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ORIGINAL PAPER

JLX001 Modulated the Inflammatory Reaction and Oxidative Stress in pMCAO Rats via Inhibiting the TLR2/4-NF-κB Signaling Pathway

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Abstract

Inflammatory reactions and oxidative stress play critical roles in cerebral ischemic injuries. Microglia are activated after ischemic injury. Activated microglia produce neurotoxic proinflammatory factors and reactive oxygen species (ROS), which have been demonstrated closely related TLR2/4-NF-κB signal pathways. This study was to evaluate the effect of JLX001 against ischemic injury and investigate the mechanisms. The permanent middle cerebral artery occlusion (pMCAO) model was employed in rats. The neurobehavioral score, brain infarction rate, brain water content, pathological changes, immunohistochemical staining, biochemical index (T-AOC, SOD, and MDA), proinflammatory factors (IL-1β, TNF-α, and NO), expression of TLR2/4 and nuclear translocation of NF-κB p65 were determined. To explore probable underlying mechanism of the neuroprotective effect of JLX001, BV-2 cells were exposed to in oxygen–glucose deprivation (OGD) for 4 h to mimic ischemic injury in vitro. The result showed that JLX001 significantly decreased neurological deficit score, infarct size, and brain edema, attenuated pathological changes, inhibited the activation of microglia, improved the process of oxidative stress, reduced the release of proinflammatory cytokines and downregulated TLR2/4-NF-κB signal pathway. Moreover, OGD reduced BV2 cell viability, induced oxidative damage, increased the release of proinflammatory factors and activated TLR2/4-NF-κB signal pathway, which was significantly reversed by the intervention of JLX001. This study demonstrates that JLX001 is effective in protecting the brain from ischemic injury, which may be mediated by regulating oxidative stress, inflammation and inhibiting TLR2/4-NFκB signal pathway.

Keywords Cyclovirobuxine D · Permanent cerebral ischemia · Microglia · TLR2/4-NF-κB · Oxidative stress

Abbreviations

CVB-D	Cyclovirobuxine D
JLX001	Cyclovirobuxine hydrochloride D
pMCAO	Permanent middle cerebral artery occlusion
OGD	Oxygen–glucose deprivation
TLRs	Toll-like receptors
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor-κB
IL-1β	Interleukin-1β
TNF-α	Tumor necrosis Factor-α
NO	Nitric oxide
ROS	Reactive oxygen species
T-AOC	Total antioxidant capacity
NADPH	Nicotinamide adenine dinucleotide phosphate

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GBDI	Ginkgo biloba diterpene lactone meglumine injection
ICA	Internal carotid artery
ECA	External carotid artery

Introduction

Cyclovirobuxine D (CVB-D, $C_{26}H_{46}N_2O$) is an alkaloid extracted from *Buxus microphylla* and widely used in China to treat cardiovascular diseases [1, 2]. Previous studies have shown that CVB-D improves left ventricular function in coronary heart disease [3] and attenuates doxorubicin-induced cardiomyopathy by suppression of oxidative damage and mitochondrial biogenesis impairment [4]. Moreover, it relieves the injury of heart failure and induced the recovery of heart function on myocardial infarction rats [5]. These provide a good foundation for further research and development of CVB-D. Considering the slightly water-soluble nature of CVB-D, we have improved its structure and adopted a fully synthetic process to produce cyclovirobuxine hydrochloride D (JLX001, $C_{26}H_{46}N_2O{\cdot}2HCl$, structure shown in Fig. 1). Earlier studies in our lab have also designated that JLX001 has a therapeutic effect on cerebral ischemia by inhibiting platelet activation and thrombosis in rats [6]. Although some of its therapeutic effects have been demonstrated, the underlying mechanism mediated by JLX001 in cerebral ischemic injury remains unknown.

Among several molecular mechanisms contributing to brain injury induced by ischemic stroke, inflammation is involved in the pathophysiology of cerebral ischemia and recognized as a major risk factor [7]. Toll-like receptors (TLRs), a classical receptor family mediating the inflammatory reaction, are involved in the pathological processes of cerebral ischemic injury [8]. As innate immune cells in the brain, microglia monitor the local brain tissue and rapidly respond to injury through initiating inflammatory reactions [9]. The TLR family is one of the most studied types of microglial activation receptors. TLR2 and TLR4 can bind to myeloid differentiation primary response 88 (MyD88), thereby free nuclear factor-κB (NF-κB) to translocate into the nucleus and activate the expression of the pro-inflammatory cytokine genes [10]. Subsequently, activated microglia release a large number of inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor (TNF-α) and nitric oxide (NO), which contribute to further brain injury [11, 12]. Many studies have demonstrated the neuroprotection via suppression of TLR-NF-κB-mediated inflammatory pathway in rats with permanent cerebral ischemia [13] or cerebral ischemia/reperfusion [14, 15]. NF-κB not only promotes the release of inflammatory cytokines but also regulates the generation of reactive oxygen species (ROS), affects the content of superoxide dismutase (SOD) and malondialdehyde (MDA) through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2/4 [16]. Activated microglia can produce a fantastic amount of free radicals and sequentially causing lipid peroxidation. Lipid peroxidation readily decomposes to liberate carbonyl fragments, the most prominent being MDA, which are highly reactive and responsible for cytotoxic effects and neuronal death [17]. Previous research showed that attenuated inflammation and improved antioxidant defences had a significant impact on stroke in rats [18, 19].

Nowadays, intravascular techniques and thrombolytic agents are the only ways to restore blood flow [20]. However, most stroke patients cannot be effectively treated due to the stenotic time window (3 to 4.5 h after onset) and reperfusion injury after thrombolysis. Considering the majority of stroke patients can't receive reperfusion timely, it is critical to evaluate the drug efficacy in permanent cerebral ischemia

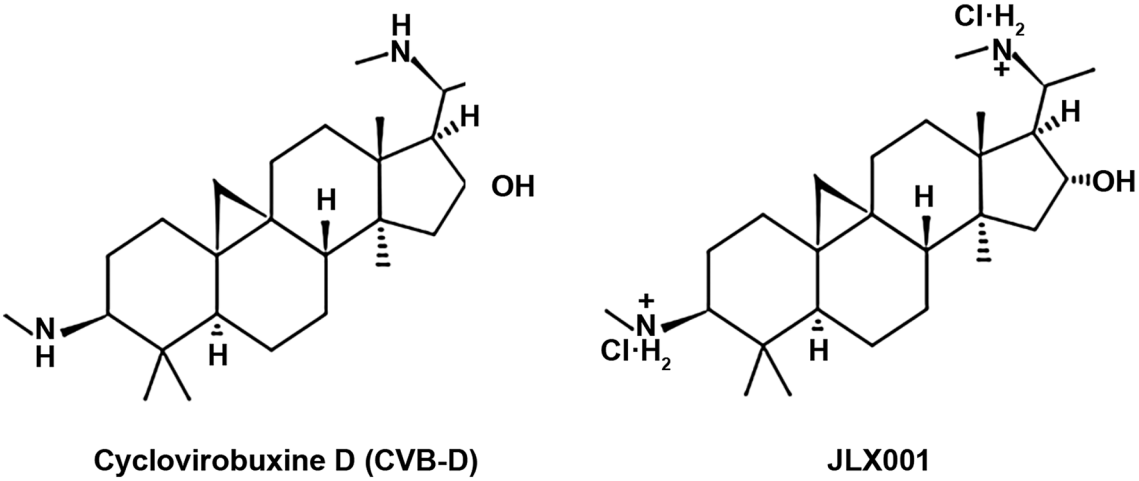

Fig. 1 Chemical structure of CVB-D and JLX001

[21]. In this study, we assessed the neuroprotective effect of JLX001 on permanent cerebral ischemia injuries in rats and oxygen–glucose deprivation (OGD) damages in microglia, and then investigated whether TLR/NF-κB signaling pathway is involved in the neuroprotective effect of JLX001. As a platelet activating factor (PAF) inhibitor, Ginkgo biloba diterpene lactone meglumine injection (GBDI) is a commercially available product used for neuroprotection, which partly related to the inhibition of oxidative damage and inflammatory response [22]. GBDI was used to compare the effect of JLX001.

Materials and Methods

Animals and Drug Administration

SPF male SD rats (250–300 g) used for the establishment of permanent middle cerebral artery occlusion (pMCAO) were purchased from Qinglongshan Animal Farm (Nanjing, China, license number: SYXK (Su) 2017–0001). Rats were housed in coops on a 12 h light–12 h dark schedule at the controlled temperature of 23 ± 2.5 °C with free access to a normal diet and tap water. All animals were cared for and treated according to institutional guidelines of China Pharmaceutical University, which conforms to the European Community guidelines (EEC Directive of 1986: 86/609/EEC). Efforts were made to minimize the number of rats and their suffering.

