.

and provides the necessary activated signal to form the acquired immune reaction. Myeloid differentiation factor (MyD88) is a key joint molecule in the TLRs signal path. MyD88 contains two domains, one is carboxyl terminal homologous binding to TLRs, the other is amino acid terminal binding to the death domain of IL-1 receptor associate kinase (IRAK), which causes IRAK phosphorylation. A series of activated reaction causes the activation of Iκbα, eventually leads to activate NF-κB and transposition, releases of inflammatory cytokines, which induces inflammatory response process.

We then determined the expression of TLR4, MyD88 and NF-κB, all important M1-like molecules, by immunostaining. Our results showed that the expression of these molecules was upregulated by LPS stimulation, and TLR4 and NF-κB expression was inhibited after AST-IV treatment (Fig. 3A, B). Similar results were observed by Western blot, which showed significantly inhibited TLR4 and NF-κB expression by AST-IV treatment at both concentrations of 1 and 5 μmol/l, while MyD88 expression was inhibited when 5 μmol/l of AST-IV

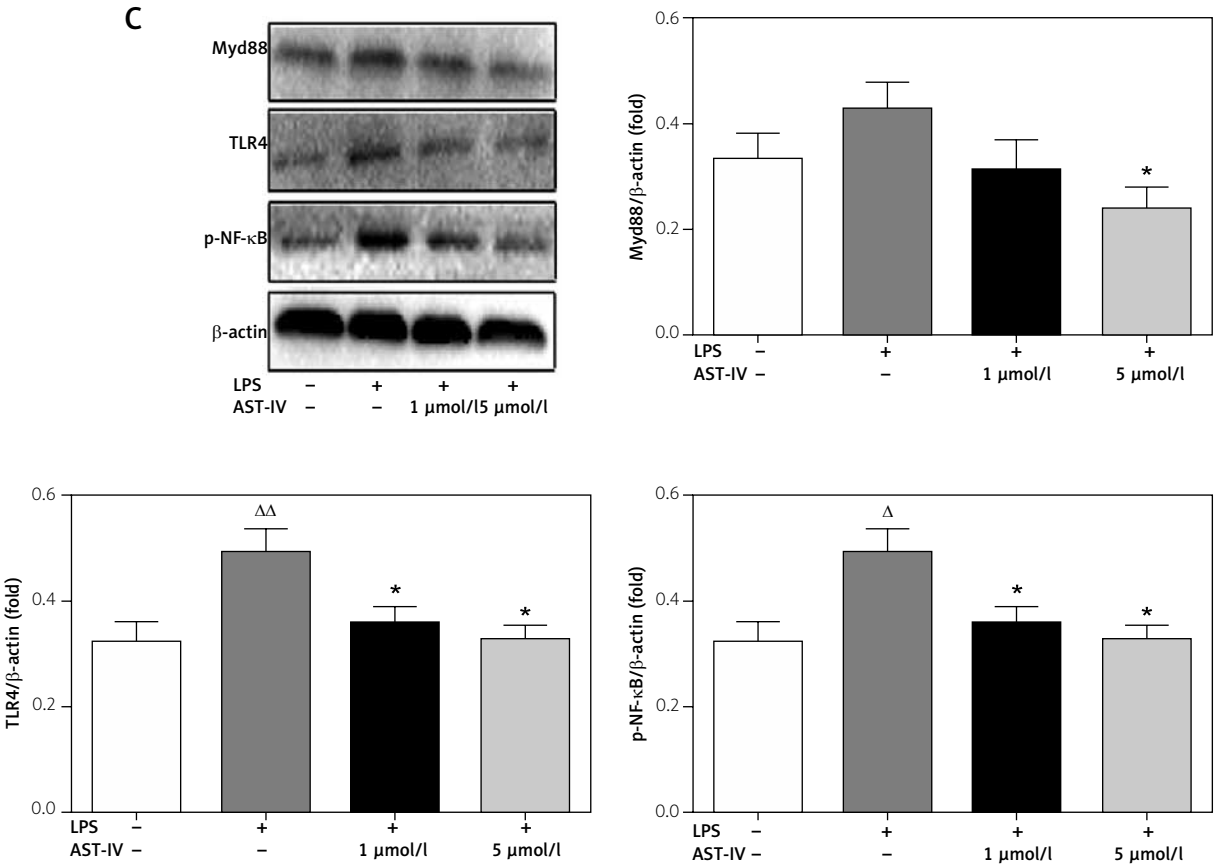

Fig. 3. Cont.

was added (Fig. 3C; $p < 0.05$). Thus, AST-IV exerts a suppressive effect on TLR4-MyD88 signalling pathway.

