Scutellarin acts via MAPKs pathway to promote M2 polarization of microglial cells

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Abstract

Scutellarin, an herbal agent, is known to possess anti-oxidant and anti-inflammatory properties. In activated microglia, it has been reported that this is achieved through acting on the MAPKs, a key pathway that regulates microglia activation. This study sought to determine if scutellarin would affect the commonly described microglia phenotypes, namely, M1 and M2, thought to contribute to pro- and anti-inflammatory roles, respectively. This is in consideration of its potential effect on the polarization of microglia phenotypes that are featured prominently in cerebral ischemia. For this purpose, we have used an experimentally induced cerebral ischemia rat model and LPS-stimulated BV-2 cell model. Thus, by Western blot and immunofluorescence, we show here a noticeable increase in expression of M2 microglia markers, namely, CD206, Arg1, YM1/2, IL-4 and IL-10 in activated microglia both in vivo and in vitro. Remarkably, scutellarin treatment markedly augmented the increased expression of the respective markers in activated microglia. It is therefore suggested scutellarin can exert the polarization of activated microglia from M1 to M2 phenotype. Because M1 microglia are commonly known to be proinflammatory, while M2 microglia are anti-inflammatory and neuroprotective effect, it stands to reason therefore that with the increase of M2 microglia which became predominant by scutellarin, the local inflammatory response is ameliorated. More importantly, we have found that scutellarin promotes the M2 polarization through inhibiting the JNK and p38 signaling pathways, and concomitantly augmenting the ERK1/2 signaling pathway. This lends its strongly support from observations in LPS activated BV-2 microglia treated with p38 and JNK inhibitors in which expression of M2 markers was increased; on the other hand, in cells subjected to ERK1/2 inhibitor treatment, the expression was suppressed. In light of the above, MAPKs pathway is deemed to be a potential target of scutellarin in mitigating microglia mediated neuroinflammation in activated microglia.

Introduction

Ischemic stroke is one of the most common life-threatening and debilitating neurological diseases affecting many world-wide. Stroke brings severe neurological impairments which may lead to perpetual disability[1]. Following a stroke, sequential pathological changes occur in the damaged brain areas. In view of this, many therapeutic strategies have been designed targeting to modify the acute pathological changes in the ischemic tissue at the cellular and molecular levels to with the aim restore homeostasis brain and functions. In the event of an ischemic stroke, microglia which act as a neuropathology sensor are readily activated[2]. It is well recognized that in response to ischemia, activated microglia undergo a series of phenotypic transformation and functional alterations, including proliferation and migration[3]. At the site of ischemic infarct and penumbral legion, activated microglia are admixed with infiltrated macrophage[4, 5] described as M1 and M2 phenotypes[6]. Polarization of activated microglia is a two-edge sword, which involves the classical M1 and alternative M2 phenotype.[7] In the ischemic stroke environment, microglia act swiftly to form of an active immune defence. They migrate readily to the site of infarct to perform phagocytic clearance of cellular debris thus serving a protective function at the early onset of the ischemic damage.[8] Additionally, in response to neuronal injury, microglia activated

primarily of M1 phenotype and release a variety of proinflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), inducible nitric oxide synthase (iNOS), nitric oxide (NO) and reactive oxygen species (ROS) etc[9].On the other hand, in adverse conditions, some activated microglia maybe polarized to M2 phenotype which is known to produce large amounts of anti-inflammatory and neurotrophic mediators, such as interleukin-10 (IL-10), interleukin-4 (IL-4), CD206 and arginase-1 (Arg-1) etc, that help mitigate the inflammatory reaction M2 microglia phenotype is regarded to be neuroprotective as they foster the progressive neural tissue reconstruction and restoration of neurological function[10]. Therefore, suppressing M1 microglia activation has been widely considered a therapeutic option to attenuate or arrest undesirable neuronal damage and promote activated micrglia polarization forward M2 phenotype. In consideration of this, it is therefore essential to fully define the underlying mechanisms of key signaling pathways that regulate microglia activation.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases that are involved in various cellular responses such as cell proliferation, differentiation and apoptosis. There are three main members of the MAPKs family, which are the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, and each member exerts different biological functions[11]. The activated ERK1/2 MAPK pathway takes part in cellular proliferation and survival; however, JNK and p38 MAPK partake in apoptosis[12]. MAPKs signaling pathway is well known to be a major regulator of proinflammatory cytokines in lipopolysaccharide (LPS)-stimulated microglia[13]. MAPKs signaling pathway regulates the production and release of proinflammatory factors by activated microglia[14]. Among them, p38 MAPK and ERK appear to be primarily involved in the production of proinflammatory mediators in activated microglia. We have reported previously that scutellarin, a herbal compound, acts via MAPKs pathway to regulate the expression of iNOS, TNF-α and IL-1β in activated microglia, however, it remained uncertain whether scutellarin is able to promote M2 microglia polarization via MAPKs pathway.[15]

Scutellarin, 5,6,4-Trihydroxyflavone-7-O-glucoronide is a flavone and the major active component of *Erigeron breviscapus* (Vant.) *Hand-Mazz*. It is widely used in China for treatment of the cerebrovascular diseases and neurodegenerative disease[16]. Increasing reports in recent years have shown the beneficial effects of scutellarin in different experimental animal and cell models owing to its anti-oxidant[17], anti-inflammatory[18], and calcium channel antagonist properties[19]. It has been reported that scutellarin can attenuate ischemic brain injury in patients and animal models. More specifically, it has been shown to significantly reduce the infarct size as well as improve neurological scores[3]. Additionally, we have shown that scutellarin was able to attenuate the expression of different proinflammatory mediators by activated microglia through MAPKs pathway, such as TNF-α, IL-1β and iNOS which are markers of M1 microglia[20]. However, It remains obscure whether scutellarin would act to promote M2 polarization to exert of anti-inflammatory function. The objective of this study was to explore if scutellarin would exert its anti-inflammatory effect though M2 polarization via regulating the MAPKs pathway in an experimentally induced stroke model as well in BV-2 microglial cells challenged with LPS. More importantly, the study may turn up the realities that the MAPKs pathway might serve as a therapeutic target to promote M2 microglia polarization for functional recovery following an ischemic stroke.

Materials And Methods

Ethics statement

This study was performed in line with the principles of the Declaration of Helsinkik. Ethical guidelines as described in the National Institutes of Health Guidelines for The Care and Use of Laboratory Animals are used in the handling and use of rats. All experimental protocols and use of animals were approved by Kunming Medical University and all efforts were made to minimize the number of rats used and their suffering.

Animals and experimental groups

Seventy-eight adult male SD rats, weighing 250-280g, were provided by experimental Animal Center of Kunming Medical University. All animals were cared for in accordance with the NATIONAL Institutes of Health Guidelines for The Care and Use of Laboratory Animals. The animals were randomly divided into sham-operated + saline (sham), middle cerebral artery occlusion (MCAO) + saline (MCAO), and MCAO + scutellarin(100mg/kg) groups[62] (Table 1).

MCAO group was anesthetized with pentobarbital sodium intraperitoneally. The middle cerebral artery was cauterized or permanently occluded in rats. To do this, a dental drill is used to drill a round hole 3 mm in diameter into the parietal bone(as reported previously by as[37]). Expose the main middle cerebral artery and cut off the blood supply. In sham rats, the same procedure was performed, but the middle cerebral artery (MCA) was not severed. If the rats died during or after surgery, the died rats were removed out of groups.

Injection of scutellarin

The rats in MCAO + scutellarin groups were given an intraperitoneal injection of scutellarin (100mg/kg dissolved in saline[62]; Cat. No. 131021, Shanghai Winherb Medical Technology, China) at 2 h before MCAO and at 12, 24, 48 and 60h after MCAO. The rats were sacrificed at 1, 3 and 7d，n=5 for each time point，after MCAO; for sham, n=3 for each time point. In our preliminary study, we found that microglia response to ischemic cerebral cortex was strongest at 3d compared to other time points. In view of this, both Western blot and immunofluorescence labeling results in this study focused on this time point.

BV-2 microglia cell culture and treatment

Bv-2 microglia (ATCC, USA) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) in a moist incubator with 5% CO2 at 37℃. The cells were divided into control, LPS induced, inhibitor treatment, LPS + scutellarin, LPS + inhibitor, LPS + scutellarin + inhibitor groups. Inhibitors of MAPKs pathway includes p38 inhibitor(America, APExBIO, Cat.SB203580), JNK inhibitor(America, MCE, Cat.HY-12041) and ERK inhibitor(America, MCE, Cat.HY-112287). In all of the experiments, the cells were seeded at a density of 6×10^5 cells/well in six-well plates. The cells were pretreated with inhibitor(p38 inhibitor at a dosage of 10μM; JNK inhibitor at dosage of 25μM; ERK

inhibitor at dosage of 7.5μM as recommended by the manufacturer's instructions) for 1h followed by washing thoroughly with phosphate buffered saline (PBS). After that, the cells were pretreated with scutellarin (0.54μM[62]) for 1 h at 37°C in a humidified incubator under 5% CO_2. After incubation, the culture medium was discarded, the cells were washed with PBS and then incubated with LPS (1 mg/ml, Sigma-Aldrich, MO, USA) for 3 h. The medium was replaced with basic DMEM before treatment. As a control, the medium was replaced by basic DMEM cultured in 95% air and 5% CO2. Finally, proteins were extracted from the cells for Western blot analysis.

