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Full-length Article

Nafamostat mesilate improves function recovery after stroke by inhibiting neuroinflammation in rats

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ABSTRACT

Inflammation plays an important role in stroke pathology, making it a promising target for stroke intervention. Nafamostat mesilate (NM), a wide-spectrum serine protease inhibitor, is commonly used for treating inflammatory diseases, such as pancreatitis. However, its effect on neuroinflammation after stroke was unknown. Hence, the effects of NM on the inflammatory response post stroke were characterized. After transient middle cerebral artery occlusion (tMCAO) in rats, NM reduced the infarct size, improved behavioral functions, decreased the expression of proinflammatory mediators (TNF-α, IL-1β, iNOS and COX-2) in a time-dependent manner and promoted the expression of different anti-inflammatory factors (CD206, TGF-β, IL-10 and IL-4) at different time points. Furthermore, NM could inhibit the expression of proinflammatory mediators and promote anti-inflammatory mediators expression in rat primary microglia following exposure to thrombin combined with oxygen–glucose deprivation (OGD). The immune-modulatory effect of NM might be partly due to its inhibition of the NF-κB signaling pathway and inflammasome activation after tMCAO. In addition, NM significantly inhibited the infiltration of macrophage, neutrophil and T lymphocytes, which was partly mediated by the inhibition of monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Taken together, our results indicated that NM can provide long-term protection of the brain against tMCAO by modulating a broad components of the inflammatory response.
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1. Introduction

Ischemic brain damage is mediated by a complex series of biochemical and molecular mechanisms. Among these mechanisms, there is increasing evidence showing that inflammation is one of the key elements in the pathobiology of ischemic stroke (Chamorro et al., 2012; Fu et al., 2015). The inflammatory response following stroke is characterized by the activation of microglia and the infiltration of circulating inflammatory cells (Jin et al., 2010), which may progress over hours to days after stroke, and have extensive influence on stroke pathology. Microglia, for example, can be either neuroprotective or neurotoxic, and recent studies have showed that promoting the anti-inflammatory activation of microglia may provide long-term protection in experimental stroke (Jin et al., 2014).

Recently, a novel concept of 'thrombo-inflammation' has been raised for ischemic stroke, which describes a close relationship between thrombotic factors and the inflammatory response (Gob et al., 2015; Nieswandt et al., 2011). The serine proteases, such as thrombin, factor Xa (FXa) and kallikrein are best known for their functions on thrombosis (Siller-Matula et al., 2011). Besides, they may also play crucial roles in inflammation. For example, thrombin promotes the activation of NOD-like receptors family, pyrin domain-containing 3 (NLRP3) inflammasome in macrophage (Rossol et al., 2012) and microglia proinflammatory activation (Lee et al., 2005). FXa and kallikrein have also been reported to be links between inflammation and thrombosis (Prassas et al., 2015; Zuo et al., 2015). It is becoming increasingly apparent that thrombin, FXa and kallikrein are increased after ischemic stroke (Thevenet et al., 2009; Chen et al., 2012; Gob et al., 2015), and due to their dual role in thrombosis and inflammation, they may be promising targets for stroke treatment.

Nafamostat mesilate (NM), a wide-spectrum serine protease inhibitor, which inhibits several serine proteases such as thrombin, plasma kallikrein and FXa, is capable of blocking a battery of components in the coagulation system and the inflammatory cascades. For example, NM inhibits the inflammatory response in intestine, heart ischemia (Gobbetti et al., 2012; Schwertz et al., 2008) and multiple

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sclerosis (Li et al., 2009), and our previous research has demonstrated that NM attenuates neuronal damage after stroke through thrombin inhibition (Chen et al., 2014). Therefore, NM might be a potential candidate to reduce 'thrombo-inflammation' in stroke.

In this study we explored the effects of NM on behavioral recovery and neuroinflammation post transient middle cerebral artery occlusion (tMCAO) in rats. We demonstrated that NM improves behavioral recovery after tMCAO in rats by inhibiting the expression of proinflammatory mediators in a time-dependent manner, promoting the expression of different anti-inflammatory mediators at different time points, and inhibiting the recruitment of circulating immunocyte, which might be partly via inhibiting the activation of NF-κB signaling pathway and NLRP3 inflammasome. In addition, an *in vitro* hypoxia model, in which cells were exposed to oxygen–glucose deprivation (OGD) in the presence of thrombin, was taken to explore the mechanism of NM on neuroinflammation.

2. Materials and methods

2.1. Animals and surgery

Adult male Sprague–Dawley (SD) rats weighing 260–280 g were obtained from Zhejiang Laboratory Animals Center (Hangzhou, China) and housed in controlled-temperature environment under a 12-h light/dark cycle and allowed free access to food and water. All animal handling and surgical procedures were approved by the Animal Research Ethics Committee of China Pharmaceutical University. tMCAO was performed as reported earlier with some modifications (Longa et al., 1989). Briefly, rats were initially anesthetized with 3% chloral hydrate (Sinopharm Chemical Reagent Co., Ltd, Shanghai, China) in 0.9% saline (Sinopharm Chemical Reagent Co., Ltd). The body temperatures were maintained at 37.0 °C with warming pads, and the cerebral blood flow (CBF) was monitored via laser Doppler flowmetry (Moor Instruments, Essex, UK). The bifurcation of the right common carotid artery was exposed, and a 3-0 poly-lysine (Sigma–Aldrich, USA) coated monofilament nylon suture was advanced through external carotid artery into the lumen of internal carotid artery to occlude the origin of the middle cerebral artery (MCA). In the ischemia phase, the blood perfusion dropped >75% of the base line was considered as successful ischemia. Rats were re-anesthetized and the suture was gently withdrawn to restore the blood flow after 2 h occlusion. And blood gases were monitored with i-STAT Portable Clinical Analyzer (Abbott, Ontario, Canada), blood pressure were monitored with biological function experiment system (Zhenghua, Anhui, China), and temperature were monitored with thermometer. These physiological factors monitored before, during, and after surgery.

2.2. Drug treatment

The specific thrombin inhibitor argatroban (Enzo Life Sciences, USA) was used as positive control. NM (Nanjing D&R Pharmaceutical Company, Nanjing, China) and argatroban were diluted in 5% glucose (Sinopharm Chemical Reagent Co., Ltd). Animals were randomly divided into six groups, including sham, vehicle, argatroban (3.4 mg/kg) and NM (0.01, 0.1 and 1 mg/kg) groups, respectively. After tMCAO surgery, rats in the vehicle group and drug-treated groups were intravenously treated with glucose or drugs respectively at the beginning of ischemia (0 h) and the moment of reperfusion (2 h). And drugs were administered again with the same time interval at 4 h and 6 h after ischemia onset.

2.3. Behavioral tests

Behavioral tests were performed at different days after MCA occlusion.

1) Corner test: the corner test was used to assess the sensorimotor deficit as described before with some modification (Jin et al., 2014). Briefly, a rat was placed between two boards with an angle of 30° and facing the corner. Both sides of the vibrissae were stimulated together when the rat entered deep into the corner. The rat then turn back to face the open side. The non-ischemic rats turned non-selectively left or right, but the tMCAO treated rats preferentially turned toward the non-impaired side. Between each trails, there was a rest period of 10 s. The turns toward the non-impaired side were recorded from ten trials for each test.
2) Grip-traction test: the modified grip-traction test was used to test the muscle strength of the rat by hanging the rat to a horizontal rope (4 mm in diameter) by its forepaws (Bona et al., 1997). Time to falling (maximum 60 s) was recorded.
3) Beam balance test: the beam was 2.5 cm in width, 80 cm in length, and 60 cm in height. The beam balance test was performed as described before (Chen et al., 2001).
4) Limb-placing test: the test was taken to test the asymmetry of the upper limb movement (Bona et al., 1997). This test consist six limb-placing tasks, with a 3-point scale each.
5) Y-maze test: the test was performed as descried previously (Tang et al., 2013). The Y-maze was constructed of black plastic walls (10 cm high), consisting of three compartments (10 cm × 10 cm) connected with passages (4 cm × 5 cm), with the floor of 3.175 mm stainless steel rods (8 mm apart). On day 1 (the training trial), each rat was placed in the conjunction area of the maze and allowed to explore the maze freely for 5 min with no electric shocks. Then two of the three arms were turned to be shocks-available but light-off, with the third one was shock-free and light-on. Each rat was trained for 10 times, the training was completed once the rat entered the shock-free arm and stayed for 30 s, which was taken as right choice. The testing trail was taken on the next day, each rat was tested for 10 times following the same procedures as on day 1. The times and the latency to enter the shock-free arm were recorded.
6) Longa test: this test was assessed using a 5-point scale as described previously (Chen et al., 2014): 0, no observable deficits; 1, failure to extend the left forepaw; 2, circling to the left; 3, falling to the left; and 4, unable to move spontaneously.

2.4. Histological assessments of the brain damage following MCAO

At 7 days after MCAO, the infarct volumes were evaluated with triphenyl-2,3,4-tetrazolium-chloride (TTC) stain. The brains were coronal cut into 2-mm-thick slices and stained with saline containing 2% TTC (Wako Pure Chemical Industries, Ltd., Osaka) at 37 °C for 10 min. The infarct areas were measured using Image J (NIH). The infarction rates were calculated as the infarction volume/the brain volume × 100%.

2.5. Western blotting

At 6 or 24 h after tMCAO, tissues collected from the MCA perfusion area (coronal between +3 and −2 mm relative to Bregma in the ipsilateral cortex) were homogenized with radio immunoprecipitation assay (RIPA) buffer (Beyotime, Hangzhou, China) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Protein concentrations were measured with bicinchoninic acid assay (BCA) kit (Beyotime). Equal amounts of protein (50 μg) of each samples was separated by using sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred to nitrocellulose filter membrane. Membranes were blocked with 2.5% (w/v)

bovine serum albumin (BSA, Sigma) in Tris-Buffered Saline with Tween 20 (0.1%, TBST) for 1 h in room temperature with slow shaking. The membrane was then incubated overnight at 4 °C with primary antibodies including iNOS (1:1000, Abcam, Cambridge, MA, USA), COX-2 (1:1000, Santa Cruz Biotechnology Inc., CA, USA), CD206 (1:1000, Cell Signaling Technology, Danvers, MA, USA), caspase-1 (1:4000, Abcam), NLRP3 (1:500, Santa Cruz Biotechnology Inc.), phosphorylation-NF-κB p65 at Ser536 (1:1000; Cell Signaling Technology Inc.), NF-κB p65 (1:1000; Cell Signaling Technology Inc.), phosphorylation-IKK at Ser180 (1:1000; Cell Signaling Technology Inc.), IKK (1:1000; Cell Signaling Technology Inc.), IκBα (1:1000; Cell Signaling Technology Inc.), and β-actin (1:1000, Santa Cruz Biotechnology Inc.). After washing four times (6 min per wash) with TBST, the membrane was incubated with secondary antibodies against the primary antibody for 1 h at room temperature. After washed, the labeled proteins were detected using the Bio-Rad system (Bio-Rad, Germany).

