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Original Research

TREM2 Alleviates Subarachnoid Hemorrhage-Induced Brain Injury through Attenuating Neuroinflammation and Programmed Cell Death *in Vivo* and *in Vitro*

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Abstract

Background: Apoptosis and pyroptosis are two types of programmed cell death related to the neuroinflammatory reaction after subarachnoid hemorrhage (SAH). Research indicates that triggering receptor expressed on myeloid cells 2 (TREM2) can regulate the SAH-induced inflammatory response. However, whether TREM2 regulates programmed cell death (apoptosis and pyroptosis) remains to be clarified. The purpose of the present study was to investigate the effects of TREM2 on cell death in SAH. **Methods**: SAH was induced in adult male C57BL/6J mice by endovascular perforation. An *in-vitro* cellular model of SAH was established by treating cocultured BV2 microglia and HT22 neuronal cells with oxyhemoglobin. TREM2 overexpression or knockdown was carried out by intraventricular lentivirus injection at 7 d before SAH induction in mice or lentiviral transfection, respectively. Neurobehavioral tests as well as western blot, reverse transcription–quantitative polymerase chain reaction (RT-qPCR), immunofluorescence, Evans blue (EB) staining, Nissl staining, and flow cytometry assays were performed to investigate the neuroprotective role of TREM2 after SAH. **Results**: After SAH, the TREM2 mRNA and protein levels were elevated in SAH mice, exhibiting a peak at 72 h. TREM2 overexpression improved the SAH-induced neurological deficits in mice, while TREM2 knockdown worsened them. In the brains of mice with TREM2 overexpression, less neuronal death and more neuronal survival were detected at 72 h post SAH. Meanwhile, TREM2 overexpression showed an inhibitory effect on microglial activation, neutrophil infiltration, and the expression of cell death marker proteins. Consistent results were obtained *in vitro*. **Conclusions**: Our research indicates the important role of TREM2 on cell death after SAH, suggesting that targeting TREM2 might be an effective approach for treating SAH.

Keywords: subarachnoid hemorrhage; TREM2; neuroinflammatory; pyroptosis; apoptosis

1. Introduction

Subarachnoid hemorrhage (SAH), which is bleeding in the subarachnoid space, is a common cerebrovascular disease. The symptoms of SAH include headache, vomiting, unconsciousness, numbness, and even seizures [1]. The overall crude incidence of SAH globally is reported to be 6.2–10.0 per 100,000 persons [2]. Up to 30% of SAH patients have a poor outcome or even death, while most survivors suffer from long-term disability or cognitive impairment [3]. SAH may result from a traumatic brain injury or a spontaneous aneurysm rupture. Family history, smoking, alcoholism, and high blood pressure have been confirmed to be the main risk factors for spontaneous SAH [4]. SAH patients are often diagnosed by computed topography and managed by stabilization and prevention of rebleeding, mostly symptomatic treatment. Many mechanisms have been proposed to explain the brain injury damage following SAH. For cerebral vasospasm, which is one typical complication of SAH, blood products are thought to be released from the SAH, which trigger the activation of the tyrosine kinase pathway and cause calcium ion release, making the smooth muscle of the cerebral arteries contract [5]. Besides, oxyhemoglobin released into the cerebrospinal fluild (CSF) can also increase the release of free radicals, endothelin-1, prostaglandin, etc., resulting in vasoconstriction. In addition, studies have shown that the inflammatory reaction, which is featured by microglial activation, inflammatory cell infiltration, and cytokine release, contributes to the pathogenesis of SAH-induced brain injury [6]. Furthermore, oxidative stress, mitochondrial dysfunction, etc. also have been widely accepted as detrimental factors in SAH-induced brain injury [7]. However, based on the current situation that SAH patients still have poor neurological function outcomes, more investigations are needed to clarify the detailed mechanism.

The triggering receptor expressed on myeloid cells 2 (TREM2), which is selectively and highly expressed on microglia, is the main receptor for inducing the anti-inflammatory response [8]. It can be activated by damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), and then it can combine with adaptor proteins, triggering the immune responses in microglia [9,10]. TREM2 also can be regulated by nuclear factors, which play an important role in inflammation [11]. Many researchers have reported the neuroprotective effect of TREM2 by promoting phagocytosis and suspending inflammation in experimental intracerebral hemorrhage [12,13]. Moreover, TREM2 overexpression can decrease the expression of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha (TNF-α) in microglia [14,15]. However, TREM2 deficiency enhances the expression of these cytokines, and, furthermore, TREM2 deficiency can affect the number of microglia [16]. After SAH, TREM2 plays a critical role in driving microglial polarization, and its neuroprotective effect might be potentially inhibited by Toll-like receptor 4, which is the main proinflammatory receptor on microglia in early SAH [17]. Additionally, in the brain, the soluble form of TREM2, sTREM2, can be cleaved by a disintegrin and metalloproteinase and released extracellularly [18]. An increased sTREM2 level has been detected in the blood and CSF from patients with Alzheimer's disease and ischemia [19,20]. Given the central role of TREM2 in the function of microglia and the brain, further investigations on the relationship between TREM2 and brain diseases, such as SAH, should be performed.