Western blot analysis for MCAO tissues and BV-2 cells

A total of 39 rats were used for Western blot analysis. The sham-operated (n=3) and MCAO rats given saline or scutellarin injections (n=5), respectively, were sacrificed at 1, 3 and 7d. The control or ischemic cortex was dissected and immersed in lysis buffer (1×RIPA lysis buffer, protease-inhibitor cocktail, and phosphatase-inhibitor cocktail) (Cell Signaling Technology) for 10 min on ice cubes. Tissue samples from various groups were homogenized with protein extraction reagent (Pierce, IL, USA) containing protease inhibitors.

For Bv-2 culture,PBS cells were washed twice, placed at 4 ° C, in lysis buffer and cell lysis for 10 min, then mechanically scraped off with a rubber scraper and centrifuged at 14000 RPM for 10 min. Protein concentrations in brain tissue and BV-2 cells were determined using bicinchoninic acid (BCA) method. Samples of supernatants containing 50μg protein of tissues or 30μg protein of BV-2 cells were loaded and heated at 95°C for 5 min and separated by sodium dodecyl sulfate-poly-acrylamide gel electrophoresis in 10% or 12% gels, in a Mini-Protein II apparatus (Bio-Rad, CA, USA). Protein bands were electroblotted onto polyvinylidene difluoride (PVDF) membrane and blocked with non-fat dried milk for 1 h. The membranes were incubated with primary antibodies against(Table 2) CD206 (rabbit monoclonal IgG 1:1000), IL-10 (rabbit polyclonal IgG 1:2500), IL-4 (mouse polyclonal IgG 1:2000), YM1/2 (rabbit polyclonal IgG 1:10000) , Arg1 (Goat polyclonal IgG 1:500) and β-actin (mouse monoclonal IgG 1:5000), p-ERK1/2(rabbit monoclonal IgG 1:4000) , p-JNK (rabbit monoclonal IgG 1:4000), and p-p38(rabbit monoclonal IgG 1:4000). Primary antibodies were diluted in Tris-Buffered Saline-0.1% Tween (TBST) overnight at 4°C. They were then incubated with the secondary antibodies, either with horseradish peroxidase (HRP) conjugated anti-rabbit IgG (dilution 1:5000) (Chemicon; Cat. NO. AP32P) or anti-mouse IgG (dilution 1:5000) (Chemicon; Cat. AP124P). Specific binding was revealed by enhancing the chemiluminescence kit according to the manufacturer's instructions. Image J software was used to quantify the frequency band intensity. All experiments were repeated at least three times.

Double immunofluorescence labeling of microglia in MCAO and BV-2 cells

Thirty-nine rats in all groups were labeled with double immunofluorescence. After deep anesthesia with 3% pentobarbital sodium intraperitoneally, the rats were infused with 2% paraformaldehyde in 0.1m phosphate buffer. The brain was taken and paraffin embedded. The coronal brain sections were cut with 8μm thickness on a microtome. Sections were rinsed with PBS. To block non-specific binding proteins,

tissue sections were incubated at room temperature (22-24 ℃) in 5% normal goat serum diluted with PBS for 1 h. After discarding the serum, the sections were incubated in a humidified chamber with primary polyclonal antibodies against CD206 (1:200), Arg1 (1:200), IL-4 (1:100), IL-10 (1:200), YM1/2 (1:400), p-ERK1/2 (1:200), p-JNK (1:200), and p-p38 (1:200), diluted with PBS overnight at 4°C. Following rinsing in PBS, sections were incubated, respectively, with fluorescent secondary antibodies: Cy3-conjugated secondary antibody (1:200; Jackson Immuno-Research; Cat. NO. 125364) and FITC (1:200; SIGMA; Cat. No. SLPB1894V) conjugated lectin (Lycopersicon esculentum) that labels both microglia and rascalar endothelial cells for 1 h at room temperature. After 3 rinses with PBS, the sections were mounted with a fluorescent mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI). Colocalization was detected by confocal microscopy（Laser confocal scanning microscope, Leica SP5）.

Bv-2 cells were immobilized with 4% paraformaldehyde 0.1 M PBS for 20 min. After washing with PBS, immunofluorescence staining was performed on the cover glass of adherent microglia. In each group, bv-2 cells were incubated with the primary antibody for 4℃ overnight. Finally, the cells were incubated in FITC (1:200; SIGMA; Cat. No. SLPB1894V) /Cy3(1:200; Jackson Immuno-Research; Cat. NO. 125364)-conjugated secondary antibodies for 1 h at room temperature. After washing, the coverslips were mounted with a fluorescent mounting medium with DAPI. Colocalization was visualized by immunofluorescence microscopy (Zeiss cell observer, Zeiss CaT #1026470987). The quantification of immunofluorescence intensity in the images was expressed as integrated density, which was quantified using Image J software; changes in intensity were then plotted as bar graphs. (Table 2)

Statistical analyses

The data were presented as mean standard deviation (M±SD). The statistical significance was assessed by one-way analysis of variance (ANOVA), followed by multiple comparisons using Dunnet's test to determine the statistical significance between groups. When $p < 0.05$, the difference was considered statistically significant. Prism 5.0 statistical software was used to analyze all data.

Results

Scutellarin promoted M2 markers CD206, YM, Arg1, IL-10 and IL-4 protein expression in cerebral cortex in MCAO rats sacrificed at 1d, 3d and 7d.

By Western blot analysis, the expression of CD206, YM, Arg1, IL-10 and IL-4 in the cerebral cortical tissues in MCAO rats was significant increased when compared with the sham group. The protein expression of M2 makers CD206, YM, Arg1, IL-10 and IL-4 was further augmented in MCAO group given scutellarin pretreatment (Fig.1,supplementary Fig.1-Fig.2), is being more pronoun in MCAO rats sacrificed at 3d (Fig.1), when compared with1d (supplementary Fig.1) and 7d (supplementary Fig.1).

Scutellarin induced immunofluorescence of M2 markers CD206, YM, Arg1, IL-10 and IL-4 in activated microglia in MCAO rats sacrificed at 1d, 3d and 7d.

Consistent with Western blot analysis, immunofluorescence labeling showed increased CD206, YM, Arg1, IL-10 and IL-4 immunoceactivity in activated microglia double labeled with lectin in MCAO rats. The immunofluorescence of all biomarkers was further enhanced in MCAO rats given scutellarin treatment. Of note, the immunofluorescence of all M2 microglia markers was most conspicuous when MCAO rats were pretreated with scutellarin and sacrificed at 3d(Fig. 2). The results of MCAO rats sacrificed at 1d and 7d are presented in the supplementary Figure 1-2.

Scutellarin upregulated expression of CD206, YM, Arg1, IL-10 and IL-4 in LPS-activated BV-2 microglia.

By Western blot analysis, CD206, YM1/2, Arg1, IL-10 and IL-4 expression in LPS-activated BV-2 microglia was increased when compared with the control group. The protein expression of M2 markers CD206, YM, Arg1, IL-10 and IL-4 was further increase in LPS group given scutellarin pretreatment (Fig.3).

Scutellarin promoted CD206, YM, Arg1, IL-10 and IL-4 immunoreactivity in LPS-activated BV-2 microglia.