2.6. Cytokine enzyme-linked immune sorbent assays (ELISA)

At 24 h after tMCAO, brain samples harvested as described above were homogenized, and the concentration of IL-1β, TNF-α, MCP-1, IL-10 and IL-4 in brain homogenates were measured by ELISA kits (RayBiotech, Norcross, GA) according to the manufacturer's recommendations.

2.7. Preparation of inflammatory cells from brain tissues

This method was a modification of the isolation protocol developed by Lee et al. (2007). Briefly, at 1, 3 and 7 days after MCAO, rats were anesthetized with 3% chloral hydrate, and then received transcardiac perfusion with 0.9% saline to remove the exclude blood cells from the vasculature of the brain. Brains were rapidly removed and immersed into ice cold phosphate-buffered saline (PBS) containing 0.2% (w/v) BSA. After removing the olfactory bulb and cerebellum, the whole ischemic hemisphere was dissociated by ophthalmic scissors, the suspension was digested with 1 mL dissociation solution (20 U/mL Papain and 0.05% DNase I; Sigma–Aldrich) for 90 min at 37 °C. The digested material was passed through a 40 μm nylon cell strainer. After centrifugation of 400*g* for 10 min, the pellet was re-suspended in 5 mL of isotonic Percoll (Amersham Pharmacia Biotech, Buckinghamshire, UK) brought to a density of 1.030 g/mL. This cells-containing solution was gently overlaid on the top of 2.5 mL of Percoll (1.095 g/mL), then pipette 2.5 mL of PBS on top of the cells-containing layer slowly, and centrifuged for 20 min at 1000 × g in room temperature avoid of brake. Cells were collected from the interface and washed once with PBS containing 0.2% (w/v) BSA.

2.8. Flow cytometric analysis

In order to analyze the number of infiltrating inflammatory cells in the ischemic hemisphere after ischemia, flow cytometric analysis was performed. Bone marrow cells were isolated as described above, cells were stained with a combination of the following anti-rat antibodies: CD45-APC (eBiosciences, San Diego, CA), His 48 Granulocyte Marker-PE (eBiosciences), CD11b/c-FITC (Biolegend, San Diego, CA), CD3- PerCP-eFluor® 710 (eBiosciences). Antibody-labeled cells were washed with PBS. Characterization of antibody-labeled cells was performed on a BD FACS calibur flow cytometer (BD Biosciences, San Diego, CA). The data obtained were then analyzed using Cell Quest software (BD Biosciences). Cells were classified as followed: (1) resting microglia ($CD45^{+}CD11b^{low}$-$His48^{-}$), (2) macrophage/activated microglia ($CD45^{+}CD11b^{high}$-$His48^{-}$), (3) neutrophil ($CD45^{+}CD11b^{high}His48^{+}$) and T lymphocyte ($CD45^{+}CD11b^{-}His48^{-}CD3^{+}$).

2.9. Real-time PCR

At 1, 3 and 7 days after tMCAO, brain samples collected as described above were processed for RT–PCR. The samples were homogenized in TRIzol reagent (Vazyme, Nanjing, China). Total RNA was extracted from the tissue according to the manufacturer's suggested protocol. The RNA was converted to cDNA using HiScript 1st Strand cDNA Synthesis Kit (Vazyme). Quantitative PCR was performed on an ABI7000 real-time PCR system (Applied Biosystems, Inc., University Park, IL, USA) using SYBR Green Master Mix (Vazyme). The cycle time values were normalized to GAPDH of the same sample. Primer sequences are shown in Table 1.

2.10. Primary microglia-enriched cultures

Primary microglial culture was prepared following the described method with some modifications (Yan et al., 2012). Briefly, after removing meninges, the cerebral cortex from postnatal SD rats (P0-P2) was digested with trypsin–EDTA (0.25%, Sigma), and then the resulting cell suspension were seeded into poly-lysine (0.05 mg/ml, Sigma) pre-coated 75 cm^2 flasks in Dulbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F12) supplement with 10% heat-inactivated fetal bovine serum (FBS). Subsequent medium was changed every 3 days. After two weeks in culture, the loosely attached microglia were isolated by gently shaking of the flask. The resulting cell suspension was then collected and seeded in DMEM/F12 supplement with 10% heat-inactivated FBS. About 95% of these cells were positive for Iba-1, a marker for microglia cell types.

To mimic the relevant situation of microglia in stroke, an *in vitro* hypoxia model was used as described (Won et al., 2015). In brief, microglia were washed once with serum-free DMEM/F12 medium, and starved with the same serum-free medium for 2 h with or without prior to exposure to OGD. OGD was induced by transfer to glucose free DMEM and maintained at 5% CO_2 and 1% O_2/N_2 in 37 °C in an OGD-chamber. For reoxygenation, the medium was replaced with DMEM/F12 medium supplemented with thrombin at 5% CO_2 and 95% air in 37 °C, when the drug treatment groups were pretreated with NM or argatroban for 5 min before thrombin. After reoxygenation for 2 or 24 h, microglia were harvested for further investigation. For NLRP3 inflammasome priming, LPS was added to a final concentration of 1 μg/mL in the OGD phase. To be mentioned, although thrombin may activate NF-κB signaling, but to highlight the effect of thrombin on 'inflammasome install', microglia was primed with LPS before thrombin treatment *in vitro*.

2.11. Statistical analysis

Results were presented as means ± SEM. For statistical analysis, the GraphPad Prism Software 6 (La Jolla, CA, USA) was used. Differences in the frequencies (corner test, beam balance test, limb-placing test, Y-maze and Longa test) were analyzed by

Table 1
Primer sequences for PCR reactions.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
TNF-α	TTCCCAAATGGGCTCCCTCT	GTGGGCTACGGGCTTGTCAC
IL-1β	TCCAGGATGAGGACCCAAGC	TCGTCATCATCCCACGAGTCA
iNOS	AGGCCACCTCGGATATCTCT	GCTTGTCTCTGGGTCCTCTG
MCP-1	CCAGAAACCAGCCAACTCTCA	AAGCGTGACAGAGACCTGCAT
IL-10	CAAGGCAGTGGAGCAGGTGA	CCGGGTGGTTCAATTTTTCATT
CD206	GGTTCCGGTTTGTGGAGCAG	TCCGTTTGCATTGCCCAGTA
TGF-β	CGTGGAAATCAATGGGATCAG	CAGGAAGGGTCGGTTCATGT
IL-4	CAGGGTGCTTCGCAAATTTTAC	ACCGAGAACCCCAGACTTGTT
ICAM-1	CTCTTGCGAAGACGAGAACC	GCCACAGTTCTCAAAGCACA
VCAM-1	ACAAAACGCTCGCTCAGATT	GTCCATGGTCAGAACGGACT
GAPDH	CAGCCTCGTCTCATAGACAAGATG	AAGGCAGCCCTGGTAACCA

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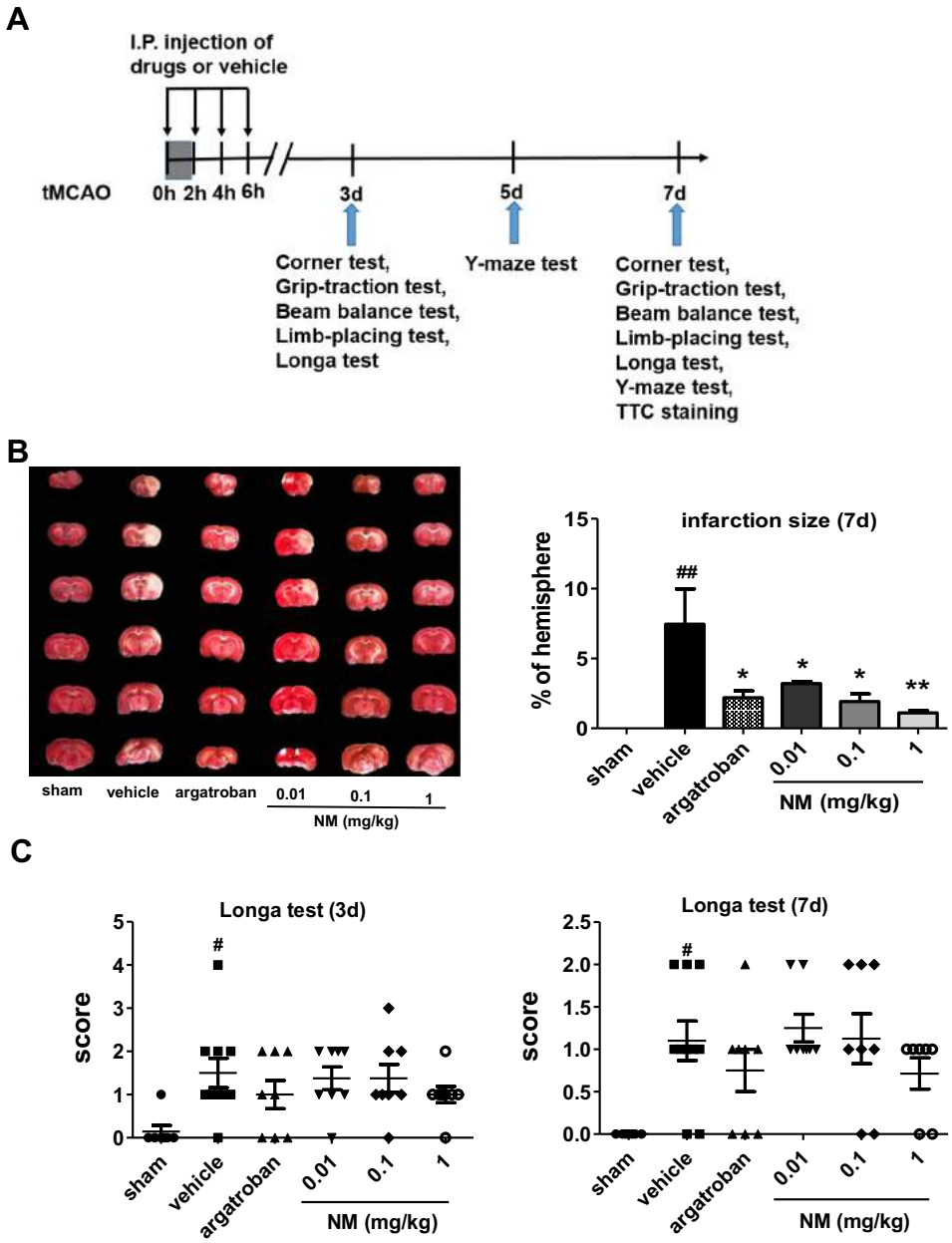

Fig. 1. Effects of NM on brain infarct volume and sensorimotor and cognitive functions. (A) The drug administration schedule and behavioral assessment timeline are illustrated schematically. (B) NM reduced cerebral infarction volume as assessed by TTC staining at 7 days after tMCAO. Excepted for the Longa test (C), NM treatment improved sensorimotor recovery as evaluated by limb-placing test (D), corner test (E), beam balance test (F) and the grip test (G) at 3 and 7 days after tMCAO. Y maze test was employed to monitor the cognitive decline. (H) Rats in the NM group showed improved cognitive function as shown with more correct choice and less latency time compared with the vehicle group at 5 days after tMCAO. (I) The protective effect of NM on cognitive function were maintained at 7 days after tMCAO. N = 7–9, results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

non-parametric Mann–Whitney test. One-way ANOVAs followed by post hoc Student-Newman-Keuls test were utilized for multiple-group comparisons of the parameters. Two-way ANOVAs followed by post hoc Student-Newman-Keuls test were utilized for multiple-factors comparisons of the parameters in Figs. 2C and D, 7 and 8A–C. All tests were considered statistically significant at $P < 0.05$.

