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# N2 neutrophils may participate in spontaneous recovery after transient cerebral ischemia by inhibiting ischemic neuron injury in rats

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ABSTRACT

Neutrophils have been traditionally considered as the major mediators of harmful inflammatory responses in ischemic stroke, whereas accumulating evidence indicates that neutrophils can be polarized into an N2 phenotype. Similar to M2 microglia, N2 neutrophils contribute to resolution of inflammation and may participate in neuroprotection. However, it remains unclear whether N2 neutrophils protect ischemic neurons and whether they are associated with long-term outcomes after transient cerebral ischemia in rats. The present study proved that N2 neutrophils protected against oxygen glucose deprivation/re-oxygenation (OGD/R)-induced primary cortical neuron injury via brain-derived neurotrophic factor/tropomyosin-related kinase B (BDNF/TrkB) signaling. In addition, in vivo studies revealed that transient middle cerebral artery occlusion (tMCAO)-induced injury exhibited spontaneous recovery over time in rats. Moreover, neutrophils could infiltrate the ipsilateral brain parenchyma from the periphery after transient cerebral ischemia. Pearson's correlation analysis indicated that the proportion of N2 neutrophils in ipsilateral brain parenchyma was negatively correlated with the number of degenerating neurons, modified Neurological Severity Score (mNSS), brain water content and infarct volume, and positively correlated with the number of surviving neurons and grip strength. In summary, the present study shows that N2 neutrophils likely participate in spontaneous recovery after transient cerebral ischemia by inhibiting ischemic neuron damage in rats, which indicates that N2 neutrophils may represent promising therapeutic target for promoting recovery after ischemic stroke.

## 1. Introduction

Stroke is the leading cause of long-term disability in adults [1,2]. Currently, the mainstay of acute ischemic stroke therapy is intravenous administration of tissue plasminogen activator, which has a narrow time window, and must be used within 4.5 h after the symptoms first start [2,3]. However, there is no effective drug in the subacute and chronic stages following ischemic stroke. Spontaneous functional recovery after stroke has been found in human patients [4], but in rodents, spontaneous recovery after transient cerebral ischemia remains unclear.

Neutrophils have been traditionally considered as the major mediators of deleterious inflammatory responses in ischemic stroke [5–7]. After brain injury, blood-brain barrier (BBB) permeability increases, allowing peripheral immune cells to infiltrate brain parenchyma [8–11]. Among these cells, neutrophils are the first leukocytes to infiltrate the ischemic brain parenchyma [5,6,12]. Whereas increasing evidence indicates that neutrophils can acquire different phenotypes, similar to M2 macrophages, neutrophils are also thought to be polarized to an N2 phenotype, which contribute to resolution of inflammation and may participate in neuroprotection [13–16]. Brain-derived neurotrophic factor (BDNF), one of the members of neurotrophin family, can be secreted by neurons both from dendrites and axons in response to neuronal activity [17] and binds to a tropomyosin-related kinase B (TrkB) receptor. Through TrkB receptor activation, BDNF influences synaptic connectivity and neuromorphological development [18]. Remarkably, BDNF can reduce infarct volume and ameliorate neurological outcome through either exogenous supplementation [19,20] or overexpression in vivo [21,22] in experimental stroke. In addition, BDNF/TrkB potentiation could be protective in acute stroke and promote neural repair at later stages [23]. However, it is not clear whether N2 neutrophils protect ischemic neurons through the BDNF/TrkB signaling

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or whether N2 neutrophils are associated with long-term outcomes after transient cerebral ischemia in rats.

In this study, the effects of N2 and N1 neutrophils on oxygen glucose deprivation/re-oxygenation (OGD/R)-induced primary cortical neuron injury were investigated in vitro to verify whether N2 neutrophils had protective effects on ischemic injured neurons. Moreover, a rat transient middle cerebral artery occlusion (tMCAO) model was used to investigate the changes in long-term outcomes after transient cerebral ischemia, as well as the correlation between the proportion of N2 neutrophils in the ipsilateral brain parenchyma and the severity of cerebral ischemic injury.

## 2. Methods

### 2.1. Animals

Male Sprague-Dawley rats weighing 280–300 g (age 8 weeks approximately), supplied by the Animal Centre of Shenyang Pharmaceutical University (Shenyang, China), were housed in standardized environment conditions (12 h alternating light and dark, 21–24 °C) with food and water available ad libitum. All experimental procedures and treatments were approved by the Ethics Committee of Shenyang Pharmaceutical University, and followed the guidelines for the Care and Use of Laboratory Animals.

### 2.2. Neuronal cell culture and oxygen-glucose deprivation (OGD)

Primary neuronal cells were derived from the cerebral cortex of newborn rats within 24 h as described previously [24]. Briefly, cerebral cortices were dissected, minced, trypsinized (0.25%) and mechanically dissociated with a Pasteur pipette. Cells were seeded into 96-well plates ($1.0 \times 10^6$ cells/mL) pre-coated with 0.01% poly-L-lysine, and cultured in Neurobasal medium (Gibco, Carlsbad, CA, USA) supplemented with 2% B27 (Gibco, Carlsbad, CA, USA) and 100 U/mL penicillin, 100 μg/mL streptomycin. Beta-III Tubulin (Santa Cruz, Dallas, TX, USA) staining of primary neuronal cells were used to assess the purity of the cell population.

For OGD treatment, cells were rinsed once with warm glucose-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA), and then refreshed with $O_2$– and glucose-free DMEM (pre balanced in an $O_2$-free chamber at 37 °C). Cells were then immediately placed in a sealed chamber (Billups-Rothenburg, Del Mar, CA) loaded with mixed gas containing 5% $CO_2$ and 95% $N_2$ for 5 min at 20 L/min. Primary neurons were cultured in O2– and glucose-free DMEM supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin and incubated at 37 °C for 4 h. After OGD exposure, the cells were maintained in Neurobasal medium supplemented with 2% B27 and 100 U/mL penicillin, 100 μg/mL streptomycin under normoxic conditions for 24 h to reoxygenation.

### 2.3. Isolation and stimulation of primary rat neutrophils

Neutrophils were isolated from rat arterial blood through rat peripheral neutrophils separation medium kit (Solarbio, Beijing, China) according to the manufacturer's instruction. To prepare neutrophil-enriched culture, neutrophils were further isolated from the erythrocyte fraction through hypotonic lysis. Neutrophils were resuspended in RPMI 1640 media (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, 100 μg/mL streptomycin, and then seeded into 6-well plates ($1.0 \times 10^6$ cells/mL) pre-coated with 0.01% poly-L-lysine. HIS48 (1:200, Abcam, Camb., UK) staining of neutrophils was used to assess the purity of the cell population. For N1 neutrophils induction, lipopolysaccharide (LPS) (Sigma-Aldrich, MO, USA) (100 ng/mL) and IFN-γ (Sigma-Aldrich, MO, USA) (20 ng/mL) were added to the neutrophil cultures for 12 h. For N2 neutrophils induction, IL-4 (Sigma-Aldrich, MO, USA) (20 ng/mL) was added to the culture for 12 h [16]. The cells were collected for phenotypic validation.

### 2.4. MTT assay

For MTT assay, cells were seeded into 96-well plates and grown in 200 μL of culture medium. After OGD treatment, MTT (Sigma-Aldrich, MO, USA) solution (0.25 mg/mL) was added into each well and incubated at 37 °C for 3 h. The supernatant was discarded and 200 μL DMSO were applied to dissolve the formazan. The absorbance was measured at 560 nm wavelength with a microplate reader, with 630 nm as reference wavelength.

### 2.5. Neutrophil-Neuron Cocultures, treatments and detection

The following experimental system was used: a co-culture system allowing cell-to-cell communication via direct neutrophil-neuronal contacts. To generate neutrophil-neuron cocultures, neurons at 11 days in vitro (DIV) cultured in 96-well plate ($1 \times 10^5$/well) were subject to 4 h of OGD (Neurons were also separately treated with LPS/IFN-γ or IL-4 as a control). Primary neutrophil ($1 \times 10^6$/well) were then seeded and cultured together with neurons in the presence of LPS (100 ng/mL) and IFN-γ (20 ng/mL), IL-4 (20 ng/mL) or TrkB antagonist ANA-12 (Selleck, Shanghai, China) (10 μM) in the culture medium (RPMI 1640 media supplemented with 10% FBS, and 100 U/mL penicillin, 100 μg/mL streptomycin) at 37 °C for 24 h. Afterwards, neutrophils were removed from the co-culture system (because neutrophils are suspension cells, they can be removed by gently rinsing with RPMI 1640 media), and neuronal viability was measured by MTT assay. To observe morphological changes of neurons, cells were stimulated as described above and fixed in 4% paraformaldehyde at 37 °C for 30 min. After rinsing, cells were blocked with 10% normal goat serum for 1 h at 37 °C and incubated with beta-III Tubulin antibody overnight at 4 °C. After being washed, cells were incubated with goat anti-mouse IgG antibody for 1 h at 37 °C. Neurons were then photographed under a laser confocal microscope (C2 Plus; Nikon).