Compatible with the Western blot analysis, immunofluorescence labeling showed increased CD206, YM, Arg1, IL-10 and IL-4 immunofluorescence in activated BV-2 microglia subjected to scutellarin pretreatment.(Fig. 4)

Effects of scutellarin on Arg1 and MAPKs signaling pathway proteins P-JNK and P-P38 in microglia

immunofluorescence double staining with M2 microglia marker Arg1(green) and activated MAPKs pathway proteins p-JNK and p-P38 (red). The results revealed small volumes of inactive cells in the control group. Microglias with many branches hardly expressed m2 microglia markers (Arg1, green), while sedentary microglia almost did not express activated MAPKs pathway proteins (p-JNK and p-P38, red). No co-expression of M2 microglia marker and activated MAPKs pathway proteins was observed. In the LPS group, there were a large number of activated microglias with large volume, approximately round shape and few branches The number of Arg1 immunopositive cells of M2 microglias increased, and the number of immunopositive cells of activated MAPKs pathway proteins p-JNK and p-P38 increased, which were co-expressed with some Arg1 positive microglias (yellow). Compared with the control group, Arg1, p-JNK and p-P38 were significantly increased in LPS group, and the expression levels of p-JNK and p-P38 were significantly decreased in LPS group. After pretreatment with scutellarin, the expression of Arg1 was significantly increased, while the expression of p-JNK and p-P38 was significantly decreased, which was statistically different from that in LPS group. (Fig 5,6)

Effects of scutellarin on Arg1 and MAPKs signaling pathway proteins p-ERK1/2 in microglia

immunofluorescence double staining with M2 microglia marker Arg1(green) and activated MAPKs pathway proteins p-ERK1/2 (red). The results revealed small volumes of inactive cells in the control group. Microglias with many branches hardly expressed m2 microglia markers (Arg1, green), while sedentary microglia almost did not express activated MAPKs pathway protein (p-ERK1/2, red). No co-expression of M2 microglia marker and activated MAPKs pathway protein was observed. In the LPS group, there were a large number of activated microglias with large volume, approximately round shape

and few branches The number of Arg1 immunopositive cells of M2 microglias increased, but the number of immunopositive cells of activated MAPKs pathway proteins p-ERK1/2 decreased, which were co-expressed with some Arg1 positive microglias (yellow). After pretreatment with scutellarin, the expression of Arg1 was significantly increased, and the expression of p-ERK1/2 was significantly increased, which was statistically different from that in LPS group. (Fig.7)

p38 Inhibitor (SB203580) increased YM, Arg1 and IL-10 expression level in LPS-activated BV-2 microglia.

SB203590 treatment resile in significantly increased YM, Arg1 and IL-10 expression level by western blot in LPS-activated microglia group. More importantly, SB203580 suppressed p-p38 protein expression level in LPS-activated BV-2 microglia that was coupled with increased expression of M2 microglia makers (YM, Arg1 and IL-10) in LPS-activated BV-2 microglia. (Fig.8)

JNK Inhibitor (HY-12041) increased YM, Arg1 and IL-10 protein expression level in LPS-activated BV-2 microglia.

By Western blot analysis, HY-12041 increased YM, Arg1 and IL-10 expression level signifcantly in LPS-activated microglia group. HY-12041 suppressed p-JNK protein expression level in LPS-activated BV-2 microglia that was coupled with increased expression of YM, Arg1 and IL-10 in LPS-activated BV-2 microglia.(Fig.9)

ERK1/2 Inhibitor (HY-112287) decreased YM, Arg1 and IL-10 protein expression level in LPS-activated BV-2 microglia.

By Western blot analysis, HY-112287 suppressed YM, Arg1 and IL-10 expression level signifcantly in LPS-activated microglia group. Remarkably, HY-112287 suppressed not only p-ERK1/2 protein expression level in LPS-activated BV-2 microglia but also decreased expression of all M2 microglia makers YM, Arg1 and IL-10 in LPS-activated BV-2 microglia.(Fig.10)

Discussion

Cerebrovascular diseases have surpassed cancer as the main risk factor endangering human health and life safety; indeed they account for a large proportion of morbidity and mortality[21]. In cerebral ischemia, neuronal death and apoptosis, glial activation and glia-mediated inflammatory response are featured prominently in the core area and penumbra of ischemia[22].

Characteristics of activated microglia polarization and its mediated inflammatory response

In ischemic stroke, microglia refracted their branching processes and acoumed an amoeboidic phenotype with a large round cell body. Meanwhile, the expression of CD11b protein in activated microglia is increased[23]. Achieved microglia in cerebral ischemia microglia have opposing functions. Activated microglia are engaged in phagocytosis in damage area and penumbra zone; at the same time, they also release different neurotophic and inflammatory factors so that the microenvironment in damaged area

and penumbra zone is effectively maintained for tissue restoration[24]. In the early stage of ischemic stroke, activated microglia were of M2 phenotype. With the progress of the disease, they gradually transformed into M1 phenotype causing inflammation and cell toxicity[25]. Therefore, microglia are activated and are functional immediately after acute ischemic stroke. They then transform into M1 phenotype within 7 days[25]. If there is no proper or adequate therapeutic interference at this stage for ischemic stroke, M1 microglia would release inflammatory factors and cytotoxic factors, such as TNF-α, iNOS and IL-1β, can further activated organization complex signaling cascade. Excessive inflammatory reaction can further activated microglia and astrocytes which would accelerate the glial cells to clean up cellular debris in damage and penumbra areas and aggravate the damage of neurons or apoptosis[26, 27]. However, excessive inflammatory response will lead to apoptosis or death of neurons in the penumbrae area that still possess the ability to recover, thus this would aggravate the cerebral infarct damage caused by ischemic stroke thus making the recovery after ischemic stroke more difficult[28, 29]. However, M2 microglia release anti-inflammatory factors, such as IL-10, TGF-β and IGF-1, which can attenuate inflammatory reaction, and effectively promote damage neuronal recovery in the penumbrae area and reduce the brain infarction volume after ischemic stroke. It would appear that IL-10 and TGF-β expression of mRNA occurs to reach the top after stroke peaks at 2-6 days; therefore, the expression level was decreased. TGF-β secreted by activated microglia not only it can protect the damage of the nervous system, but it can also promote proliferation of glial cells and strengthen the biological function of microglia[30]. Therefore, the present study who aimed to identify an effective therapeutic strategy to promote polarization of M1 microglia into M2 microglia considerate to be neuroprotective in view of their antiinflammation note.

Effect of scutellarin on polarization of microglia

Activated microglia are classified into classical activated state (M1) and alternative activated state (M2) [31]. In cerebral ischemia, M1 microglia are activated and show increased expression of toll-like receptors. The cell body appear to be more rounded and hypertrophic. Microglia reduced their processes and produce different proinflammatory factors, such as IL-1β, IL-6 and TNF-α, as well as CCL2 and among other cytokines chemokines. All that can cause excessive inflammatory reaction can have toxic effects on neurons, and tissue damage[32].

M2 microglia are divided into the M2a, M2b, M2c and M2a sub-phenotypes and are induced by IL-4 and IL-13. They exhibit smaller and few protrusions, which produce high levels of anti-inflammatory factors such as IL- 4, IL-13 and transforming growth factor beta (TGF-β). This is accompanied by reduced expression of interleukin 12 (IL-12) but a high expression of IL-10. M2 phenotype inhibits excessive inflammatory reaction and promote tissue repair and neuron regeneration, which can prevent excessive inflammatory reaction mediated by M1 microglia. Markers of M2 phenotype include arginase-1 (Arg1), CD206[33]. When IL-1β and LPS are applied at the same time, or when exposed to IgA immune complexes, microglia can activate to become M2b phenotype with immune function. The markers are shared by both M1 microglia and M2a microglia. Meanwhile,the expression of signal transduction and transcription activating factor 1 (STAT1), and nuclear transcription factor κB (NF-κB) is increased. Thus,

M2b phenotype has the dual functional roles of pro-inflammatory/anti-inflammatory. After phagocytosis of apoptotic cells, microglia showed an activated anti-inflammatory M2c phenotype. M2c microglia can help tissue remodeling and cell regeneration after the down-regulation of inflammatory response, and its markers such as CD16, CD206 and TGF-β[34].

In this study, Western blot results showed that scutellarin effectively promoted expression of M2 microglia markers CD206, IL - 10, IL - 4 YM1/2 and Arg1 in LPS-activated BV2 microglia. Furthermore, by immunofluorescence staining, LPS-activated microglia increased production of more neurotrophic factors (YM1/2 and Arg1) and neuroprotective factors (IL-10 and IL-4). Of note, the expression of M2 microglia makers was increased after pretreating the cells with scutellarin. Along with BV-2 microglia, we investigated the experimental results by using a rat model of middle cerebral artery occlusion[35, 36]. In agreement with our previous study, we found that scutellarin can significantly reduce the cerebral infarction volume of MCAO rat model. Additionally, scutellarin inhibited effectively inflammatory cytokines and cytotoxic factors secreted by M1 microglia[37]. The present research have shown that scutellarin, not only can it effectively inhibit the M1 microglia phenotype to reduce and excessive microglia-induced inflammatory reaction, but it can also promote M2 microglia polarization. In the later, it is conceivable that large amounts of nerve neurotrophic factors and neuroprotective factors wouldbe produced promote the neurofruction of nervous tissue. Already, many fuclies have shown that polarization microglia to M2 phenotype is pivotal to the functional recovery after ischemic stroke.

