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Original Research

Neuroprotection of Emodin by Inhibition of Microglial NLRP3 Inflammasome-Mediated Pyroptosis

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Abstract

Background: Neuroinflammation triggered by chronic cerebral ischemia-induced microglial pyroptosis is a significant contributor to vascular cognitive impairment. It has been shown that emodin possesses anti-inflammatory and neuroprotective properties, however, it's potential molecular and signaling transduction pathway remains to be illuminated. This study researched the neuroprotective mechanisms of emodin focussing on emodin effects on lipopolysaccharide/adenosine triphosphate (LPS/ATP)-caused pyroptosis in BV2 cells and HT-22 hippocampal neurons. **Methods**: To explore the neuroprotective effect of emodin, Emodin was applied to BV2 cells, HT-22 hippocampal neurons, and BV2/HT-22 co-cultures stimulated with LPS/ATP to evaluate the cell morphology, levels of inflammatory factors, NLRP3 inflammatory inflammasome activity and focal pyroptosis-related protein expression, as same as neuronal apoptosis. **Results**: Emodin alleviated LPS/ATP-induced pyroptosis of BV2 cells by preventing the activity of the NLRP3 inflammasome and the cleavage of pyroptosis executive protein Gasdermin D (GSDMD). Furthermore, levels of interleukin (IL)-18, IL-1β and tumor necrosis factor (TNF)-α were reduced, the apoptosis of HT-22 hippocampal neurons was attenuated, and cell viability was restored. **Conclusions**: Emodin can antagonize microglial neurotoxicity by inhibiting microglial pyroptosis, thereby exerting anti-inflammatory and neuroprotective effects.

Keywords: emodin; microglia; neuroinflammation; NLRP3 inflammasome; pyroptosis

1. Introduction

As a stroke or vascular brain injury, vascular cognitive impairment (VCI) is induced by cerebrovascular lesions as well as their risk factors, which includes a range of cognitive impairments, from vascular moderate cognitive impairment to vascular dementia [1]. Since there is now no effective therapy for VCI patients, VCI is becoming a major public health issue worldwide [2]. VCI is a progressive disease with the neuroinflammatory response playing an significant function in the pathological changes of the vascular-neural cascade [3].

Pyroptosis, known as cellular inflammatory necrosis, is a recently identified pattern of cell demise related to inflammation [4]. NLRP3 inflammasome (nucleotide-binding oligomerization domain-like receptor protein3 Inflammasome), an important regulator of pyroptosis, is a multimeric protein complex composed of the receptor protein NLRP3, apoptosis-associated speckle-like protein (ASC) and cysteine aspartate-specific protease 1 precursor (pro-caspase-1), which is mainly expressed in astrocytes or microglia [5,6]. As innate immune cells of the nervous system, microglia cells have a crucial function in the central nervous system (CNS) homeostasis [7]. Following ischemic brain injury, cells sent damage-associated molecular pattern (DAMPs) signals, which are recognized by NOD-like receptor proteins of the NLRP3 inflammasome [8]. The NOD-like receptor proteins NLRP3 recruits ASC to activate caspase-1, which in turn, activates pro-IL-18 and pro-IL-1β to produce inflammatory cytokines IL-18 and IL-1β to induce a series of inflammatory responses. Also, activated inflammasomes specifically cleave gasdermin D (GSDMD) to produce an active N-terminal domain (GSDMD-N) which tightly binds to the plasma membrane and forms holes 10 to 20 nm in diameter in the cell membrane, disrupting the osmotic pressure and causing the swelling, rupture, and death of neurons and immune cells in the brain, as well as the release of a significant number of inflammatory factors [9]. These factors activate microglia and astrocytes, leading to a positive feedback inflammatory cascade, that greatly aggravates injury to the neurons in the white matter and CA1 region of the hippocampus caused by chronic cerebral ischemia, thereby resulting in cognitive dysfunc-

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tion [10]. There are studies suggesting that microglial pyroptosis plays a critical part in the neurotoxicity and neuroinflammation after cerebral ischemia by promoting excitatory amino acid and oxidative toxicity, so it has become an important target in the cerebral ischemic injury remedy [11].

Emodin (1,3,8-trihydroxy-6-methylanthraquinone, Fig. 1a) is an anthraquinone compound extracted from natural plants, which has been confirmed to have many pharmacological functions like anti-cancer [12], anti-inflammation [13], anti-oxidation [14], anti-viral [15], anti-bacterial [16], immunosuppression [17], and regulation of lipid metabolism [18]. Emodin can ameliorate ischemic brain injury via its anti-inflammatory and neuroprotective actions, however the exact mechanism and target remain unclear [19]. Previous studies about emodin mostly focus on microglial activation [20], while there are few reports on microglial pyroptosis.

This study was designed from the perspective of microglial pyroptosis and neuronal injury. By establishing a co-culture model of BV2 microglial cells and HT-22 hippocampal neurons combined with LPS/ATP stimulation, we explored the potential effects of emodin in inhibiting neuroinflammation and exerting neuroprotection to achieve insight into the *in vitro* molecular and signaling mechanism of emodin.