3. Results

3.1. NM reduced brain infarct volume and improved sensorimotor and cognitive function

Among the 396 rats underwent the tMCAO surgery, 15 rats were excluded for unsuccessful occlusion (CBF fell less than 75% of baseline) and 7 rats were excluded for unsuccessful reperfusion (CBF raised to less than 70% of baseline). 64 rats died either during (n = 15) or after surgery (n = 49). The rats that died after surgery were evenly distributed among groups (the vehicle group: n = 12, the argatroban group: n = 7, the NM (0.01 mg/kg) group: n = 11, the NM (0.1 mg/kg) group: n = 9, the NM group (1 mg/kg): n = 10). During surgery, the CBF, blood gasses, body temperature and blood pressure were within normal ranges for all animals and did not differ among groups (data not shown).

At first we observed the effect of NM on brain infarct volume at 7 days after tMCAO. Compared with the vehicle group, the infarct sizes were smaller in the positive control argatroban group ($P < 0.05$) and the NM groups (0.01 mg/kg, $P < 0.05$; 0.1 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$) as assessed by TTC staining (Fig. 1B).

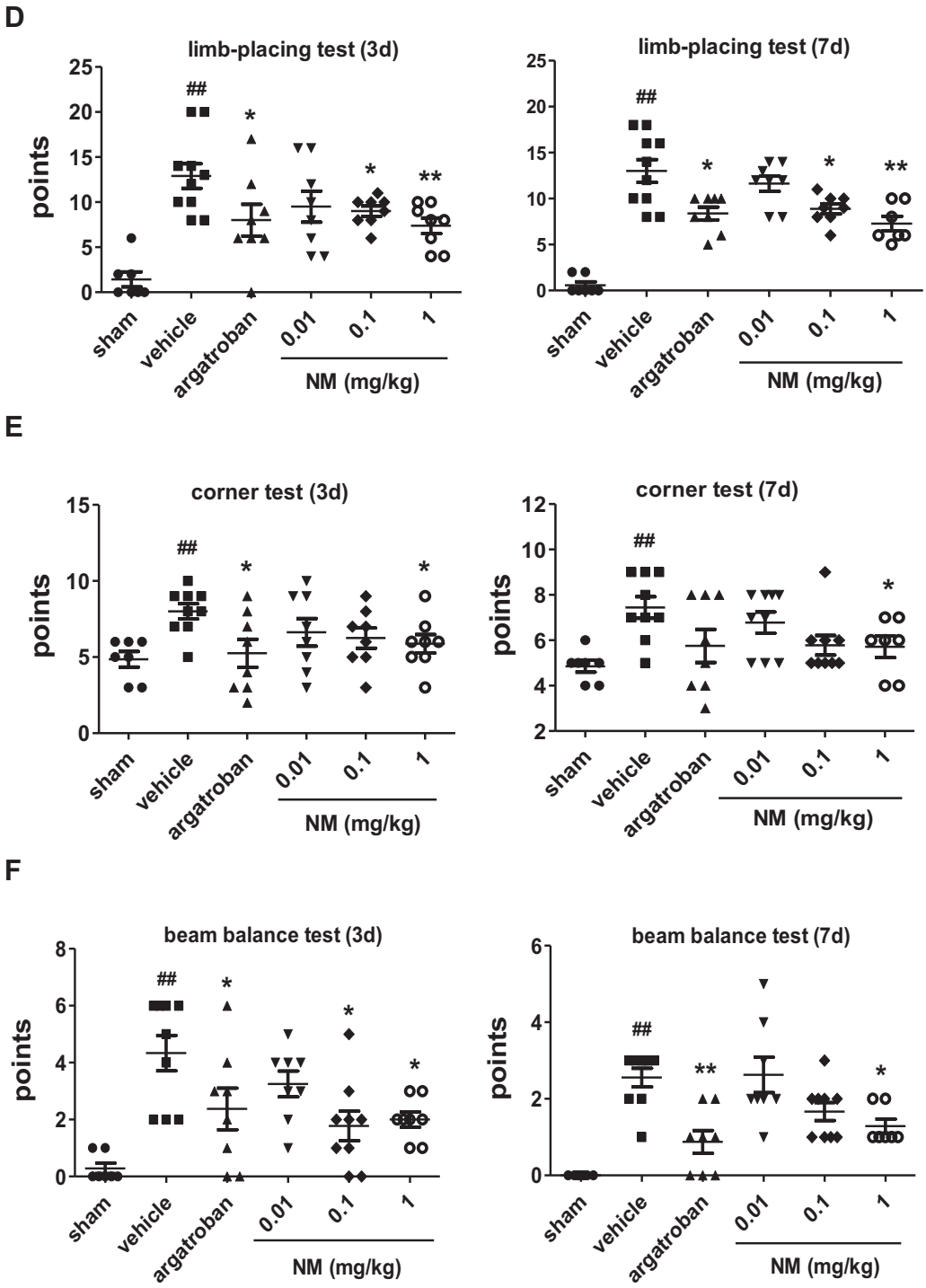

Fig. 1 (*continued*)

Next, a battery of behavior tests was conducted to explore the effects of NM on sensorimotor and cognitive recovery after tMCAO. The behavioral assessment timeline was shown in Fig. 1A. At 3 or 7 days after tMCAO, the vehicle treated rats exhibited significant deficits in mobility of the left limbs as shown in the Longa test ($P < 0.05$) and the limb-placing test ($P < 0.01$) (Fig. 1C and D). At 3 or 7 days after tMCAO, the limb-placing test showed that the forelimb mobility of the tMCAO rats were markedly improved by NM (0.1 mg/kg: $P < 0.05$; 1 mg/kg: $P < 0.01$) or argatroban ($P < 0.05$) compared with vehicle (Fig. 1D).

The corner test was applied to monitor the sensorimotor asymmetry, at 3 or 7 days after tMCAO, rat in the sham group turned non-selectively left or right, the vehicle treated rat performed more right turns after tMCAO ($P < 0.01$, versus sham) (Fig. 1E). Whereas the sensorimotor asymmetry was ameliorated by NM (1 mg/kg) (at day 3, $P < 0.05$; at day 7, $P < 0.05$) or argatroban (at day 3, $P < 0.05$; at day 7, $P > 0.05$) compared with vehicle (Fig. 1E).

In the beam balance test, rats in the vehicle group could not balance on the beam and fall off quickly after tMCAO. At 3 days after tMCAO, performance in the test was significantly improved in rats

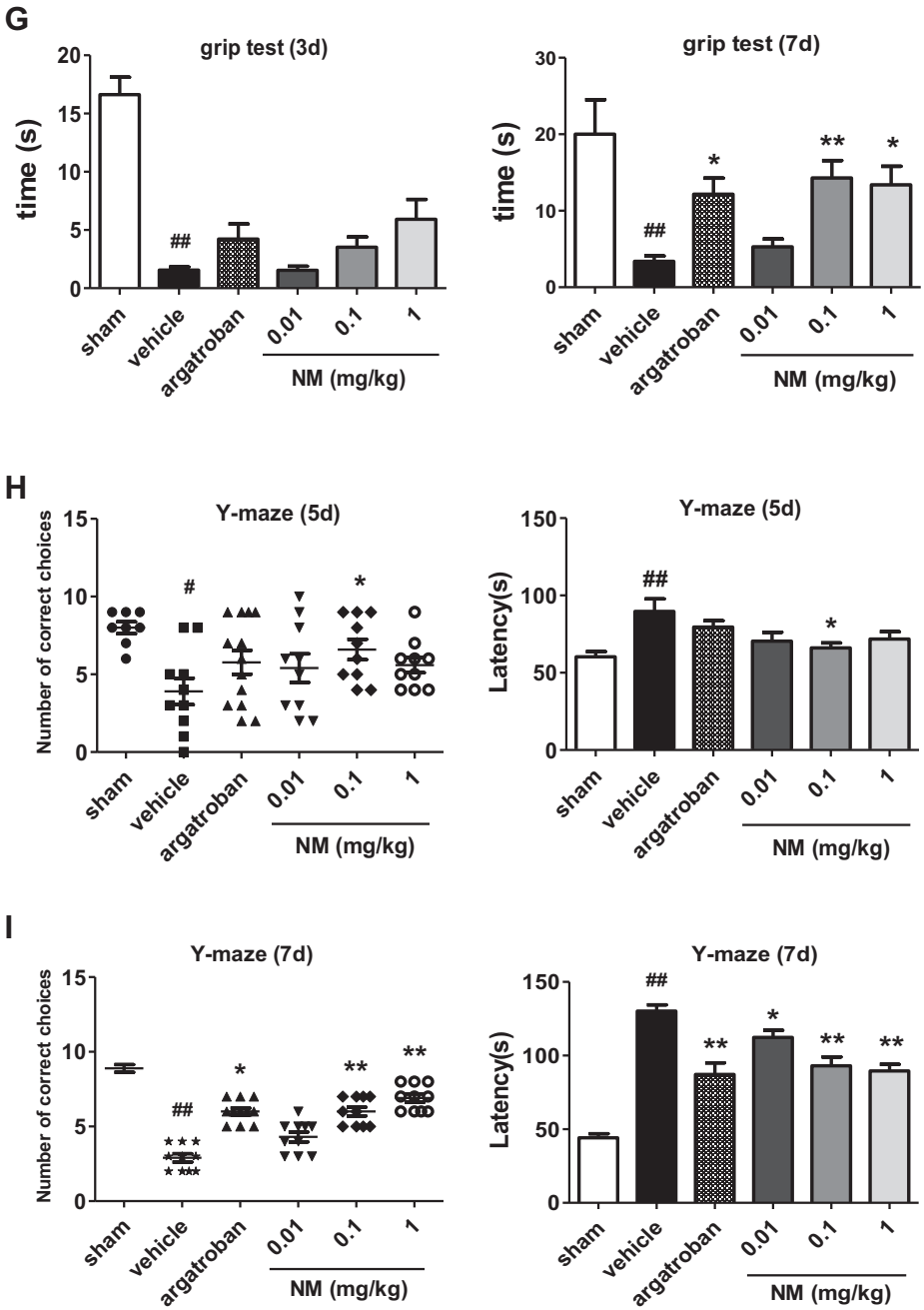

Fig. 1 (*continued*)

treated with NM (0.1 and 1 mg/kg) or argatroban ($P < 0.05$, respectively) (Fig. 1F). At 7 days after tMCAO, performance in the test was markedly improved in rats treated by NM (1 mg/kg, $P < 0.05$) or argatroban ($P < 0.01$) (Fig. 1F). Besides, as shown in the grip test, the muscle strength of the tMCAO rats was markedly improved by NM (0.1 mg/kg, $P < 0.01$; 1 mg/kg, $P < 0.05$) or argatroban ($P < 0.05$) compared with vehicle at 7 days after tMCAO (Fig. 1G).