JLX001 (molecular formula: $C_{26}H_{46}N_2O{\cdot}2HCl$, purity 99.39%, Mr: 475.58) was kindly provided by Jiangsu Jinglixin Technology Co., Ltd. (Jiangsu, China, 20171019). GBDI was bought from Jiangsu Kangyuan Pharmaceutical Co., Ltd. (Jiangsu, China, 160601). 2, 3, 5-triphenyltetrazolium chloride (TTC) was the product of Bioengineering Co., Ltd. (Shanghai, China, C428BA0042). MTT was bought from Beyotime Biotech. Co., Ltd. (Shanghai, China, 041416160612). Glucose-free Dulbecco's minimum essential medium (DMEM) was bought from Gibco by life technologies™ (New York, America, 11966–025). All other reagents are commonly used in the laboratory and commercially available.

Drugs were dissolved in 0.9% NaCl and injected via caudal vein 3 h after the establishment of pMCAO, once a day for 5 days, the administration volume was 0.2 mL/100 g body weight, and the injection rate was 1 mL/min. Grouping is as follows: (1) Sham group; (2) Ischemia injury model group; (3) Positive control group of GBDI (2.5 mg/kg); (4) JLX001 high dose group (1.2 mg/kg); (5) JLX001 middle dose group (0.6 mg/kg); (6) JLX001 low dose group (0.3 mg/kg). Sham group and model group were treated with the same volume of 0.9% NaCl.

Establishment of pMCAO Model

After 12 h of fasting, rats were anesthetized by intraperitoneal injection (i.p) with 3% chloral hydrate (300 mg/kg). Body temperature was maintained at 37 ± 0.5 °C with a heating pad and lamp until rats wake up. The pMCAO model was established as previously described [23, 24]. Briefly, the right common carotid artery (CCA) was exposed, and the other abdominal muscle and sternocleidomastoid muscle were pulled outward. The occipital artery and superior thyroid artery, branches of the external carotid artery (ECA), were isolated and ligated. Carefully separate the internal carotid artery (ICA) and clip the common carotid artery and internal carotid artery. Subsequently, a 40 mm 3–0 surgical monofilament nylon suture was inserted from ECA to ICA to occlude all blood supply sources of the right middle cerebral artery. Stitch the skin and keep warm to wake up.

Neurological Deficit Score, Water Content and Infarct Size

1 h after the last administration, the neurological deficit score was scored on a five-point scale as previously reported [25]: (0) no apparent neurological deficits; (1) unable to extend the contralateral forelimb if pulled by tail; (2) the resistance decreases as the shoulder moves toward the contralateral side; (3) spontaneous contralateral circling; and (4) paralysis, no spontaneous activity. The investigator who evaluated all the results of the experiment was blind to the group to which each rat belongs. A preliminary neurobehavioral score was performed 3 h after pMCAO, and qualified rats (neurological score during 2–3 points) were randomly divided into the treatment and model groups, and the dead rats during treatment were discarded. The mortality of rats was 30–45% (6–9 of 20 rats) in 5 days in our experiment. There was no significant difference in mortality between groups.

TTC can react with succinate dehydrogenase in normal cells to produce hyperthyroidism (red). So, a TTC staining was used to determine infarct volume. Rats were sacrificed after the neurological deficit assessment, and the brain was removed, weighed (to obtain brain wet weight) and coronally sliced into 2.0 mm-thick sections. All slices were immersed in 1% TTC at 37 °C for 15 min, and then the infarcted brain tissue appeared white, whereas the noninfarcted region appeared red. The coronal slices were photographed with a digital camera and analyzed by an image processing software (ImageJ software) [26]. Infarct volume of the whole brain was calculated according to the formula:

$$\text{Infarct size (\%)} = \frac{(\text{contralateral area} - \text{ipsilateral non infarct area})}{2 \times \text{contralateral area}} \times 100\%$$

Brain sections were then dried at 110 °C for 24 h to obtain dry weight. Brain water content was calculated according to the formula:

$$\text{Water content (\%)} = \left(1 - \frac{\text{Brain dry weight}}{\text{Brain wet weight}}\right) \times 100\%$$

Biochemical Assays in Rats

One hour after the last administration, blood samples (1 mL per rat) were extracted from orbital venous plexus followed by anticoagulation with 3.8% trisodium citrate (1: 9, v/v). After centrifugation at 3000 rpm for 15 min, the upper layer of liquid (plasma) collected for the following test.

Then brains were removed and rinsed with iced saline. Forebrain tissue (0.5 g) in ischemic ipsilateral hemisphere was homogenized with normal saline (1: 9, w/v). The homogenate was then centrifuged at a 3000 rpm for 10 min at 4 °C, after which the supernatant was obtained to determine the level of SOD and MDA. Another part of the homogenate was centrifuged at 12,000 rpm for 15 min at 4 °C, and the supernatant was taken to determine the activity of Total antioxidant capacity (T-AOC). All samples were stored at − 20 °C.

T-AOC activities, SOD activities and MDA levels in plasma and brain tissue homogenate were all determined spectrophotometrically using appropriate assay kits following the manufacturer's protocols. All test kit was bought from Beyotime Biotech. Co. (Shanghai, China).

Analysis of TNF-α, IL-1β and NO in Rats

One hour after the last administration, blood samples (n = 10) were extracted from orbital venous plexus followed by anticoagulation with 3% EDTA (1: 9, v/v). After centrifugation at 4000 rpm for 15 min, collect the upper layer of liquid (plasma) to test the content of TNF-α and IL-1β using a rat TNF-α or IL-1β enzyme-linked immunosorbent assay (ELISA) kits (NeoBioscience, Shenzhen, China) according to manufacturer's protocols. Then, the brain was removed and homogenized with cell and tissue lysate (1: 9, w/v (Beyotime Biotech. Co., China)). After centrifugation at 12,000×*g* for 5 min at 4 °C, the supernatant was collected to determine the content of NO in the brain according to the manufacturer's protocols from the assay kit (Beyotime Biotech. Co., China).

Histopathologic Analysis in Rats

Hematoxylin and eosin (H&E) staining is the most basic and widely used technique in histology and pathology research and usually used to evaluate the pathological changes in ischemic injury. One hour after the last administration, rats were anesthetized with 3% chloral hydrate (300 mg/kg, i.p) and perfused with 50 mL 0.9% NaCl injection and 50 mL 4% paraformaldehyde. Whole brains were removed and post-fixed in 4% paraformaldehyde overnight at 4 °C. The coronal section of the brain was taken, dehydrated, embedded in paraffin, and produced (4 μm), stained with hematoxylin and eosin. Each section was photographed with the optical microscope at a magnification of × 40/400 and evaluated for the pathological score by a pathologist [27]. Four categories of pathological findings were scored as follows: 0, normal; (1) focal loss of individual neurons; (2) multiple or large foci of neuronal loss along with reactive gliosis, involving one-half or less of the area of ipsilateral hemisphere; (3) severe infarction, neuronal loss and reactive gliosis, involving greater than half of the cerebral hemisphere.

Immunohistochemistry

Immunohistochemistry was performed to study the changes of Iba-1, a marker of microglia activation. Brain sections were incubated overnight at 37 °C, blocked with 5% bovine serum albumin (BSA) for 20 min at 37 °C, then incubated with primary antibody of Iba-1 (1: 200, Abcam, China, Ab15690) at 4 °C for 18 h. After washing with tris buffered saline (TBS), the sections were incubated with goat anti-rabbit IgG for 50 min at 4 °C and then observed with the diaminobenzidine (DAB) system. The sections were observed under a microscope (OLYMPUS CX31 Japan), and the number of Iba-1 positive cells in hippocampus regions was calculated using ImageJ software to quantify glial activation. Three high-resolution (400 ×) images of the same area were randomly selected for each animal, and the average number of positive cells in these three fields was used as an indicator of animal microglia activation [28].

BV2 Cells Culture and Drug Administration

BV2 microglial cells were obtained from the School of Pharmacy (China Pharmaceutical University). BV2 microglia were cultured in DMEM medium (Jiangsu KeyGen Biotechnology Co., Ltd., China, 20180606) containing inactivated 10% fetal bovine serum (Gibco by life technologies™, America, 1495527) and seeded (cell density: 5×10^4 cells/

mL) into culture plates and cultured to confluence. All cells were cultured in a constant temperature incubator at 37 °C, 5% (volume fraction) CO_2 + 95% (volume fraction) air, saturated humidity conditions.

BV2 cells were challenged by OGD to mimic ischemic injury in vivo. Briefly, after attachment, the culture medium was changed to glucose-free DMEM and placed in a 37 °C, 5% CO_2, 94% N_2, 1.0% O_2 incubator for hypoxia. BV2 cells were incubated with GBDI (25 mg/L) and different concentrations of JLX001 (3, 1, 0.3 μM) during 4 h of OGD.