Apoptosis and pyroptosis are two types of programmed cell death related to the neuroinflammatory reaction in intracranial hemorrhagic disease [21,22]. The activation mechanisms of apoptosis include both intrinsic and extrinsic pathways. Generally, when apoptosis begins, the TNF-α or Fas pathway is activated, and the proapoptotic (Bax, Bak, Bid, etc.) and antiapoptotic (Bcl2, Bcl-xl) members in the Bcl family are balanced [23]. The proapoptotic homodimers can cause caspase activators (e.g., cytochrome c) to be released in the mitochondria. Caspases play a central role in the apoptotic cascade. The initial caspases, including caspases 2, 8, and 9, are activated and then activate the effector caspases, such as caspases 3, 6, and 7, through proteolytic cleavage. Finally, the effector caspases degrade the intracellular proteins to execute the cell death program [24]. Meanwhile, pyroptosis is initiated by the formation of pyroptosomes (also known as inflammasomes) in response to intracellular danger signals [25]. The innate immune system in the body can recognize PAMPs and DAMPs, which trigger the formation of pyroptosomes. The pyroptosomes then activate another set of caspases, including caspases 1, 4, and 5, by autocatalytic cleavage. Activated caspase 1 can cleave pro-IL-1β into IL-1β, and pro-IL-18 into IL-18. It can also cleave gasdermin D (GSDMD) into the N-terminal form of GSDMD (GSDMD-N) and the C-terminal form of GSDMD (GSDMD-C). GSDMD-N can form oligomers and transmembrane pores, which allow the release of IL-1β and IL-18 [26]. In previous studies, neuronal apoptosis has been found in intracerebral hemorrhage, and TREM2 activation attenuates neuroinflammation [12]. Since there is a connection between inflammation and programmed cell death, especially apoptosis and pyroptosis, the role of TREM2 in apoptosis and pyroptosis in brain injury, such as SAH, needs to be thoroughly investigated. Furthermore, the cerebral spreading of microglia-induced inflammation after SAH leads to secondary brain injury, and interventional reduction of microglial activation reduces neuronal cell death [12,27]. Whether manipulation of TREM expression can change programmed cell death remains to be determined.

The present study aimed to clarify the effects of TREM2 in neuroinflammation and programmed cell death in *in-vivo* and *in-vitro* SAH models. By using lentiviral transfection for bidirectional intervention of TREM2 expression, we compared the results of TREM2 knockdown and overexpression on the cognitive status, neuroinflammatory response, and programmed cell death in mice. We hypothesized that TREM2 activation would attenuate the neuroinflammatory reaction and interfere with programmed cell death as well as alleviate brain injury in experimental SAH; therefore, TREM2 may serve as a pharmacological target in SAH therapy.

2. Materials and Methods

2.1 Animals and Treatments

Male C57BL/6J mice (6–8 weeks old, 20–25 g) were from GemPharmatech Co., Ltd. (Nanjing, China). All mice were housed under a 12-h light/dark cycle and had access to food and water *ad libitum*. Animal handling and all of the related experimental procedures were carried out according to the National Institutes of Health guidelines and approved by the Animal Ethics Review Committee of Wannan Medical College (approval number: WNMC-AWE-2023293).

To explore the expression pattern and localization of TREM2 in the brain, mice were randomly divided into six groups: Sham, SAH 1 d, SAH 2 d, SAH 3 d, SAH 5 d, and SAH 7 d (n = 6). To evaluate the neurological function in mice after SAH, mice were randomly divided into four groups: Sham, SAH, SAH+sh-NC, and SAH+sh-TREM2. To verify the role of TREM2 in programmed cell death, mice were randomly divided into four groups: Sham, SAH, SAH+sh-NC, and SAH+sh-TREM2. The brain water content, open field test, neurological evaluation, western blot, reverse transcription–quantitative polymerase chain reaction (RT-qPCR), and immunofluorescence assays were performed at 72 h after SAH.

The SAH model was built via endovascular perforation, as reported previously [28]. The animals were first anesthetized by isoflurane (Sigma-Aldrich, St. Louis, MO,

USA) inhalation. Then, the right common carotid artery, internal carotid artery, and external carotid artery were exposed clearly under a microscope. A MACO nylon suture (0.2 ± 0.01 mm in diameter, Beijing Cinontech Co., Ltd., Beijing, China) with a sharp tip was inserted into the external carotid artery and then slowly moved into the internal carotid artery. When resistance was felt, the suture was further advanced by 2 mm. Next, the bifurcation of the internal carotid artery was punctured, followed by the immediate retrieval of the suture bolt. Afterwards, ligation of the external carotid artery and skin was done sequentially. For the sham mice, the same operation was performed without puncture of the internal carotid artery. Lentiviral vectors were administered intraventricularly to induce TREM2 knockdown or overexpression at 7 d before SAH induction.

2.2 Behavior Assessment

At 72 h after the operation, the animals were evaluated by the modified Garcia Neuroscore [29,30]. The Garcia Neuroscore includes six subtests: spontaneous activity, limb extension, forepaw outstretching, climbing, side stroking, and vibrissae touch. The mice were graded, and the total score was calculated, ranging between 0 (greatest deficits) and 18 (without deficits).

2.3 Open Field Test

The open field test was performed in a 40 cm × 40 cm square with 40-cm-high walls (RWD Life Science Co., Ltd., Shenzhen, China). The 5-min activity of the mice was recorded by a video camera, and the total distance travelled and the percentage of active space were analyzed by software.

2.4 Evans Blue (EB) Diffusion Assay

The mice were injected with 0.4 mL of 1% EB (Acmec Biochemical Technology (ACMEC), Shanghai, China) solution through the tail vein and sacrificed 0.5 h later. A 100-mg sample of brain tissue was taken and homogenized. After centrifugation at 1000 *g* for 15 min, the supernatant was mixed with acetone (supernatant:acetone = 3:7) and incubated at room temperature for 24 h. The centrifuged supernatant (2000 g, 15 min) was used for the determination of the optical density at 620 nm. The content of EB was calculated according to the EB standard curve.

2.5 Brain Water Content Measurement

The mice were sacrificed at 72 h after SAH surgery, and the brains were removed. The wet weight (W) was immediately recorded. Then, the specimens were put in an oven at 105 °C for 24 h, and the dry weight (D) was also recorded. The brain water content was calculated by the following formula: [(W–D)/W] × 100%.

2.6 Immunohistochemistry

The mouse brain tissues were put in 4% paraformaldehyde for 72 h after dissection. Then, the tissues were embedded with paraffin and cut into sections (4 μm). Nissl staining (Beyotime, Shanghai, China) was performed according to the manufacturer's instructions. Normal neurons have relatively big cell bodies and are rich in cytoplasm, with one or two big round nuclei, whereas damaged cells have shrunken cell bodies, condensed nuclei, a dark cytoplasm, and numerous empty vesicles.