2. Materials and Methods

2.1 BV2 Cell Culture and Treatment

BV2, a line of mouse microglial cells, was purchased from Guangzhou Jennio Biotech Co., Ltd (Guangzhou, China) and cultured in DMEM at a density of 2×10^5/mL with 10% fetal bovine serum added as a supplement (Cat. no. 12800017 and 10099141, Invitrogen, Carlsbad, CA, USA) in an incubator for cell culture. Cells were treated with emodin or NLRP3 inhibitor MCC950 (Cat. no. HY-14393 and HY-12815, both from Monmouth Junction, NJ, USA) at doses of 5, 10, 20, or 50 μM, respectively for 18 h prior to the addition of LPS (1 μg/mL; Cat. no. L4391, Sigma-Aldrich, St. Louis, MO, USA). ATP (5 mM; Cat. no. A1852, Sigma-Aldrich, St. Louis, MO, USA) was used on the cultures 6 h after LPS application and incubated for 30 min. The treatment-induced morphological changes in BV2 cells were observed with an inverted phase-contrast microscope (Model AxioVert 200M, Zeiss, Oberkochen, Germany) and in order to do further study, the cells and supernatants were then collected.

2.2 HT-22 Cell Culture and Treatment

The cell line of mouse hippocampus neurons HT-22 (Jennio Biotech, Guangzhou, China) was cultured in DMEM in a cell culture incubator. The cell cultures were treated with emodin (20 μM) for 18 h, then LPS (1 μg/mL) was added and incubated for another 6 h.The cells were then treated with ATP (5 mM) and cultured for 30 min. In parallel, the upper compartment of a transwell plate included DMEM containing BV2 cells suspended at a density of 5×10^5 cells/mL, the lower compartment was used to hold HT-22 cells and then cultured for 1 h before the addition of emodin. The cocultures were then stimulated with LPS and ATP as above and the lower compartment's cells and supernatants were gathered for additional examination.The ratio of BV2 cells to HT-22 cells was 1:1.

2.3 Cell Counting Kit8 (CCK8) Assay

To detect the toxicity of emodin or LPS and ATP co-stimulation, 5×10^3 cells per well of plated BV2 or HT-22 cells were cultured in 96-well plates with 100 μL medium overnight. Then, the culture had successive emodin treatments or MCC950, LPS, and ATP at indicated concentrations. 10 μL of the CCK-8 reagent (Cat. no. CK04, Dojindo, Kumamoto, Japan) was added into each well after 24 h, and they were left to incubate for 2 h in dark. A microplate reader (Model Synergy 2, BioTek, Winooski, VT, USA) was used to measure the absorbance at 450 nm.

2.4 Western Blot Analysis

Proteins from cultured BV2 cells were extracted with lysis buffer (2% SDS, 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 4% β-mercaptoethanol, 1 mM phenylmethanesulfonyl and 50 mM DTT). Protein samples (30 μg/lane) were electroblotted onto polyvinylidene fluoride membranes after being separated by 8% or 12% SDS-PAGE (1658033, Bio-Rad, Hercules, CA, USA). The membranes were treated with primary antibodies against NLRP3, cleaved caspase-1, ASC, or GSDMD-N (all diluted at 1:500; ab263899, ab179515, ab307560, and ab209845, from Abcam, Cambridge, UK) at 4 °C overnight after being blocked with 5% skimmed milk. As an internal standard, anti-β-actin antibody (1:3000; ab8224, Abcam) was applied.The membranes were scanned using an Odyssey laser scanning system (LI-COR, Lincoln, NE, USA) after incubating with appropriate fluorescence-conjugated secondary antibodies (1:5000; 926-32211 and 925-68070, LI-COR, Lincoln, NE, USA) for 1 h at room temperature. By using Image J software's (version 2.0, LOCI, University of Wisconsin, Madison, WI, USA) densitometric analysis, the protein bands were measured and normalized to the internal standard β-actin.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

Following the manufacturer's instructions, the levels of TNF-α, IL-1β, or IL-18 in BV2 or HT-22 cell culture supernatants were determined by ELISA (Cat. no. BMS607-3 and BMS6002, eBioscience, San Diego, CA, USA, for TNF-α and IL-1β, and Cat. no. YJ003057, Mlbio, Shanghai, China, for IL-18). The absorbance was measured using a microplate reader at 450 nm (Model Synergy 2, BioTek, YT, USA).