MTT Assay

BV2 cells viability in response to OGD and JLX001 treatment was assessed by MTT assays, respectively. After OGD, 20 μL MTT solution (5 mg/mL) was added to each well for cell viability assay. After incubation for 4 h at 37 °C, 5% CO_2, the medium was carefully decanted off and dissolved with 150 μL of DMSO at room temperature for 15 min. The absorbance was measured by an enzyme-linked immunosorbent at 570 nm to reflect cell viability indirectly. The experiment was repeated 3 times with 6 replicate wells per set.

Biochemical Assays in BV2 Cells

After 4 h of OGD, the culture supernatant of each group was collected immediately for the detection of T-AOC activity, SOD activity, MDA level, TNF-a level, IL-1β level and NO content following the manufacturer's protocols.

Western Blot

Total proteins and nuclear proteins in ipsilateral brain tissues and BV2 cells were extracted and purified using RIPA lysate by the manufacturer's instructions (Beyotime Biotech. Co., China). After protein quantification, the same amount of protein (60 μg) was separated on 10% or 12% Sodium dodecyl sulfate (SDS) polyacrylamide gels, transferred to polyvinylidene fluoride (PVDF) membranes, and probed with anti-TLR2 (1: 1000, Abways, China), anti-TLR4 (1: 500, Wanleibio, China), anti-NF-κB p65 (1: 500, Abways, China), anti-β-actin (1: 3000, Abways, China) and anti-Histon3 (1: 1000, Abways, China), then incubated with horse radish peroxidase (HRP)-conjugated secondary antibody (1: 5000, Abways, China). The level of protein expression was determined with a hypersensitive chemiluminescence reagent (Beyotime Biotech. Co., China) and exposed on a chemiluminescence imaging system (Bio-Rad, America). The optical density of protein bands was quantitatively analyzed with ImageJ software.

Statistical Analysis

Data are shown as mean ± SD and statistically analyzed using SPSS 19.0 software. One-way analysis of variance (ANOVA) was utilized for mathematical comparisons between different groups, and a value of $P < 0.05$ was acknowledged statistically significant.

Result

Effect of JLX001 on Neurological Score, Brain Water Content, and Infarct Size

The neurological score, brain water content and infarct size were evaluated to assess the overall treatment effect of JLX001 against ischemic injury in rats. As shown in Fig. 2a, JLX001 (1.2 mg/kg) reduced neurologic deficits in response to pMCAO ($P < 0.01$) compared with the model group. Besides, JLX001 (1.2, and 0.6 mg/kg) slightly reduced brain edema in comparison with the model group. There was no significant difference between groups ($P < 0.05$) (Fig. 2b).

TTC staining showed that JLX001 (1.2 and 0.6 mg/kg) significantly lessened the cerebral infarction ($P < 0.01$) compared with the model group (Fig. 2c, d, $P < 0.01$). These results indicated that JLX001 is effective in treating ischemic stroke in rats and can be further studied as a potential drug for stroke.

JLX001 Improved Pathological Damage

After a five-day administration, H&E staining was used to assess the pathological damage of brain tissue after pMCAO in rats. As shown in Fig. 3, No remarkable neuronal abnormalities were observed in the hippocampus dentate gyrus (a) and cortex (a') from the sham group. However, 5 days after pMCAO, the model group showed evidently pathological damage, such as loose cortical interstitial, foamy softening structure and neuronal degeneration including nuclear pyknosis, shrinkage and neuronal loss in cerebral cortex and hippocampus. After treatment with JLX001, the morphology and the construction of neuron cells tend to be healthy (Fig. 3b–f, b'–f'), indicating that JLX001 may have a specific neuroprotective effect on ischemic injury. Simultaneously, compared with model group, JLX001 (0.6 and 1.2 mg/kg) attenuated pMCAO-induced pathological changes and significantly reduced the pathological scores (Fig. 3g, $P < 0.01$), further confirming the neuroprotective effect of JLX001.

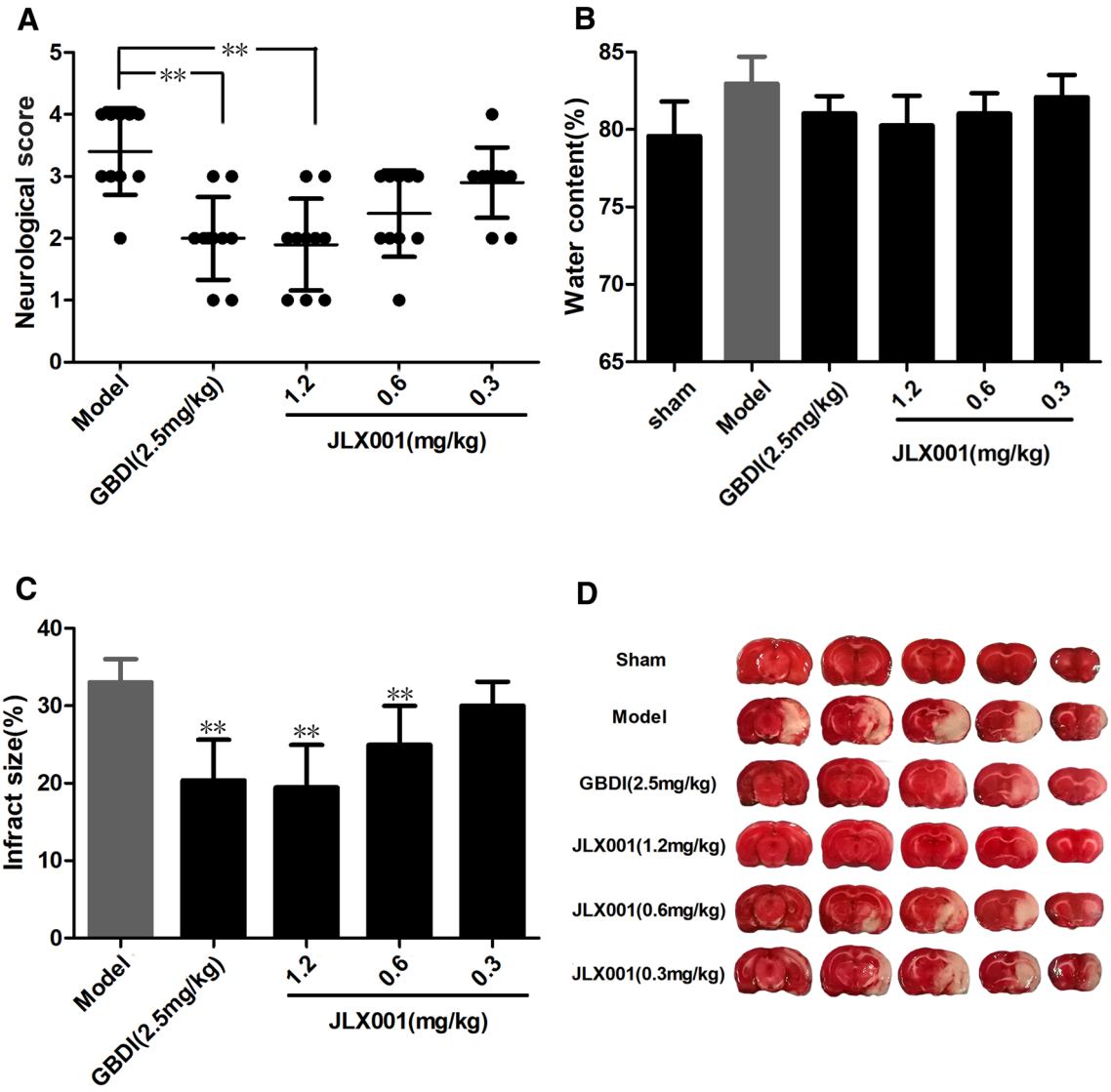

Fig. 2 Effect of JLX001 on the neurological score, brain water content and infarct size in rats subjected to pMCAO. **a** Neurological score. **b** Brain water content. **c** Infarct size. **d** Representative TTC-stained sections. Data are shown as mean ± SD, n = 10 rats per group, $^{**}P < 0.01$ versus model group

Assays of T-AOC, SOD, and MDA in Brain Tissues and Plasma

Compared with the sham group, the activities of T-AOC and SOD in plasma were significantly decreased ($P < 0.01$), and the MDA level in plasma was significantly increased in the model group ($P < 0.01$). Five days after continuous administration, JLX001 (1.2 mg/kg) significantly enhanced the activities of T-AOC and SOD ($P < 0.01$), and decreased the level of MDA ($P < 0.01$) in plasma compared with the model group (Fig. 4a–c).