2.7 Cell Culture

Microglial BV-2 cells were cultured in Dulbecco's modified Eagle medium. Lentivirus (Hanbio Co., Ltd. Shanghai, China) bearing TREM2 shRNA or the whole-length TREM DNA sequence was used for the construction of cell lines with TREM2 interference or overexpression, respectively. The sequence of siRNA used for TREM2 knockdown was ACAGTCATCGCAGATGACACCCTTG. TREM2 overexpression was done with Mouse-tagged ORF Clone Lentiviral Particle (NM-031254, NCBI). Lentiviral transfection was screened by puromycin for 2–3 passages until the establishment of stably transfected cell lines. All cell lines were validated by short tandem repeat profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO_2.

BV-2 microglial cells and HT22 neuronal cells were cocultured by using polycarbonate membrane transwell chambers (pore size of 1.0 μm, JET BIOFIL, Guangzhou, China). BV2 cells were placed in the upper chamber (interfering cells), and HT22 cells were placed in the lower chamber (effector cells). The SAH model was simulated by oxygenated hemoglobin (40 μM, MilliporeSigma, Burlington, MA, USA) treatment for 24 h.

2.8 Immunofluorescence

Frozen brain sections (7 μm) and cultured neurons were both used for immunofluorescence staining. The slices were treated with 0.3% Triton X-100 for 30 min and 5% donkey serum for 1 h, sequentially. Then, anti-ionized calcium-binding adapter molecule 1 (Iba-1) (1:200, Cell Signaling Technology, 20825, USA), anti-neuronal nuclear protein (NeuN) (1:200, Abcam, ab177487, UK), anti-glial fibrillary acidic protein (GFAP) (1:200, Abcam, ab68428, UK), anti-myeloperoxidase (MPO) (1:200, Santa Cruz Biotechnology, sc-390109, USA), or anti-CD68 (1:250, Abcam, ab201844, UK) antibody was incubated with the tissue slices at 4 °C overnight. After washing with phosphate-buffered saline (PBS), the brain slices were incubated with anti-TREM2 antibody (1:200, Abcam, ab86491, UK). After washing with PBS–Tween, the slices were incubated with the proper fluorescently labeled secondary antibody. Subsequently, 4′,6-diamidino-2-phenylindole (DAPI; 1:2000, MilliporeSigma, Burlington, MA, USA)

was added and incubated with the tissue slices for 4 min. The slices were washed, dried, sealed, and observed under a fluorescence microscope.

2.9 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

A One Step TUNEL Assay Kit (KeyGen BioTECH, Nanjing, China) with either brain tissue or cultured cells was used, according to the manufacturer's instructions.

2.10 Flow Cytometry

An annexin V-adenomatous polyposis coli (APC)/7-aminoactinomycin D (7-AAD) apoptosis detection kit (KeyGen BioTECH) was employed for staining cells, and flow cytometry was adopted to detect and analyze the cells. The cells were cultured in 60-mm dishes. After oxygenated hemoglobin (40 µM) treatment for 24 h, the lower HT22 cells were digested and collected with 0.25% EDTA-free trypsin. Then, the cells were washed twice with PBS (centrifugation at 1000 rpm, 5 min), and 5×10^5 cells were collected. The cells were suspended in 500 µL of binding buffer. After mixing with 5 µL of annexin V-APC, 5 µL of 7-AAD was added, and the cells were incubated for 5–15 min in the dark at room temperature. The cells were immediately assayed by flow cytometry to analyze the percentages of apoptotic cells.

2.11 Western Blot

Temporal cortical tissues or cultured neurons were collected and lysed in radioimmunoprecipitation assay buffer (Beyotime, Shanghai, China) for 10 min, and then the supernatant was collected after centrifugation at 12,000 *g* for 15 min. The protein concentrations were determined by using a detergent-compatible protein assay (Beyotime, Shanghai, China).

After separation by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk for 2 h, the membrane was then serially incubated with primary antibodies (TREM2, GeneTex, GTX53229, 1:1000; cleaved caspase 3, ImmunoWay, YC0006, 1:1000; cleaved caspase 1, ImmunoWay, YC0003 1:1000; GAPDH, Proteintech, 60004-1-lg, 1:50,000; Bcl-2, Abcam, ab182858, 1:2000; Bax, Proteintech, 50599-2-lg, 1:2000; GSDMD-N, ImmunoWay, TY7991, 1:1000; IL-1β, Cell Signaling Technology, 12426S, 1:1000) at 4 °C overnight. Then, the membrane was washed with Tris-buffered saline (TBS)–Tween (3×10 min) and cultured with the proper secondary antibody for 1 h at room temperature. After washing with TBS–Tween, the protein on the membrane was detected by an enhanced chemiluminescence kit (Millipore) and recorded by G:BOX chemiXR5 (Syngene Europe, Cambridge, UK).

2.12 RT-qPCR

Total RNA was extracted and converted to cDNA, according to the manufacturer's guidelines. The primers used are listed in Table 1. PCR was performed in a 20-µL reaction mixture, including 10 µL of $2 \times$ SYBR Green PCR Master Mix, 1 µL of cDNA, 2 µL of primers, and 7 µL of ddH_2O. The amplification procedure was set as follows: 5 min at 95 °C; 40 cycles of 15 s at 95 °C, 20 s at 60 °C, and 40 s at 72 °C; 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C. Afterwards, the dissolution curve was generated, and the real-time data were collected and analyzed. All data were normalized according to the *GAPDH* value with the $2^{-\Delta\Delta Ct}$ method.

2.13 Statistical Analysis

All experimental results are shown as the mean $\pm$ standard deviation. Analysis of variance (ANOVA) was used for comparing different groups. Tukey's multiple comparisons test was used for checking the significant difference from the ANOVA result. $p < 0.05$ was considered a statistically significant difference.

3. Results

3.1 SAH Animal Model Establishment

A total of 251 mice were used for this study, including 209 mice that were exposed to SAH. All mice in the sham group survived. The total mortality rate was 17.70% (37/209). SAH was successfully induced in 156 mice. Sixteen mice were excluded from this study due to a lack of hemorrhage. The typical brains of SAH and sham mice are shown in Fig. 1a.