Scutellarin acts via MAPKs pathway to promote M2 polarization

MAPKs signaling pathway plays an important role in the development of nervous system, and mediates neuroinflammatory response. Recent studies have shown that MAPKs signaling pathways are involved in central neuropathy, such as ischemic stroke and Alzheimer's disease, more especially, they play an important role in the pathophysiological processes such as apoptosis and inflammatory response[38]. As a classic signaling pathway of the nervous system, many studies have shown that the expression of various components of MAPKs signaling pathway is significantly altered during the ischemic stroke. It has been reported that this may be involved in the regulation of cell damage or repair[39-42]. Immediately after the cerebral ischemia, the G protein-coupled receptor activates protein kinase C (PKC) pathway, adenylate cyclase (AC) pathway, Phosphatidylinositol kinase (P13-K) pathway, tyrosine receptor pathway and calmodulin (Ca2+ CaM) pathway among others, which this activates the MAPKs signaling pathway, followed by the next cascade reaction[43]. Studies have found that MAPKs signaling pathway plays an important biological role in both transient and permanent ischemia in ischemic encephalopathy. Many signaling cascades have been reported at different sites of ischemia and hypoxia injury. If the neurons and glial cells in the central brain region were damaged first, the activation sequence of signal transduction pathway was ERK (2min to 2h after injury, mainly neurons)[39, 40], c-jun (30min to 6h after injury, mainly neurons)[44] and p38 (2d to 4d after injury, mainly glial cells)[45, 46]. It is described that the activation of the pathways mediated above can promote the death of neurons. In the rat MCAO model, the expression of ERK, JNK and p38 was significantly increased at different times after ischemia, respectively, thus confirming that cerebral ischemia can readily trigger the phosphorylation of MAPKs.

The results suggested that MAPKs signaling pathway is involved in the signal transduction of the early pathophysiological mechanism of cerebral ischemia[47]. In a recent study, it has been reported that the expressions changes of signal cascade reaction in the damaged core area and the surrounding penumbral area were not completely consistent. The phosphorylated JNK was significantly increased in the central area, while the p38 activity was significantly increased in the penumbral area[48]. Wang et al. showed that the expression of phosphorylated ERK1/2 was increased in the ischemic core and peripheral areas after 30 min in MCAO mouse models[44]. In our previous studies both *in vivo* and *in vitro*, Western blot results showed that in the rat MCAO model, the expression of p-p38 p-JNK and p-ERK in the cerebral cortex was significantly increased. Additionally, the MAPKs signaling pathway protein p38 was phosphorylated and increased in the LPS-activated BV-2 microglia. Concomitantly, the expression of p-JNK and p-ERK was also significantly increased. Immunofluorescence staining showed that p-p38 p-JNK and p-ERK expression was highly expressed in the rat MCAO model and LPS-activated microglia[37]. All this further demonstrated that MAPKs signaling pathway is involved in a series of pathophysiological changes after cerebral ischemia. Separately, Su et al. found in their studies that MAPKs signaling pathway was closely related to the polarization of M2 macrophages/microglia. When p38 and JNK inhibitors were used, the expression of p-p38 and p-JNK was significantly down-regulated. At this time, the expression of CD206 and Arg1, markers of M2 macrophages/microglias. When p38 and JNK were inhibited, macrophages/microglia would polarize to the M2 macrophages/microglia. However, when ERK protein was inhibited, the expressions of markers CD206 and Arg1 of M2 macrophages/microglias were significantly reduced, indicating that the number of M2 macrophages/microglias would be reduced when ERK protein was inhibited[49]. Together their results suggest that MAPKs signaling pathway is closely related to not only the occurrence and development of M1 microglia mediated inflammatory response, but also exerts neurotrophic and neuroprotective effects mediated by M2 microglia in cerebral ischemia injury.

Scutellarin possesses anti-oxidative and anti-inflammatory properties. It acts against anti-neuronal apoptosis, thus exerting neuroprotective effects. Presently, different preparations of scutellarin are widely used in the clinical treatment of cardiovascular diseases such as hypertension, angina pectoris, coronary heart disease, stroke etc. However, its complex mechanism of action remains yo be fully explored. Arising from the many studies using different experimental paradigms, it is evident that the pharmacological activity scutellarin involves multiple signaling pathways. Adding to this conundrum or the mechanism and precise target of pharmacological action of scutellarin which the remained obscure. In study by Tang H, et al., in the ischemia reperfusion injury in rats, it was reported that the expression of glycine γ-aminobutyric acid (GABA) and Tau protein (Tau) improved the activities of $Ca2^{+}$-ATP kinase, Na^{+}, K^{+}-ATP kinase and neuron injury[50]. In this study, it was found that scutellarin not only inhibited inflammatory response through MAPKs signaling pathway, but also promoted the polarization of M2 microglia through MAPKs signaling pathway, thus showing its effect of neuroprotection and nerve repair. *In vivo*, it was found that the expression of specific markers in activated M2 microglia was increased after the treatment of rat MCAO model with scutellarin. This was also evident by immunofluorescence staining, which showed that M2 marker-positive cells co-expressed with microglia.

The present results have shown that in LPS stimulated BV-2 microglia pretreated with scutellarin, the expression of the M2 markers are significantly increased. Of note in BV-2 microglia pretreated with p38 inhibitor and JNK inhibitor, the M2 microglia marker expression was significantly increased; on the other hand, with ERK1/2 inhibitor, the expression of M2 markers was decreased. By double immunofluorescence staining. Meanwhile, it was found that the number of M2 microglia marker-positive cells was increased. The number of p-p38 and p-JNK positive cells was decreased, whereas that of p-ERK1/2 positive cells was increased after scutellarin pretreatment. The present study showed that the p38 pathway is the most important member of the MAPKs family to control the inflammatory response. It can promote the activation and aggregation of white blood cells, and regulate the activity of transcription factors and inflammatory factors, thus playing a key role in the regulation of inflammatory response. In microglia activation, MAPKK is activated through some intermediate link. Activated-MAPKK regulates the activity of p38 phosphorylase, leading to phosphorylation of p38 and p-p38 is then froslocated to the nucleus to activate inflammatory factors such as TNF-α, iNOS and etc[39, 51, 52]. Concurrently, p38 and JNK regulate the M1/M2 polarization through the cytomembrane-5, -nucleotide enzyme, shifting making the activated microglia polarization toward the M1 microglia and showing pro-inflammatory effects[53]. Zhang, et al. found in their study in LPS-activated microglia that 4,5, 7-trihydroxy flavonone is dependent on MAPKs signaling pathway to promote the polarization of M2 microglia, especially the p38 and JNK signaling pathways. When p38 and JNK signaling pathway proteins are inhibited, leading to signaling pathway inactivation, the selective activators of p38 and JNK inhibit 4,5, 7-trihydroxy flavonone is promoting the polarization of microglia to M2 microglia[54]. Qu, et al. found that dexmedetomidine can effectively promote M2 polarization of activated microglia by inhibiting p38 and JNK, thereby reducing the inflammatory response in Parkinson's and Alzheimer's rat models[55]. The above results suggest that p38 and JNK signaling pathways can inhibit the polarization of M2 microglia, thus inhibiting the neurotrophic and neurorepair functions of activated microglia. This would ultimately aggravate the neuroinflammatory response and cause greater neural damage. As an effective inhibitor of p38 and JNK signaling pathways. We showed here that scutellarin can significantly promote the polarization of M2 microglia. Therefore, inhibiting p38 and JNK signaling pathways by scutellarin may prove to be a potential therapeutic strategy to filt the M2 polarization that neuroprotective essential for tissue repair.

ERK1/2, the first member of MAPKs signaling pathway family, is closely related to cell survival and proliferation[56]. ERK can regulate cell biological functions such as cell growth, proliferation, differentiation and apoptosis, and may play an important role in cerebral neuropathy and regulate the development of the disease. However, the exact role of ERK 1/2 in the death or survival of nerve cells is still controversial. Su et al reported that high glucose environment and TGF-β could effectively promote the polarization of activated microglia to M2 microglia. However, when Su et al inhibited ERK protein, the expression of markers CD206 and Arg1 of M2 macrophages/microglia was significantly reduced. This indicated that when ERK was inhibited, M2 macrophages/microglia were significantly decreased[49]. Separately, it was found that apoptotic SKOV3 cells stimulated the polarization of M0 macrophages to M2 macrophages by activating the ERK signaling pathway, and promoted the proliferation and migration of ovarian cancer cells by Zhang et al. When the ERK1/2 signaling pathway was inhibited, the number of

M2 macrophages decreased, and the proliferation and migration ability of ovarian cancer cells also decreased[57]. Cao demonstrated that hypoxia of tumor cells selectively promoted the polarization of macrophages to M2 microglia through the activation of ERK signal, thereby enhancing the metastasis of non-small cell lung cancer[58]. These studies confirmed that ERK signaling pathway plays a crucial role in the process of M2 polarization of microglia/macrophages. We have shown in the present study that scutellarin could promote the expression more M2 microglia markers in LPS-activated BV-2 microglia. More importantly, we have shown that scutellarin also promoted the expression of p-ERK1/2. In LPS stimulated BV-2 microglia pretreted with the ERK1/2 inhibitor HY-112287, the expression of p-ERK1/2 was decreased, and remarkable expression of M2 microglia markers was also down-regulated. This is contrary to the findings of Li, Zhang and Shan, who reported activation of ERK1/2 signaling pathway can inhibit the polarization of microglia/macrophages to M2, and conversely inhibition of ERK1/2 signaling pathway can promote the polarization of microglia/macrophages to M2[55, 59-61]. Discrepancy in results may be attribute to the difference between experimental conditions and detection indicators, but the undulying mechanism requires further investigation .