### 2.6. Western blotting

Western blotting analysis was performed as described previously [25]. Briefly, cells were stimulated as described in 2.5, total proteins in neurons were collected using RIPA lysis buffer (Beyotime, Shanghai, China) according to the manufacturer's instructions. Equal amounts of proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membrane (PVDF). The membranes were blocked with 5% skimmed milk and were incubated with primary antibodies against BDNF (1:1000, Abcam, Camb, UK), TrkB (1:1000, Cell Signaling Technology, MA, USA), and β-actin (1:1000, Santa Cruz Biotechnology, TX, USA) overnight at 4 °C. Afterward, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; Thermo Fisher Scientific, MA, USA) for 1 h at room temperature. The bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific, MA, USA) and were analyzed with ImageJ software.

### 2.7. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA samples from neutrophils were extracted using TRIzol Regent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. For cDNA synthesis, 2 μg of total RNA was reverse transcribed using the PrimeScriptTM RT reagent kit with gDNA Eraser (Perfect Real Time) (Takara, Otsu, Japan). qPCR was carried out using the SYBR green-based qRT-PCR kit (Takara, Otsu, Japan) on a CFX Connect Real Time PCR Detection System (Bio-Rad, CA, USA). The primers used for qRT-PCR (GenePharma, Suzhou, China) are listed in Table 1. After an initial denaturation step of 95 °C for 30 sec, 40 cycles

**Table 1**
List of primer sequences for qRT-PCR were used in the study.

| Gene | Primer sequences | PCR products length |
|---|---|---|
| *ACTB* | 5′-GAAGTACCCCATTGAACACGG-3′<br>5′-TGGGTCATCTTTTCACGGTTG-3′ | 175 |
| *NOS2* | 5′-TTGGAGCGAGTTGTGGATTG-3′<br>5′-TGAGGGCTTGCCTGAGTGA-3′ | 124 |
| *Tnf* | 5′-GCCGATTTGCCATTTCAT-3′<br>5′-CAGTCGCCTCACAGAGCAA-3′ | 214 |
| *Il1b* | 5′-TGTTTCCCTCCCTGCCTCT-3′<br>5′-CACTGGTCCAAATTCAATTCATC-3′ | 163 |
| *Mrc1* | 5′-GCAGATCATGAGTGTTGCTTTCA-3′<br>5′-TGTGTCGTTTTTACACTCCCATTT-3′ | 127 |
| *Arg1* | 5′-GGCCCATTCACCTGAGTTT-3′<br>5′-TGATTACCTTCCCGTTTCGT-3′ | 262 |
| *Chi313* | 5′-AGAACCGGCAGACATTCATC-3′<br>5′-AGGAGGGCTTCCACGAGAC-3′ | 107 |

of PCR were performed. Each cycle consisted of a melting step at 95 °C for 15 sec and an annealing extension step at 60 °C for 40 sec. Data were quantified using the $2^{-\Delta\Delta Ct}$ method and normalized to *ACTB* expression.

### 2.8. Middle cerebral artery occlusion and reperfusion

Surgical anesthesia was induced with 5% isoflurane in oxygen, and maintained with 2.5% isoflurane. Through a ventral midline incision, the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA) were isolated. ECA was coagulated distal to the carotid bifurcation. A 4–0 nylon filament (the diameter of the filament is 0.26 mm), with its tip embedded in paraffin (the diameter of the tip is 0.34 mm), was inserted from the right ECA stump to the right ICA until it reached the origin of the middle cerebral artery and secured in position with nylon ties [26]. The middle cerebral artery was occluded for 2 h and the filament was withdrawn to restore blood flow. Sham-operated rats received the same surgical procedures with the exception of filament insertion. Rats were kept in a heat blanket after surgery. The animals were sacrificed after reperfusion at the indicated times.

### 2.9. Measurement of neurological scores, cerebral infarct volume and brain water content

We determined neurological scores, cerebral infarct volume and brain water content as described previously [24]. Briefly, neurological function was evaluated with the modified Neurological Severity Score (mNSS) test. Each function is graded on a scale of 0 to 18 (normal score, 0; maximal deficit score, 18). Higher scores indicate more severe behavioral deficits. To investigate cerebral infarct volume, rats were sacrificed at the indicated time and their brains were quickly removed and weighed, and then sliced into six 2-mm-thick coronal sections. The sections were incubated in a 1% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) (Sigma-Aldrich, MO, USA) at 37 °C for 20 min and fixed in 4% PFA in PBS overnight, and then photographed. Normal tissue stained red, whereas the infarct area remained white. Afterward, the slices were dried at 60 °C for 72 h and then weighed, and the brain water content was determined using a wet/dry method as previously described [27]. Infarcted areas on the posterior surface in each brain slice were measured using Image J software (NIH, Bethesda, MD, USA) by subtracting the intact area of the ipsilateral hemisphere from the total area of the contralateral hemisphere to correct for brain swelling. Then the total infarct volume was calculated by linear integration of the corrected lesion areas. The infarct volume percentage was calculated with the formula: total infarct volume/contralateral hemisphere volume × 100%.

### 2.10. Grip strength test

Grip strength tests were conducted on the 1st, 3rd, 7th, 14th and 30th days post-tMCAO. Forelimb strength was measured using an electronic digital force gauge grip-strength meter (Columbus Instruments, OH, USA) that measured the peak force exerted by the animal while gripping the sensor bar as previously described [28,29]. Briefly, rats volunteered to grip a T-bar with forelimb and the rat was pulled backwards. The maximum strength out of 3 trials was used in the data analysis.

### 2.11. Rota rod

Rats were subjected to the rota rod task as previously described [30]. Briefly, prior to tMCAO, animals were acclimated to the rotating spindle of the rota rod apparatus (Rota-Rod 47700, Ugo Basile, Italy). Animals were placed on the rotating spindle, set to a constant 8 rotations per minute (RPM), until they demonstrated the ability to remain on the spindle for 60 s. The animals were then subjected to a baseline test trial on the accelerating spindle (4–40 RPM) over 5 min. The acceleration trial was repeated on the 1st, 3rd, 7th, 14th and 30th days post-tMCAO, and the latency to fall off the rota rod was recorded.

### 2.12. Immunofluorescence

The rats were perfused transcardially with pre-cooled 0.9% saline followed by 4% paraformaldehyde (PFA) in deep anesthesia. The brains were removed and post-fixed in 4% PFA for 24 h, and then were immersed in 20% and 30% sucrose solutions for dehydration. For immunofluorescence, frozen sections (20 μm) were blocked with 10% normal goat serum (ZSGB-BIO, Beijing, China) containing 0.3% Triton X-100 (Solarbio, Beijing, China) for 1 h at room temperature, and then were incubated with mouse anti-NeuN antibody (1:1000, Millipore, Kenilworth, NJ, USA) overnight at 4 °C and subsequently with goat anti-mouse IgG antibody (Alexa Fluor 488, 1:400) (ZSGB-BIO, Beijing, China) for 1 h at 37 °C. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, MO, USA) for 20 min at room temperature. The slices were then mounted with coverslips using antifade reagent (Solarbio, Beijing, China) and were sealed with nail polish. Images were acquired with a laser confocal microscope (C2 Plus; Nikon).

### 2.13. Fluoro-Jade B (FJB) histochemistry

To assess degenerating neurons, we stained tissue sections with a polyanionic fluorescein derivative, FJB (Histo-Chem Inc., Jefferson, AR). Staining was performed according to the manufacturer's protocol. Brain tissue sections were dried on gelatin coated slides at 50 °C for 45 min, immersed in a solution containing 0.2% sodium hydroxide in 80% alcohol for 5 min, followed by 2 min in 70% alcohol and 2 min in distilled water. Slides were transferred to a solution of 0.06% potassium permanganate for 10 min, rinsed for 2 min in distilled water, and then placed in 0.0001% FJB for 20 min, before rinsing in distilled water (3 × 2 min each). The slides were placed on a slide warmer at 50 °C for 30 min, cleared in 100% xyleneand (3 × 2 min each), and then coverslipped with DPX (Sigma-Aldrich, MO, USA).

### 2.14. Flow cytometry

Blood, bone marrow, and brain were collected from each animal for flow cytometric analyses as reported previously [31], with some modifications.

Blood was collected from abdominal aorta and leukocytes were purified using red blood cell lysis buffer (Biolegend, CA, USA), then washed with phosphate-buffered saline (PBS) containing 2% FBS (Gibco, Carlsbad, CA, USA) (300 g, 10 min), and resuspended on 100 μL

of PBS containing 2% FBS with Fc Block reagent (Biolegend, CA, USA) for staining and FACS analysis.

For bone marrow-derived cells, the tibia and femur bones were flushed with PBS containing 2% FBS using a syringe to obtain bone marrow cells. To remove red blood cells, bone marrow cells were treated with a red blood cell lysis buffer followed by washing with PBS containing 2% FBS, and resuspended on 100 μL of PBS containing 2% FBS with Fc Block reagent for staining and FACS analysis.

The ischemic brain hemispheres were removed and freed from meninges, then gently shredded in a small amount of ice-cold DMEM-high medium (Gibco, Carlsbad, CA, USA) containing 2% FBS. The suspension was digested with a warm trypsin/DNAse solution (DMEM + 0.25% Trypsin (Gibco, Carlsbad, CA, USA) + 50 U/mL DNase (Sigma-Aldrich, MO, USA)) and incubated at 37 °C for 30 min with gentle agitation. The mixture was filtered on 200 μm nylon mesh filters and centrifuged at 300 g for 10 min. Pellets were resuspended in 4 mL of 70% Percoll (Solarbio, Beijing, China) and overlaid on the top of a gradient containing 4 mL of 30% of Percoll. The gradient was centrifuged at 500 g for 25 min at room temperature without the use of a brake. Cells were collected from the 30% to 70% interface and washed with PBS containing 2% FBS (300 g, 10 min), then resuspended on 100 μL of PBS containing 2% FBS with Fc Block reagent for staining and FACS analysis.