3.2 TREM2 Expression Was Increased in SAH Mice

By performing western blot and RT-qPCR assays, we determined the protein and mRNA levels of TREM2. Compared to the levels in the sham mice, the results indicated that the TREM2 protein and mRNA levels were significantly increased in the SAH mice at 2 d after SAH, peaked at 3 d, and decreased at 5 d (Fig. 1b–d). Co-immunostaining of TREM2 with Iba1, GFAP, or NeuN showed that TREM2 was mainly expressed in the microglia (Iba1) after SAH (Fig. 1e). Only trace TREM2 immunostaining signals were observed in astrocytes (GFAP) and neurons (NeuN) (Fig. 1e). These results indicate that TREM2 plays a role in SAH, which may be mediated by microglia.

3.3 TREM2 Overexpression Rescued Impaired Neural Function

Since TREM2 exerted an immediate response in SAH, we wondered whether the change in the TREM2 level after SAH was protective or destructive. Thus, we manipulated the expression level of TREM2 in the mouse brain by shRNA knockdown and overexpression intraventricularly. First, we evaluated the neurobehavior of the mice. The Gar-

Table 1. Primers for quantitative real-time polymerase chain reaction (RT-qPCR).

Gene name	Primer sequence
Cleaved caspase 3	Forward: TGGAGGCTGACTTCCTGTATGC Reverse: GAACCACGACCCGTCCTTTGA
Bcl-2	Forward: GCTACGAGTGGGATGCTGGAGA Reverse: GGTTGCTCTCAGGCTGGAAGGA
Bax	Forward: CCAGGATGCGTCCACCAAGAAG Reverse: CCGTGTCCACGTCAGCAATCAT
Cleaved caspase 1	Forward: GGACTGACTGGGACCCTCAAGT Reverse: GGCAAGACGTGTACGAGTGGTT
GSDMD-N	Forward: ACTGAGGTCCACAGCCAAGAGG Reverse: CCACTCGGAATGCCAGGATGCT
IL-1β	Forward: TCGCAGCAGCACATCAACAAGA Reverse: CCACGGGAAAGACACAGGTAGC
GAPDH	Forward: AAGGTCGGTGTGAACGGATT Reverse: TGAGTGGAGTCATACTGGAACAT

cia Neuroscores of the SAH mice were found to be lower than those of the sham mice (Fig. 2a). In addition, the sh-TREM2 mice were shown to have a lower score after SAH compared to the mice that only underwent SAH. These results indicate that TREM2 may play a protective role after SAH.

Afterwards, the brain water content of all mice was also determined (Fig. 2b). The brain water content of the SAH mice was significantly increased, while sh-TREM2 treatment aggravated it. By immunohistochemistry and an EB diffusion assay, we found that the blood–brain barrier (BBB) permeability (Fig. 2c), neuronal degeneration (Fig. 2f), neutrophil infiltration (MPO^+, Fig. 2d), and microglia activation ($CD68^+$, Fig. 2e) were all increased in the SAH mice. sh-TREM2 showed a deteriorating effect on neutrophil infiltration and phagocytic activation, while oe-TREM2 showed a rescuing role in these deteriorating effects, confirming the protective role of TREM2 in the neurons of SAH mice.

In the open field test (Fig. 2g–i), the SAH mice travelled significantly less than the sham mice, while the sh-TREM2 mice were shown to be less active than the SAH mice. However, the oe-TREM2 mice exhibited a better performance in the open field test, showing a greater total travelling distance and a higher activity compared to those of the SAH mice. Therefore, TREM2 was demonstrated to rescue the impaired neural function in the SAH mice.

3.4 TREM2 Knockdown Worsened Inflammation and Programmed Cell Death in the SAH Mice

TREM2 was shown to suspend inflammation. Besides, cell apoptosis and pyroptosis also were shown to follow brain damage after SAH. Here, to clarify the regulatory role of TREM2 in SAH, we further detected cell apoptosis and pyroptosis in the brain. As shown in Fig. 3a–c, the levels of cleaved caspase 3, Bax, cleaved caspase 1, GSDMD-N, and IL-1β were all increased in the SAH mouse brain, while the level of Bcl-2 was decreased, indicating the promotion of cell apoptosis and the pyroptotic effect of SAH on the brain neuronal cells. The TUNEL staining results also showed an increase in cell apoptosis after SAH in the mouse brain (Fig. 3d). However, when TREM2 was knocked down in the SAH mouse brain, cell apoptosis and pyroptosis were worsened, indicating that TREM2 may regulate cell apoptosis and pyroptosis and thus exert a neuroprotective effect.

3.5 TREM2 Showed a Neuroprotective Effect in an in-vitro SAH Cell Model

To confirm the protective effect of TREM2 on neurons, we adopted an *in-vitro* SAH cell model for further investigation. By lentiviral transfection, sh-TREM2 (by shRNA) and oe-TREM2 (by overexpression of TREM2) stable BV-2 cell lines were constructed. After the coculture of BV-2 microglia and HT22 neuronal cells, cell apoptosis and inflammation were detected in HT22 neurons. As shown in Fig. 4a–c, the levels of cleaved caspase 3, Bax, cleaved caspase 1, GSDMD-N, and IL-1β were increased in the SAH neurons, while the level of Bcl-2 was decreased, indicating the promotion of cell apoptosis and pyroptosis by SAH on neuronal cells. The annexin V-APC/7-AAD staining and TUNEL staining results also showed an increase of cell apoptosis in HT22 neurons (Fig. 4d–f). However, when TREM2 was overexpressed in BV-2 cells, neuronal cell apoptosis and pyroptosis were alleviated, indicating that TREM2 may regulate cell apoptosis and pyroptosis and thus exert a neuroprotective effect.