Likewise, similar results were observed in brain tissue homogenate. JLX001 (1.2 mg/kg) significantly converted the changes in T-AOC activity ($P < 0.01$), SOD activities ($P < 0.01$) and MDA levels ($P < 0.05$) stimulated by pMCAO (Fig. 4d–f).

JLX001 Attenuates the Release of TNF –α, IL-1β and NO in Rats

To explore the influence of JLX001 in the inflammatory response, we examined the production of proinflammatory mediators (IL-1β, TNF-α, and NO) in rats. The results showed that pMCAO injury led to a significant rise of IL-1β (Fig. 5a), TNF-α (Fig. 5b) in plasma and NO (Fig. 5c) in the brain in comparison with the sham group ($P < 0.01$). Administration with JLX001 (1.2 and 0.6 mg/kg) obviously depressed the level of IL-1β, TNF-α, and NO compared with

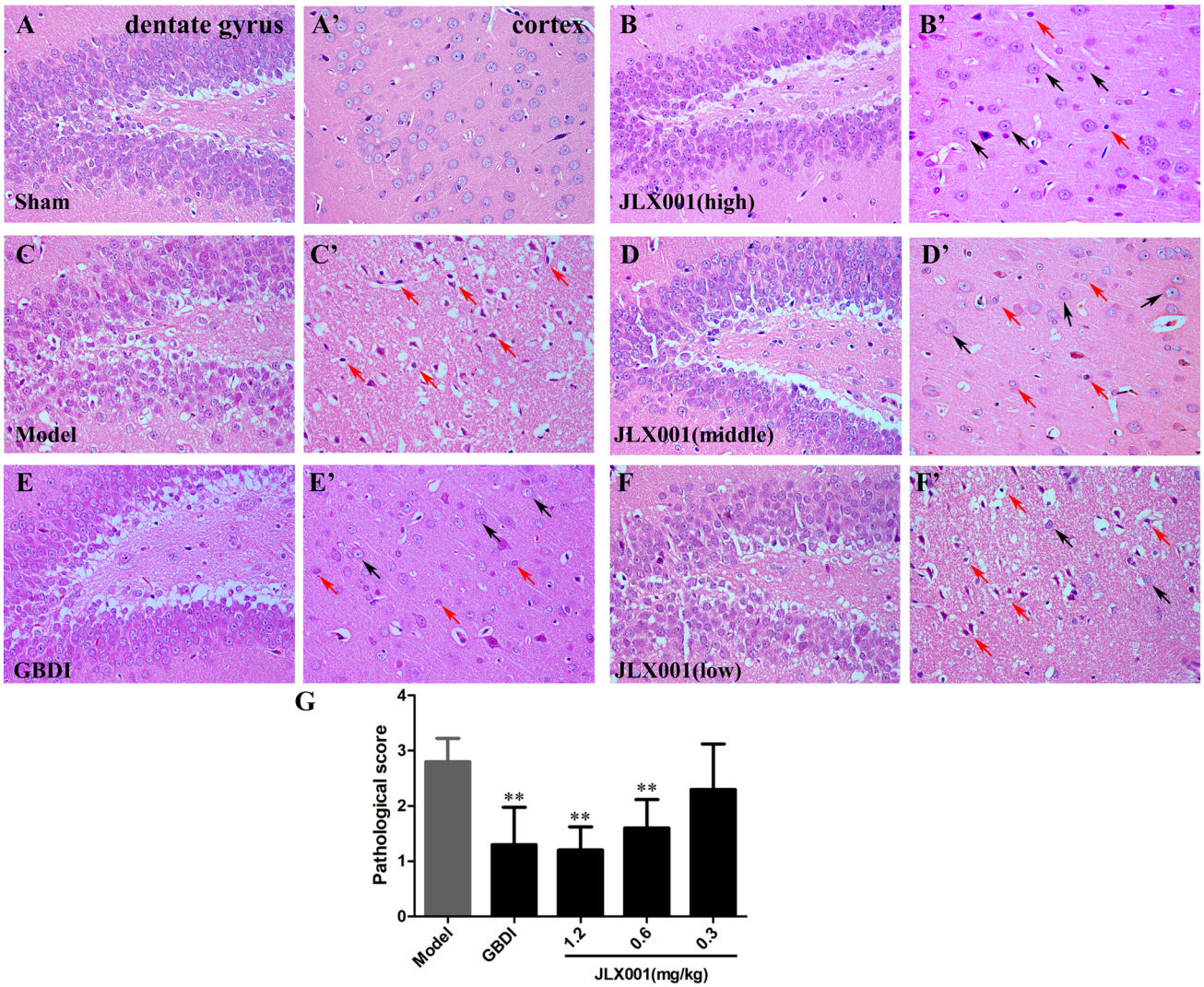

Fig. 3 JLX001 attenuated the morphological alterations in the hippocampus dentate gyrus (**a**–**f**) and cortex (**a'**–**f'**) after pMCAO, healthy neurons (black arrow) and damaged neurons (red arrow). **g** Pathological scores for each group. Data are shown as mean ± SD, n = 10 rats per group. Data are shown as mean ± SD, Magnification, ×400. $^{**}P < 0.01$ versus model group

the model group ($P < 0.01$). Besides, JLX001 (1.2 mg/kg) showed a better effect than GBDI ($P < 0.05$).

JLX001 Reduces the Expression of TLR2/4 and Inhibits the Nuclear Translocation of NF-κB p65 in Rats

We next investigated alterations of proteins involved in the intracellular TLR2/4 signaling pathways in Rats. As shown in Fig. 6b, c, the expression of TLR2 and TLR4 was significantly enhanced in ischemic cerebrum tissue 5 days after pMCAO in comparison with the sham group ($P < 0.01$). JLX001 (1.2 mg/kg) significantly downregulated the expression of TLR2 ($P < 0.05$) and TLR4 ($P < 0.01$).

NF-κB is a downstream signaling molecule of TLR2/4 and is activated by TLR2/4. Activation of NF-κB plays a crucial role in initiating inflammation, which is characterized by the translocation of p65 to the nucleus. As shown in Fig. 6d, pMCAO increased the expression of NF-κB p65 in the nucleus of ischemic rat brain in comparison with the sham group ($P < 0.01$), which was significantly reversed by JLX001 (1.2 and 0.6 mg/kg, $P < 0.01$).

JLX001 Inhibits Ischemia-Induced Microglial Activation

To futher explore the mechanism of JLX001 in protecting against ischemic stroke, we used immunohistochemical