Effect of AST-IV on LPS-induced microglia polarization

We then studied whether AST-IV can trigger the polarization of BV-2 microglia, and shift from inflammatory M1 cells toward anti-inflammatory M2 cells. As shown in Figure 4, LPS stimulated the upregulation of IL-1β and iNOS ($p < 0.01$, $p < 0.000$), and the treatment of AST-IV effectively inhibited the expression of IL-1β and iNOS (Fig. 4; $p < 0.05$, $p < 0.05$, $p < 0.000$). In contrast, AST-IV treatment upregulated Arg-1 expression (Fig. 4, $p < 0.05$, $p < 0.01$). The results clearly indicated that AST-IV treatment converted LPS-induced inflammatory M1 phenotype toward an anti-inflammatory M2 phenotype.

Effect of AST-IV on cytokine production of microglia

To further address the effect of AST-IV on the microglia phenotype, cytokines IL-6, TNF-α, IL-4 and IL-10 in BV-2 microglia culture supernatants were measured by ELISA. LPS stimulated BV-2 cells to produce higher amounts of IL-6 ($p < 0.01$), TNF-α ($p < 0.05$) but reduced IL-10 ($p < 0.01$) compared to non-stimulated cells (Fig. 5). The production of IL-6 and TNF-α was significantly inhibited when treated by AST-IV (all $p < 0.01$). In addition, treated by AST-IV increases IL-10 production compared to that treated with LPS (Fig. 5, $p < 0.05$). Meanwhile, AST-IV treatment had a tendency to increase IL-4