4. Discussion

In the brain, neurons are responsible for neuronal functions, while microglia serve an auxiliary role. Microglia can exert either a protective or deleterious impact

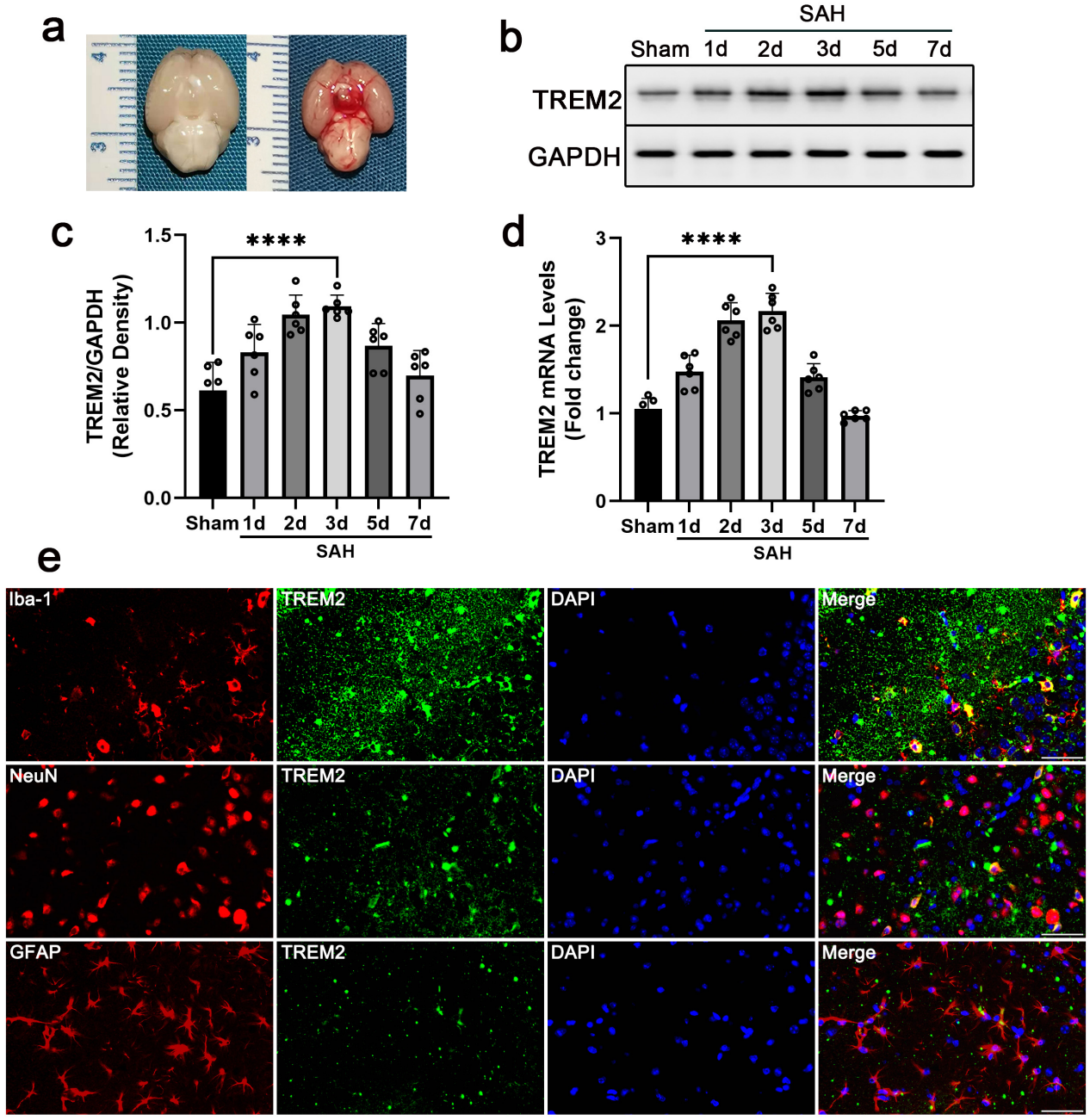

Fig. 1. Increased basal cortex TREM2 expression after subarachnoid hemorrhage (SAH). Male C57BL/6J mice were assigned into the sham or SAH group (n = 6). The SAH model was built by endovascular perforation. Brain samples were obtained at the indicated time points. The protein and mRNA levels of TREM2 were determined by western blot and RT-qPCR, respectively. (a) Typical mouse brains of sham and SAH mice post SAH. (b) Western blot result of TREM2 protein in the basal cortex at 72 h post SAH. GAPDH was used as the loading control. (c) Quantitative analysis of the western blot results. (d) RT-qPCR results of TREM2 mRNA at the indicated time points. (e) Immunofluorescence staining of TREM2 (green) in the basal cortex at 72 h post SAH, while Iba1 (microglia), NeuN (neuron), and GFAP (astrocyte) are shown in red. The cell nuclei stained with DAPI are shown in blue. ****$p < 0.0001$. Scale bar = 50 µm.

upon neurons, depending on the time and conditions [31]. In SAH, neuroinflammation caused by microglial activation is tightly connected to the secondary injury. A therapeutic strategy targeting neuroinflammation has been reported to reduce neuronal damage and to improve neurological dysfunction [32]. However, until now, the underlying mechanism has not been elucidated. Recently, evidence has shown that TREM2, a specific anti-inflammatory receptor