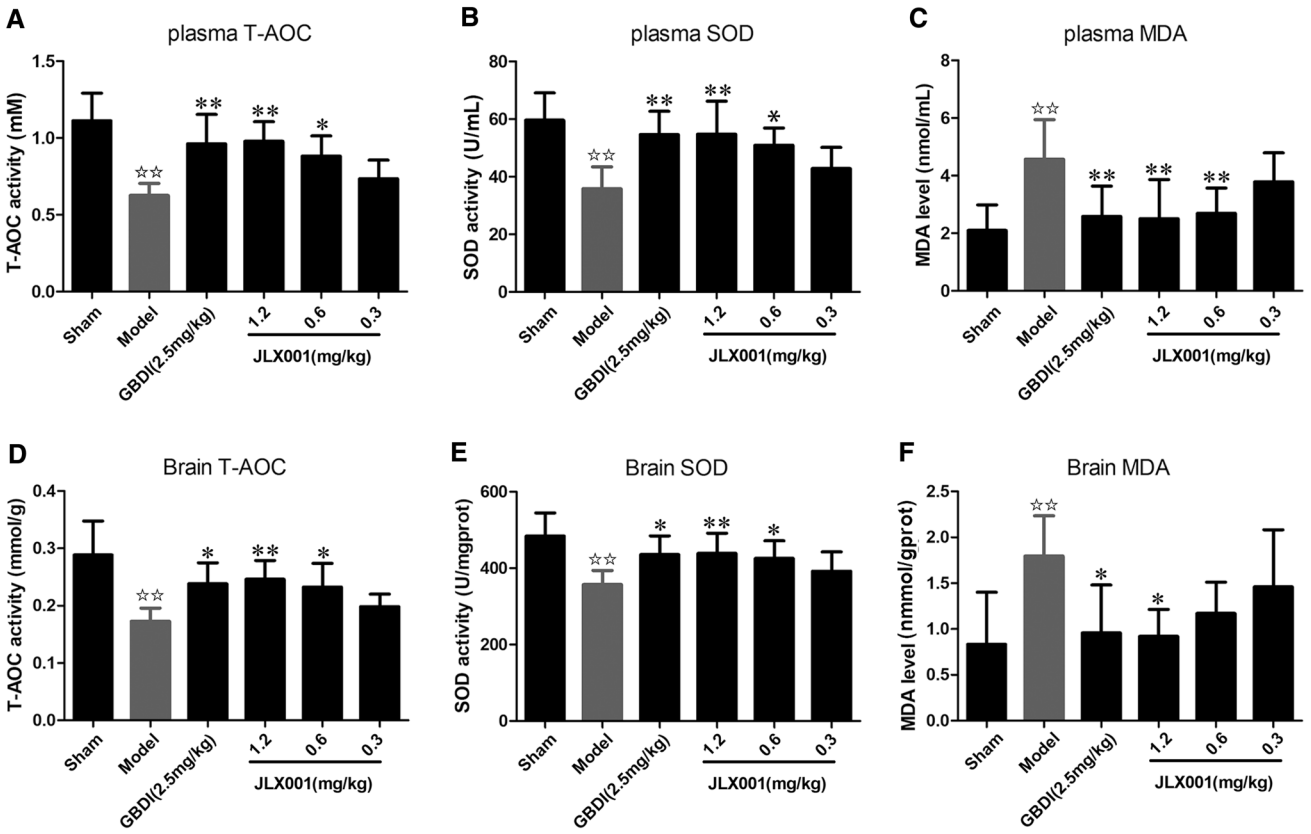

Fig. 4 Assays of T-AOC, SOD, and MDA in plasma and brain tissue. **a–c** The levels of T-AOC, SOD, and MDA in plasma. **d–f** The levels of T-AOC, SOD, and MDA in brain tissue. Data are shown as mean ± SD, n = 10 rats per group, $^{\star\star}P < 0.01$ versus sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group

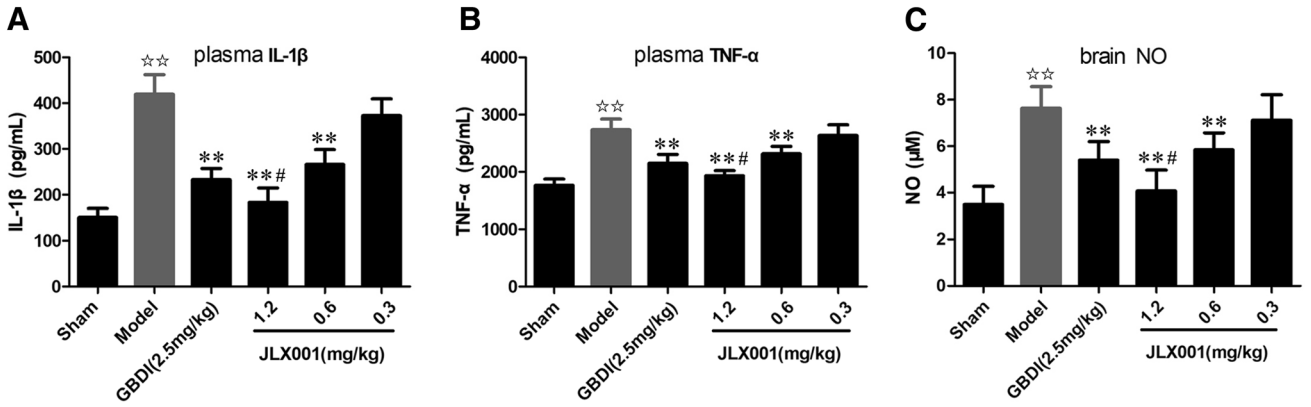

Fig. 5 JLX001 attenuates the release of IL-1β, TNF-α, and NO in rats. **a** and **b** The levels of IL-1β and TNF-α in plasma. **c** The content of NO in brain. Data are shown as mean ± SD, n = 10 rats per group, $^{\star\star}P < 0.01$ versus sham group. $^{**}P < 0.01$ versus model group. $^{\#}P < 0.05$ versus GBDI group

analysis to assess microglial activation in rats. Iba-1 is a microglia-specific protein antibody that can be used to label activated microglia (red arrow). The results showed that Iba-1 was not evident in the sham group (Fig. 7a, a'). However, Iba-1 immunoreactivity was prominent in ischemic ipsilateral and contralateral hippocampal cortex regions of

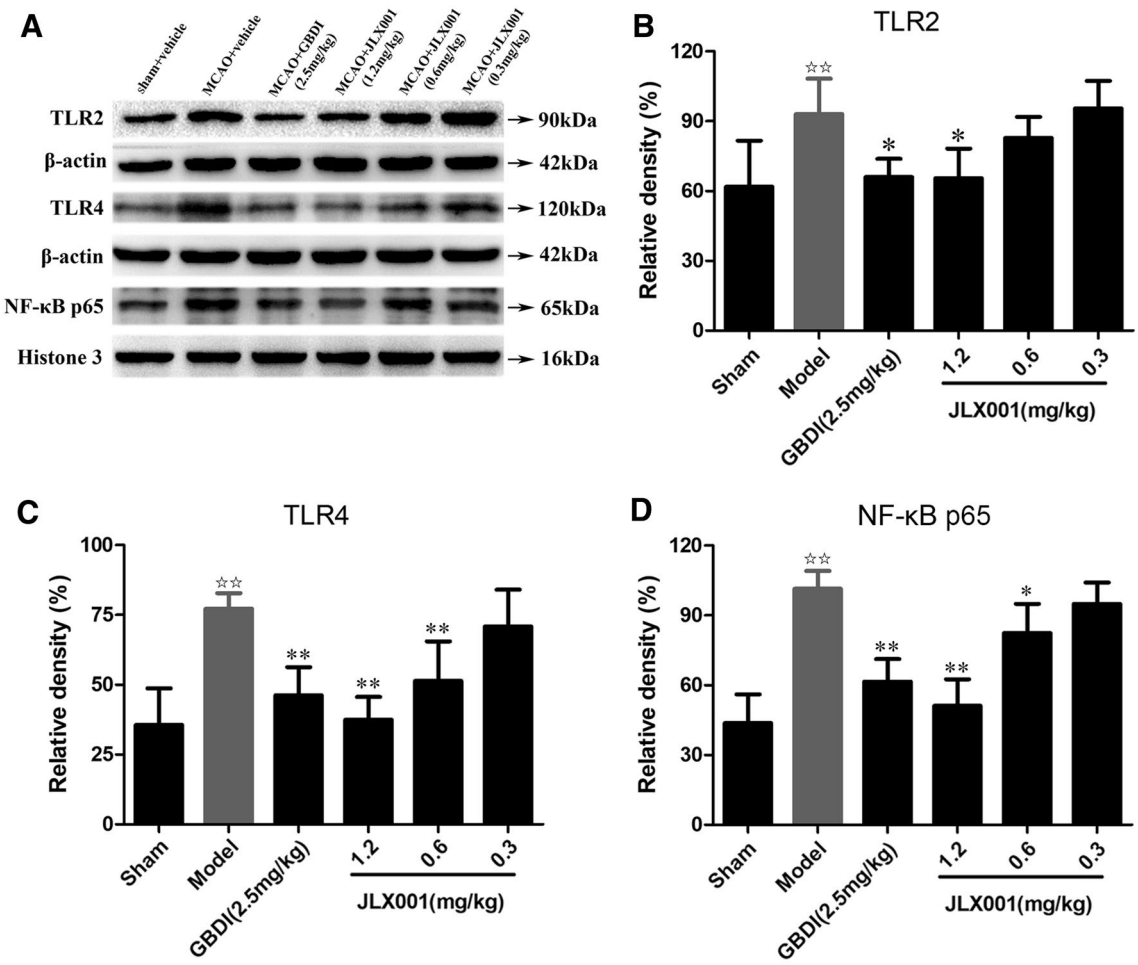

Fig. 6 JLX001 reduces the expression of TLR2/4 and inhibits the nuclear Translocation of NF-κB p65 in Rats. **a** Representative protein expression bands of TLR2, TLR4, and NF-κB p65 in rat brain after pMCAO were detected by western blot, and the data were shown in (**b–d**). Data are shown as mean ± SD, n = 6 rats per group, $^{\star\star}P < 0.01$ versus sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ versus model group

the model group, indicating noticeable activation of microglia, which was significantly inhibited by treatment with GBDI or JLX001 (Fig. 7b–f, b'–f'). As shown in Fig. 7g, h, compared with model group, JLX001 (1.2 and 0.6 mg/kg) significantly decreased the number of Iba-1 positive cells in both ischemic ipsilateral and contralateral cortex regions ($P < 0.01$), which indicated the neuroprotective effect of JLX001 on cerebral ischemia may be related to the inhibition of microglia activation.