The Y-maze showed that rats perform significant cognitive deficits after tMCAO (Fig. 1H and I). At 5 days after tMCAO, rats in the NM (0.1 mg/kg) group performed with more correct choice, accompanied by significantly less latency time ($P < 0.05$, versus vehicle), while other drug treatment groups showed no significant effect (Fig. 1H). And at 7 days after tMCAO, rats performed with more correct choice in the NM groups (0.1 and 1 mg/kg, $P < 0.01$, respectively) and the argatroban group ($P < 0.05$), and less latency time in the NM groups (0.01 mg/kg, $P < 0.05$; 0.1 mg/kg, $P < 0.01$; 1 mg/kg, $P < 0.01$) and the argatroban group ($P < 0.01$) (Fig. 1H and I). These observations indicated that NM reduced brain infarct volume and improved the sensorimotor and cognitive functions.

3.2. NM altered the expression profiles of inflammation mediators after tMCAO

To explore the effect of NM on the inflammatory response after tMCAO, the expression profiles of proinflammatory and anti-inflammatory factors were explored. At 1 day after tMCAO, the protein levels of the proinflammatory factors, including TNF-α, IL-1β, iNOS and COX-2 were significantly elevated in the vehicle group ($P < 0.01$, respectively, versus sham), which were significantly attenuated by NM (0.01 mg/kg) (TNF-α: $P < 0.05$; COX-2:

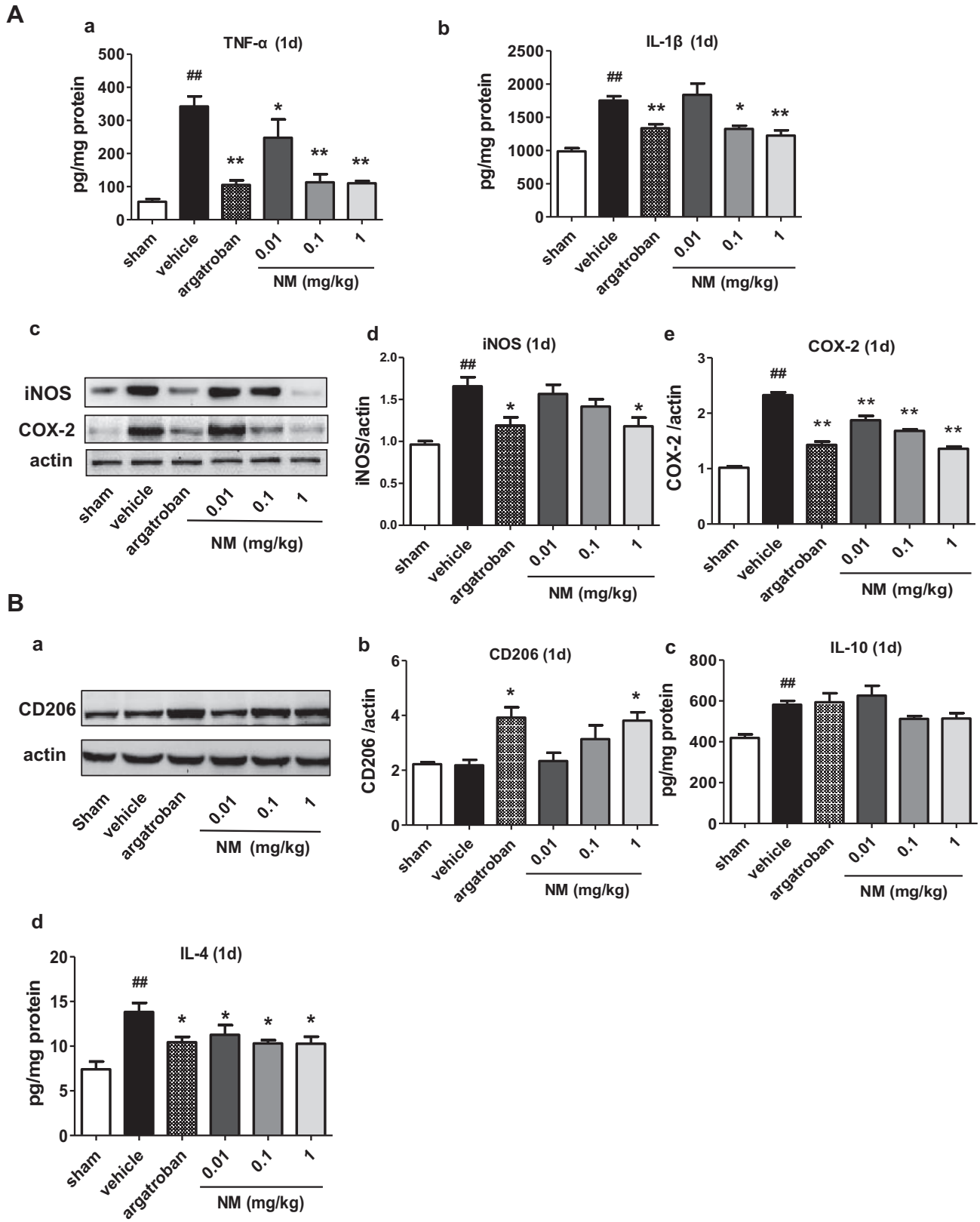

Fig. 2. The effect of NM treatment on the expression profiles of inflammation mediators at 1, 3 and 7 days after tMCAO. (A (a–e)) NM reduced the protein levels of proinflammatory mediators, including TNF-α, IL-1, iNOS and COX-2 in the ischemic cortex at 1 day after tMCAO (n = 4–7). (B (a–d)) The effect of NM treatment on the protein levels of CD206, IL-10 and IL-4 in the ischemic cortex at 1 day after stroke (n = 4). (C (a–c)) NM reduced the mRNA levels of TNF-α, iNOS and IL-1β at 3 and 7 days after tMCAO (n = 4). (D (a–d)) NM increased the mRNA levels of CD206, IL-10, TGFβ and IL-4 at 3 days after tMCAO, and up-regulated the mRNA level of CD206 without significantly affecting IL-10, TGFβ and IL-4 at 7 days after tMCAO (n = 4). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

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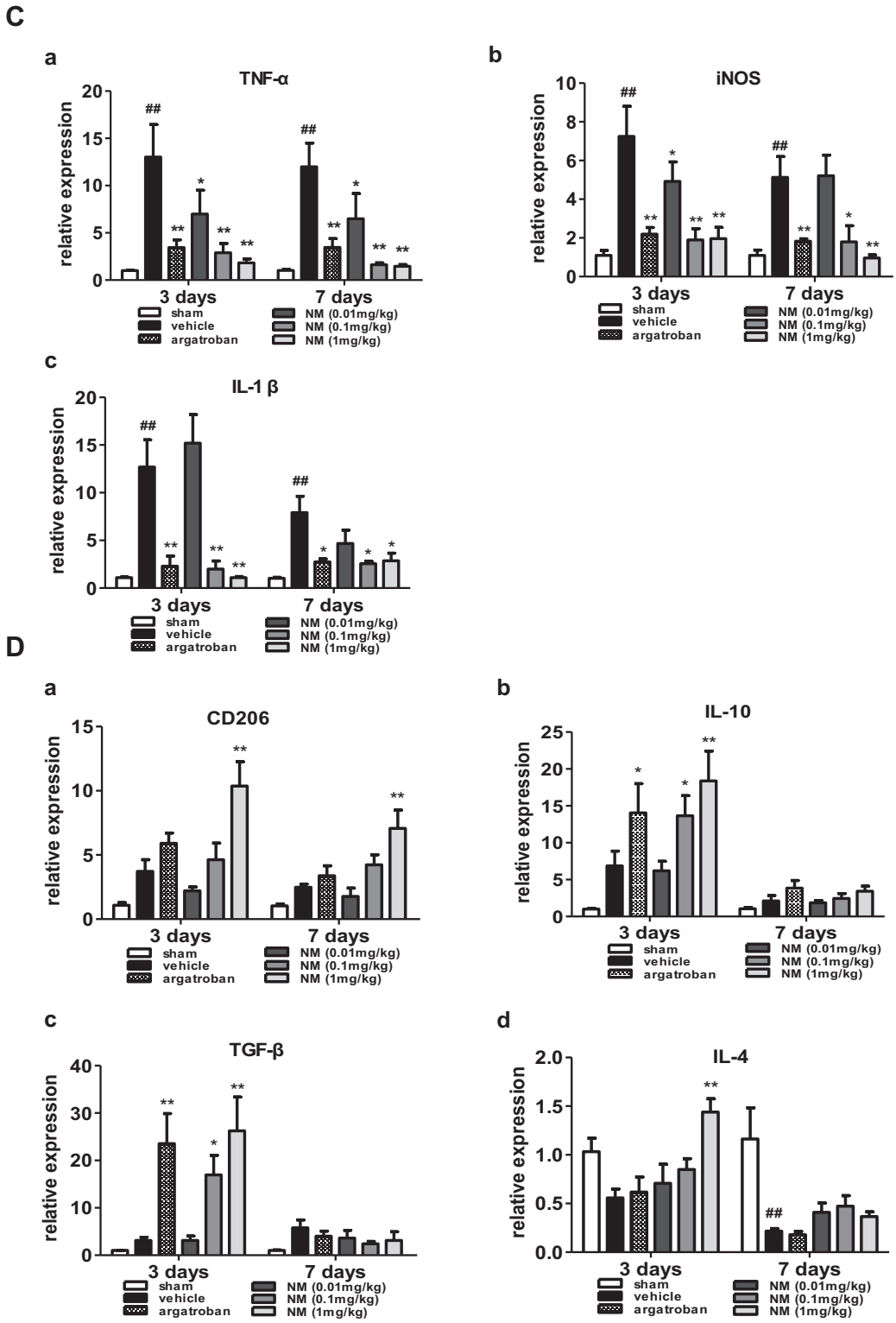

Fig. 2 (*continued*)

$P < 0.01$), NM (0.1 mg/kg) (TNF-α: $P < 0.01$; IL-1β: $P < 0.05$; COX-2: $P < 0.01$), NM (1 mg/kg) (TNF-α: $P < 0.01$; IL-1β: $P < 0.01$; iNOS: $P < 0.05$; COX-2: $P < 0.01$) and argatroban (TNF-α: $P < 0.01$; IL-1β: $P < 0.01$; iNOS: $P < 0.05$; COX-2: $P < 0.01$) (Fig. 2A).

At 1 day after tMCAO, the level of CD206 was not altered after tMCAO ($P > 0.05$), whereas NM (1 mg/kg) or argatroban treatment markedly increased the expression of CD206 ($P < 0.05$, versus vehicle) (Fig. 2B (a, b)). In addition, compared with vehicle, NM (0.01, 0.1 and 1 mg/kg) or argatroban did not affect IL-10 ($P > 0.05$) (Fig. 2B (c)), but decreased the protein level of IL-4 ($P < 0.05$, respectively) (Fig. 2B (d)).