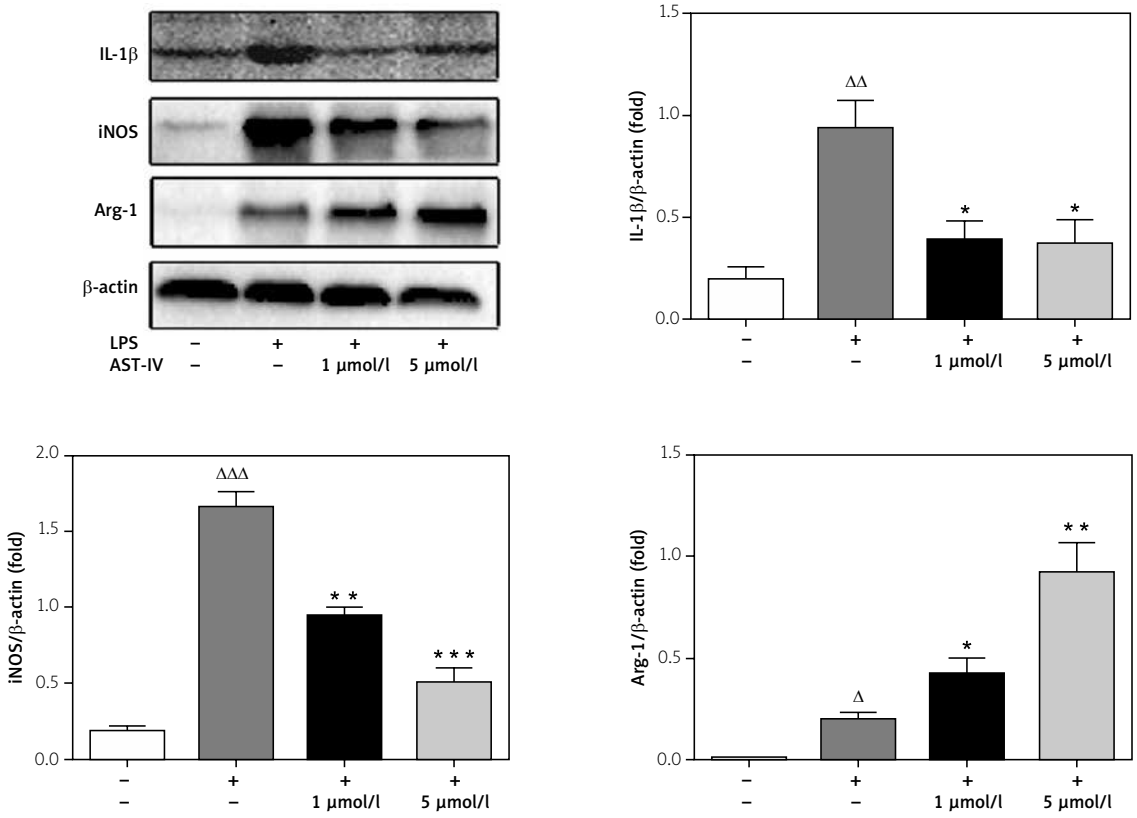

Fig. 4. AST-IV effectively inhibited the expression of IL-1β and iNOS and upregulated Arg-1 expression. The BV-2 cells were cultured in 6-well plates with LPS (1 μg/ml)/AST-IV (1 μmol/l and 5 μmol/l) for 24 h. The protein was extracted from BV-2 cells, and the expression of IL-1β, iNOS and Arg-1 were measured using Western blotting. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs. LPS(–) AST-IV(–), $^{\Delta\Delta\Delta}p < 0.000$ vs. LPS(+) AST-IV(–), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.000$ vs. LPS(+) AST-IV(–).

production, while it did not reach statistical significance (Fig. 5).

BV-2-conditioned medium treated with AST-IV exhibits a neuroprotective effect

Finally, we collected BV-2 microglia-conditioned medium treated with AST-IV (1 and 5 μmol/l) and determined its neuroprotective effect when exposing cultured PC12 neurons, using MTT assay. The results showed that, while supernatants of LPS-stimulated microglia induced neuron death, AST-IV-conditioned medium significantly increased cell viability compared to LPS-stimulated BV-2 microglia medium (Fig. 6, $p < 0.05$, $p < 0.01$).

Discussion

Microglia are unique resident immune cells and major cellular source of inflammatory mediators in the CNS, and a key player in the inflammatory processes related to neurodegenerative diseases [11]. Microglial activation is a double-edged sword in the CNS which are related to both the immune response and maintaining homeostasis [6,14]. Many studies have demonstrated that microglia/macrophages undergo M1 or M2 polarization in response to different environmental stimulation. M1 microglia/macrophages can secrete a large number of inflammatory mediators and upregulate IL-1β and iNOS expression. These changes can create an inflammatory microenvironment and then promote the