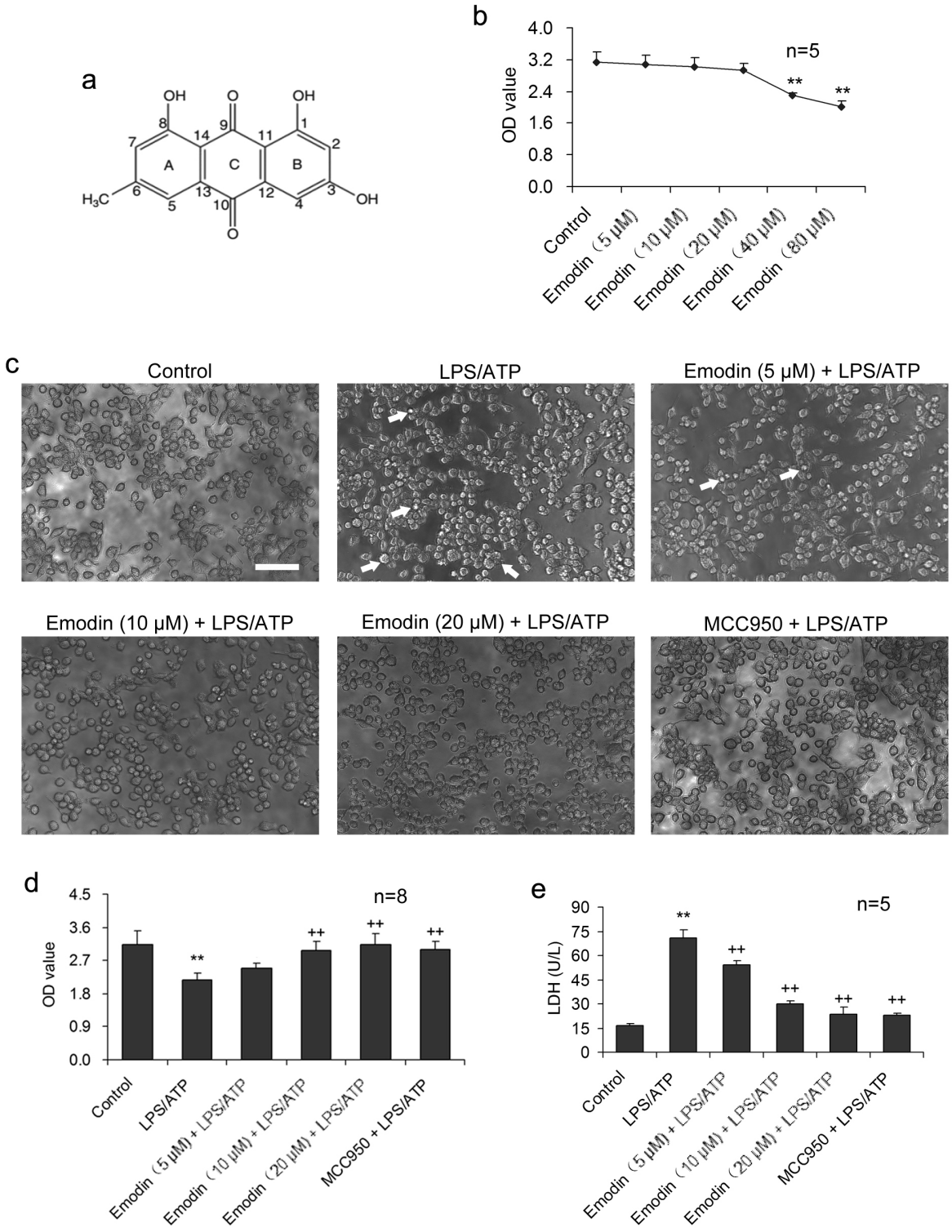

Fig. 1. Emodin suppresses LPS/ATP-caused injury in BV2 cells. BV2 cells were stimulated with LPS and ATP in the presence or absence of emodin. (a) The molecular structure of emodin. (b) BV2 cells were treated with emodin (0, 5, 10, 20, 40, 80 μM) for 24 h, and cytotoxicity was tested by the CCK-8 assay. (c) The morphological changes of treated BV2 cells were observed using an inverted phase-contrast microscope. The damaged cells showing cellular swelling, irregular shrinkage, or unclear cell border were indicated by the arrows. Scale bar = 50 μm. The viability of BV2 cells was assessed by the CCK-8 (d) and LDH assays (e). Values are expressed as the mean $\pm$ SD of five or eight separate experiments. Data were evaluated by one-way ANOVA followed by Newman Keuls post hoc test. ** $p < 0.01$ compared with the control group, + $p < 0.05$, ++ $p < 0.01$ compared with LPS + ATP group.

2.6 Immunofluorescent Staining

4% paraformaldehyde was used to fix the BV2 cells for 20 min at room temperature, followed with incubation in PBS with 0.3% Triton X-100 and 3% normal goat serum for 30 min. Then, cells were incubated with the primary antibody against ASC (1:100; ab307560, Abcam) overnight at 4 °C, followed by incubation with FITC-conjugated secondary antibody (1:400; ab6717, Abcam) for 18 h at 4 °C. To mark the nuclei, DAPI was used as a counterstain on BV2 cells, and the stained cells were quantified in five randomly selected visual areas on each coverslip under fluorescence microscopy (DMLB, Leica, Heidelberg, Germany). The ratio of DAPI-labeled cells to ASC-positive cells in each field of view was calculated for statistical analysis.