At 3 or 7 days after tMCAO, the mRNA level of TNF-α was significantly increased in the ischemic regions of the vehicle group ($P < 0.01$, versus sham), which was markedly decreased by NM

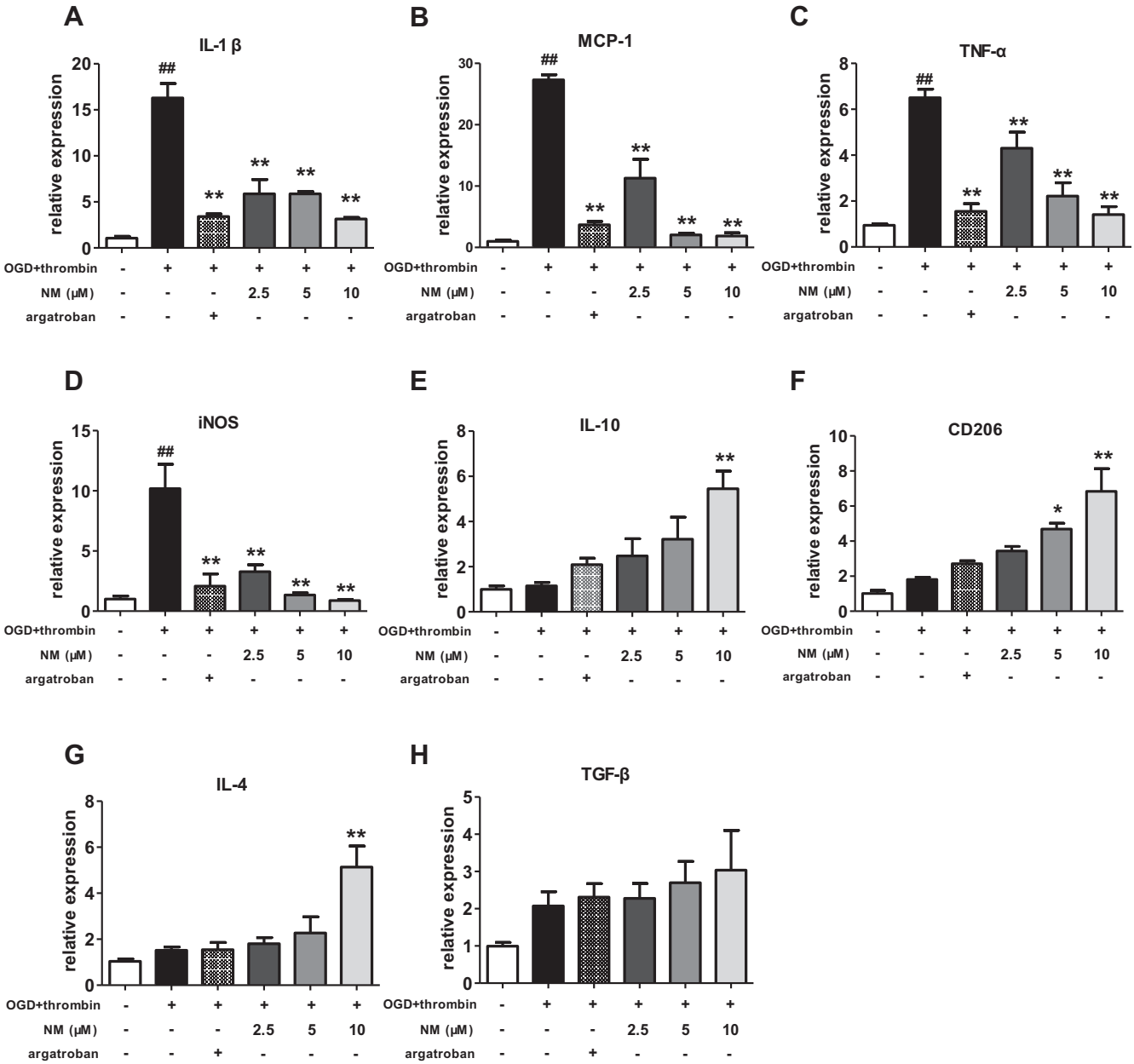

Fig. 3. Effects of NM on the expression of inflammatory mediators in microglia *in vitro*. Microglia in the culture were treated by OGD in the presence of thrombin. (A–D) The mRNA levels of proinflammatory mediators (IL-1β, MCP-1, TNF-α and iNOS, respectively) and (E–H) the levels of anti-inflammatory mediators (IL-10, CD206, IL-4 and TGF-β, respectively) were examined by RT-PCR (n = 3). Results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the control group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the OGD + thrombin treated group.

(0.01 mg/kg, $P < 0.05$; 0.1 mg/kg, $P < 0.01$; 1 mg/kg, $P < 0.01$) or argatroban ($P < 0.01$) (Fig. 2C (a); Time × Drug interaction: F (5,36) = 0.049, $P > 0.05$).

Besides TNF-α, significantly increased mRNA level of iNOS was observed in the vehicle group at 3 or 7 days after tMCAO ($P < 0.01$, versus sham) (Fig. 2C (b)). Compared with vehicle, the mRNA level of iNOS was decreased by NM (0.1 and 1 mg/kg) or argatroban at 3 days after tMCAO ($P < 0.01$, respectively) (Fig. 2C (b)), and these decrease were maintained at 7 days after tMCAO in the NM groups (0.1 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$) and the argatroban group ($P < 0.01$) (Fig. 2C (b); Time × Drug interaction: F(5,36) = 0.6287, $P > 0.05$).

Remarkable increased IL-1β was also observed in the vehicle group at 3 or 7 days after tMCAO ($P < 0.01$, versus sham), which was attenuated by NM (0.1, 1 mg/kg) or argatroban at both 3 days ($P < 0.01$, respectively) and 7 days ($P < 0.05$, respectively) after tMCAO (Fig. 2C (c); Time × Drug interaction: F(5,36) = 5.423, $P < 0.01$).

Moreover, NM (1 mg/kg) significantly increased the mRNA levels of the anti-inflammatory mediators, including CD206, IL-10, TGF-β and IL-4 versus the vehicle group at 3 days after tMCAO ($P < 0.01$, respectively), but at smaller doses, it had a less effect (0.1 mg/kg: CD206, $P > 0.05$; IL-10, $P < 0.05$; TGF-β, $P < 0.05$; IL-4, $P > 0.05$) (Fig. 2D (a–d)). At 7 days after tMCAO, NM (1 mg/kg) remarkably increased the mRNA level of CD206 compared with the vehicle group ($P < 0.01$) (Fig. 2D (a)), without affecting the expression of IL-10, TGF-β and IL-4 ($P > 0.05$) (Fig. 2D (b–d)), whereas NM (0.1 and 0.01 mg/kg) or argatroban did not affect the expression of CD206, IL-10, TGF-β and IL-4 ($P > 0.05$, respectively) (Fig. 2D; Time × Drug interaction: CD206, F(5,36)

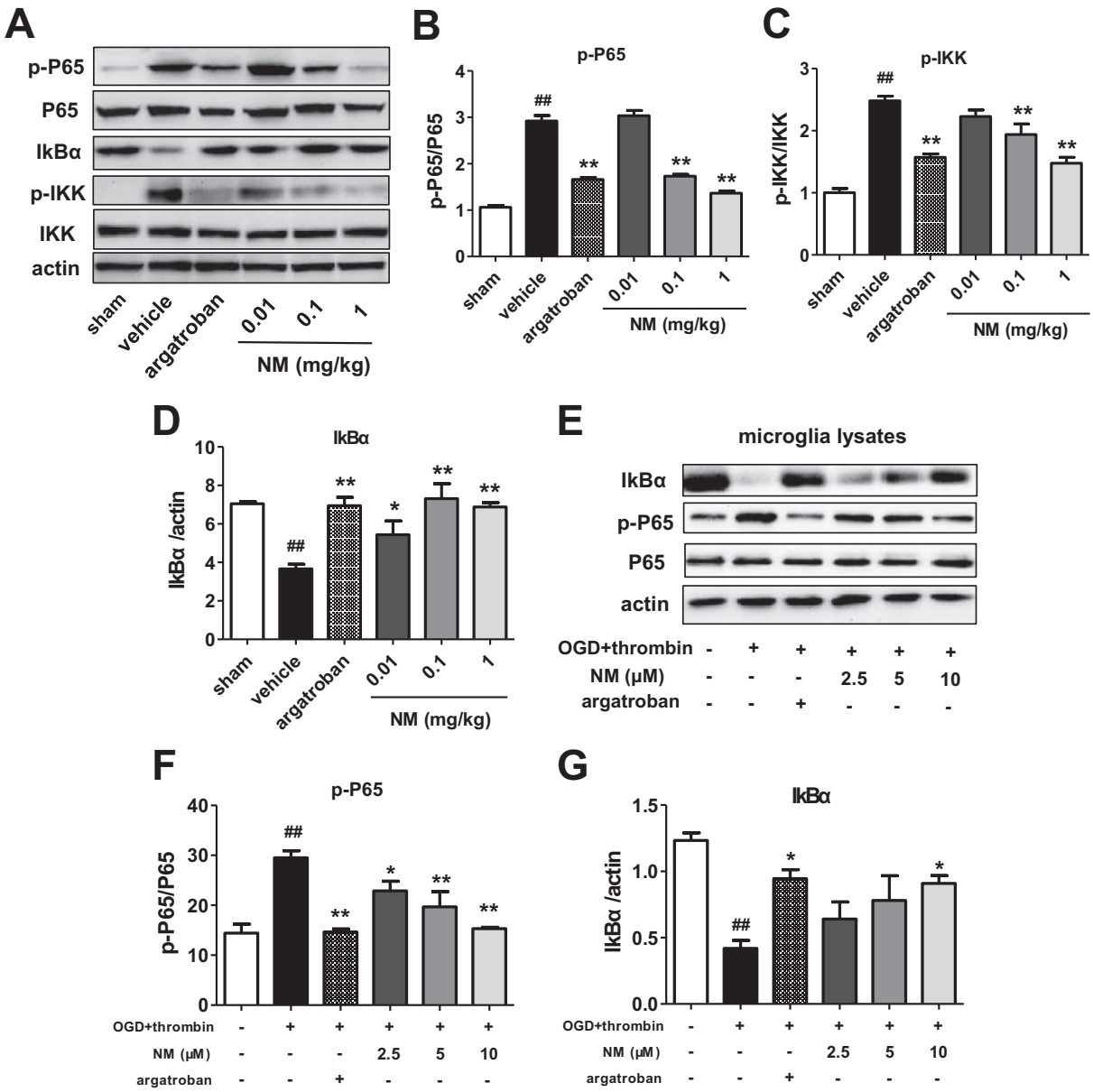

Fig. 4. Effect of NM on the activation of NF-κB signaling pathway *in vivo* and *in vitro*. (A, B) NM ameliorated the phosphorylation of p65 (n = 3). (C) NM dampened the phosphorylation of IKK (n = 3). (D) NM inhibited the degradation of IκBα (n = 3). To mimic hypoxia/reoxygenation in ischemic stroke, the primary rat microglia were exposed to OGD for 2 h followed by reoxygenation in the presence of thrombin for 2 h *in vitro*. (F) NM reduced the phosphorylation of p65 in primary microglia after treated by OGD in the presence of thrombin (n = 3). (G) NM ameliorated the degradation of IκBα in primary microglia after treated by OGD in the presence of thrombin (n = 3). Results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group (the control group *in vitro*). $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group (the OGD + thrombin group *in vitro*).