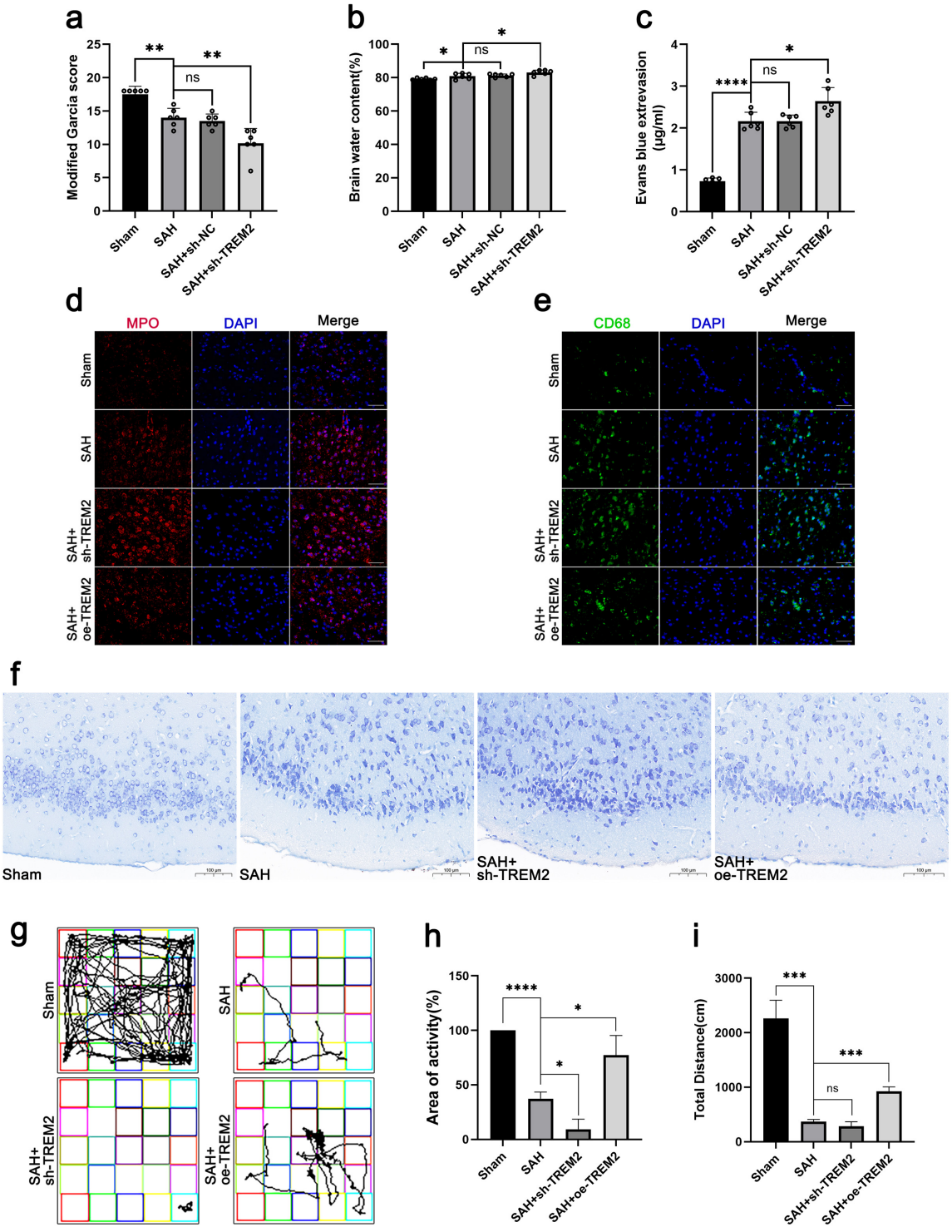

Fig. 2. TREM2 overexpression rescued impaired neural function. C57BL/6J mice were randomly divided into five groups: sham, SAH, SAH nc-TREM2, SAH sh-TREM2, and SAH oe-TREM2. Seven days before SAH model construction, lentivirus (8 μL) was injected into the right ventricle using an automatic brain stereotaxic apparatus. Neurobehavior assessment and the open field test were performed at 72 h after SAH. After sacrifice, the brain water content of the mice was evaluated. Evans blue (EB) staining, Nissl staining, and immunofluorescence staining of MPO and CD68 were performed. (a) Neurobehavior score. (b) Brain water content. (c) EB staining of the blood–brain barrier. (d,e) Immunofluorescence staining of MPO and CD68. Scale bar = 50 μm. (f) Nissl staining. Scale bar = 100 μm. (g–i) Open field test. Typical pictures of the route (g), total traveled distance (h), and percentage of area of activity (i) are shown. ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