All cells were incubated with conjugated antibodies CD45-APC (1:40, AbD Serotec, Oxford, UK), CD11b/c- PE/Cy7 (1:80, Biolegend, CA, USA), HIS48- FITC (1:200, Abcam, Camb, UK), and CD163-PE (1:10, AbD Serotec, Oxford, UK) at 4 °C for 30 min. Cells were then washed with PBS containing 2% FBS twice, and analyzed on a FACSCalibur flow cytometer and flowJo software (Tree Star Inc., Ashland, OR, USA). The unstained cells and/or cells stained with isotypematched IgG were used as negative controls. Neutrophils were defined as $CD45^+CD11b/c^+HIS48^+$, and N2 neutrophils were defined as $CD45^+CD11b/c^+$ $HIS48^+$ $CD163^+$.

### 2.15. Evaluation of BBB permeability

BBB permeability was evaluated by Evans Blue (EB) leakage as described previously [32], with some modifications. Briefly, EB dye (Sigma-Aldrich, MO, USA) (2% in saline; 4 mg/kg; filter sterilized) was injected into the rat tail vein. Dye circulated 2 h before rats were anesthetized and then transcardially perfused with saline to flush away the blood and EB in blood vessels. Then the brains were removed. To detect leakage in the ischemic hemispheres, the ischemic hemispheres were removed and weighed before being cut into small pieces and then immersed in N,N-dimethylformamide (Sigma-Aldrich, MO, USA) (2 mL N,N-dimethylformamide per 1 g of brain tissue) at 50 °C for 72 h for EB extraction. The solution containing the small pieces of brain was then centrifuged at 350 g for 15 min to collect the supernatant. The EB content of the supernatant was detected using a spectrophotometer (wavelength 630 nm). The EB concentration was normalized to tissue weight (ng/g).

### 2.16. Cytokine-induced neutrophil chemoattractants (CINCs) enzyme-linked immune-sorbent assay (ELISA)

CINCs have four isoforms: CINC-1, CINC-2α, CINC-2β, and CINC-3 [33,34]. They are members of the CXC chemokine family, and potent chemotactic factors for neutrophils [35]. The higher the expression level of CINCs, the stronger the chemotaxis to neutrophils. The ipsilateral cortex and striatum tissues were homogenized with 0.9% saline at a ratio of 1:10, and centrifuged at 12,000*g* for 10 min, and then collected the supernatants. Levels of CINCs in supernatants were measured by commercial ELISA kits (MEIMIAN, Jiangsu, China) according to the manufacturer's instructions. Data were expressed as pg/mL.

### 2.17. Statistical analysis

Data were presented as mean ± standard errors (SEM) and analyzed by SPSS 17.0 statistical software. Except for mNSS scores, data were analyzed by using independent samples *t*-test between two groups and one-way analysis of variance (ANOVA) followed by LSD or Dunnett T3 post-hoc test for multiple group comparison. For mNSS scores, statistical analyses were performed with Kruskal-Wallis one-way ANOVA on ranks, followed by Dunn's method. Statistical significance was assumed at $P$ values $< 0.05$. The presence of correlations between variables was analyzed using Pearson's correlation analysis.

## 3. Results

### 3.1. Neutrophils exhibit high plasticity in vitro

Neutrophils are heterogeneous and can be polarized into either N2 phenotype or N1 phenotype [16,36]. Flow cytometry and qRT-PCR were used to evaluate individual neutrophil phenotype. For N2 neutrophils induction, IL-4 (20 ng/mL) was used to stimulate peripheral blood neutrophils for 12 h. The data showed that $HIS48^+CD163^+$ and $HIS48^+CD163^-$ cells accounted for 86.2% and 13.7%, respectively (Fig. 1a), and the expression of anti-inflammatory markers (*Arg1*, *Mrc1* and *Chi313*) in IL-4-induced neutrophils was significantly increased (Fig. 1b), which indicated that N2 neutrophil induction was successful. For N1 neutrophils induction, LPS (100 ng/mL) and IFN-γ (20 ng/mL) were used to stimulate peripheral blood neutrophils for 12 h. The data revealed that $HIS48^+CD163^+$ and $HIS48^+CD163^-$ cells accounted for 24.2% and 75.6%, respectively (Fig. 1a), and the expression of pro-inflammatory markers (*Tnf*, *NOS2* and *Il1b*) in LPS and IFN-γ-induced neutrophils was significantly increased (Fig. 1c), which indicated that N1 neutrophils were successfully induced in vitro.

### 3.2. Effects of N2 and N1 neutrophils on OGD/R-induced primary cortical neuron damage

Next, we explored whether N2 neutrophils had protective effects on primary cortical neuron injury induced by OGD/R in vitro, simulating the effects of N2 neutrophils in ipsilateral brain parenchyma on ischemic injured neurons after transient cerebral ischemia in rats. As shown in Fig. 2a, compared with the NO OGD group, the viability of primary cortical neurons in the OGD 4 h/ R 24 h group was significantly reduced. N2 neutrophils markedly improved the decline of neuronal viability induced by OGD/R. On the contrary, N1 neutrophils decreased the viability of no OGD neurons, and exacerbated OGD/R-induced reduced neuronal viability (Fig. 2b). Moreover, as shown in Fig. 2c to e, the dendrites of primary cortical neurons in Me (NO OGD) group were continuous and complete, after OGD 4 h/ R 24 h, the mean length of neurites was shorter and the number of beta-III Tubulin-positive neurons decreased. Importantly, N2 neutrophils obviously ameliorated OGD/R-induced shortening of mean neurite length and decreased number of beta-III Tubulin-positive neurons, while N1 neutrophils reduced the mean neurite length of no OGD neurons, and further aggravated OGD/R-induced neuronal damage. Notably, IL-4, LPS + IFN-γ and unstimulated neutrophils (N0) had no effect on the viability and morphology of no OGD and OGD/R neurons (Fig. 2a to c). Taken together, the data showed that N2 neutrophils had protective effects on primary cortical neuron damage induced by OGD/R, while N1 neutrophils could further aggravate neuronal damage.

### 3.3. N2 Neutrophils protect against OGD/R-induced neuron injury via BDNF/TrkB signaling

To investigate the molecular mechanisms by which N2 neutrophils protected against OGD/R-induced neuron damage, we examined BDNF/TrkB signaling. The results showed that OGD/R significantly