= 0.9735, $P > 0.05$; IL-10, $F(5,36) = 3.802$, $P < 0.01$; TGF-β, $F(5,36) = 6.517$, $P < 0.01$; IL-4: $F(5,36) = 3.634$, $P < 0.01$). These results showed that NM had a neuroinflammation modulatory effect after stroke.

3.3. NM inhibited the expression of proinflammatory mediators and promoted anti-inflammatory mediators expression in microglia treated by OGD in the presence of thrombin

Microglia is one of the main cellular sources of inflammatory mediators after stroke, to explore the effect of NM on microglia activation under hypoxia/re-oxygenation condition, the mRNA levels of proinflammatory and anti-inflammatory mediators were investigated in primary rat microglia treated by OGD in the presence of thrombin *in vitro*. The mRNA levels of proinflammatory mediators, including IL-1β, MCP-1, TNF-α and iNOS were significantly increased after OGD + thrombin treatment ($P < 0.01$, respectively, versus control), which were counteracted by NM or argatroban ($P < 0.01$, respectively) (Fig. 3A–D). Moreover, OGD + thrombin treatment did not affect the mRNA levels of anti-inflammatory mediators, including IL-10, CD206, IL-4 and TGF-β ($P > 0.05$, respectively, versus OGD) (Fig. 3E–H). However, NM (10 μM) but not argatroban elevated the mRNA levels of IL-10, CD206 and IL-4 ($P < 0.01$, respectively, versus OGD) (Fig. 3E–G). The present observation indicated that NM inhibits the expression of proinflammatory mediators while promotes the expression of anti-inflammatory mediators in microglia after the treatment of OGD and thrombin.

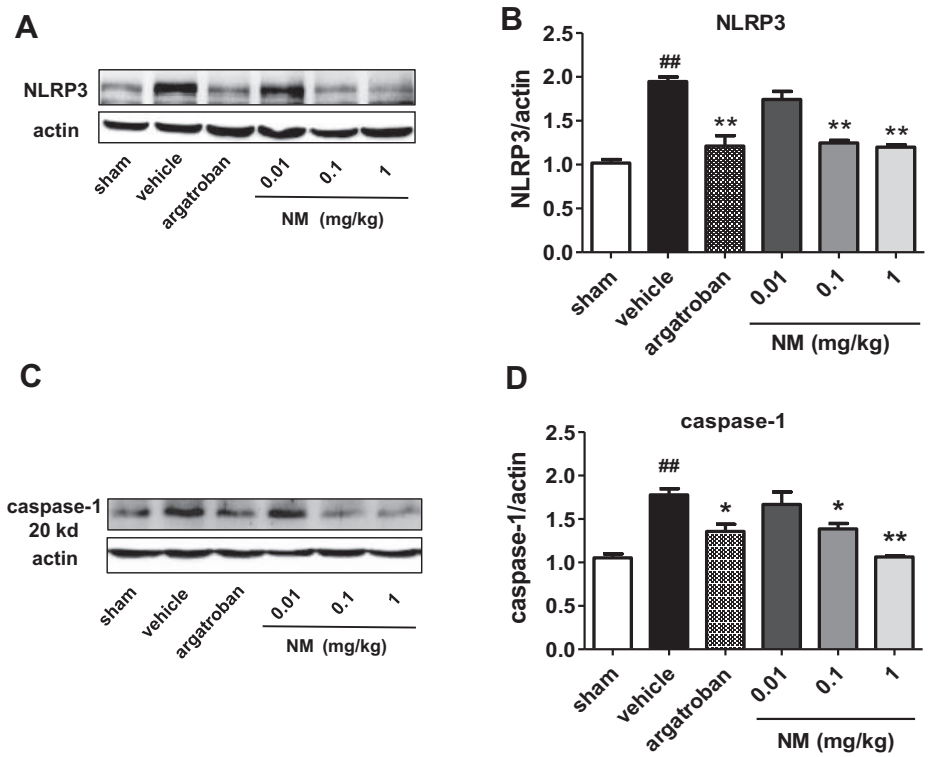

Fig. 5. Effects of NM on the activation of NLRP3 inflammasome after tMCAO. (A, B) NM reduced the protein level of NLRP3 after stroke (n = 3). (C, D) NM dampened tMCAO-induced caspase-1 activation (n = 3). Results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

3.4. NM inhibited the activation of NF-κB signaling pathway after tMCAO and in primary microglia treated with OGD in the presence of thrombin

NF-κB is the key transcription factor controlling microglia activation, and plays an important role in the expression of many proinflammatory mediators in stroke. However, whether the NF-κB signaling pathway is involved in the effect of NM on microglia polarization was not clear. The *in vivo* data showed that NM (0.1 and 1 mg/kg) or argatroban attenuated the phosphorylation of p65 induced by tMCAO ($P < 0.01$, respectively, versus vehicle) (Fig. 4A and B), accompanied by decreased phosphorylation of IKK ($P < 0.01$, respectively) (Fig. 4A and C), and reduced degradation of IκBα ($P < 0.01$, respectively) (Fig. 4A and D).

To confirm the effect of NM on NF-κB signaling pathway in microglia, the primary microglia was treated by OGD in the presence of thrombin, and the activation of the NF-κB signaling was illustrated by Western blot. Marked p65 phosphorylation and IκBα degradation were observed in the thrombin + OGD group compared with the control group ($P < 0.01$, respectively) (Fig. 4E–G). In contrast, NM suppressed the phosphorylation of p65 and IκBα degradation (p65: 2.5 μM, $q = 3.753$, $P < 0.05$; 5 μM, $q = 5.558$, $P < 0.01$; 10 μM, $q = 8.032$, $P < 0.01$. IκBα: 2.5 μM, $P > 0.05$; 5 μM, $q = 3.426$, $P > 0.05$; 10 μM, $q = 4.659$, $P < 0.05$), suggesting that thrombin + OGD treatment activated NF-κB signaling in microglia, whereas NM ameliorated the activation of NF-κB signaling pathway.

3.5. NM inhibited the activation of NLRP3 inflammasome after tMCAO

The secretion of IL-1β is an important feature of microglia proinflammatory activation, and as showed in Fig. 2A, NM inhibited IL-1β secretion after tMCAO. In addition, the maturity and secretion of IL-1β depend on the activation of inflammasomes, and thrombin promotes NLRP3 inflammasome activation in macrophage (Rossol et al., 2012). Therefore, one possible mechanism underlying IL-1β inhibition was that NM dampened the activation of NLRP3 inflammasome. Our results showed that the expression of cleaved caspase-1 and NLRP3 was significantly increased in the vehicle group compared with the sham group ($P < 0.01$, respectively), which were significantly decreased by NM (0.1 mg/kg) (NLRP3, $P < 0.01$, caspase-1, $P < 0.05$), NM (1 mg/kg) (NLRP3, $P < 0.01$, caspase-1: $P < 0.01$) and argatroban (NLRP3: $P < 0.01$; caspase-1: $P < 0.05$) (Fig. 5A–D), suggesting the involvement of NLRP3 inflammasome in the inflammation-modulatory effect of thrombin.

3.6. NM inhibited thrombin-stimulating NLRP3 inflammasome activation in vitro

It has been reported that microglia is the main cellular source of NLRP3 in the brain, but the effect of thrombin on NLRP3 inflammasome activation in microglia and whether NM could reverse its function have not been observed. Our results displayed that thrombin induced IL-1β secretion in LPS-primed microglia (1 U/mL: $q = 6.605$, $P < 0.001$; 2 U/mL: $q = 7.085$, $P < 0.001$; 5 U/mL: $q = 8.784$, $P < 0.001$; 10 U/mL: $q = 25.80$, $P < 0.001$; 20 U/mL: $q = 16.68$, $P < 0.001$), and NM suppressed this effect (2.5 μM: $q = 3.575$, $P > 0.05$; 5 μM: $q = 4.706$, $P < 0.05$; 10 μM: $q = 6.141$, $P < 0.01$; 20 μM: $q = 7.220$, $P < 0.01$) (Fig. 6A, B). In line with these results, thrombin could promote the cleavage of caspase-1 (5 U/mL: $P < 0.01$; 10 U/mL: $P < 0.01$, versus LPS), while NM significantly inhibited this process (2.5 μM: $P < 0.01$; 5 μM: $P < 0.01$; 10 μM: $P < 0.01$, versus thrombin (10 U/mL)) (Fig. 6C, E). To further investigate the relevant effect of thrombin on inflammasome activation in ischemic stroke, the effect of thrombin on OGD