2.7 TUNEL Assay

After fixation and permeability as above, the HT-22 cells were incubated with primary antibody against NeuN (1:400; ab104224, Abcam) for 24 h at 4 °C. Cells were immediately incubated with Alexa Fluor 594-conjugated secondary antibody (1:400; ab150120, Abcam) for 18 h at 4 °C. To visualize apoptotic cells, the Fluorescein *in situ* cell death detection kit (Cat. no. 11684795910, Roche, Basel, Switzerland) was used in the TUNEL experiment as directed by the manufacturer. All images were visualized by fluorescence microscopy (DMLB, Leica, Germany) at 200$\times$ magnification. For quantification, TUNEL-positive cells and NeuN-positive cells were counted in five randomly selected visual areas on each coverslip to calculate the proportion of cells with TUNEL staining to the total number of $NeuN^+$ and $TUNEL^+$ cells in a given field of view.

2.8 Lactate Dehydrogenase (LDH) Assay

LDH is an enzyme that is normally found in the cytoplasm and is released into the medium when the cell membrane is disrupted, thus, the quantification of LDH release can be used to evaluate the integrity of cell membranes. After LPS and ATP treatment, using an LDH Assay kit (Cat. no. K726, BioVision, Mountain View, CA, USA) in accordance with the manufacturer's instructions, the cell supernatants were gathered for LDH measurement. LDH oxidizes lactate to produce NADH, reacting with WST, which can be measured at 450 nm optical density using an absorbance plate reader. Therefore, the volume of LDH that cells secreted was measured at 450 nm optical density with an absorbance plate reader (Model Synergy 2, BioTek, VT, USA), and then, the values were interpolated using the calibration curve and the LDH release finally was calculated and expressed as the value of U/L, which represents the degree of cell necrosis.

2.9 Cell Viability

The vitality of the HT-22 cell was assessed using Syto-13/PI, two dyes with various membrane permeabilities. Syto-13 is used to identify viable cells, whereas a non-permeant membrane red dye called PI is used to spot cells that have changed membrane integrity and are either necrotic or in the advanced stages of apoptosis. Briefly, cells were incubated with 0.1 μM Syto-13 (Cat. no. S7575, Molecular Probes, Eugene, OR, USA) and 10 μg/mL PI (Cat. no. P4170, Sigma-Aldrich, MO, USA) in PBS at 37 ºC for 5 min and visualized using a laser confocal microscope (TCS SPE, Leica, Heidelberg, Germany). Both viable cells and necrotic cells in five visual fields on each slide were counted to determine the typical proportion of PI+ necrotic cells.

2.10 Statistical Analysis

Data are reported as mean $\pm$ SD, and one-way ANOVA is used to examine them before the Newman-Keuls post hoc test. Statistical analysis was conducted using the Statistics Package for Social Science (SPSS, 16.0, IBM Corp., Chicago, IL, USA) and differences were deemed statistically significant at $p < 0.05$.

3. Results

3.1 Emodin's Cytotoxicity to BV2 Cells

Emodin had no conspicuous cytotoxicity to BV2 cells during 24 h treatment at 20 μM (Fig. 1b), therefore, 20 μM was selected as the greatest concentration for the studies that followed.

3.2 Emodin Inhibited the Injury Caused by LPS/ATP in BV2 Cells

The impact of emodin on LPS/ATP-induced BV2 cell pyroptosis was investigated under the inverted phase-contrast microscope (Fig. 1c). LPS/ATP caused the formation of abnormal cells, such as dysfunction of pores and cellular membrane damage, as well as irregular shrinkage or formation of closed-cell foams or bubbles. Necrosis was considerably increased. Emodin treatment (5, 10, and 20 μM) reduced these effects and maintened the normal morphology of LPS/ATP-treated BV2 cells in a dose-dependent manner (Fig. 1c). This was accompanied by increased cell viability (Fig. 1d) and alleviated LPS/ATP-induced BV2 membrane damage (Fig. 1e).

3.3 Emodin Reduced the Production of Pro-Inflammatory Cytokines

IL-18 and IL-1β, two proinflammatory cytokines, play a vital role in pyroptosis and are released as a consequence of caspase-1 and 'inflammasome' activation. TNF-α, as an inflammatory factor in the brain, can directly activate microglia to enhance the inflammatory response in the CNS by affecting the BBB permeability, thereby invading the center and causing CNS inflammation. LPS/ATP significantly elevated the level of IL-1β, IL-18, and TNF-α, while emodin treatment (10 and 20 μM) remarkably suppressed the production of IL-1β, IL-18, and TNF-α com-

parison with the control group. Our findings show that emodin suppresses the proinflammatory cytokine production induced by LPS/ATP stimulation in a dose-dependent manner (Fig. 2).