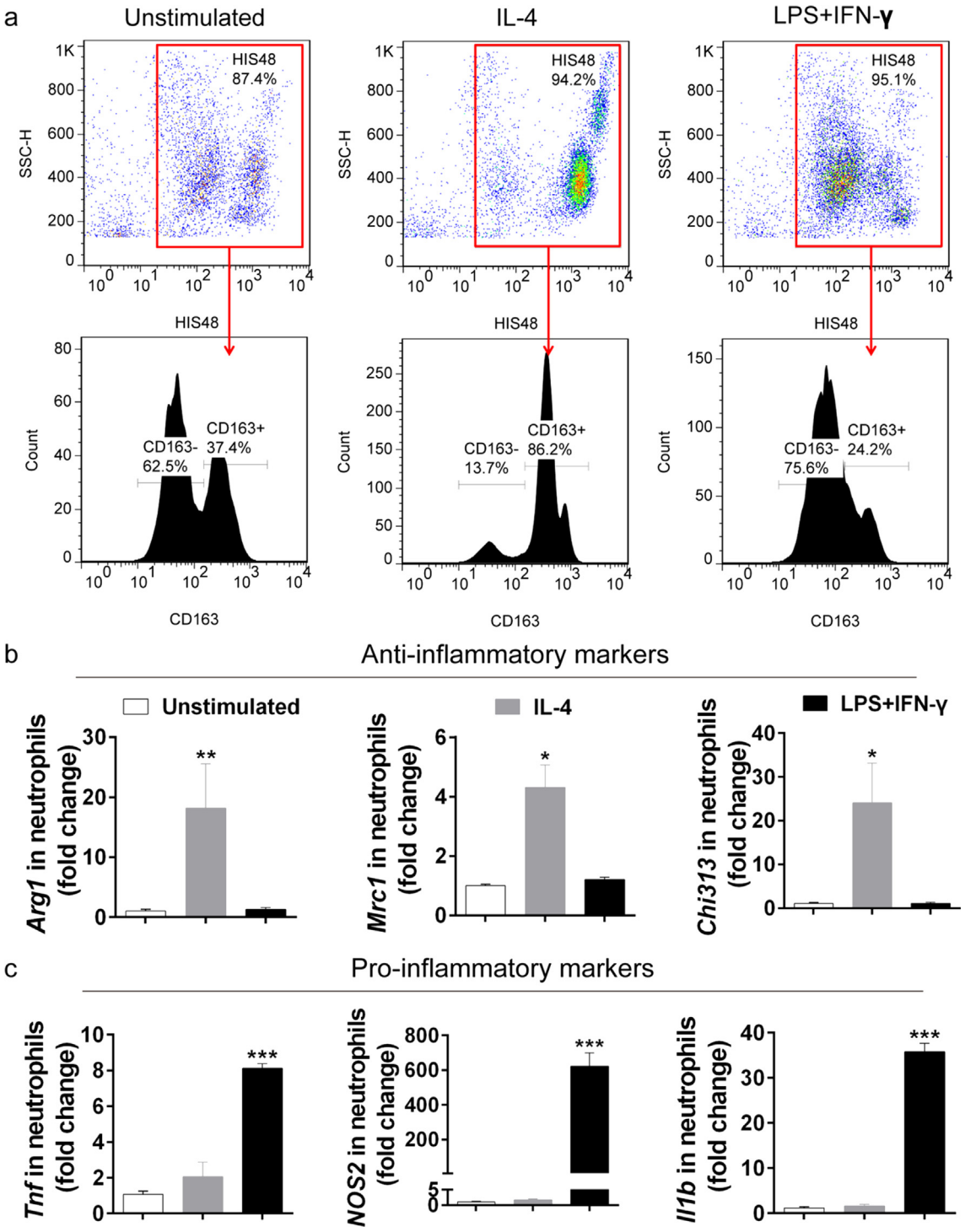


**Fig. 1.** IL-4 polarizes neutrophils to an N2 phenotype, while LPS and IFN-γ polarizes neutrophils to an N1 phenotype. (a) Representative flow cytometry plots illustrated gating strategy to confirm neutrophil N2 and N1 phenotypes. (b, c) IL-4 (20 ng/mL) treatment upregulated the expression of anti-inflammatory markers; on the contrary, LPS (100 ng/mL) and IFN-γ (20 ng/mL) upregulated the expression of pro-inflammatory markers in neutrophils. (n = 3/condition; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus unstimulated).

decreased the protein expression levels of BDNF and TrkB in primary cortical neurons compared with NO OGD group, while N2 neutrophils obviously inhibited the OGD/R-induced decrease in BDNF and TrkB expression (Fig. 3a to c). Therefore, we hypothesized that N2 neutrophils protect against OGD/R-induced neuron damage through BDNF/TrkB signaling. Next, we validated the above hypothesis using the TrkB antagonist ANA-12. As shown in Fig. 3d, e, ANA-12 (10 μM) had no significant effect on morphology and viability of normal neurons. Immunofluorescence results showed that OGD/R significantly shortened the mean length of neurites and decreased the number of beta-III Tubulin-positive neurons compared with NO OGD group, while N2 neutrophils improved OGD/R-induced neuronal injury. Notably, ANA-12 (10 μM) markedly attenuated the protective effect of N2 neutrophils on ischemic neurons (Fig. 3f to h). Therefore, these data indicated that N2 neutrophils protected against OGD/R-induced neuron damage via BDNF/TrkB signaling.

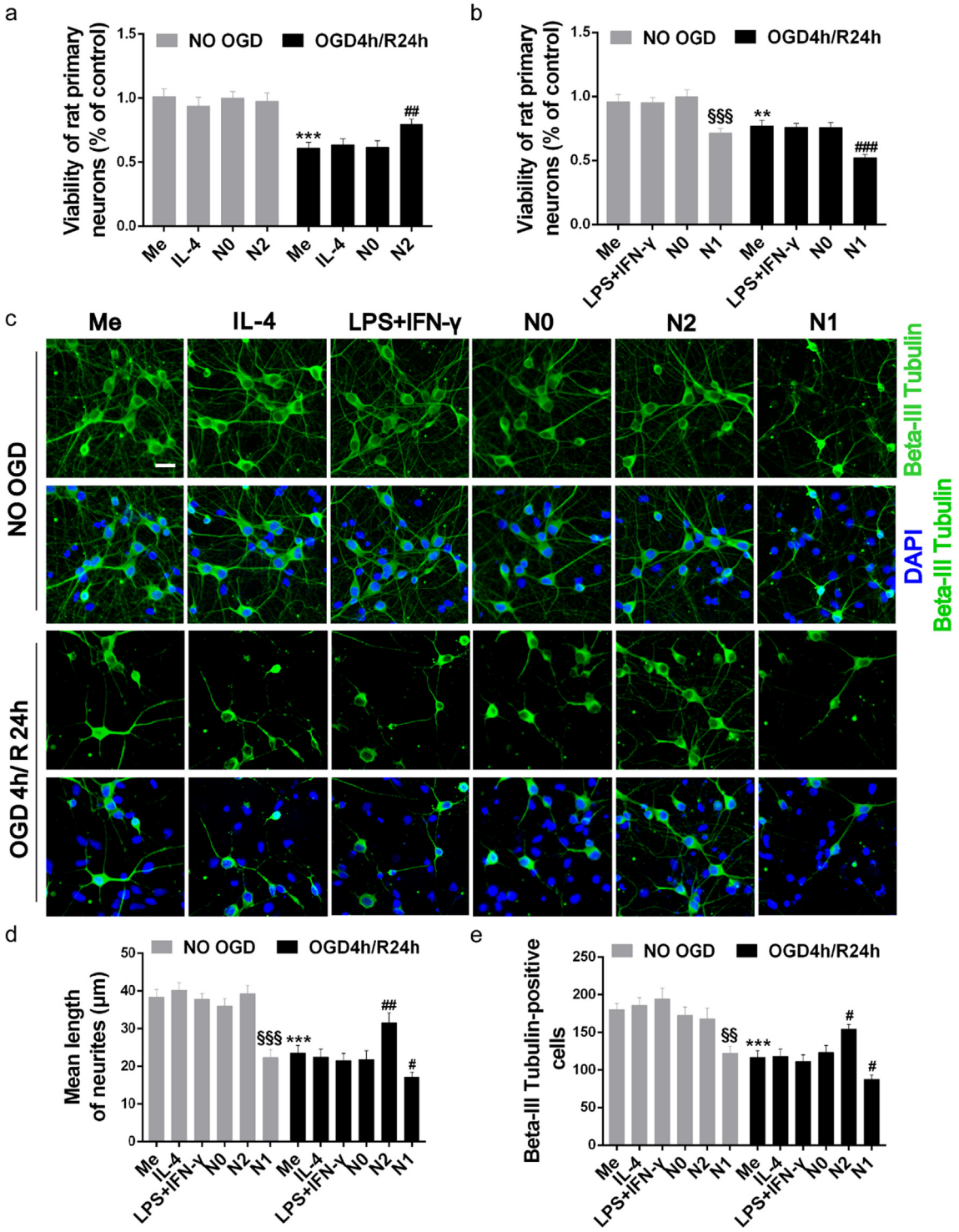


**Fig. 2.** Effects of N2 and N1 neutrophils on OGD/R-induced primary cortical neuron injury. (a, b) Effects of N2 and N1 neutrophils on viability of normal neurons and OGD/R neurons (n = 3/condition). (c) Representative images of the effects of N2 and N1 neutrophils on morphology of primary cortical neurons. Scale bar = 20 μm. (d) Measurements and statistical analyses of mean neurite length. (n = 30 cells/group). (e) Statistical analyses of the number of beta-III Tubulin-positive neurons (n = 3/condition). $^{**}p < 0.01$, $^{***}p < 0.001$ versus Me (NO OGD); $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus Me (OGD 4 h/ R 24 h); §§$p < 0.01$, §§§$p < 0.001$ versus N0 (NO OGD). OGD/R, oxygen glucose deprivation/re-oxygenation; Me, media; N0, unstimulated neutrophils; N2, neutrophils stimulated by IL-4; N1, neutrophils stimulated by LPS and IFN-γ.

### *3.4. The injury induced by tMCAO exhibits spontaneous recovery over time in rats*

To investigate whether N2 neutrophils are associated with long-term outcomes after transient cerebral ischemia in rats. First, we examined the changes in ischemic stroke outcomes on day 1, 3, 7, 14 and 30 after tMCAO. The results showed that infarct volume (Fig. 4a, b), brain water content (Fig. 4c), mNSS (Fig. 3d), grip strength (Fig. 4e), rotarod performance (Fig. 4f), and the surviving and degenerating neurons in ipsilateral cortex and striatum (Fig. 4g) exhibited