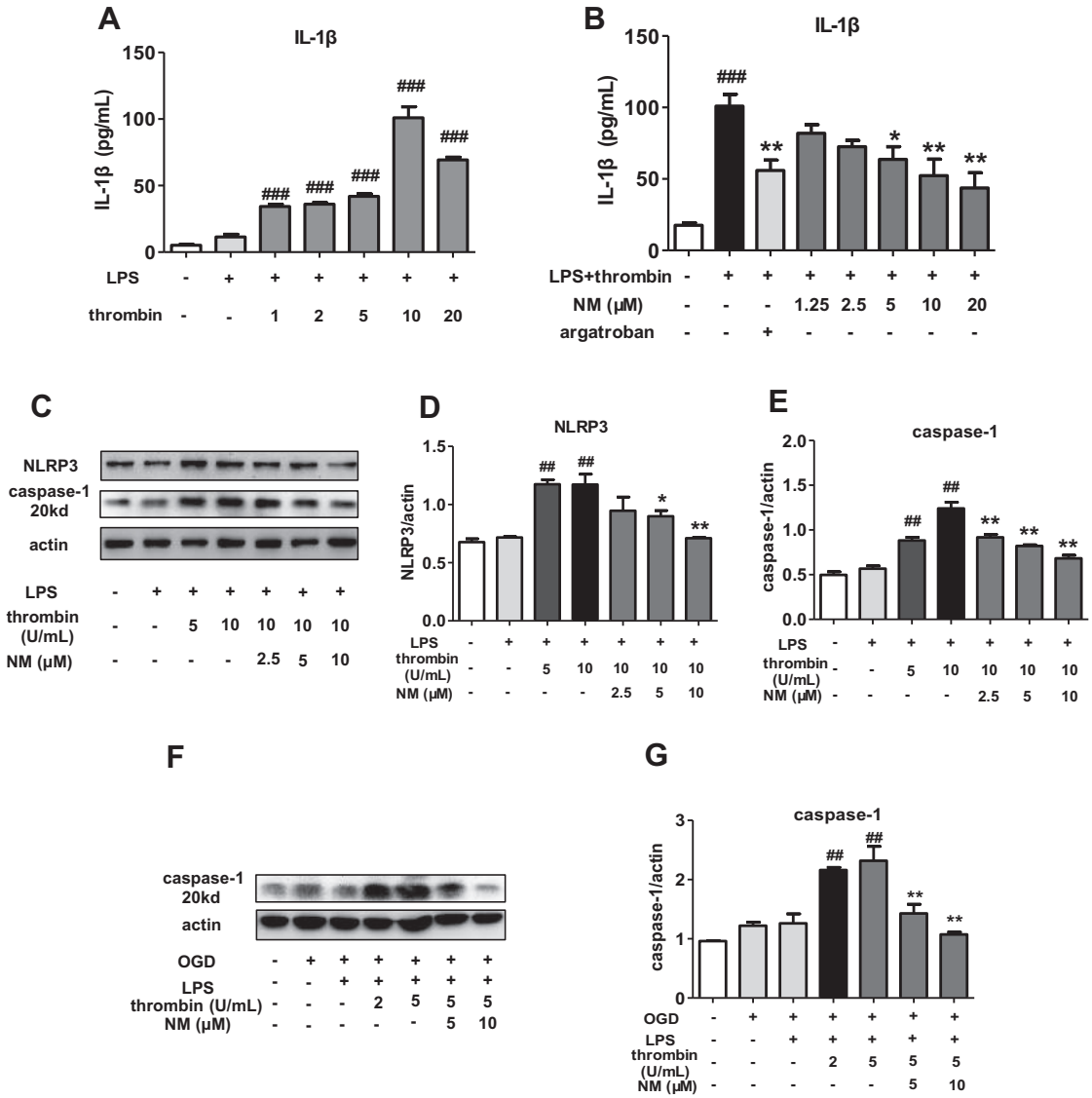

Fig. 6. Effect of NM on thrombin-stimulated NLRP3 inflammasome activation in primary rat microglia *in vitro*. (A) Thrombin-induced IL-1β secretion (n = 3). (B) NM decreased thrombin-induced IL-1β secretion (n = 3). (C–E) Thrombin induced the expression of NLRP3 and activation of caspase-1 in primary rat microglia and the effect of NM on thrombin treatment (n = 3). $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus the LPS primed control group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus thrombin (10 U/mL)-treated LPS-primed group. (F, G) Thrombin-induced caspase-1 activation in OGD-treated microglia in the presence of thrombin, and NM dampened this effect (n = 3). $^{\#\#}P < 0.01$ versus the LPS-primed OGD group. $^{**}P < 0.01$ versus the thrombin (5 U/mL)-treated LPS-primed OGD group.

treated microglia were evaluated *in vitro*. The results showed that thrombin + OGD treatment significantly increased the activation of caspase-1 compared with the control group (thrombin (2 U/mL): $P < 0.01$; thrombin (5 U/mL): $P < 0.01$), and NM ameliorated the activation (5 μM: $P < 0.01$; 10 μM: $P < 0.01$, versus thrombin (5 U/mL)) (Fig. 6F, G). The results suggested that thrombin promote NLRP3 inflammasome "installing activation" in microglia, while NM might reduce NLRP3 inflammasome activation by inhibiting thrombin after tMCAO.

3.7. NM decreased inflammatory cells invading to the ischemic hemisphere after tMCAO

The infiltration of circulating inflammatory cells is an important component of the inflammatory response following stroke. Hence we sought to investigate whether NM contributed to the reduction in the recruitment of inflammatory cells using flow cytometric analysis.

At 1 day after tMCAO, the percentage of resting microglia was not significantly affected by stroke in all groups, but it was significantly decreased in the vehicle group at 3 or 7 days after tMCAO ($P < 0.01$, versus sham) (Fig. 7A and B). At 3 days after tMCAO, the percentage of resting microglia was markedly increased by NM (0.01 mg/kg, $P < 0.05$; 0.1 mg/kg, $P < 0.01$; 1 mg/kg, $P < 0.01$) and argatroban ($P < 0.05$) (Fig. 7B). And at 7 days after tMCAO, the percentage of resting microglia was significantly increased in the NM (0.1 mg/kg) group ($P < 0.05$, versus vehicle) (Fig. 7B; Time × Drug interaction: resting microglia, $F(10,50) = 6.738$, $P < 0.01$).

Compared with the sham group, intensive accumulation of activated macrophage/microglia were observed in the ischemic hemisphere of the vehicle group at 1, 3 or 7 days after tMCAO ($P < 0.01$, respectively) (Fig. 7A and C). At 1 day after tMCAO, the accumulation was significantly dampened by NM (0.01 mg/kg, $P < 0.05$; 0.1 mg/kg, $P < 0.05$; 1 mg/kg, $P < 0.01$) and argatroban ($P < 0.05$) (Fig. 7C). At 3 days after tMCAO, NM (0.1 and 1 mg/kg) significantly dampened the accumulation of activated macrophage/microglia

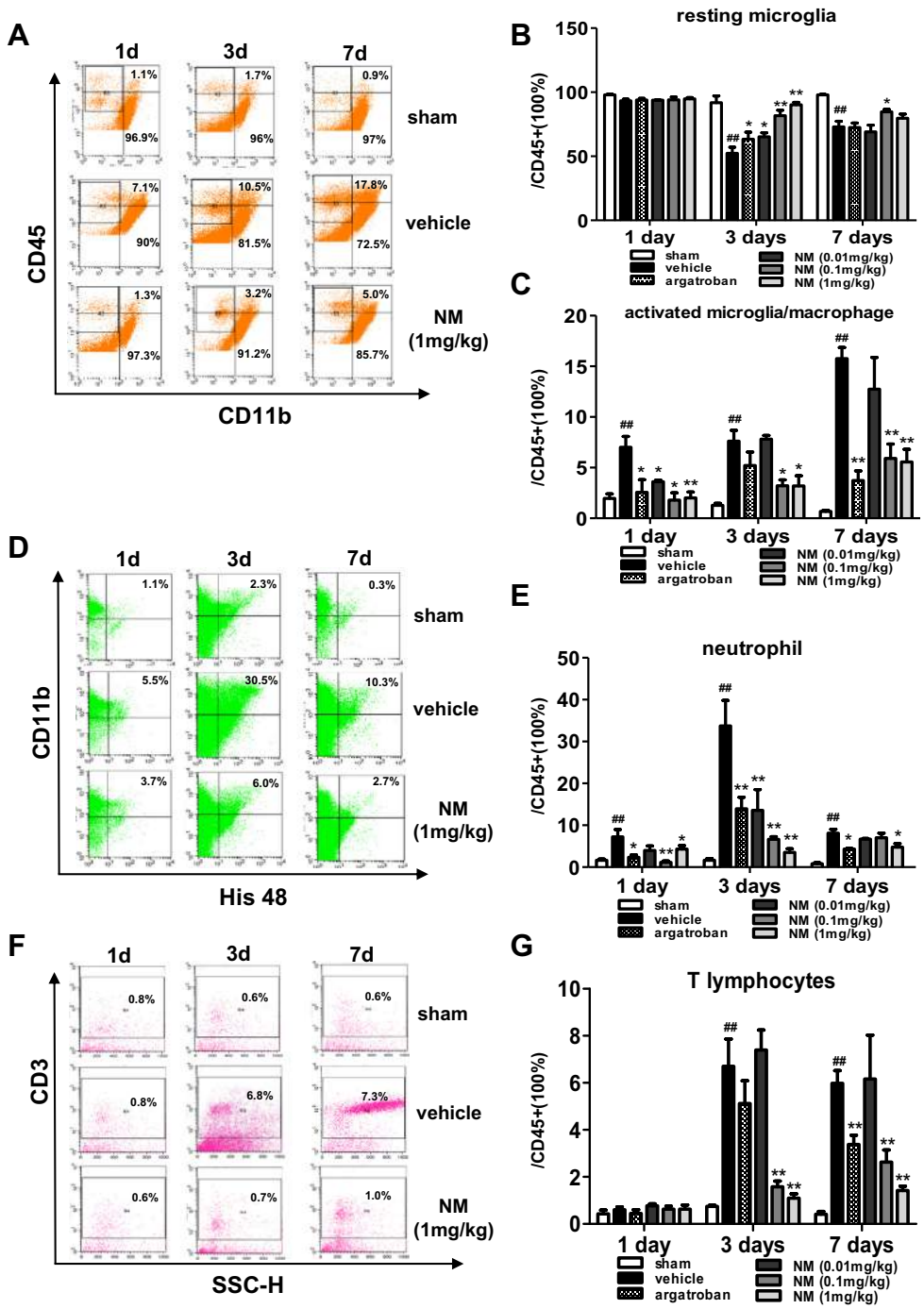

Fig. 7. Effect of NM on inflammatory cells invading the ischemic hemisphere after tMCAO as determined by flow cytometric analysis. (A, B) The $CD11b^{+}His48^{-}CD45^{low}$ cells (resting microglia) in the hemisphere at 1, 3 and 7 days respectively after tMCAO. (C) The $CD11b^{+}His48^{-}CD45^{high}$ cells (activated microglia/macrophage) in the ischemic hemisphere. (D, E) The number of $CD11b^{+}His48^{+}CD45^{high}$ cells (neutrophil) invading to the ischemic hemisphere. (F, G) The number of $CD11b^{-}CD45^{+}CD3^{+}$ cells (T lymphocyte) invading to the ischemic hemisphere at 1, 3 and 7 days respectively after tMCAO. n = 3–4, results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

($P < 0.05$, respectively, versus vehicle), and the decrease were maintained at 7 days after tMCAO in the NM (0.1 and 1 mg/kg) groups and the argatroban group ($P < 0.01$, respectively, versus vehicle) (Fig. 7C; Time × Drug interaction: activated macrophage/microglia: $F(10,50) = 4.570$, $P < 0.01$).

Intensive accumulation of neutrophil can also be observed in the vehicle group at 1, 3 or 7 days after tMCAO ($P < 0.01$, versus sham) (Fig. 7D and E). Compared with the vehicle group, the accumulation of neutrophil was remarkable dampened by NM (0.1 mg/kg, $P < 0.01$; 1 mg/kg, $P < 0.05$) and argatroban ($P < 0.05$) at 1 day after tMCAO (Fig. 7E). At 3 days after tMCAO, the accumulation of neutrophil was decreased in the NM (0.01, 0.1 and 1 mg/kg) groups or the argatroban group ($P < 0.01$, respectively, versus vehicle) (Fig. 7E). Decreased neutrophil infiltration was still observed in the NM (1 mg/kg) group and the argatroban group at 7 days after tMCAO ($P < 0.05$, respectively, versus vehicle) (Fig. 7E; Drug interaction: neutrophil, $F(10,50) = 7.292$, $P < 0.01$).