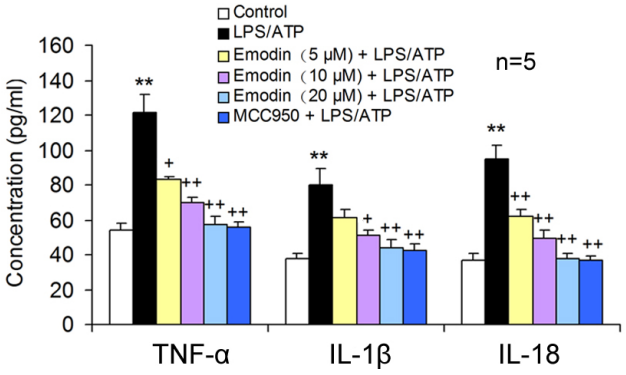

Fig. 2. Emodin decreased the pro-inflammatory cytokine release in LPS/ATP-induced BV2 cells. BV2 cells were co-stimulated with LPS and ATP after 18 h pretreatment with emodin or MCC950. The TNF-α, IL-1β, and IL-18 was detected by ELISA. Values are represented by the mean $\pm$ SD of five independent experiments. Data were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. ** $p < 0.01$ compared with the control group, + $p < 0.05$, ++ $p < 0.01$ compared with LPS + ATP. group

3.4 Emodin Prevented the LPS/ATP-Induced Pyroptosis of BV2 Cells by Inhibiting the Activation of the NLRP3 Inflammasome

NLRP3 receptor protein does not possess pro-inflammatory activity. NLRP3 combines with caspase-1 and ASC to form the NLRP3 inflammatory body, which becomes proinflammatory. The activation of the NLRP3 inflammasome was examined using green fluorescence to label ASC and blue fluorescence to locate the nuclei in BV2 cells. Compared to the control group, a large number of NLRP3 inflammasomes were observed in the LPS/ATP stimulated cells, suggesting that LPS/ATP could activate NLRP3 inflammasomes. MCC950, the NLRP3 inflammatory body specific inhibitor, significantly inhibited the NLRP3 inflammatory body activation. Emodin also suppressed the formation of the NLRP3 inflammasome in a dose-dependent manner (Fig. 3a,b), suggesting that emodin can suppress the NLRP3 inflammatory body activation.

Pyroptosis is carried out by the pyroptosis-executing protein GSDMD and is triggered by NLRP3 activation. It was showed that emodin pretreatment significantly restrained the pyroptosis-related protein levels of NLRP3, ASC, caspase-1, and GSDMD-N in LPS/ATP-treated BV2 cells, similar to the effect of MCC950 (Fig. 3c,d). This suggests that emodin can prevent LPS/ATP-caused BV2 cell pyroptosis by inhibiting NLRP3 inflammasome activation.

3.5 Emodin Protected HT-22 Neurons from BV2 Cell Pyroptosis-Mediated Toxicity

Under normal conditions, there were no significant differences in LDH, optical density (OD) values measured by CCK8, and apoptotic rates between the HT-22 neuron group and the HT-22 neuron co-cultured group (Fig. 4). After LPS/ATP treatment, the release of LDH was significantly enhanced in the co-culture group (Fig. 4f), neuronal OD values were prominently decreased (Fig. 4e), and the neuronal apoptosis rate was improved (Fig. 4c,d), suggesting that microglial pyroptosis aggravates the degree of neuronal injury under LPS/ATP conditions.

The supernatants obtained from the co-culture models were analyzed to confirm the neuronal damage caused by excessive inflammatory mediator release, showing that LPS/ATP stimulation resulted in markedly increased concentrations of TNF-α, IL-1β, and IL-18 (Fig. 5). Since emodin can inhibit neuroinflammation associated with microglial pyroptosis in BV2, we investigated whether emodin could reduce microglial pyroptosis-mediated neuronal injury. Emodin added to the culture medium of LPS/ATP-treated HT-22 neurons co-cultured with BV2 cells significantly decreased LDH levels (Fig. 4f), increased neuronal viability (Fig. 4e), and significantly decreased TNF-α, IL-1β, and IL-18 (Fig. 5). The TUNEL assay and Syto-13/PI assay revealed a reduced apoptosis rate of neurons (Fig. 4a–d). Taken together, these findings imply that emodin can protect neurons via suppressing LPS/ATP-induced microglial pyroptosis.

4. Discussion

This report describes the novel neuroprotective mechanism of emodin by antagonizing the neuroinflammatory response mediated by microglia pyroptosis. Emodin inhibited LPS/ATP-induced activation of NLRP3 inflammasome, preventing pyroptosis executive protein gasdermin D (GSDMD) cleavage, and suppressing LPS/ATP-caused pyroptosis in BV2 cells. Furthermore, emodin decreased levels of the inflammatory mediators TNF-α, IL-18, and IL-1β, reduced the apoptosis of HT-22 hippocampal neurons, and restored cell viability. This novel anti-inflammatory and neuroprotective mechanism indicates that emodin has potential clinical application in the remedy of neuroinflammation and nerve injury.