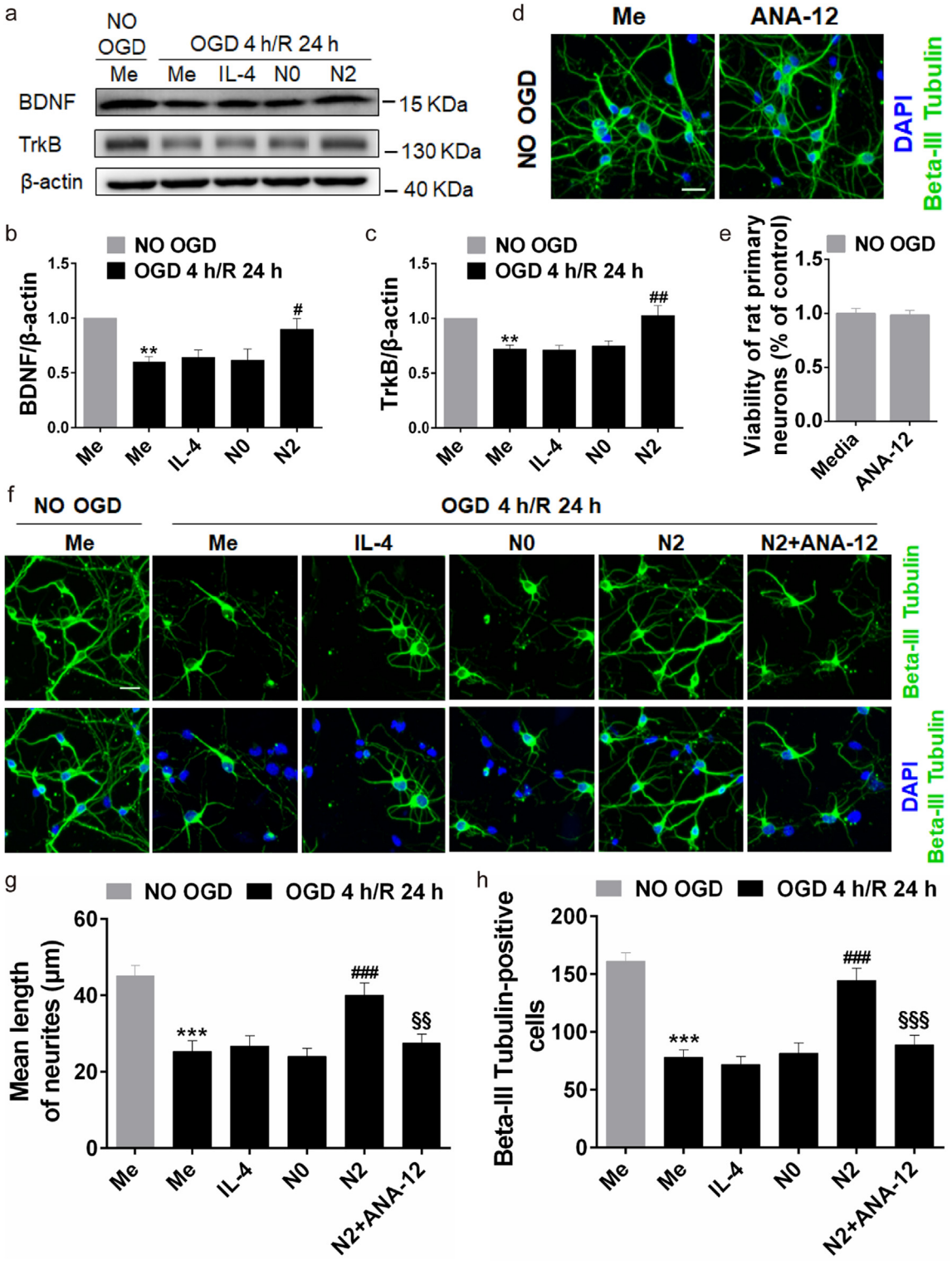


**Fig. 3.** N2 neutrophils protect against OGD/R-induced neuron injury via BDNF/TrkB signaling. Representative western blots (a) and statistical analyses (b, c) of BDNF and TrkB in primary cortical neurons (n = 3/condition). (d) Effects of TrkB antagonist ANA-12 (10 μM) on morphology of normal neurons (n = 3/condition). Scale bar = 20 μm. (e) Effects of TrkB antagonist ANA-12 (10 μM) on viability of normal neurons (n = 3/condition). (f) Representative images of neurite of primary cortical neurons cultured in different conditions. Scale bar = 20 μm. (g) Measurements and statistical analyses of mean neurite length. (n = 30 cells/group). (h) Statistical analyses of the number of beta-III Tubulin-positive neurons (n = 3/condition). $^{**}p < 0.01$, $^{***}p < 0.001$ versus Me (NO OGD); $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus Me (OGD 4 h/ R 24 h); §§$p < 0.01$, §§§$p < 0.001$ versus N2 (OGD 4 h/ R 24 h). Me, media; N0, unstimulated neutrophils; N2, neutrophils stimulated by IL-4.

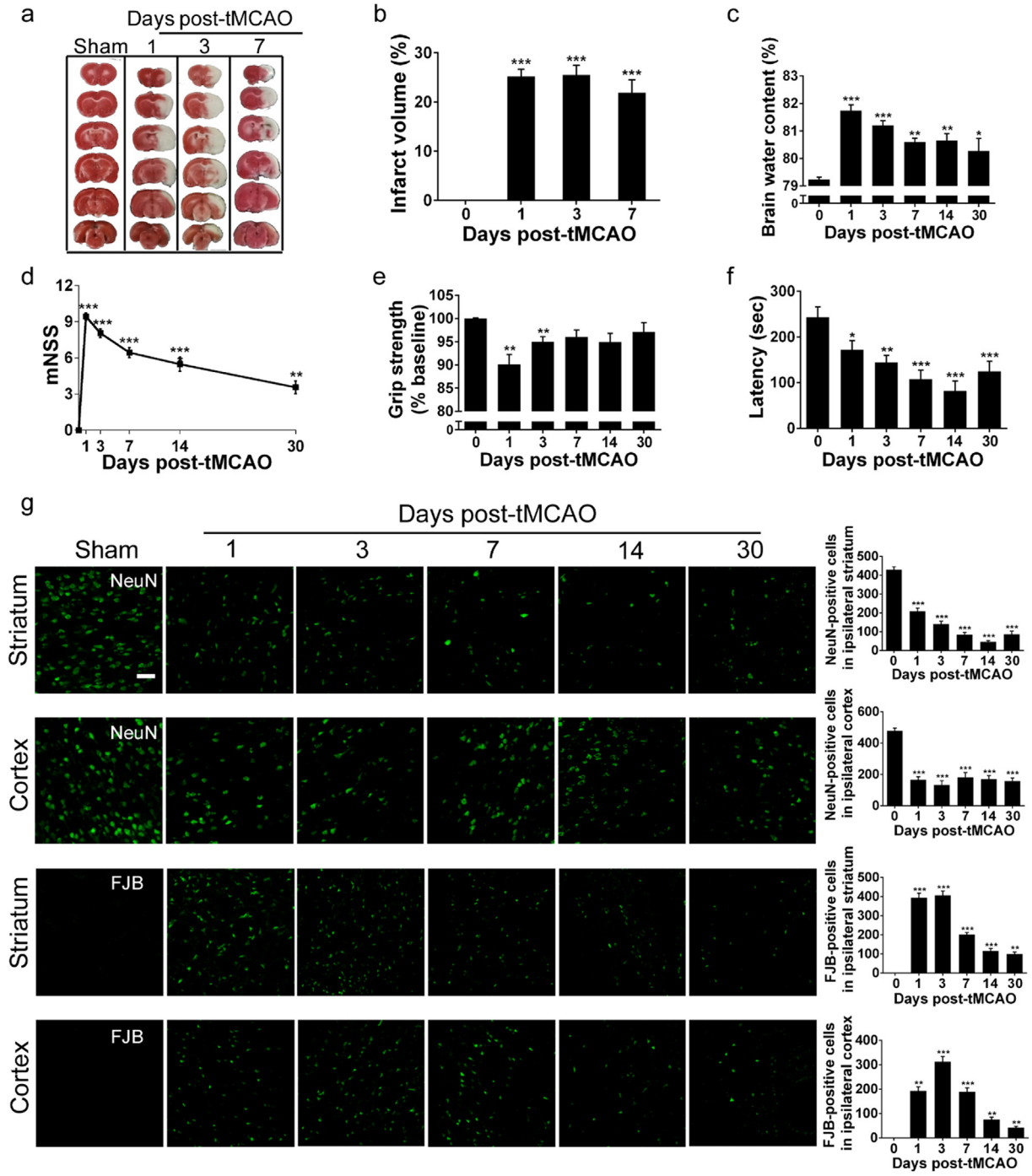


**Fig. 4.** The damage induced by tMCAO exhibits spontaneous recovery over time in rats. (a) Representative images of cerebral infarct volume at 1, 3 and 7 days after tMCAO. (b) Statistical analysis of cerebral infarct volume was conducted using Image-Pro Plus 6.0. (c, d) Assessments of brain water content and mNSS at 1, 3, 7, 14 and 30 days after tMCAO. (e, f) Assessments of forelimb grip strength and the latency on accelerated rotarod at 1, 3, 7, 14 and 30 days after tMCAO. (g) Representative images of immunofluorescence staining for NeuN and FJB staining in ipsilateral striatum and cortex at 1, 3, 7, 14 and 30 days after tMCAO and statistical analysis of surviving neurons (NeuN-positive) and degenerating neurons (FJB-positive) was conducted using ImageJ. Scale bar = 20 μm. (n = 6 to 8; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus day 0).

spontaneous recovery over time.