Conclusion

All in all, the present results have shown that LPS-activated BV-2 microglia and rat cerebral ischemia-activated microglia overexpressed M2 microglia markers CD206, Arg1, YM1/2, IL-4 and IL-10. However, in MCAO and BV-2 microglia given scutellarin pretreatment the expression of M2 microglia makers. It is justified to suggest that this would reduce the microglia mediated inflammatory response, because M2 microglia are known to exert neuroprotective effect and promote tissue repair, after cerebral ischemia injury essential for neurological recovery. The detection of MAPKs signaling pathway and along with the pretreatment of p38 inhibitors (SB203580), JNK inhibitors (HY-12041) and ERK1/2 inhibitors (HY-112287) confirmed that scutellarin may directly promote the polarization of M2 microglia and its expression of neurotrophic and protective mediators by inhibiting the JNK and p38 signaling pathways. Moreover, scutellarin promote the polarization of M2 microglia by augmenting the ERK1/2 signaling pathway (Fig. 11).

Abbreviations

BCA bicinchoninic acid

CNS central nervous system

DAPI 4’, 6-diamidino-2-phenylindole

DMEM Dulbecco’s modified Eagle’s medium

ERK1/2 extracellular signal-regulated kinase1/2

FCS fetal calf serum

GEB Gastrodia elata Blume

GSK-3β Glycogen synthase kinase-3 beta

HRP horseradish peroxidase

IL-10 interleukin-10

IL-4 interleukin-4

IL-1β interleukin-1β

iNOS inducible nitric oxide synthase

JNK c-Jun N-terminal kinase

LPS lipopolysaccharide

MAPKs mitogen-activated protein kinases

MCAO middle cerebral artery occlusion

PBS phosphate buffered saline

p-JNK phosphorylated c-Jun N-terminal kinase

p38 MAPK p38 mitogen-activated protein kinase

p-p38 phosphorylated p38

p-ERK1/2 phosphorylated extracellular signal-regulated kinase1/2

PVDF polyvinylidene difluoride

SD rats Sprague-Dawley rats

TNF-α Tumor necrosis factor-α

Arg-1 arginase-1

Declarations

Consent to participate

Not applicable.

Ethics statement

All experiments were approved by the Experimental Animal Care and Use Committee of Kunming Medical University, and were in agreement with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Availability of data and material

The date and materials supporting the conclusions of this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

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Zhi Qi	Western Blot
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Tables

Table 1 Surgical procedures and number of rats used in various treatments

	Sham-operated + saline group(sham)	MCAO + saline group (MCAO)	MCAO + scutellarin(100mg/kg) group
Double immunofluorescence	n=9	n=15	n=15
Western blot	n=9	n=15	n=15
Total	n=18	n=30	n=30

Table 2 Antibodies used for Western blotting and immunostaining

Anti-body	Host	Source	Catalog Number
CD206	Rabbit Monoclonal	Proteintech, Chicago, CA, USA	18704-1-ap
IL-10	Rabbit Monoclonal	Abcam, Cambridge, CA, UK	ab9969
IL-4	Mouse Monoclonal	Santa Cruz, CA, USA	Sc-53084
Arg1	Goat Monoclonal	Abcam, Cambridge, CA, UK	ab60176
YM1/2	Rabbit Monoclonal	Abcam, Cambridge, CA, UK	ab192029
β-actin	Mouse Monoclonal	Proteintech, Chicago, CA, USA	66009-1-lg
p-ERK1/2	Rabbit Monoclonal	Abcam, Cambridge, CA, UK	ab124956
p-p38	Rabbit Monoclonal	Cell Signaling Technology, Boston, CA, USA	4631S
p-JNK	Rabbit Monoclonal	Abcam, Cambridge, CA, UK	ab124956
Cy3	Rabbit	Proteintech, Chicago, CA, USA	SA00009-2
Cy3	Mouse	Proteintech, Chicago, CA, USA	SA00009-1
Cy3	Goat	Proteintech, Chicago, CA, USA	SA00009-3
FITC	Rabbit	Proteintech, Chicago, CA, USA	SA00003-2

Figures

Figure 1

CD206, YM, Arg1, IL-10 and IL-4 protein expression was increased in MCAO rats sacrificed at 3d given scutellarin treatment.

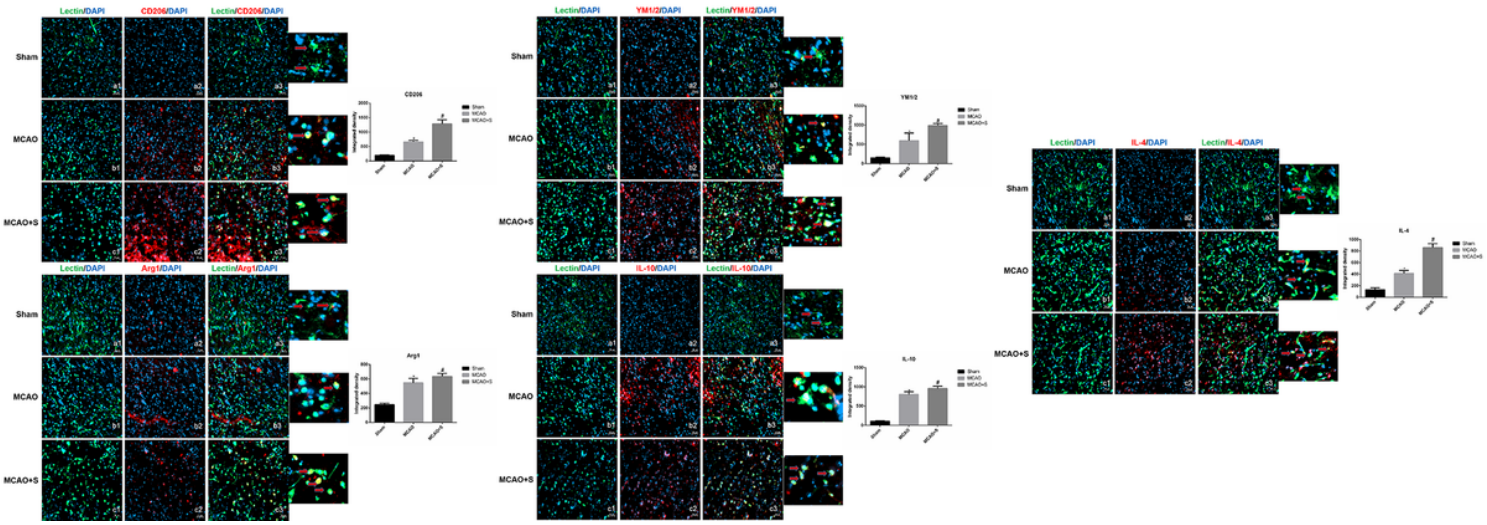

Figure 2

Scutellarin induced immunofluorescence of M2 markers CD206, YM, Arg1, IL-10 and IL-4 in activated microglia in MCAO rats sacrificed at 3d