The anti-inflammation effect of emodin has been reported recently but the precise mechanism remains largely unknown. Park *et al.* [21] demonstrated that emodin surpressed microglia activity and decreased the pro-inflammatory cytokine release. Liu *et al.* [22] reported that emodin inhibited NLRP3 inflammasome activation and reduced IL-1β levels in LPS-induced J774A cell inflammation. Pyroptosis can trigger an inflammatory response after ischemia and aggravate brain injury [23]. As a key protein in the pyroptosis pathway, the NLRP3 inflammasome takes part in the initiation of pyroptosis and the process of

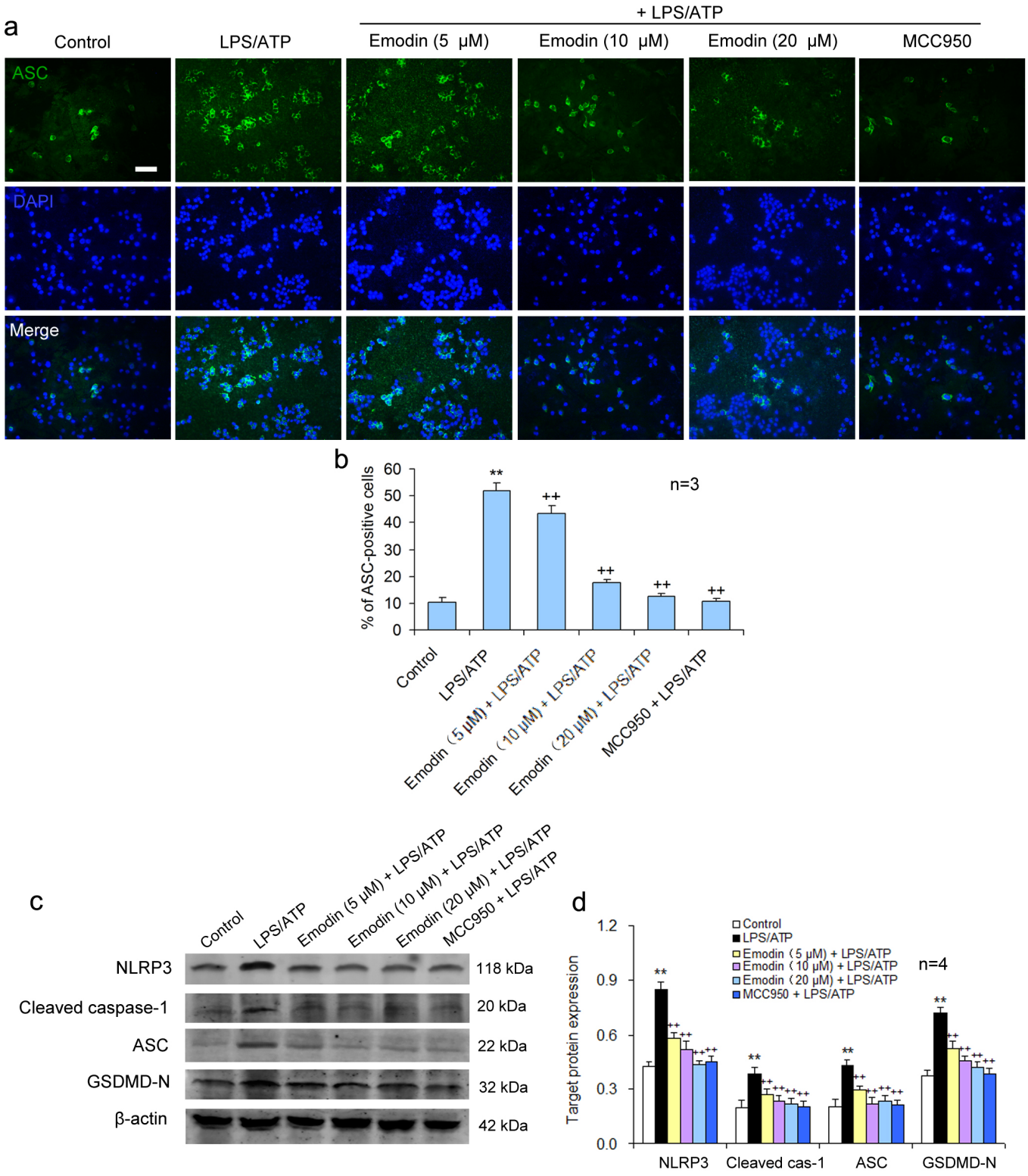

Fig. 3. Emodin suppresses pyroptosis via NLRP3/caspase-1 axis inhibition in BV2 cells. BV2 cells were treated as above. (a) BV2 cells were subjected to immunofluorescent staining for ASC and the nuclei were stained using DAPI. Scale bar = 50 μm. (b) The ASC-positive cell number was counted and the ratio of ASC-positive cells to DAPI-labeled cells was calculated. (c,d) The protein expression of NLRP3, cleaved caspase-1, ASC, and GSDMD-N were estimated by western blotting. Values are expressed as the mean ± SD of three or four independent experiments. Data were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. ** $p < 0.01$ compared with the control group, + $p < 0.05$, ++ $p < 0.01$ compared with LPS + ATP group.