### 3.5. Neutrophils infiltrate the ipsilateral brain parenchyma from periphery and peak at day 3 after tMCAO

Next, flow cytometry was used to quantitatively analyze the number of neutrophils ($CD45^{+}CD11b/c^{+}HIS48^{+}$ cells) in the ipsilateral brain parenchyma, blood and bone marrow at day 1, 3, 7, 14 and 30 post-tMCAO. As shown in Fig. 5a to d, the number of neutrophils in bone marrow was the lowest at day 1–3 post-tMCAO (Fig. 5b), while the number of neutrophils in ipsilateral brain parenchyma peaked at day 1–3 post-tMCAO (Fig. 5d). To clarify whether there was any association between brain and peripheral neutrophils, a Pearson's correlation analysis was performed. The number of neutrophils in the ipsilateral brain parenchyma showed very strong negative correlation with the number of neutrophils in the bone marrow (r = -0.87, $p < 0.001$)

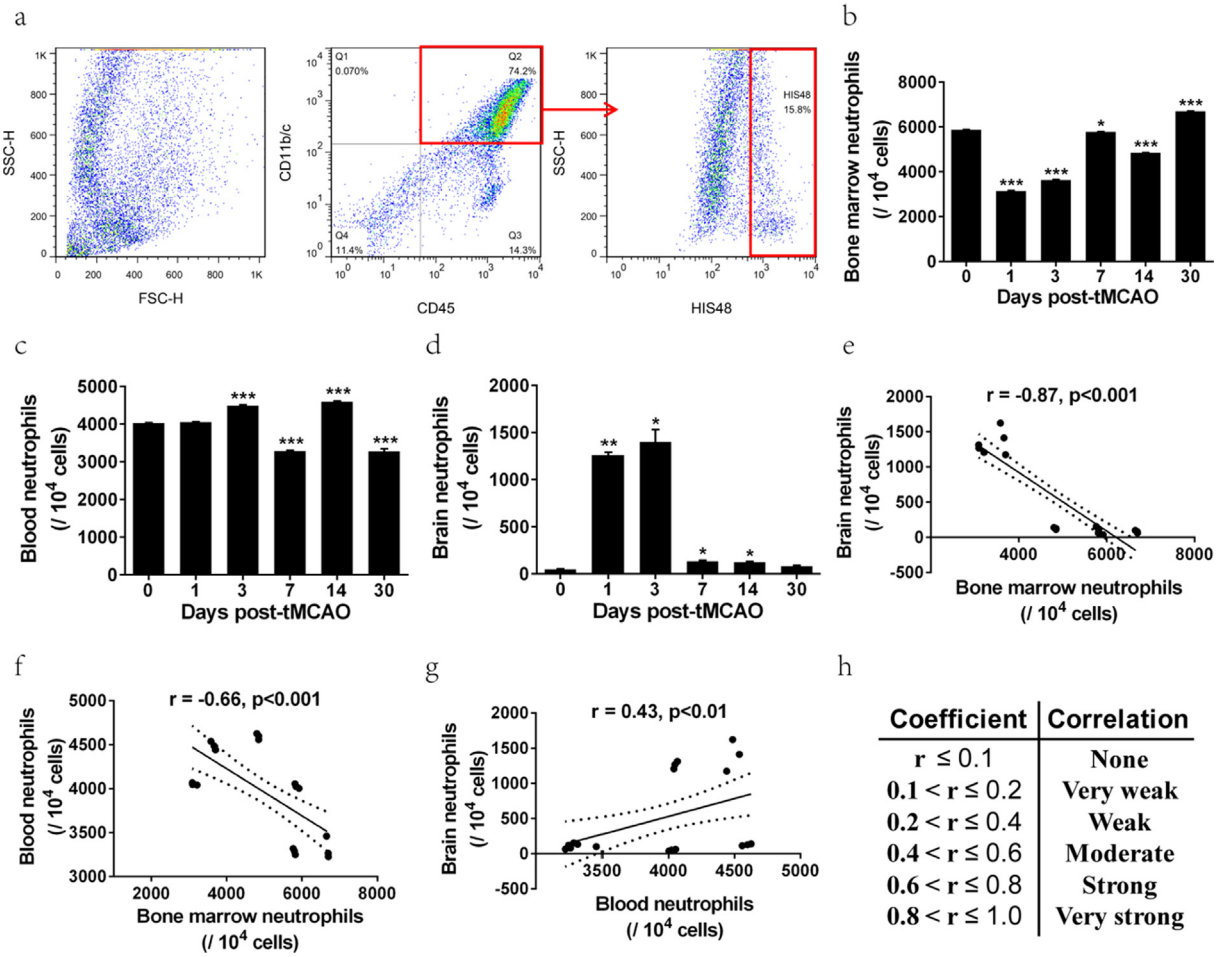


**Fig. 5.** Neutrophils infiltrate the ipsilateral brain parenchyma from periphery and peak at day 3 post-tMCAO. (a) Gate strategy for neutrophils ($CD45^+CD11b/c^+HIS48^+$) (the ipsilateral brain parenchyma on the 3rd day after tMCAO was taken as an example). (b to d) Flow cytometric quantification of neutrophils in bone marrow, blood, and the ipsilateral brain parenchyma at 1, 3, 7, 14 and 30 days after tMCAO. (n = 6 to 8; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus day 0). (e) Correlation between brain neutrophils and bone marrow neutrophils. (f) Correlation between blood neutrophils and bone marrow neutrophils. (g) Correlation between brain neutrophils and blood neutrophils. The letter "r" stands for Pearson correlation coefficient. (h) The correspondence between coefficient and correlation.

(Fig. 5e), the number of blood neutrophils was negatively correlated with the number of bone marrow neutrophils (r = -0.66, $p < 0.001$) (Fig. 5f) and positively correlated with the number of ipsilateral brain parenchyma neutrophils (r = 0.43, $p < 0.01$) (Fig. 5g). These results suggested that neutrophils could infiltrate the ipsilateral brain parenchyma from periphery and increased to a peak at 3 days post-tMCAO.

### 3.6. BBB permeability and CINCs expression levels are positively correlated with the number of neutrophils in ipsilateral brain parenchyma

Neutrophils do not exist in the brain parenchyma due to the presence of BBB under normal physiological conditions, so why do neutrophils infiltrate the brain parenchyma after transient cerebral ischemia? First, we detected BBB permeability after tMCAO by assessing EB extravasation. The results showed that the EB leakage was the highest at day 1–3 post-tMCAO (Fig. 6a, b), and there was a very strong positive correlation between the number of neutrophils ($CD45^+CD11b/c^+HIS48^+$ cells) in ipsilateral brain parenchyma and EB leakage (r = 0.92, $p < 0.001$) (Fig. 6c). This indicated that the increase of BBB permeability after tMCAO may contribute to the infiltration of neutrophils in brain parenchyma. In addition, the direction in which neutrophils migrate is mainly determined by CINCs. Therefore, we detected the expression level of CINCs in ipsilateral striatum and cortex. CINCs expression levels in ipsilateral striatum (Fig. 6d) and cortex (Fig. 6e) were the highest at day 1–3 post-tMCAO, which was significantly positively correlated with the number of neutrophils in ipsilateral brain parenchyma (r = 0.63, $p < 0.001$; r = 0.84, $p < 0.001$) (Fig. 6f, g). Taken together, our data demonstrated that the infiltration of neutrophils from the periphery into the ipsilateral brain parenchyma was largely due to increased permeability of the BBB and the chemotaxis of CINCs in ipsilateral cortex and striatum to neutrophils.

### 3.7. The proportion of N2 neutrophils in ipsilateral brain parenchyma is positively correlated with spontaneous recovery after transient ischemic injury

Flow cytometry was used to determine the proportion of N2 neutrophils ($HIS48^+CD163^+$) in ipsilateral brain parenchyma. As shown in Fig. 7a, the proportion of N2 neutrophils in ipsilateral brain parenchyma at day 1, 3, 7, 14 and 30 after tMCAO was 49%, 34%, 83%, 48% and 51%, respectively. These results suggested that the proportion of N2 neutrophils in ipsilateral brain parenchyma peaked at day 7 after transient cerebral ischemia in rats. Next, the correlation between the proportion of N2 neutrophils in ipsilateral brain parenchyma and cerebral ischemic outcomes was investigated. As shown in Fig. 7b, the results revealed that the proportion of N2 neutrophils in ipsilateral brain parenchyma was negatively correlated with the number of degenerating neurons in ipsilateral cortex (r = −0.52, $p < 0.001$) and