Effect of JLX001 on the Viability of BV2 Cells

To further validate the effect of JLX001 on microglia, BV2 cells were exposed to OGD for 4 h to mimic ischemic injury in vitro. We first confirmed the occurrence of ischemic damage by comparing the viability of the OGD model group and the control group and then examined the effects of different doses of the drug (Fig. 8a). OGD significantly reduced cell viability compared with the control group ($P < 0.01$). JLX001 at concentrations 1, 3 and 5 μM remarkably increased the cell viability compared with the OGD group ($P < 0.01$). Besides, JLX001 at the level of 3 μM surprisingly increased the cell viability compared with the GBDI group ($P < 0.05$).

Assays of T-AOC, SOD, and MDA in BV2 Cells

Assays of T-AOC, SOD, and MDA were conducted in microglia exposed to OGD to estimate the inhibitory impact of JLX001 on oxidative stress. As shown in Fig. 8b–d, OGD significantly reduced the activities of T-AOC and SOD and increased the level of MDA in the culture supernatant of BV2 cells ($P < 0.01$) compared with the control group. While a significant increase in T-AOC and SOD activities and decreased MDA level were observed when the cells were

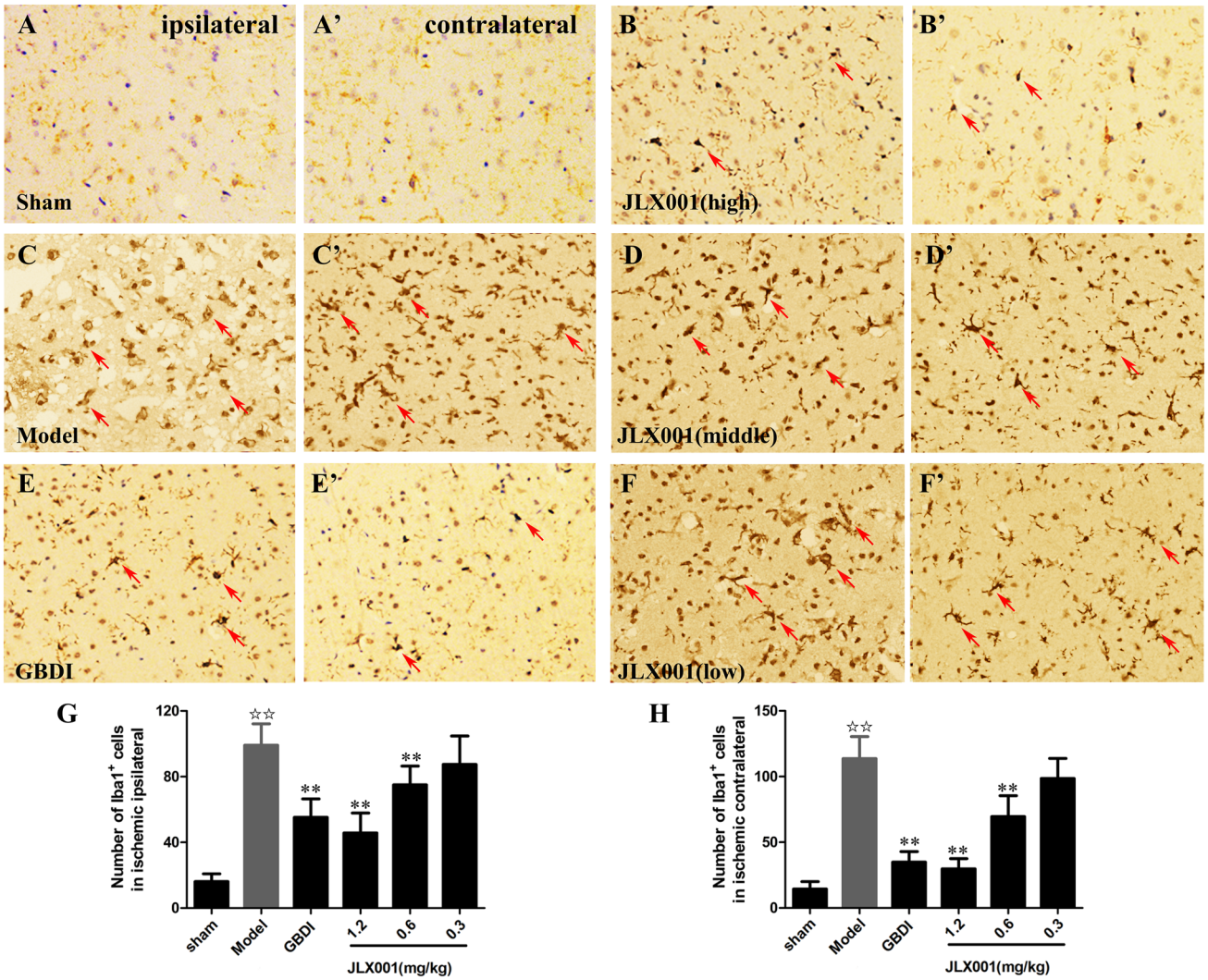

Fig. 7 JLX001 inhibited the activation of microglial cells in hippocampal cortex in rats subjected to pMCAO. Representative images of Iba1-immunopositive cells in ischemic ipsilateral (**a**–**f**) and contralateral (**a**'–**f**') regions and the quantification of the number of $Iba1^+$ cells in both areas (**g** and **h**). Data are shown as mean ± SD, n = 10 rats per group, the counting was repeated for three different fields. Magnification, × 400. $^{☆☆}P < 0.01$ versus control group. $^{**}P < 0.01$ versus OGD group

treated with JLX001 at 1 and 3 μM ($P < 0.05$, $P < 0.01$) in comparison with OGD group.

JLX001 Attenuates the Release of TNF –α, IL-1β and NO in BV2 Cells

To further verify the effect of JLX001 on the inflammatory response, we examined the level of IL-1β, TNF-α and NO in culture supernatant. Likewise, these results showed that OGD conspicuous increased the secretion of IL-1β (Fig. 9a), TNF-α (Fig. 9b) and NO (Fig. 9c) in the culture supernatant compared with the control group ($P < 0.01$), which was significantly decreased by JLX001 (1 and 3 μM) ($P < 0.01$). Similarly, the effect of JLX001 (3 μM) in inhibiting the release of the inflammatory factor was better than GBDI ($P < 0.05$).

JLX001 Reduces the Expression of TLR2/4 and Inhibits the Nuclear Translocation of NF-κB p65 in BV2 Cells

The TLR4 receptor is mainly present in microglia and exacerbates inflammatory responses by activating NF-κB nuclear transcription. As shown in Fig. 10b, c, expression of TLR2 and TLR4 in BV2 microglia exposed to OGD was significantly increased compared with the control group. Simultaneously, the expression of NF-κB p65 in the nucleus and the OGD group, respectively increased relative to the corresponding control (Fig. 10d, $P < 0.01$). JLX001 (1 and 3 μM)

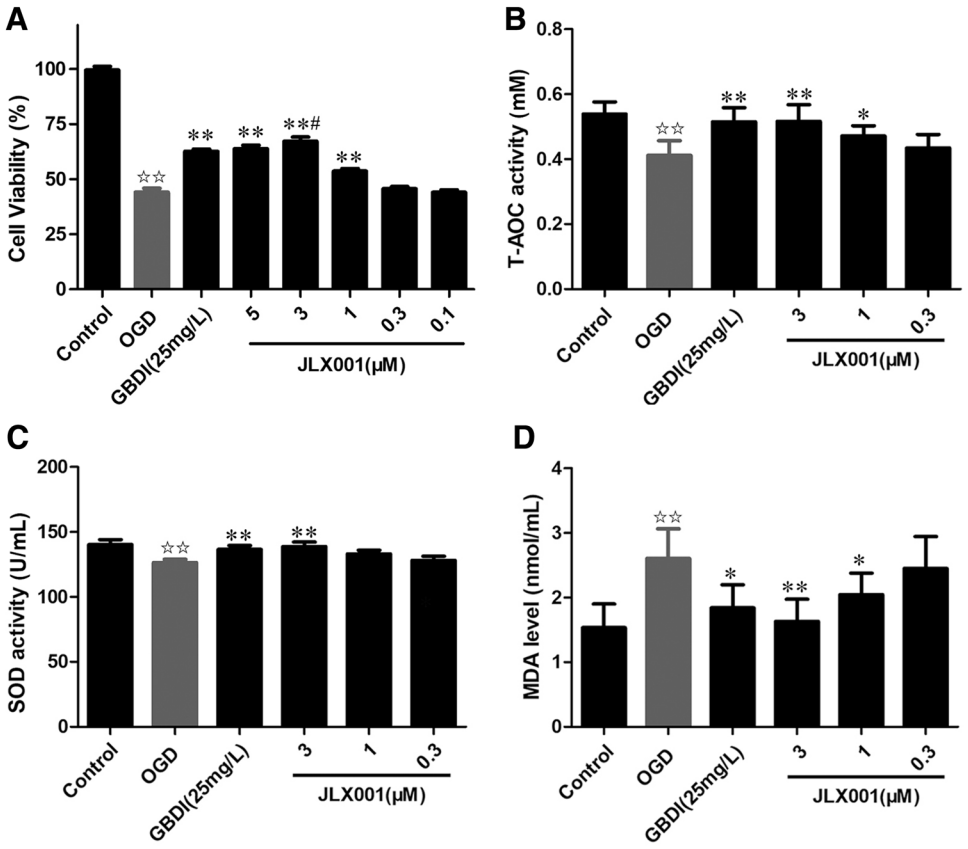

Fig. 8 Viability of microglial cells, and assays of T-AOC, SOD, and MDA in BV2 cells. **a** Cells were treated with JLX001 at dosages ranging from 0.1 to 5 μM during 4 h OGD. Cell viability was measured by MTT assay. Each group: n = 6. The experiment was repeated for three times. **b** T-AOC activity. **c** SOD activity. **d** MDA level. Each group: n = 10, Data are shown as mean ± SD, $^{☆☆}P < 0.05$, $^{☆☆}P < 0.01$ versus control group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus OGD group. $^{\#}P < 0.05$ versus GBDI group