No significant T lymphocyte infiltration was observed in the ischemic hemisphere of the vehicle group until 3 days after tMCAO (at day 1, $P > 0.05$; at day 3, $P < 0.01$; at day 7, $P < 0.01$), while the

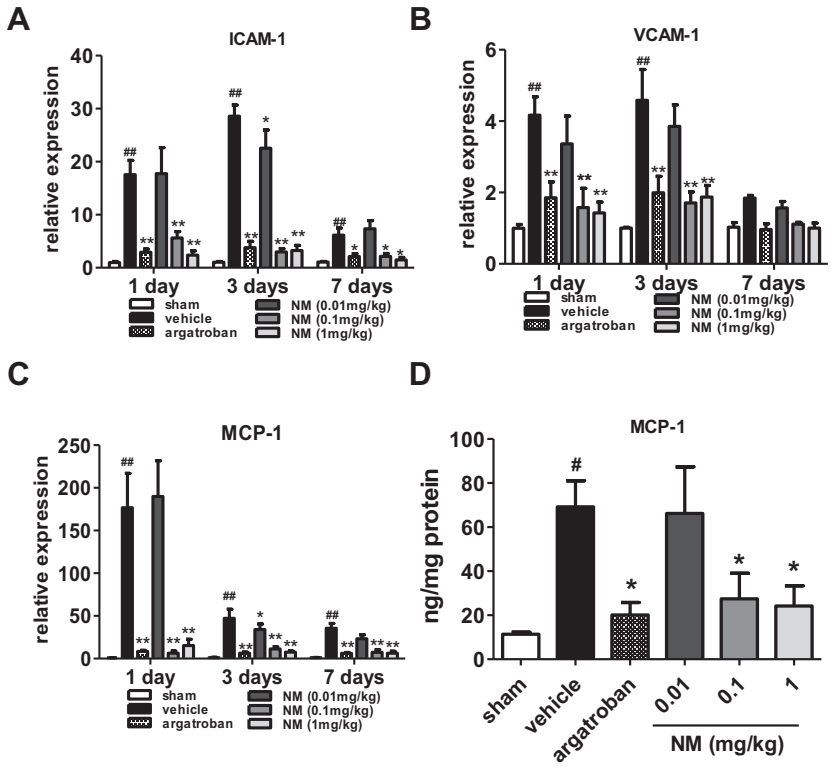

Fig. 8. The effect of NM treatment on the expressions of chemokines and cell adhesion molecules after tMCAO. (A–C) NM reduced the mRNA levels of ICAM-1, VCAM-1 and MCP-1 in the ischemic cortex at 1, 3 and 7 days after tMCAO (n = 4–5). (D) Effects of NM on the protein level of MCP-1 at 1 day after tMCAO (n = 6–7). Results are expressed as means ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the sham group. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the vehicle group.

infiltration of T lymphocyte was decreased by NM (0.1 and 1 mg/kg) ($P < 0.01$, respectively) and argatroban (at day 3, $P > 0.05$; at day 7, $P < 0.01$) (Fig. 7F, G; Time × Drug interaction: T lymphocyte, $F(10,50) = 6.760$, $P < 0.01$).

3.8. NM decreased the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1)

The infiltration of inflammatory cells is facilitated by chemokines and cell adhesion molecules. Next, it was tested whether the anti-infiltration effect of NM was related to decreasing the expression of chemokines and cell adhesion molecules.

At 1, 3 or 7 days after tMCAO, the mRNA levels of ICAM-1 were significantly increased in the vehicle group compared with the sham group ($P < 0.01$, respectively) (Fig. 8A). At 1 day or 3 days after tMCAO, NM (0.1, 1 mg/kg) or argatroban significantly decreased the mRNA level of ICAM-1 ($P < 0.01$, respectively, versus vehicle) (Fig. 8A). And at 7 days after tMCAO, the inhibitory effects were still maintained in the NM (0.1, 1 mg/kg) groups and the argatroban group ($P < 0.05$, respectively) (Fig. 8A; Time × Drug interaction: $F(10,60) = 5.810$, $P < 0.01$).

VCAM-1 was significantly increased in the vehicle group at 1 or 3 days after tMCAO ($P < 0.01$, respectively, versus sham) (Fig. 8A), whereas NM (0.1, 1 mg/kg) or argatroban markedly decreased the increase at both 1 and 3 days after tMCAO ($P < 0.01$, respectively, versus vehicle) (Fig. 8B; Time × Drug interaction: $F(10,60) = 1.637$, $P > 0.05$).

Significantly increased MCP-1 were also observed in the vehicle group at 1, 3 or 7 days after tMCAO ($P < 0.01$, respectively, versus sham), whereas NM (0.1, 1 mg/kg) or argatroban markedly decreased the mRNA level at all the three time points ($P < 0.01$, respectively, versus vehicle) (Fig. 8B). In addition, NM (0.01 mg/kg) remarkably decreased the mRNA level of MCP-1 at 3 days after tMCAO ($P < 0.05$, versus vehicle) (Fig. 8B; Time × Drug interaction: $F(10,60) = 7.557$, $P < 0.01$). Furthermore, the protein level of MCP-1 was significantly decreased in the NM (0.1 and 1 mg/kg) group or the argatroban group at 1 day after tMCAO compared with the vehicle group ($P < 0.05$, respectively) (Fig. 8D).

4. Discussion

Recently years, several studies have implied the relationship between thrombin and the inflammatory response in stroke, but only few detailed mechanisms have been provided. In this paper, we demonstrated that thrombin contributes to the proinflammatory activation of microglia by promoting the installing of NLRP3 inflammasome for the first time, and NM may provide long-lasting protection against ischemic cerebral damage by inhibiting thrombin. These findings provide a better understanding of the role of thrombin in stroke, and indicate that small-molecular thrombin inhibitor act as new candidate for ischemic stroke treatment, and indicate new therapeutic mechanism for anticoagulation therapy after stroke.

In this paper, we demonstrated that NM, a wide-spectrum serine protease inhibitor, reduced the infarct size and improved the sensorimotor and cognitive recovery in a rat model of tMCAO. We further provided evidence indicating that the inflammation-modulatory effect of NM contributes to the beneficial effects of NM on tMCAO-treated rats.

It has been shown that NM has inhibitory effects on the enzymatic activities of kallikrein, FXa and thrombin (Schwertz et al., 2008). Among these serine proteases, thrombin, which is activated by the kallikrein/tissue factor-FXa axis, is well known for its effects in thrombosis cascades and inflammatory reactions (Santilli and Davi, 2009). Upregulation of thrombin activity is observed in the

pre-infarction region after ischemic stroke (Chen et al., 2012), and there are studies which indicate that thrombin contributes to brain injury after ischemic stroke (Sheehan and Tsirka, 2005). Furthermore, our previous work has suggested that NM attenuates neuronal damage and brain infarct volume by inhibiting thrombin (Chen et al., 2014). And in this paper, we have now shown that NM modulated the neuroinflammatory response by thrombin inhibition after tMCAO. In particular, NM modulated the activation of microglia and inhibited the recruitment of circulating inflammatory cells after tMCAO.

Microglia is an important component of the inflammatory response following tMCAO, it may promote or inhibit inflammation in different conditions, and has dual roles in functional recovery (Jin et al., 2014). In addition, proinflammatory microglia classically produce a number of proinflammatory mediators (such as iNOS and TNF-α) after activation of the NF-κB signaling pathway, and are important players in causing brain damage and inhibiting the recovery after brain injury. Conversely, anti-inflammatory microglia may show enhanced production of anti-inflammatory mediators (such as IL-4 and CD206), and may protect the brain from the damage and promote recovery after stroke.

It has been shown that thrombin induces the expression of iNOS and TNF-α in microglia (Huang et al., 2008; Lee et al., 2005). Moreover, microglia in the ischemic area of MCAO-treated animals tend to migrate to the perivascular areas (Jolivel et al., 2015), where the activity of thrombin is increased (Chen et al., 2010, 2012), implying that thrombin might influence the activation of microglia following tMCAO. Our *in vivo* results indicated that NM reduces the expression of proinflammatory mediators in a time-dependent manner, and elevates the expression of different anti-inflammatory mediators at different time points. In line with the *in vivo* results, thrombin heightened the expression of proinflammatory mediators in microglia after OGD, whereas NM alleviated these effects, and increased the mRNA levels of anti-inflammatory mediators *in vitro*. These observations elucidated that thrombin might contribute to microglia proinflammatory activation subsequent to tMCAO, while NM might modulate the expression of both proinflammatory mediators and anti-inflammatory mediators in microglia by inhibiting thrombin *in vivo*.

NF-κB pathway is the key factor that promotes the proinflammatory activation of microglia (Crack et al., 2006; Harari and Liao, 2010). The inhibition of NF-κB signaling shifts microglia from proinflammatory profile to anti-inflammatory profile following tMCAO (Aslanidis et al., 2015; Ni et al., 2015). In the present study we have verified that the NF-κB pathway and NLRP3 inflammasome are responsible for the effect of NM on microglia activation. Consistent with previous study (Fujiwara et al., 2013), our results showed that NM inhibited the NF-κB pathway by suppressing the degradation of IκBα and the phosphorylation of p65 *in vivo* and *in vitro*.

One of the most important features of proinflammatory microglia is the secretion of IL-1β, which requires the activation of inflammasome. Recent studies have highlighted the detrimental role of NLRP3 inflammasome in ischemic stroke (Ito et al., 2015). The NLRP3 inflammasome is activated by various stimuli, and it has recently been reported that thrombin promotes the activation of NLRP3 inflammasome in macrophage (Rossol et al., 2012). In this paper, we put forward that thrombin triggered the activation of NLRP3 inflammasome in microglia with or without OGD treatment for the first time. Our results further showed that NM inhibited the activation of NLRP3 inflammasome *in vivo* and *in vitro*. The present observation together suggest that NF-κB signaling and NLRP3 inflammasome might take part in the effect of NM on the activation of microglia and brain protection from ischemic insult.

Besides the activation of resident microglia, a time-restricted invading of circulating inflammatory cells to the ischemic area is also involved in the inflammatory cascades after stroke (Jin et al., 2010). Adhesion molecules and chemotactic factors such as ICAM-1, VCAM-1 and MCP-1 facilitate the process (Frijns and Kappelle, 2002). Our results showed that NM reduced the infiltration of macrophage, neutrophil and T lymphocyte to the ischemic penumbra, and reduced the expression of ICAM-1, VCAM-1 and MCP-1, suggesting that NM might attenuate the recruitment of inflammatory cells, which is mediated by reducing the expression of adhesion molecules and chemotactic factors.

One limitation of the present study is that the drugs were given immediately after ischemia onset. However, to mimic the clinical situation, therapeutic time-window experiments are needed to make a comprehensive view of the effects of NM on stroke. Besides, young rats have been used in our *in vivo* study. Previous studies indicate that stroke is more common in aged people (Mozaffarian et al., 2016), and aging may affect the innate immunity system in the brain, aged rats would be helpful to explore the immunomodulatory role of NM in future studies.

In summary, NM promotes recovery after stroke, and inhibits the physiological inflammatory responses after stroke by influencing multiple cellular and molecular components of the immune system, which supports the concept of immune interventions in stroke (Fu et al., 2015). Therefore NM could be a potential candidate for modulating the inflammatory response after stroke.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

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