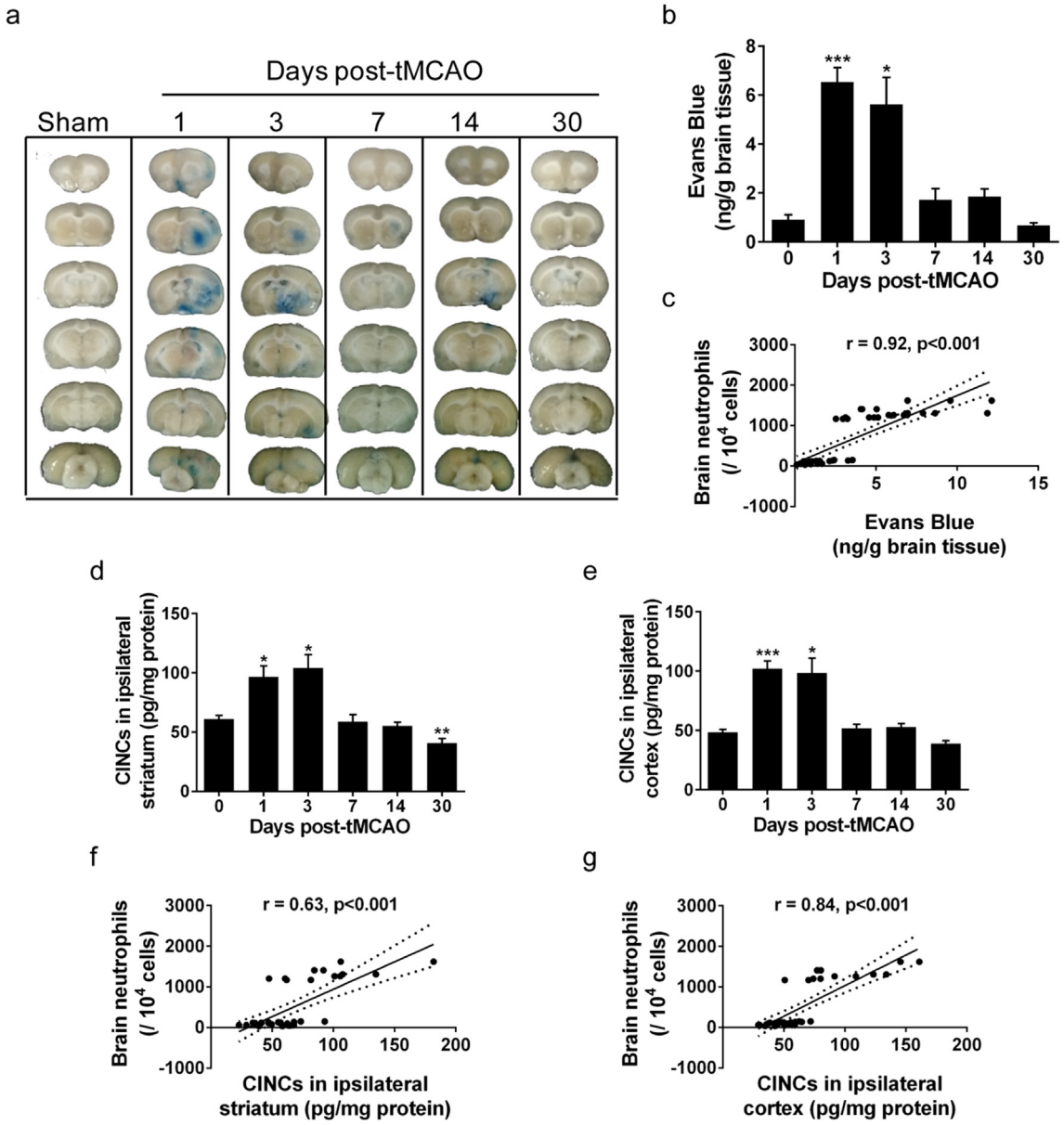


**Fig. 6.** BBB permeability and CINCs expression levels are positively correlated with the number of neutrophils in ipsilateral brain parenchyma. (a) Representative brain images show EB leakage at 0, 1, 3, 7, 14 and 30 days after tMCAO. (b) Quantitative analysis of EB leakage at 0, 1, 3, 7, 14 and 30 days after tMCAO (n = 6 to 8; $^{*}p < 0.05$, $^{***}p < 0.001$ versus day 0). (c) Correlation between the number of neutrophils in ipsilateral brain parenchyma and EB leakage. ELISA showed CINCs expression levels in ipsilateral striatum (d) and ipsilateral cortex (e) at 0, 1, 3, 7, 14 and 30 days after tMCAO (n = 6 to 8; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus day 0). (f) Correlation between the number of neutrophils in ipsilateral brain parenchyma and CINCs expression levels in ipsilateral striatum. (g) Correlation between the number of neutrophils in ipsilateral brain parenchyma and CINCs expression levels in ipsilateral cortex. The letter "r" stands for Pearson correlation coefficient.

striatum ($r = -0.56$, $p < 0.001$), mNSS ($r = -0.52$, $p < 0.001$), brain water content ($r = -0.60$, $p < 0.001$) and infarct volume ($r = -0.48$, $p < 0.05$), and positively correlated with grip strength ($r = 0.49$, $p < 0.001$) and the number of surviving neurons in ipsilateral cortex ($r = 0.55$, $p < 0.01$). These results indicated that the proportion of N2 neutrophils in ipsilateral brain parenchyma was significantly positively correlated with spontaneous recovery after transient ischemic stroke. Whereas the number of surviving neurons in ipsilateral striatum ($r = -0.15$, $p > 0.05$) and rotarod performance ($r = 0.05$, $p > 0.05$) had no significant correlation with the proportion of N2 neutrophils in ipsilateral brain parenchyma.

## 4. Discussion

The present study showed that N2 neutrophils had protective effects on OGD/R-induced primary cortical neuron damage in vitro. In addition, tMCAO-induced injury exhibited spontaneous recovery over time in rats, and the proportion of N2 neutrophils in ipsilateral brain parenchyma was significantly positively correlated with spontaneous recovery after transient ischemic stroke (Fig. 8). These results suggested that N2 neutrophils might be considered as a promising therapeutic target for promoting recovery after cerebral ischemia.

Polymorphonuclear leukocytes (PMN), predominantly neutrophils, were first identified in ischemic brain regions in dogs with air embolus-induced focal cerebral ischemia by Hallenbeck et al. [37]. Subsequently, numerous studies confirmed that neutrophils could infiltrate

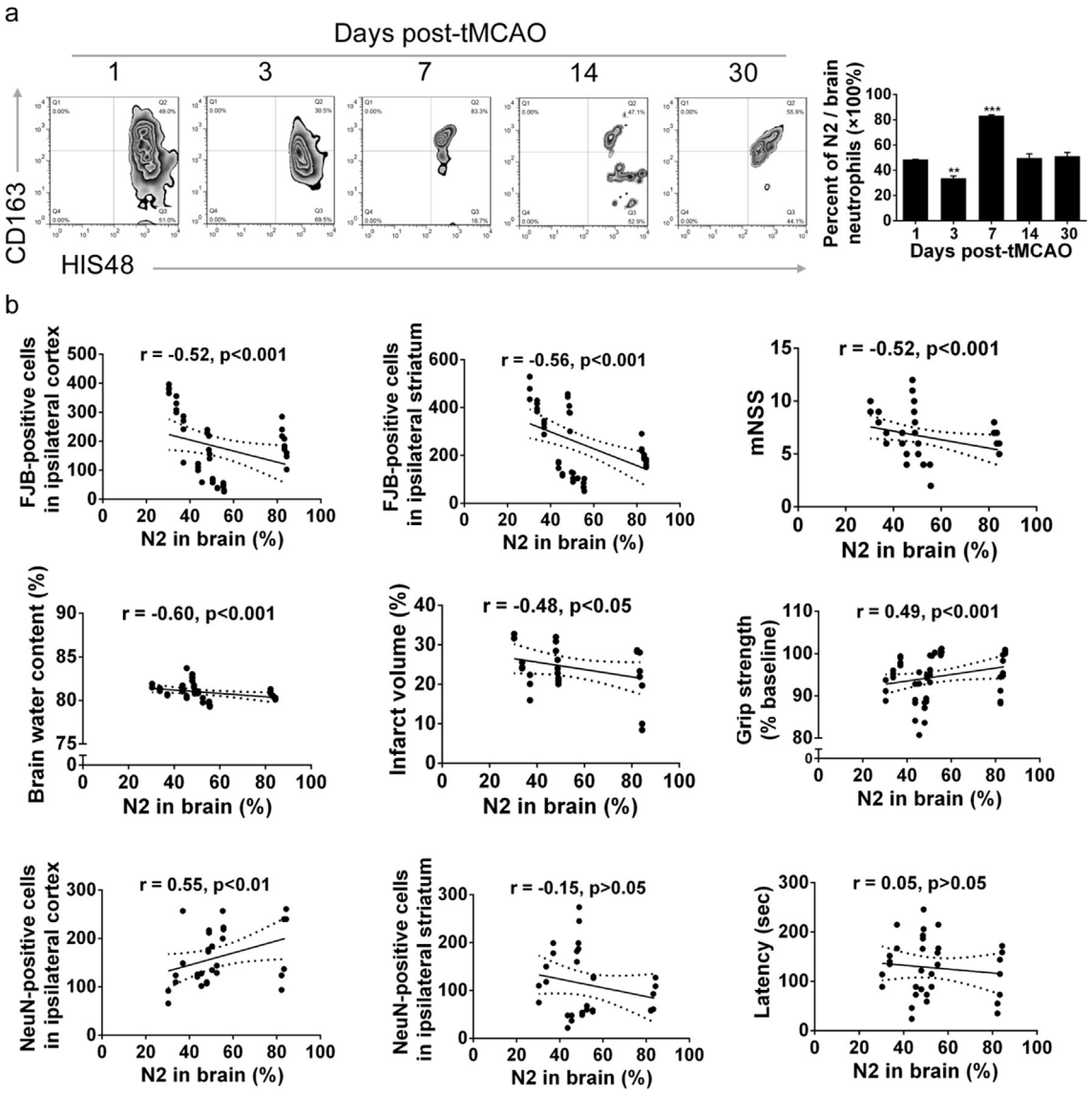


**Fig. 7.** The proportion of N2 neutrophils in ipsilateral brain parenchyma is positively correlated with spontaneous recovery after transient ischemic injury. (a) Representative zebra plot and flow cytometric quantification of the proportion of N2 neutrophils in the ipsilateral brain parenchyma at 1, 3, 7, 14 and 30 days after tMCAO. (n = 6 to 8; $^{**}p < 0.01$, $^{***}p < 0.001$ versus day 1). (b) The proportion of N2 neutrophils in ipsilateral brain parenchyma was negatively correlated with the number of degenerating neurons in ipsilateral cortex and striatum, mNSS, brain water content and infarct volume, positively correlated with grip strength and the number of surviving neurons in ipsilateral cortex, and no significant correlation with the number of surviving neurons in ipsilateral striatum and rotarod performance. The letter "r" stands for Pearson correlation coefficient.