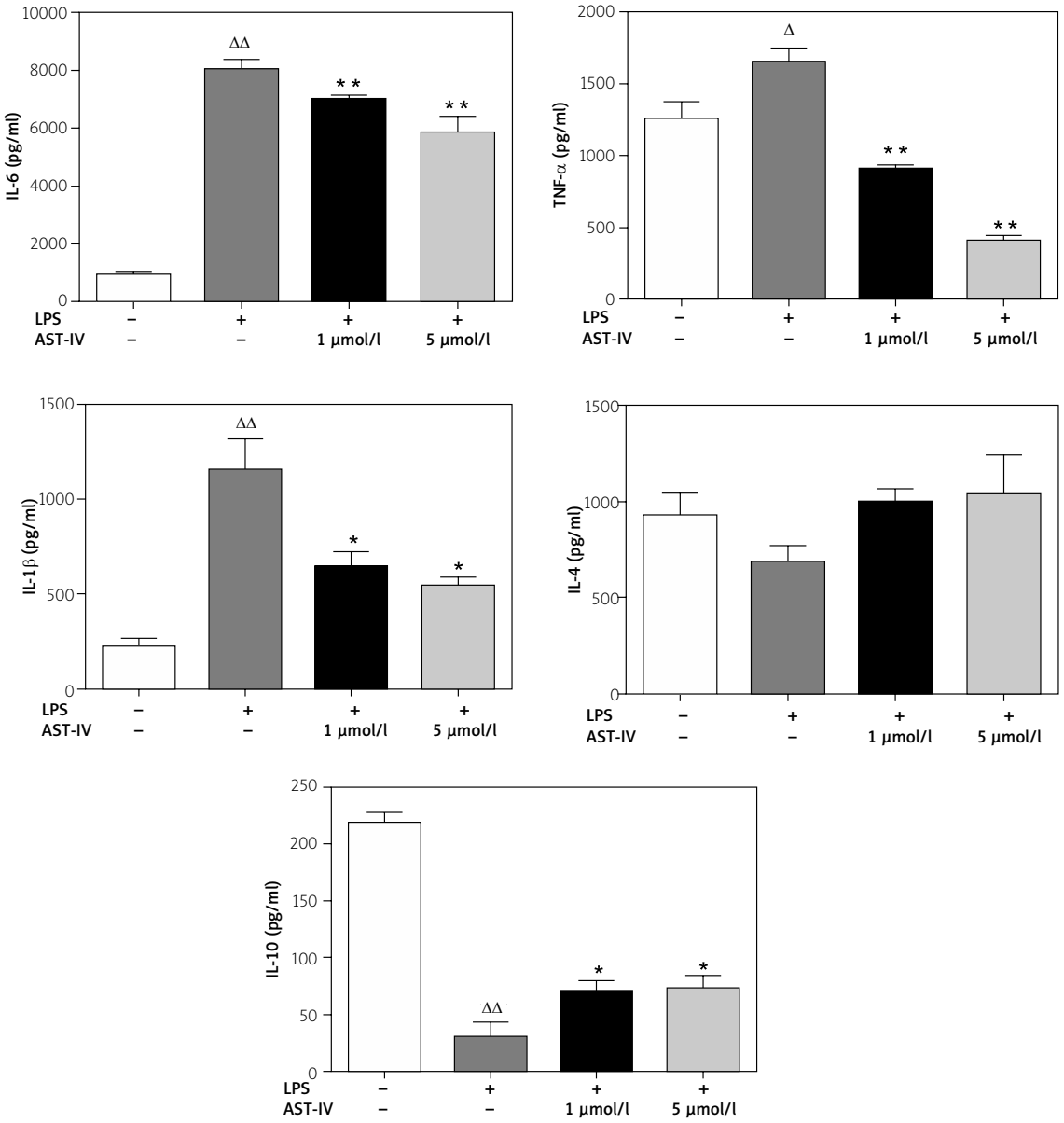

Fig. 5. AST-IV decreased the production of IL-6 and TNF-α and increased IL-10 production in BV-2 microglia compared to those treated with LPS. BV-2 cells were treated with LPS (1 μg/ml)/AST-IV (1 μmol/l and 5 μmol/l) for 24 h. The levels of IL-6, TNF-α, IL-1β, IL-4 and IL-10 in culture supernatants were measured by ELISA assay. Quantitative results are expressed as mean ± SEM from three independent experiments with similar results. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$ vs. LPS(–) AST-IV(–), $^{**}p < 0.01$ vs. LPS(+) AST-IV(–).

pathogenesis of neurodegenerative diseases [15]. On the other hand, M2-polarized microglia/macrophages upregulate Arg-1 expression and increase anti-inflammatory IL-10 and IL-4 production, which can inhibit an inflammatory response and promote neuroprotection [2,28,33]. Inhibiting microglial activation and the release of pro-inflammatory mediators and promoting microglia polarization towards M2 phenotype may, therefore, be crucial for the treatment and neurodegenerative diseases.

The morphology and function of microglia adapt to their ever-changing surroundings. Microglia in the resting state, participants in CNS homeostasis, continuously scan the surrounding extracellu-

lar space and communicate directly with neurons, astrocytes, and blood vessels [19]. When tumours, brain damage, infection, stroke attract, microglia transform into activated morphology, the phenotype, for responding to neuroinflammation. Activated microglia triggers a complex molecular cascade including nitric oxide, oxygen free radicals, proteases and inflammatory cytokines production, a complex inflammatory response, and the blood-brain barrier disruption, finally necrotic and apoptotic cell death [27]. Resting BV-2 microglia are characterized by round bodies, smooth-surface clear in cell edge and cytoplasmic bright, whereas activated BV-2 cellular morphology like a broken egg or amoeboid cell shape, swollen cell body, blur in the edge, and dim cytoplasm [3]. The present study observed that AST-IV treated LPS-induced BV-2 cells retained the shape that is characteristic of non-stimulated microglia, indicating an inhibitory effect of this small molecule on microglia activation.

The activated microglia release inflammatory cytokines including IL-6, TNF-α, and other cytotoxic molecules such as NO [22]. Here, we found that IL-6, IL-1β and TNF-α levels increased in the LPS-treated only group, while AST-IV treatment could inhibit these abnormal increases and enhance IL-10 secretion. These results demonstrate that AST-IV treatment exerts neuroprotective effects possibly through its anti-inflammatory properties. Furthermore, we selected the markers of M1 polarization, i.e. iNOS and IL-1β, as well as the markers of M2 polarization, i.e. Arg-1. While M1 markers were significantly declined, M2 markers were significantly increased in AST-IV treatment compared with LPS treatment. Thus, AST-IV can shift microglia from M1 to M2 phenotype.