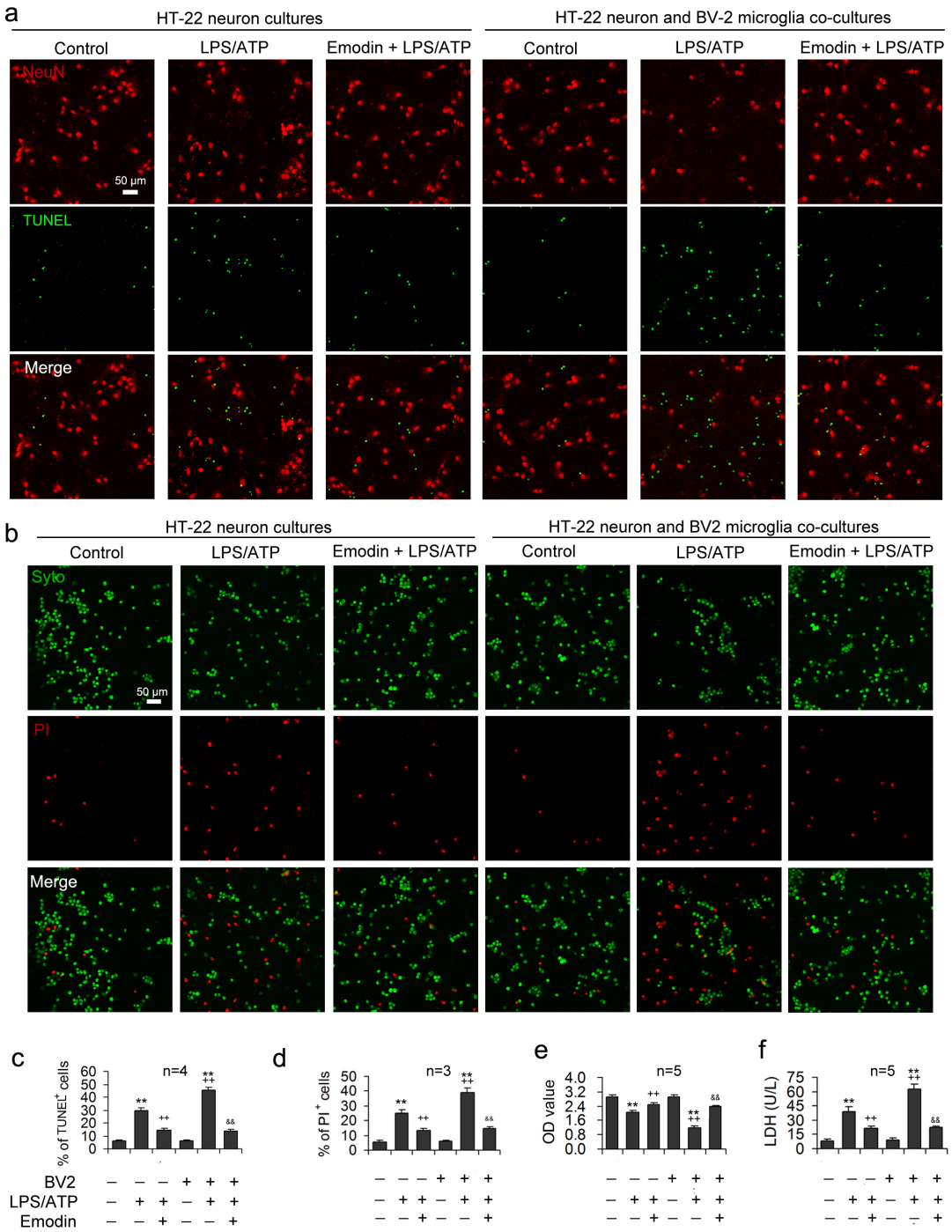

Fig. 4. Emodin alleviates LPS/ATP-induced neuronal toxicity by inhibiting inflammasome-mediated pyroptosis. LPS and ATP were applied to the HT-22 neuronal mono-cultures or the transwell co-cultures of HT-22 neurons and BV2 microglia after emodin pretreatment. NeuN and TUNEL double-staining was performed in HT-22 cells to assess the neuroprotection influence of emodin. Representative images are demonstrated in (a) and statistical data for the proportion apoptotic cells expressed as the ratio of TUNEL-labeled cells to the sum of $NeuN^+$ and $TUNEL^+$ cells are shown in (c). (b) The HT-22 cells were double stainned with SYTO-13/PI. (d) The percentage of PI-labeled cells in the sum of $SYTO\text{-}13^+$ and PI^+ cells was reported in the histogram. Scale bar = 50 μm. The HT-22 cell viability was detected using the CCK-8 (e) and LDH assays (f). All the data were analyzed by one-way ANOVA followed by Newman Keuls post hoc test. ** $p < 0.01$ compared with the control group, ++ $p < 0.01$ compared with LPS + ATP group in HT-22 mono-cultures, && $p < 0.01$ compared with LPS + ATP group in HT-22 and BV2 co-cultures.