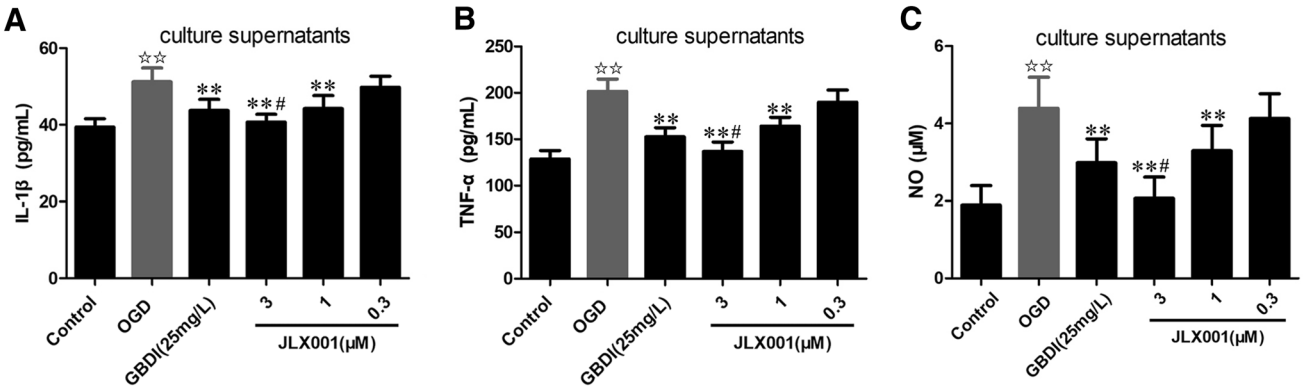

Fig. 9 JLX001 attenuates the release of IL-1β, TNF-α, and NO in BV2 cells. **a** and **b** The levels of IL-1β and TNF-α in culture supernatant after OGD. **c** The content of NO in culture supernatant after OGD. Data are shown as mean ± SD, n = 10, $^{☆☆}P < 0.01$ versus control group. $^{**}P < 0.01$ versus OGD group. $^{\#}P < 0.05$ versus GBDI group

significantly downregulated the expression of TLR2, TLR4 and NF-κB p65 ($P < 0.01$).

Discussion

Aerobic life depends on the molecular oxygen regenerated by ATP, but only in a narrow range of oxygen concentrations, which destroyed by free radicals from ischemic tissue. Oxidative stress is immediately triggered right after cerebral ischemia, which may result in the activation of TLR2/4-NF-κB signal pathway followed by the explosion of inflammatory cytokines aggravating ischemic injury [29]. As shown in Fig. 11, when the body is stimulated by ischemia, the nuclear transcription of NF-κB is promoted by activating MyD88-dependent signaling pathway, which ultimately leads to the occurrence of inflammatory reaction and increase in oxidative stress. Then, cerebral vascular

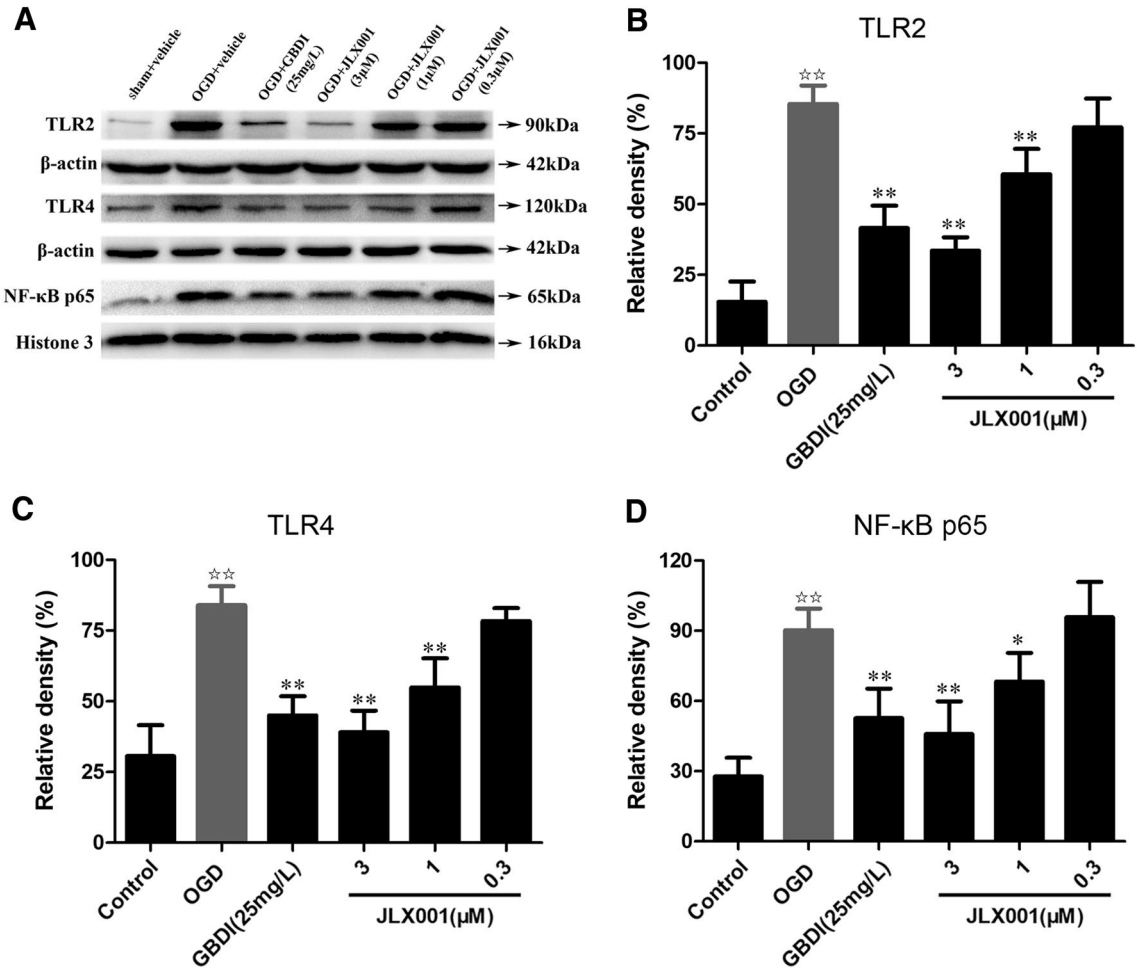

Fig. 10 JLX001 reduced the expression of TLR2/4 and inhibited the nuclear translocation of NF-κB p65. **a** Representative protein expression bands in BV2 cells after OGD. **b–d** Relative density of TLR2, TLR4, and NF-κB p65. Data are shown as mean ± SD n = 6, $^{\star\star}P < 0.01$ versus control group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus OGD group

endothelial cells, microglia, etc. release lots of inflammatory cytokines and free radicals, which in turn amplify the cascade effect of inflammatory damage, leading to cells degeneration and death. Consequently, we hypothesized that JLX001 modulated the inflammatory reaction and oxidative stress in permanent ischemic injury via inhibiting the TLR2/4-NF-κB signaling pathway in microglia.

CVB-D has a protective effect against oxidative stress, and energy metabolism in rats of myocardial injury have been shown in previous research [30]. Considering the complexity of Chinese herbal ingredients and the instability of the source, we first synthesized JLX001 from the beginning and evaluated its effects on permanent cerebral ischemia. Our data showed that JLX001 reduced the neurological score, brain infarct size, brain edema and improved pathological changes, which demonstrated that JLX001 protected brains against ischemic injury. Besides, HE staining results indicated that JLX001 exerted the protective action by decreasing inflammatory cell infiltration and neuronal loss in the brain. High levels of SOD can protect cerebral ischemia injury, catalytically reduce superoxide anion (O^{2-}) to hydrogen peroxide, and inhibit lipid peroxidation [19]. Further results suggested that JLX001 significantly enhanced the activities of T-AOC and SOD, and decreased the level of MDA.