AST-IV has been reported to exert anti-inflammatory and neuroprotective effects in various disease models [21,31]. AST-IV-induced anti-inflammatory effects indicate its potential application in the treatment of neuroinflammatory and neurodegenerative diseases [12,34]. AST-IV can attenuate the H_2O_2-induced apoptosis of neuronal cells and exert protective effects against neurodegenerative diseases via the p38 MAPK pathway, and attenuate glutamate-induced neurotoxicity in PC12 cells through Raf-MEK-ERK pathway [24,37]. In the present study we showed that AST-IV reduces microglial activation and attenuate microglia-mediated neuronal damage. These results provide evidence that the mechanism

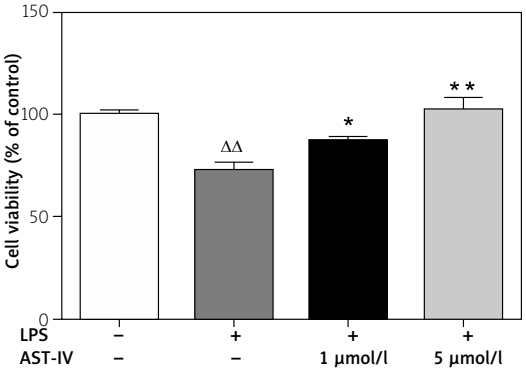

Fig. 6. AST-IV-conditioned medium increases the survival of cultured PC12 neurons. BV-2 cells were cultured with LPS (1 μg/ml)/AST-IV (1 μmol/l and 5 μmol/l) for 24 h, and conditioned media were collected. PC12 neurons were then incubated for 24 h with the different conditioned media. PC12 neurons viability were quantified with MTT. Quantitative results are expressed as mean ± SEM from five independent experiments with similar results. $^{\Delta\Delta}p < 0.01$ vs. LPS(–) AST-IV(–), $^{*}p < 0.05$, $^{**}p < 0.01$ vs. LPS(+) AST-IV(–).

underlying the neuroprotective effects of AST-IV may occur through inhibiting microglial activation and shifts microglia from the M1 to M2 phenotype.

The diversity of the macrophage function is related to many factors, including cytokines, chemokines, local micro environmental conditions, protein kinases, hormones, TLR ligands, complement and other endogenous molecules [1,17,32]. Among them, TLRs play an important role in the innate immune system [8]. Although TLR4 expressed on multiple cell lines, such as microglia, astrocytes and neurons, its expression is highest on microglia [20]. LPS is recognized by TLR4 on the surface of microglia and induces TLR4 activation, which subsequently recruits the adapter protein MyD88 and leads to the rapid activation of NF-κB. Overactivation of the NF-κB signalling pathway causes the excessive production of pro-inflammatory cytokines and triggers an array of microglia responses leading to the release of inflammatory mediators [16,30]. A recent study has shown that LPS can shift microglia from M0 to M1 phenotype by stimulating the activation of TLR4/p-38/p-JNK/NF-κB signalling pathway [36]. LPS binds to TLR4 on the surface of microglia cells, resulting in the overexpression of proinflammato-

ry genes and over-secretion of pro-inflammatory molecules by NF-κB activation [25]. The activation and nuclear translocation of NF-κB is a key step in LPS-stimulated microglial activation, and regulates the expression of a large number of inflammatory genes [9]. It has been reported that AST-IV exerted its anti-inflammatory effect via inhibition of glucocorticoid receptor-mediated NF-κB signalling [23], and attenuated release of inflammatory cytokines is related to inhibiting the TLR4/NF-κB signalling pathway [35]. In this study, AST-IV suppressed the protein expression of TLR4, MyD88 and NF-κB of microglia, which probably resulted in the decreased production of pro-inflammatory molecules, and shifted these cells to the M2 phenotype.

In conclusion, the present study demonstrates that AST-IV exerts anti-inflammatory effects and attenuates microglia-mediated neuronal damage. These effects are possible through inhibiting TLR4/MyD88/NF-κB signalling pathways, thus reducing the expression of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, iNOS, and inducing the expression of anti-inflammatory molecules, e.g., IL-10 and Arg-1, and regulating microglia polarization from inflammatory M1 to an anti-inflammatory M2 state. Our observations suggest that AST-IV has the potential to be used as a therapeutic agent for neurodegenerative diseases.

Acknowledgments

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Disclosure

The authors report no conflict of interest.

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