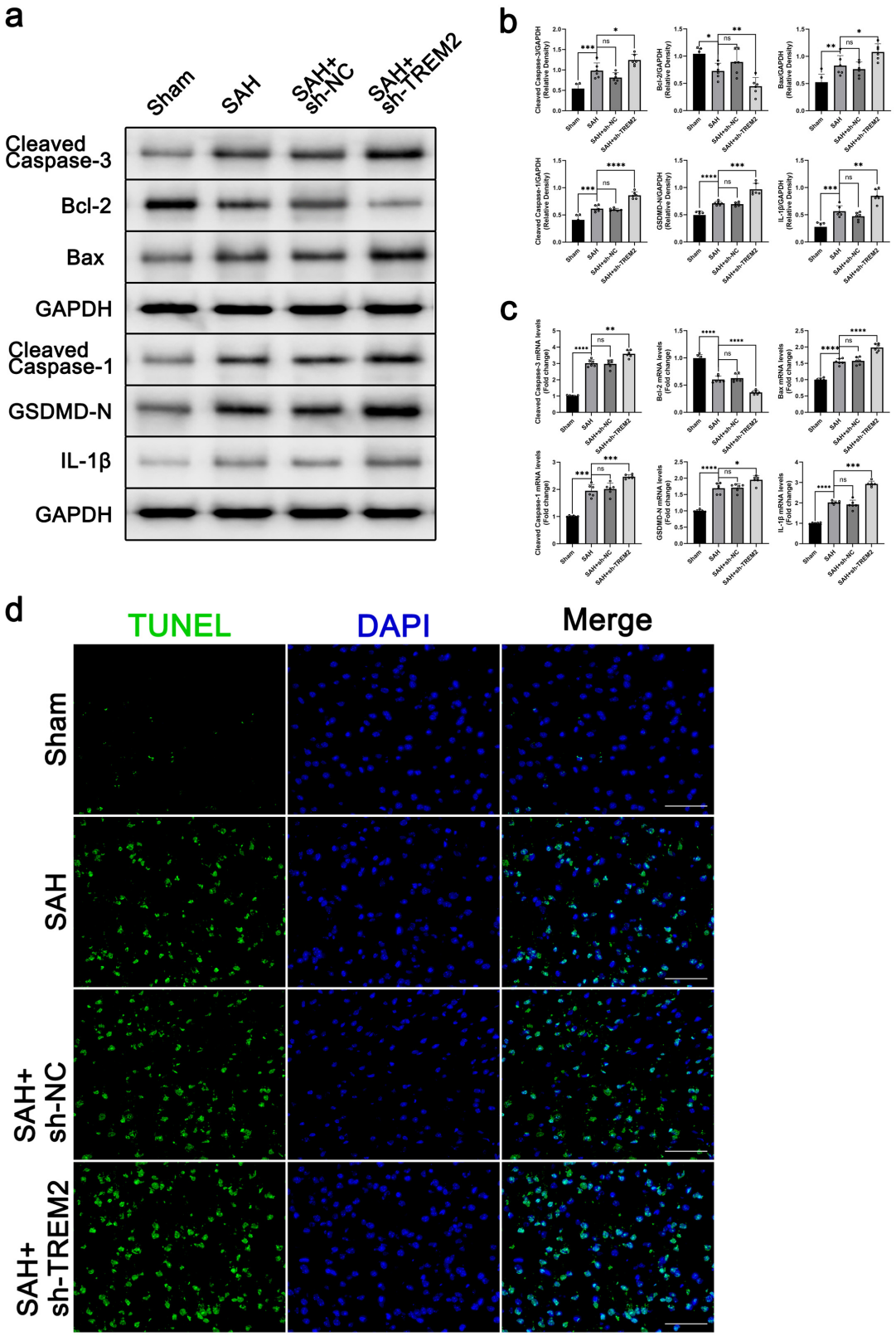

Fig. 3. TREM2 knockdown worsened inflammation and cell death in SAH mice. C57BL/6J mice were randomly divided into four groups: Sham, SAH, SAH+sh-NC, and SAH+sh-TREM2. At 72 h post SAH, the brain samples were prepared for western blot and RT-qPCR assays for cleaved caspase 3, Bcl-2, Bax, cleaved caspase 1, GSDMD-N, and IL-1β as well as terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. (a) Typical blots. (b,c) Quantification of the western blot and RT-qPCR results. (d) TUNEL staining. Scale bar = 50 µm. ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