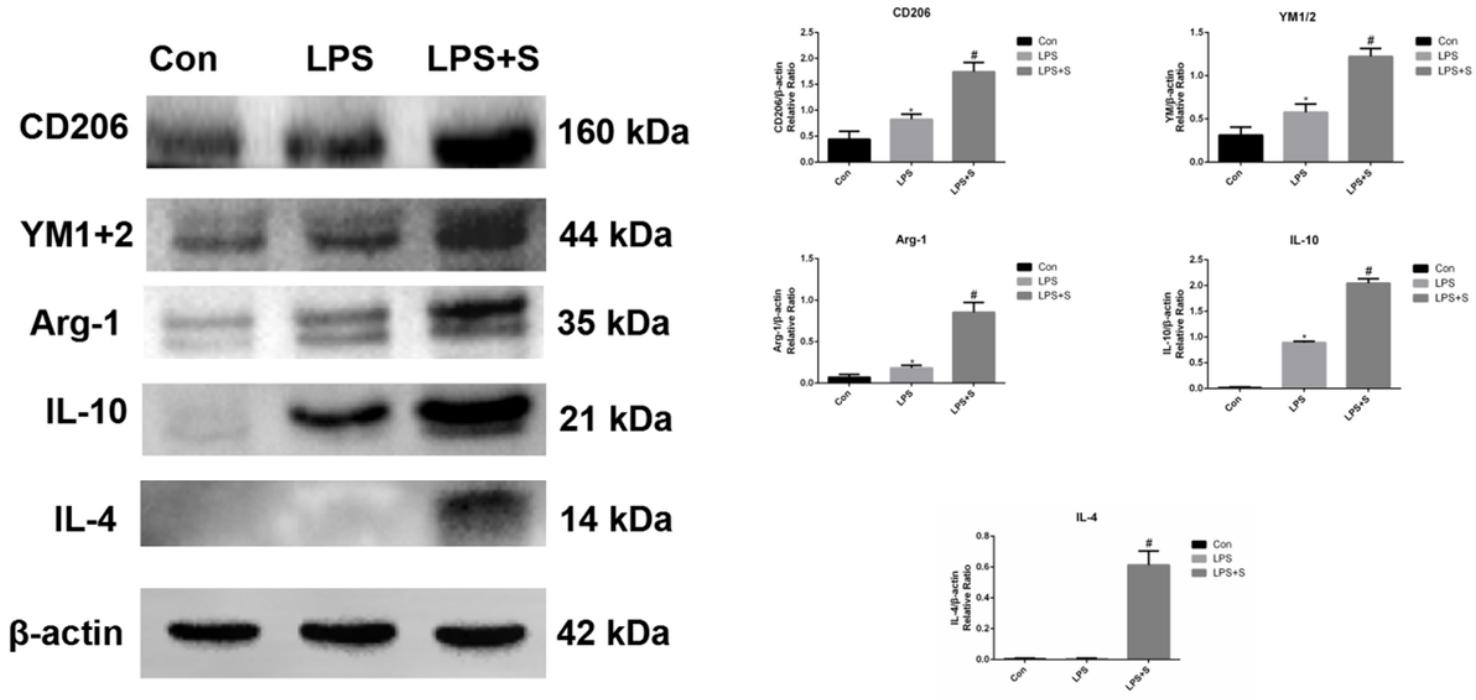

Figure 3

Scutellarin upregulated expression of CD206, YM, Arg1, IL-10 and IL-4 in LPS-activated BV-2 microglia

Figure 4

Scutellarin promoted CD206, YM, Arg1, IL-10 and IL-4 immunoreactivity in LPS-activated BV-2 microglia

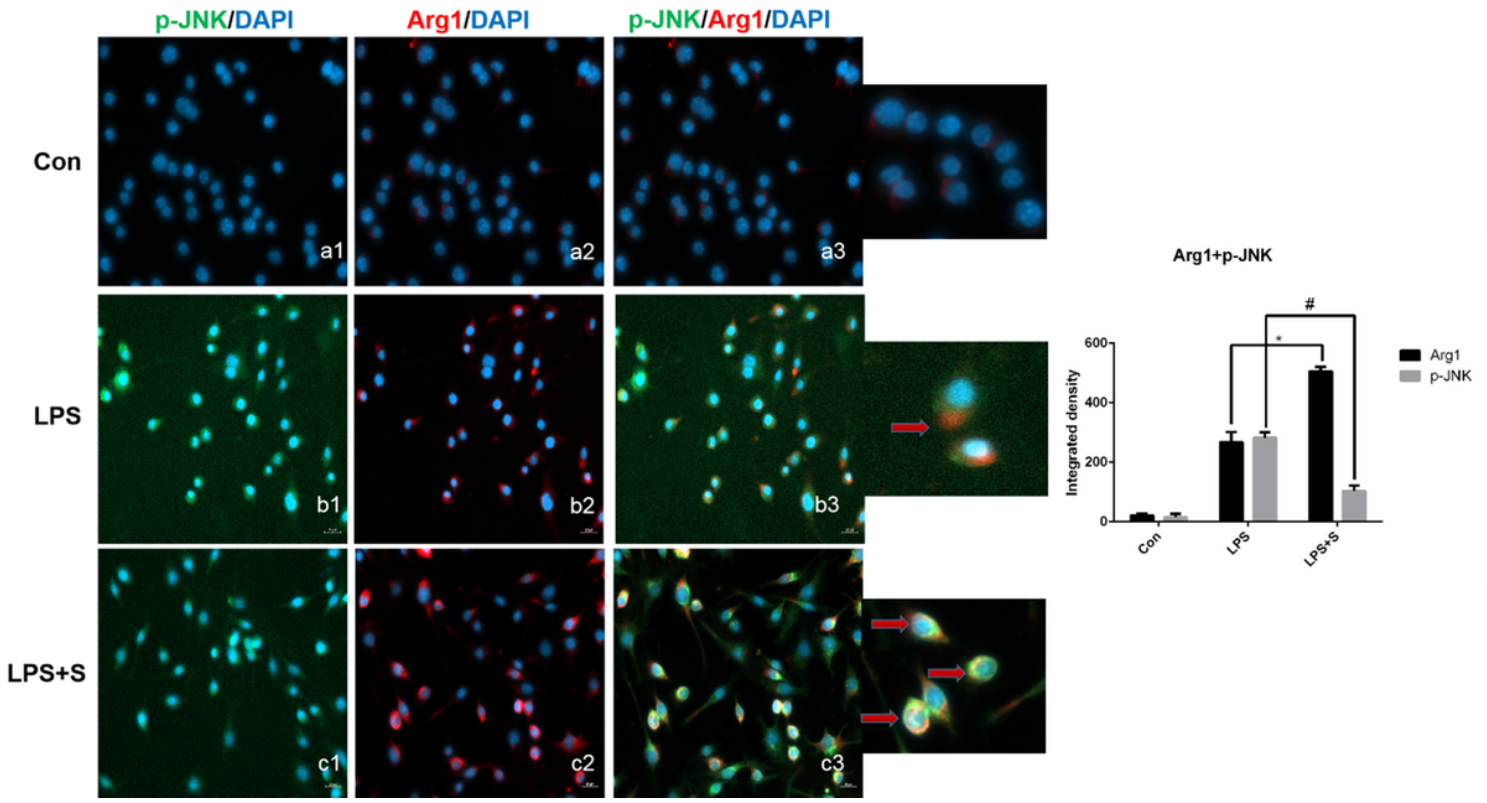

Figure 5

Effects of scutellarin on Arg1 and MAPKs signaling pathway proteins P-JNK in microglia