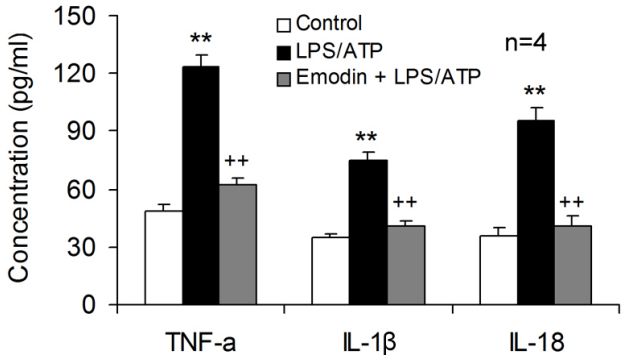

Fig. 5. Emodin is neuroprotective by inhibiting inflammatory mediator release from microglia. Emodin was applied to the transwell co-cultures of HT-22 neurons with BV2 microglia, followed by LPS and ATP co-stimulation. Cell culture supernatants in lower compartment were harvested to determine the concentration of TNF-α, IL-18, and IL-1β. All the data in the histograms were from four independent experiments and analyzed by one-way ANOVA followed by Newman Keuls post hoc test. ** $p < 0.01$ compared with the control group, ++ $p < 0.01$ compared with LPS + ATP group.

neuroinflammation. Recent reports have demonstrated that inhibiting NLRP3 inflammasomes or targeting key proteins in the pyroptosis pathway can reduce the level of brain inflammation, relieve brain parenchymal injury and alleviate the neurological defects caused by cerebral ischemia [24]. The present study demonstrated that LPS/ATP successfully caused BV2 cell pyroptosis and released the inflammatory factors, while emodin suppressed pyroptosis by inhibiting the assembly and the NLRP3 inflammasome activation by attenuating the expression of pyroptosis-related proteins in LPS/ATP-treated BV2 cells. As a pyroptosis suppressor, the production of TNF-α, IL-18, and IL-1β was reduced by emodin. Interestingly, 20 μM emodin treatment resulted in a similar inhibitory effect to that of NLRP3 inhibitor MCC950, indicating that emodin can inhibit the inflammatory response by restraining microglial pyroptosis.

A increasing body of research has confirmed the existence of bidirectional interactions between neurons and microglia [25]. Neuronal injury is associated with neuroinflammation resulting from abnormal glial function. Under pathological conditions, the pro-inflammatory cytokines produced by microglia have cytotoxic effects on neurons [26] and conversely, DAMPs released by neurons are activation signals of microglia [27]. An animal experiment successfully alleviated stroke-induced progressive cognitive impairment by silencing microglia to suppress neuroinflammation [28]. The present study showed that after LPS/ATP treatment, the viability of HT-22 hippocampal neurons in the co-cultured group was observably decreased comparison with that of the non-co-cultured group. The severity of cell injury and the rapidity of neuronal apoptosis were markedly increased in the co-culture group, suggesting the interaction between microglia and neurons in pathological conditions. It has been demonstrated that emodin has a protective impact on neurons [29]. Du *et al.* [30] reported that emodin could exert a protective effect on HT-22 hippocampal cells by activating the PKC pathway to reduce the inflammatory response in Alzheimer's disease model mice. To further elucidate the neuroprotective mechanism of emodin, we used emodin at a concentration of 20 μM to interfere with the LPS/ATP-induced co-culture model, showing that emodin can ameliorate the apoptosis of HT-22 cells and increase cell survival. The main advantage of this study is that it confirms our experimental hypothesis that "emodin exerts a protection effect on neurons by suppressing microglial pyroptosis".

Emodin also reduced the release of pro-inflammatory cytokine TNF-α which is associated with microglia polarization. During ischemic damage, the over-activation of the microglia into the M1 form leads to the release of large amounts of proinflammatory cytokines, causing damage to neurons and surrounding tissues. Therefore, we speculate that emodin's inhibitory effect on neuroinflammation may also be related to the regulation of microglia-polarized phenotypes. Nonetheless, this *in vitro* study has some limitations due to the complication of neuroinflammation and nerve injury caused by chronic cerebral ischemia, so further *in vivo* studies will be conducted to provide more evidence for the clinical administration of emodin.

5. Conclusions

In conclusion, emodin exerts neuroprotective effects on LPS/ATP-induced HT-22 hippocampal neurons, possibly by suppressing NLPR3 inflammasome-initiated pyroptosis in BV2 cells and antagonizing microglial neurotoxicity. This novel mechanism of emodin may be a potential treatment for neuroinflammation.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CC and XWY designed the research study. WJ and ZL performed the research. SW, LLX, and JFL provided help and advice on the TUNEL experiments. WJ and TM analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The protocol was approved by the Ethics Committee of Nanjing University of Chinese Medicine (approval number: 202112-012).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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