Plenty of research demonstrated knockout of TLR2 or TLR4 reduced ischemic brain injury in an experimental cerebral ischemic model, indicating TLR2/4 have a detrimental effect [31, 32]. NF-κB, a most critical transcription factor locating downstream of TLR2/4, mediates inflammatory reactions after ischemic injury. NF-κB aggravates ischemic injury through upregulating many proinflammatory factors including IL-1β, TNF-α, and inducible nitric oxide Synthase (iNOS) [33]. Our results demonstrated the secretion of IL-1β, TNF-α, and NO were markedly decreased after JLX001 treatment compared with the

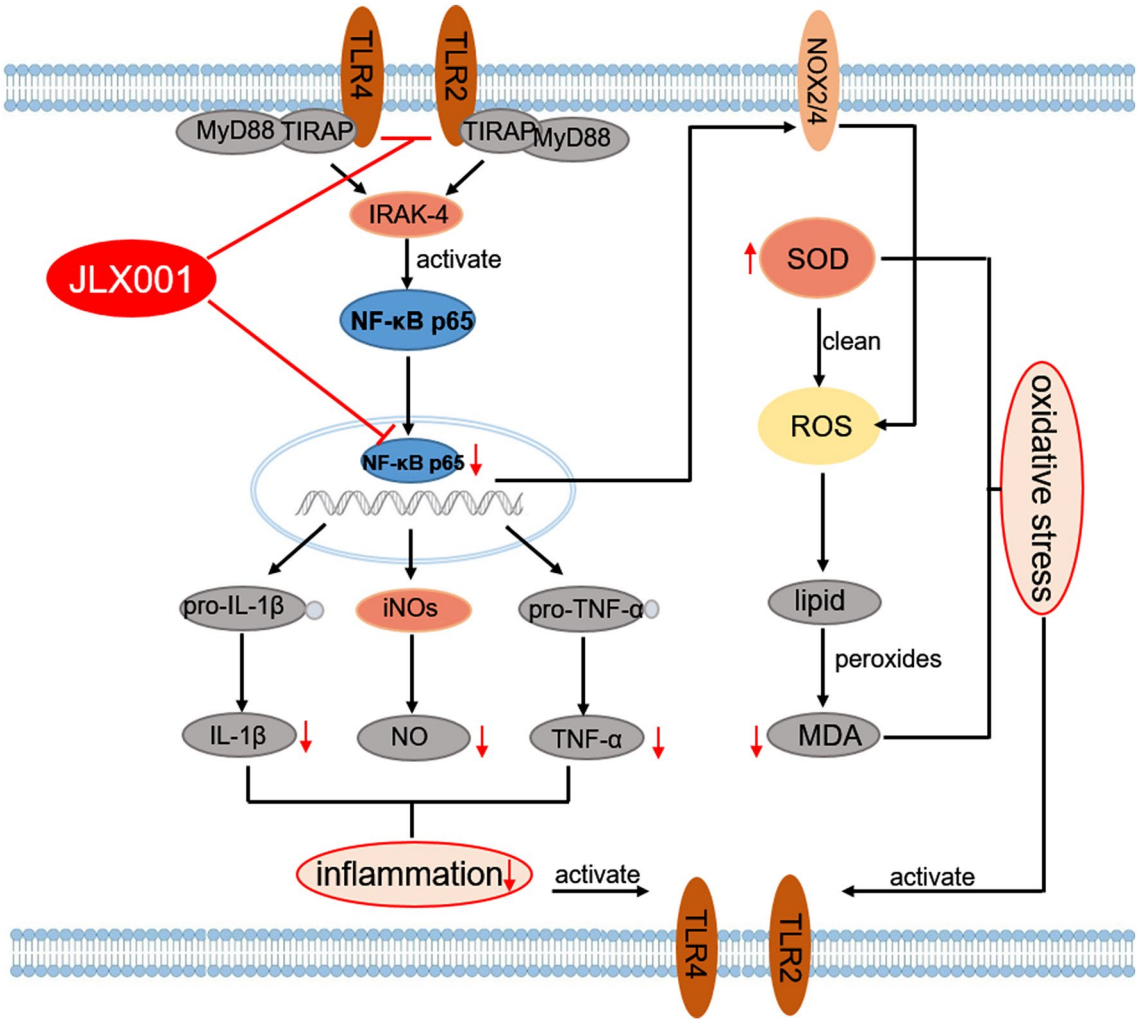

Fig. 11 Diagram of possible mechanism and the effect of JLX001 on ischemic injury (red arrow). *MyD88* myeloid differentiation primary response 88; *TIRAP* toll/interleukin-1 receptor domain-containing adapter protein; *IRAK* IL-1R-associated kinase; *ROS* reactive oxygen species, *NOX2/4* NADPH oxidase 2/4 (Color figure online)

model group, and the protein level of TLR2/4 and nuclear translocation of NF-κB p65 in the brain were dramatically up-regulated. These results proved that the anti-inflammatory effects and the inhibition of oxidative stress of JLX001 might be related to the inhibition of the TLR2/4-NF-κB pathway. The anti-inflammatory effects of CVB-D in LPS-stimulated murine macrophages in vitro [34], at least in part supported the above results.

Microglia, the primary immune effector cells in the brain and spinal cord, plays a vital function in the activation of the immune inflammation, whose activation depends on the interaction between the receptors and ligands [35, 36]. TLR2 and TLR4 are highly expressed in the microglia and are closely correlated to the activation of the NF-κB pathway during ischemic injury [37]. To evaluate the effect of JLX001 on microglial activation, Iba-1, a marker indicating microglial activation was quantified by immunohistochemistry. JLX001 reduced Iba-1 expression in both ischemic ipsilateral and contralateral hippocampal cortex regions after pMCAO. These results proposed that the neuroprotective effect of JLX001 on cerebral ischemia may be related to microglia.

Activated microglial cells are thought to be the central origin of inflammatory mediators and crucially involved in neuronal damage in the penumbra in ischemic stroke [38]. The previous study showed that inhibited the activation of microglia through regulation of TLR4/NF-κB pathway, and attenuated the release of pro-inflammatory cytokines could be a potential treatment for CNS injury [39]. To further explore the role of JLX001 on microglia, BV2 microglia were exposed to OGD to imitate the ischemic condition in vitro. JLX001 treatment significantly enhanced the activity of T-AOC and SOD, reduced the content of MDA, TNF-α, IL-1β, and NO and inhibited

the expression of TLR2, TLR4 and the nuclear expression of NF-κB p65 in BV2 cells. The result of the experiment in vitro confirmed the conclusion drawn from the animal experiment, indicating that the beneficial effects of JLX001 in cerebral ischemia injury are at least partly mediated by inhibiting the oxidative stress and TLR2/4-NF-κB pathway in microglia, but its exact mechanism needs further confirmation.

Although the expression of TLR2/4 and NF-κB in the ischemic brain tissues are increased, it cannot be concluded that these proteins are restricted to microglia [40]. Besides, the expression of proinflammatory mediators has been reported in reactive astrocytes which are featured prominently in ischemic brain tissue [41]. In addition, the activated microglia have been defined as either classic (pro-inflammatory; M1-like) or alternative (anti-inflammatory or protective; M2-like) under pathophysiological conditions [42]. Although future translational studies are required, M2-like microglia therapies are attractive for managing stroke based on their protective functions. Drug evaluations including JLX001 should take M1/M2-like cellular polarization into consideration.

Conclusions

In conclusion, JLX001 has therapeutic effects on permanent cerebral ischemic injury due to suppressing neuroinflammation and attenuating oxidative stress by inhibiting TLR2/4-NF-κB signaling pathway. This investigation provides evidence supporting JLX001 as a novel and potent candidate for the treatment of ischemic stroke in the future. Of course, the effect of JLX001 to microglia requires more experimental support, and the role of JLX001in other cells such as reactive astrocytes still needs further investigations.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest

Research Involving Animals SPF male SD rats were purchased from Qinglongshan Animal Farm (Nanjing, China, license number: SYXK (Su) 2017-0001). Rats were housed in coops on a 12 h light–12 h dark schedule at the controlled temperature of 23 ± 2.5 °C with free access to a normal diet and tap water. All animals were cared for and treated according to institutional guidelines of China Pharmaceutical University, which conforms to the European Community guidelines (EEC Directive of 1986: 86/609/EEC). Efforts were made to minimize the number of rats and their suffering.

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