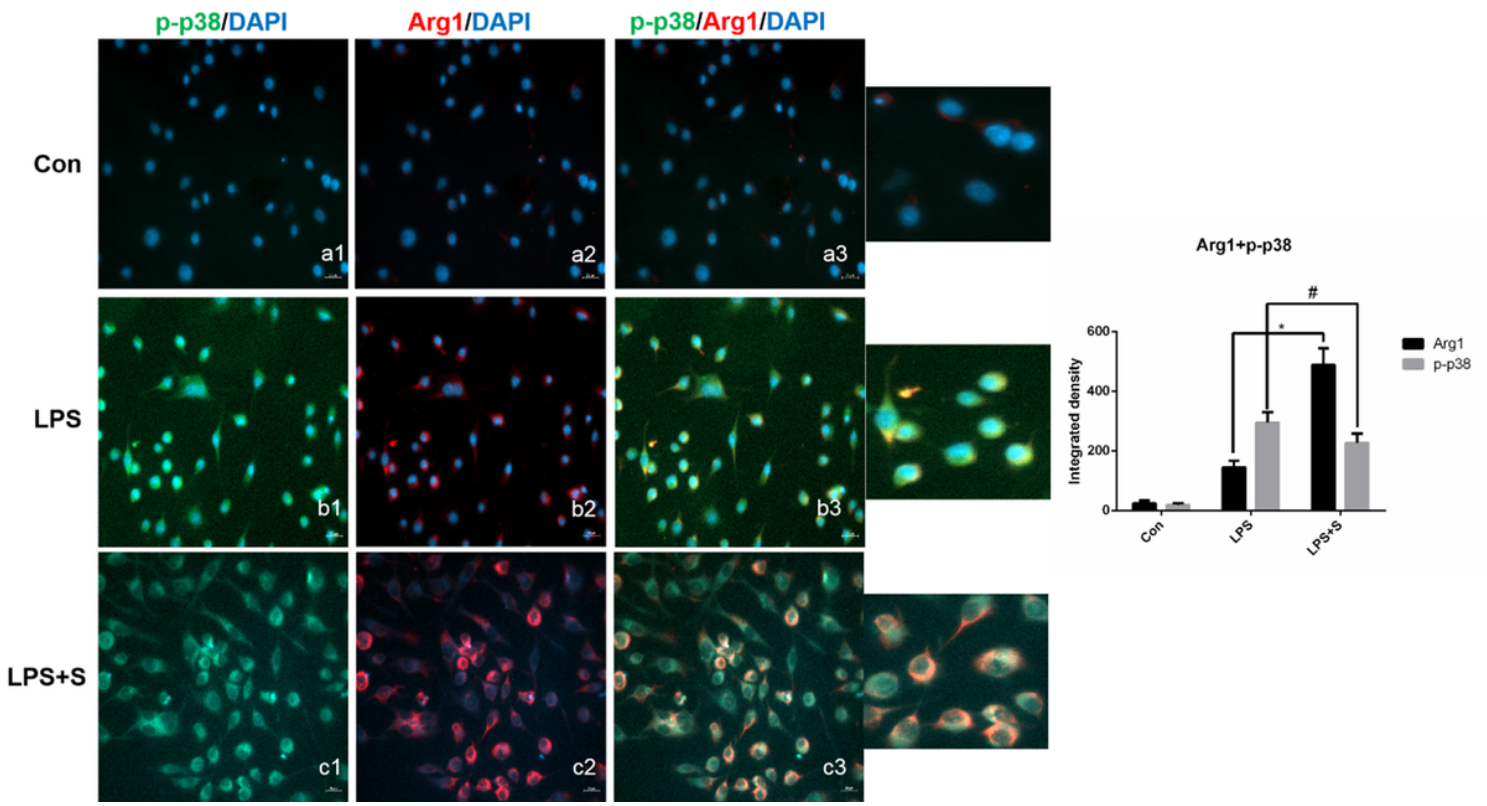

Figure 6

Effects of scutellarin on Arg1 and MAPKs signaling pathway proteins p-P38 in microglia

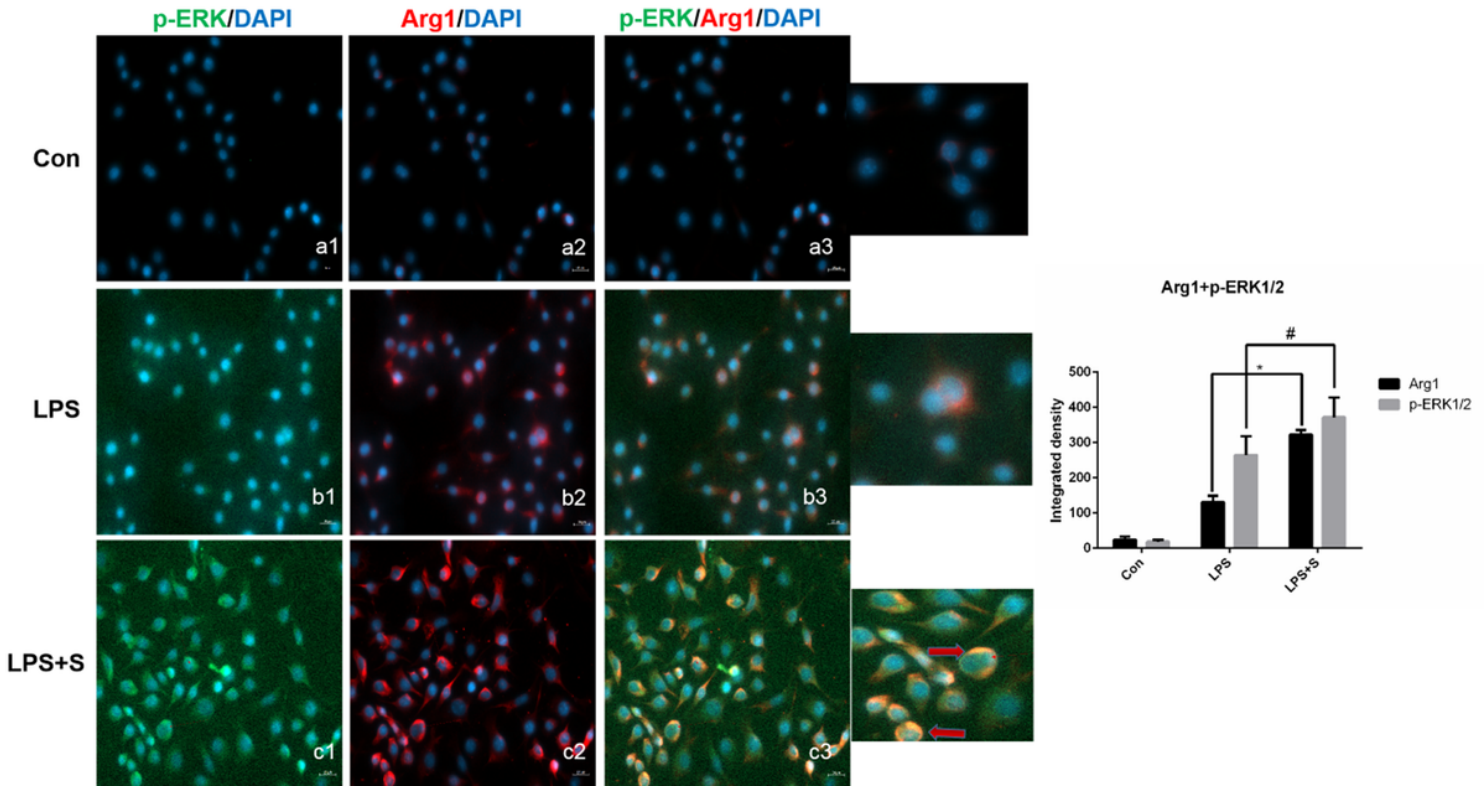

Figure 7

Effects of scutellarin on Arg1 and MAPKs signaling pathway proteins p-ERK1/2 in microglia

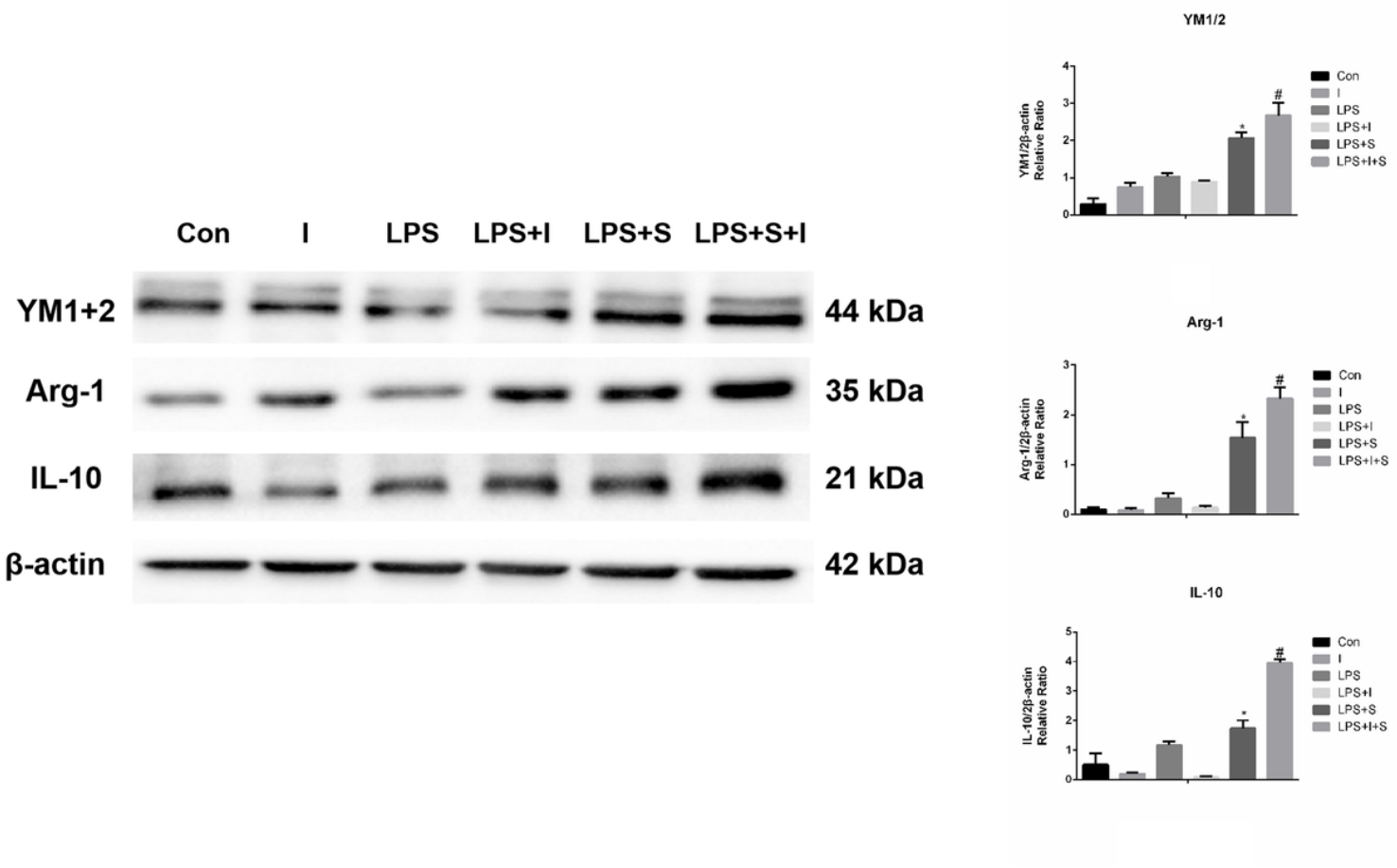

Figure 8

p38 Inhibitor (SB203580) increased YM, Arg1 and IL-10 expression level in LPS-activated BV-2 microglia