the ischemic brain parenchyma in experimental animal models [7,38–40] and humans with ischemic stroke [40–42]. Studies showed that neutrophils in ischemic brain hemisphere reached maximal levels at 1–3 days after cerebral ischemia, and then decreased over time [5,6]. Consistently, our research showed that neutrophils infiltrated the ipsilateral brain parenchyma and peaked at day 3 after tMCAO in rats (Fig. 2d). In the early stages, neutrophils infiltration into brain parenchyma after cerebral ischemia is considered harmful, and indeed, early studies revealed that neutrophil depletion could reduce infarct volume in ischemic stroke [6,7]. Yet, subsequent studies demonstrated that experimental neutropenia has equivocal efficacy [43] or no effect [44,45] on infarct volume in ischemic stroke, and neutrophil mobilization from bone marrow induced by granulocyte colony stimulating factor (G-CSF) didn't worsen neurological outcomes in animal ischemic stroke models [46] or in a clinical phase II trial (AXIS) [47]. Moreover, clinical trials aiming at neutrophils adhesion to endothelial ICAM-1 [48–50] or preventing neutrophils infiltration into brain parenchyma by neutralizing the aMb2-integrin [51] have failed to alleviate the severity of stroke. All these data suggest that the migration of neutrophils into the brain parenchyma after cerebral ischemia may not be entirely harmful. In fact, neutrophils are heterogeneous, similar to macrophages, neutrophils have been proved to shift toward an N1 or N2 phenotype [16,36]. Yonggang Ma et al. showed that N1 neutrophils exerted a pro-inflammatory response, which positively correlated with infarct wall thinning following myocardial infarction [16], while N2 neutrophils contribute to resolution of inflammation and may participate in neuroprotection [13–15]. Hence, the confirmation of N1 and N2 neutrophils demonstrates that neutrophils infiltrating the brain parenchyma after cerebral ischemia may play a dual role, which might explain why therapies targeting neutrophils egress were largely ineffective. So, whether neutrophils play a protective or injurious role after cerebral ischemia depends on the proportion of N1 and N2 neutrophils in the brain, which is mainly determined by the pathological changes in different stages after cerebral ischemia. Therefore, we

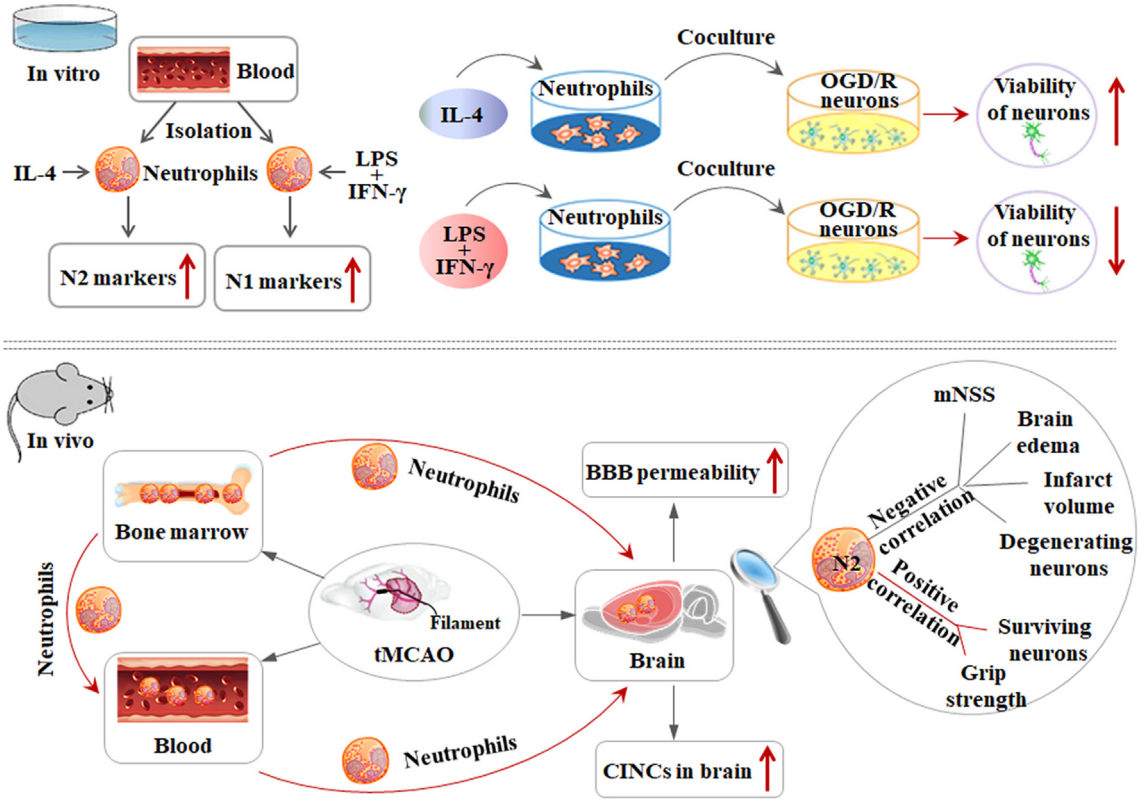


**Fig. 8.** Graphical abstract. LPS, lipopolysaccharide; IFN-γ, interferon-gamma; OGD/R, oxygen glucose deprivation/re-oxygenation; tMCAO, transient middle cerebral artery occlusion; BBB, blood-brain barrier; CINCs, cytokine-induced neutrophil chemoattractants; mNSS, modified neurological severity score.

investigated the correlation between the proportion of N2 neutrophils and long-term outcomes after transient cerebral ischemia. Our findings indicated that the proportion of N2 neutrophils in ipsilateral brain parenchyma was significantly positively correlated with spontaneous recovery after transient ischemic injury. Notably, we demonstrated for the first time that N2 neutrophils had protective effects on OGD/R-induced damage of primary cortical neurons in vitro, which was consistent with a recent report by Cai et al., they confirmed that skewing neutrophils toward N2 phenotype was protective to cerebral ischemic stroke [15].

Macrophages and lymphocytes have been shown to possess the ability to switch phenotypes, and studies showed that neutrophils share this capacity [13,14,16,36,52]. Neutrophil polarization has been identified in cancer [36], myocardial infarction [16], bacterial infection [52], and ischemic stroke animal models [13–15]. These studies consistently indicated that, similar to M1 and M2 macrophages, N1 neutrophils exert pro-inflammatory and tissue damage roles, while N2 neutrophils play resolution of inflammation and tissue repair roles. In parallel, in these studies, the selection of the markers of N1 and N2 neutrophils were the same as those of M1 and M2 macrophages, respectively. N1 neutrophils were defined as $Ly6G^+CD206^-$ or $Ly6G^+Ym1^-$, and N2 neutrophils were defined as $Ly6G^+CD206^+$ or $Ly6G^+Ym1^+$ in mice. CD163, the hemoglobin scavenger receptor, is one of the major changes in the macrophage switch to alternative activated phenotypes in inflammation [53]. Therefore, in the present study, N2 neutrophils were defined as $HIS48^+CD163^+$, and N1 neutrophils were defined as $HIS48^+CD163^-$, according to the markers of M1 and M2 in rats, respectively [53–56]. In addition, according to [16], neutrophils were induced to polarize toward N2 or N1 by IL-4 or LPS and IFN-γ respectively in vitro. In this study, flow cytometry analysis revealed that 86.2% of IL-4-induced neutrophils were $CD163^+$, and 75.6% of neutrophils induced by LPS and IFN-γ were $CD163^-$. These results indicated that $HIS48^+CD163^+$ and $HIS48^+CD163^-$ could represent N2 and N1 neutrophils, respectively.

In this study, the results indicated that N2 neutrophils contributed to spontaneous recovery after ischemic stroke. However, the limitations of this study mainly lie in that most conclusions are based on the correlation between data, and further experiments need to demonstrate the real causative relevance of the events. In addition, N2 neutrophils may not be the only ones involved in spontaneous recovery following ischemic stroke. Studies demonstrated that M2 microglia/macrophages played neuroprotective roles in cerebral ischemia [57–59]. Thus, M2 microglia/macrophages may also contribute to spontaneous recovery after ischemic stroke.

In the present study, the results revealed that the proportion of N2 neutrophils in ipsilateral brain parenchyma was not correlated with the number of NeuN-positive cells in ipsilateral striatum or the latency to fall off the rotarod. From the data, the number of NeuN-positive cells in ipsilateral striatum and the latency to fall off the rotarod began to recover from the 30th day after tMCAO. Their recovery period began later than other outcomes. This may be due to the fact that different indexes exhibit different spontaneous recovery kinetics after cerebral ischemia. In addition, TTC staining could be used to detect brain infarct within 7 days after stroke [32,60,61], however, it is not sensitive to assess brain tissue loss in long term. Therefore, we only examined the infarct volume at 1, 3 and 7 days after tMCAO in rats (Fig. 4a, b).

In summary, the present study indicates that N2 neutrophils may be involved in spontaneous recovery after transient cerebral ischemia by inhibiting ischemic neuron injury in rats, suggesting that N2 neutrophils may be a promising therapeutic target for promoting recovery after ischemic stroke.

## Declaration of Competing Interest

All the authors declare that there is no conflict of interests regarding the publication of this paper.

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