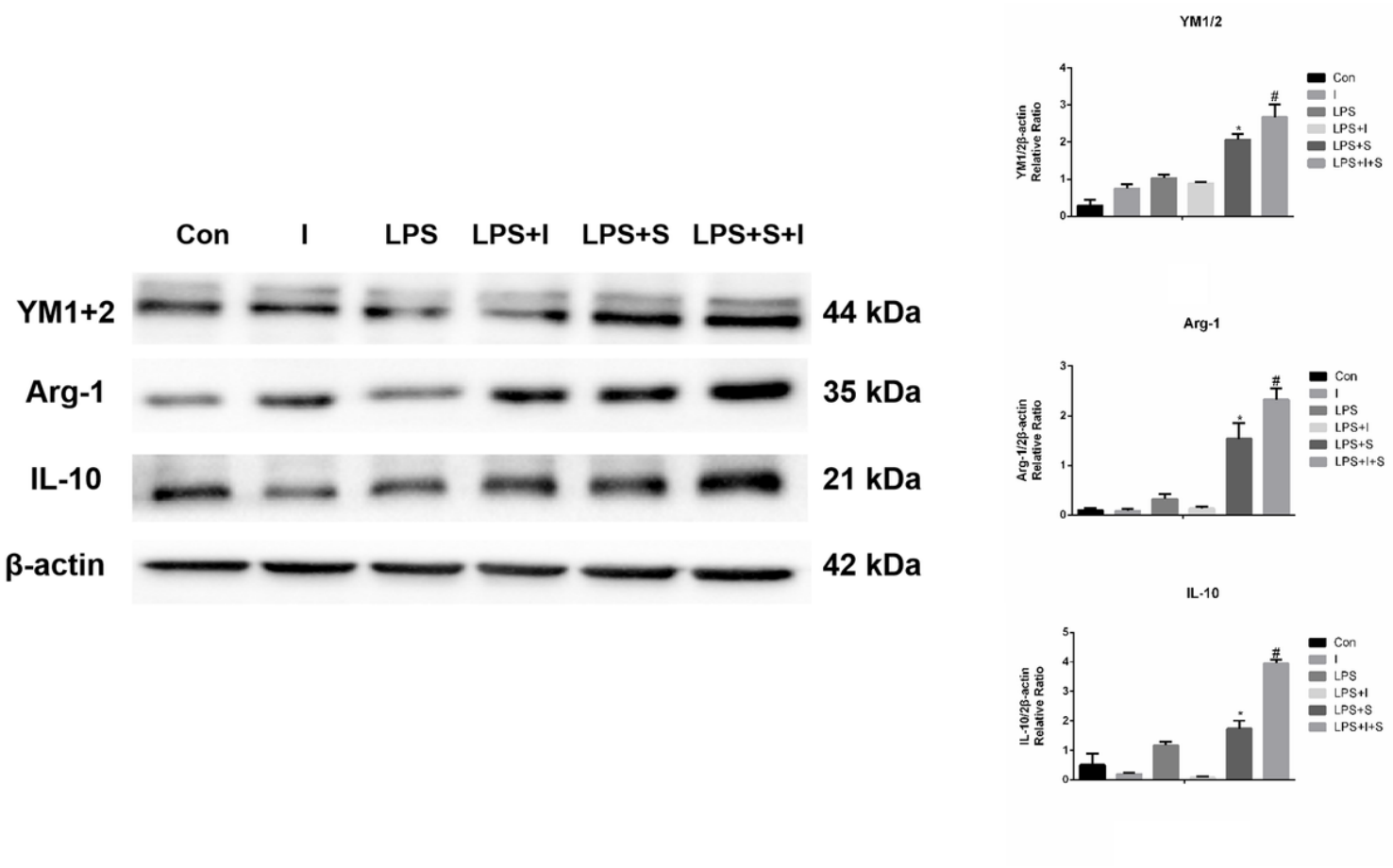

Figure 9

JNK Inhibitor (HY-12041) increased YM, Arg1 and IL-10 protein expression level in LPS-activated BV-2 microglia

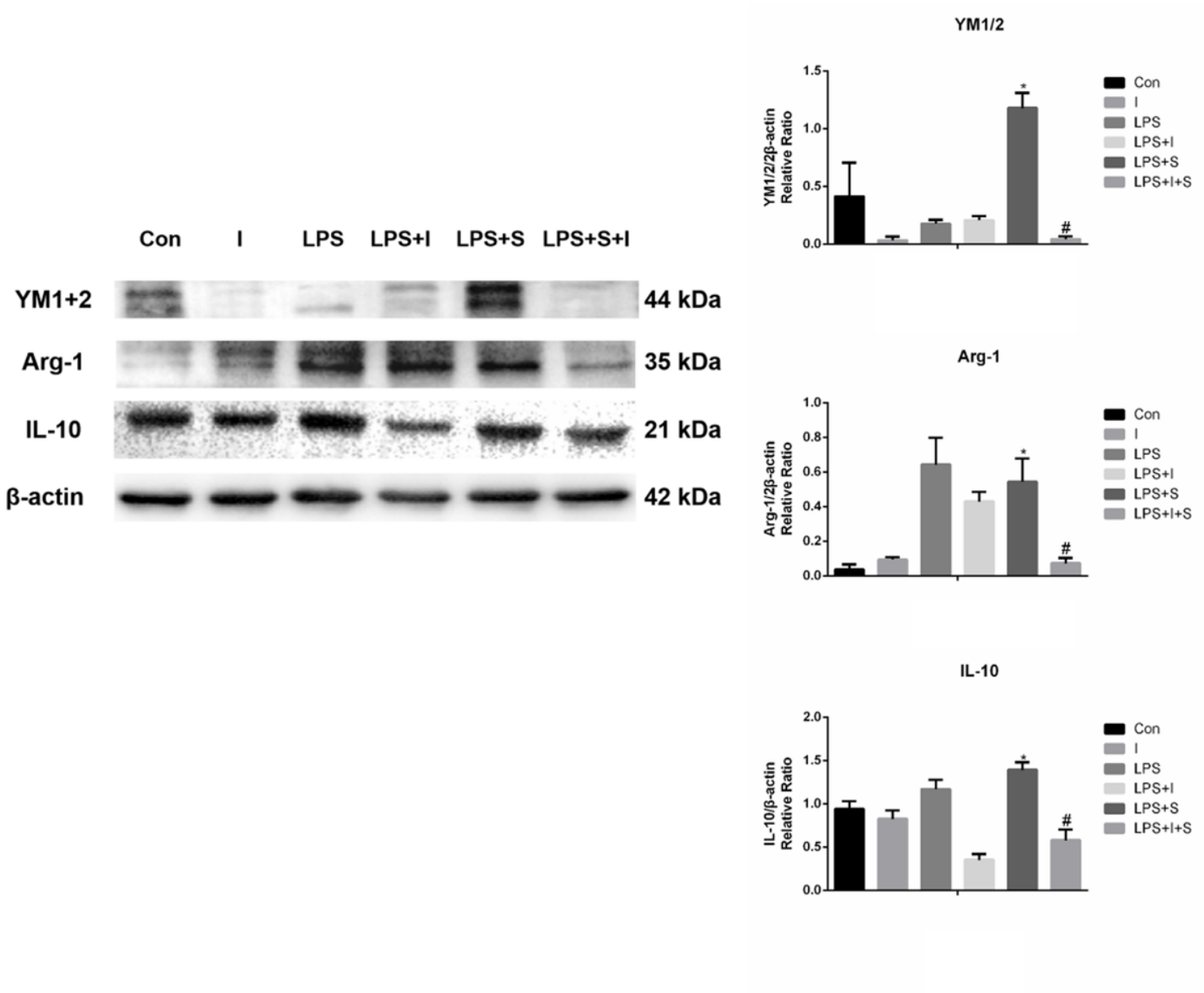

Figure 10

ERK1/2 Inhibitor (HY-112287) decreased YM, Arg1 and IL-10 protein expression level in LPS-activated BV-2 microglia

Figure 11

Pattern diagram of scutellarin acting on MAPKs signaling pathway

Supplementary Files

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- SupplementaryFig.1.tif
- SupplementaryFig.2.tif