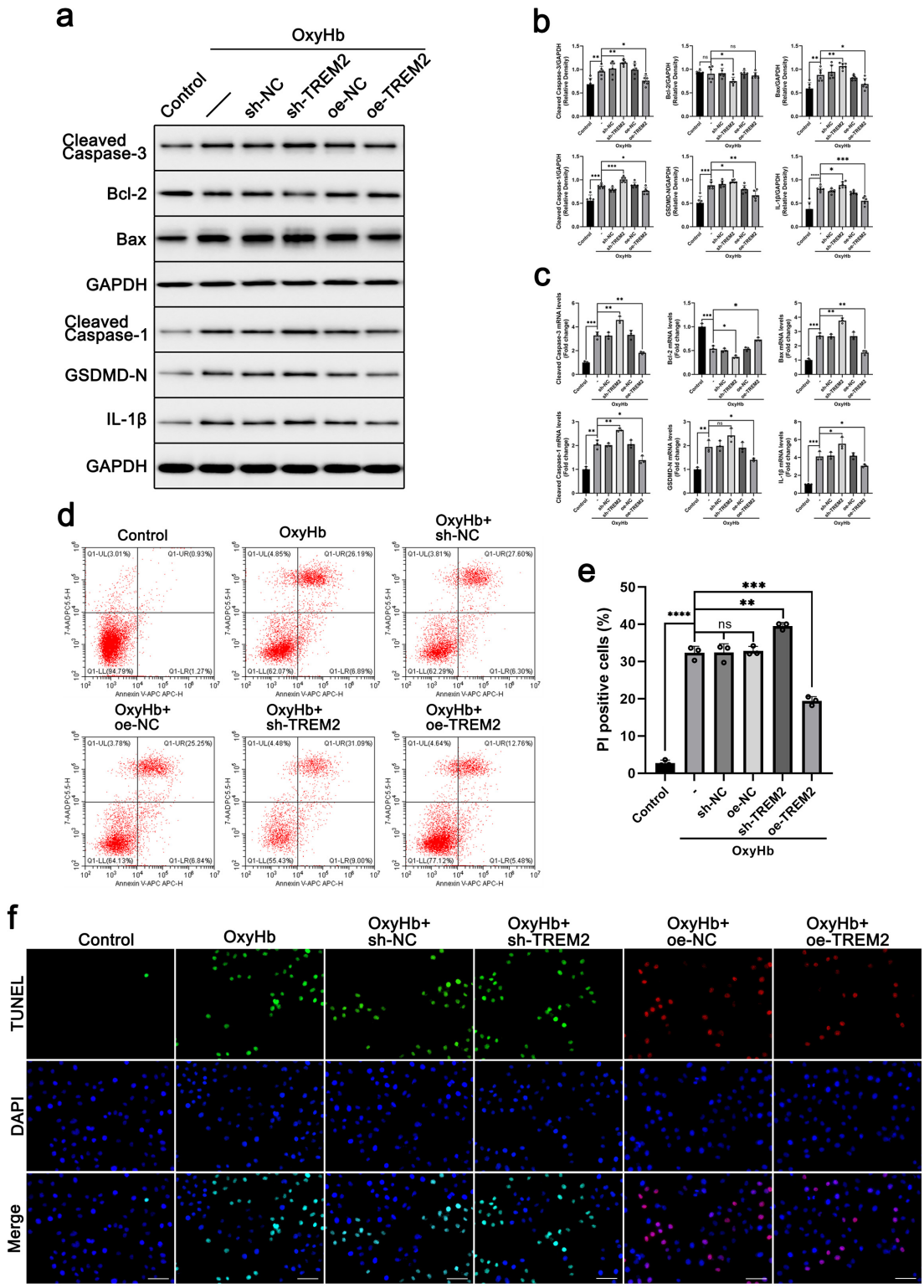

Fig. 4. TREM2 showed a neuroprotective effect in an *in-vitro* SAH cell model. sh-TREM2 (by shRNA) and oe-TREM2 (by overexpression of TREM2) stable BV-2 cell lines were constructed by lentiviral transfection. BV-2 and HT22 neuronal cells were cocultured, and the SAH model was simulated by adding 40 μM oxygenated hemoglobin for 24 h. The protein levels of cleaved caspase 3, Bcl-2, Bax, cleaved caspase 1, GSDMD-N, and IL-1β in HT22 neuronal cells were determined by western blot analysis. Cell apoptosis was analyzed by both annexin V-APC/7-AAD and TUNEL staining. (a) Typical blots. (b,c) Quantification of the western blot results. (d) Flow cytometry results. (e) Quantification of the flow cytometry results. (f) TUNEL staining. Scale bar = 50 μm. ns, no significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

in the microglia, plays a pivotal and protective role in modulating neuroinflammation [33]. Here, we explored the protective effect of TREM2 in the early secondary brain damage after SAH. By using the SAH mouse model, we determined the expression level of TREM2 after SAH. By comparing the behavioral and biochemical results from the mice with TREM2 knockdown (sh-TREM2) and overexpression (oe-TREM2), we found that the cognitive performance of the mice with oe-TREM2 after SAH was better than that in those without TREM2 alteration after SAH, while the cognitive performance of the mice with sh-TREM2 after SAH was worse than that in those without TREM2 alteration after SAH. The water brain content, BBB permeability, and programmed cell death (including apoptosis and pyroptosis) were all deteriorated in the sh-TREM2 mice compared with those in the mice without TREM2 alteration. This result was confirmed by an *in-vitro* SAH cell model. The overexpression of TREM2 in microglia can decrease the cell population in apoptosis in cocultured SAH neurons, while downregulated TREM2 expression in microglia can increase the population of cocultured SAH neurons in apoptosis. Both apoptosis and pyroptosis were manipulated by the alteration of TREM2 expression. Our results provide more evidence of the protective function of TREM2 in brain injury induced by SAH and especially underline its role in programmed cell death, including apoptosis and pyroptosis.

The TREM2 pathway has been confirmed to play roles in several diseases. For example, variants of TREM2 have been found to be associated with neurodegeneration, such as in Alzheimer's disease [33]. In addition, TREM2 has been demonstrated to be involved in the microglial activation against amyloid plaques, which are the main characteristic of Alzheimer's disease. Loss of TREM2 function also has been shown to reduce the microglial responses to amyloid plaques, which become more toxic [34]. Evidence indicates that the overexpression of TREM2 can decrease the formation of amyloid plaque and alleviate cognitive deficits [35], and this process is mediated by microglia. The sTREM2 level in the CSF has been suggested to be used as a biomarker of Alzheimer's disease [36]. In cancer, TREM2 is normally overexpressed and used as a marker for macrophages and monocytes [37]. In hepatocellular carcinoma, the disruption of TREM2 expression promotes tumor development and exacerbates inflammation in the liver. In the intestine, TREM2 is expressed in human monocyte dendritic cells and is limited to inflamed sites, contributing to the pathogenesis of inflammatory bowel diseases [38]. Besides, in many types of strokes, such as ischemic stroke, TREM2 can reduce inflammation via the Toll-like receptor signaling pathway, thus promoting the migration, survival, and regeneration of microglia [39]. Previous studies and our present results all showed that the expression of TREM2 responded quickly to SAH, reaching a peak at 24–72 h after brain injury [17]. The pathophysiological changes following SAH, including erythrocyte leakage to the subarachnoid space and resident microglia/macrophage (Mi/MΦ) activation, may contribute to the upregulation of TREM2 [40]. TREM2 can then promote the phagocytic activity of Mi/MΦ, displaying its regulatory role in brain injury after SAH. Furthermore, to the best of our knowledge, we confirmed for the first time that TREM2 is involved in neuronal cell death after SAH. In the SAH mice, the neuronal cells exerted more cell death compared to the control. However, when TREM2 was overexpressed in the brain, the cell death (including apoptosis and pyroptosis) was decreased in neurons; thus, TREM2 displayed a neuroprotective function. All of the markers for cell apoptosis (cleaved caspase 3, Blx-2, and Bax) and pytoptosis (cleaved caspase 1, GSDMD-N, and IL-1β) showed a significant change in the SAH+sh-TREM2 mice compared to the SAH mice, indicating that TREM2 modulates cell death. The same results were obtained from the *in-vitro* experiments. In cocultured microglia and neurons, the expression level of TREM2 in the microglia could significantly influence the neuronal cell death (apoptosis and pyroptosis). Since all previous research emphasized the role of TREM2 in inflammatory regulation, here, we proposed that TREM2 can also affect cell death, and it may even result from the interplay of inflammation and cell death. Kawabori *et al*. [41] have suggested that the clearance of apoptotic neurons is induced by TREM2-mediated attenuation of neuronal inflammation. Nevertheless, further investigations regarding the mechanism should be carried out in the future.

Since the neuroprotective function of TREM2 in SAH and other related disorders has been confirmed in many studies, TREM2 is thought to be a good therapeutic target. However, TREM2 displays a distinct role in different disorders and even at different stages of one disease; therefore, the modulation of TREM2 should be based on clarification of the related mechanism. For example, coupling TREM2 and apolipoprotein to promote phagocytosis of impaired neurons or clearance of amyloid plaque by microglia may be used to treat Alzheimer's disease [42,43]. Regulating the release rate of sTREM2 is another strategy to cope with the pathology of Alzheimer's disease [44]. In stroke, whether TREM2 is involved in erythrocyte and metabolite clearance still needs to be investigated. Besides, since both TREM2 overexpression and activation can enhance the phagocytic ability of Mi/MΦ, the choice of strategy needs to be further validated. TREM2 also has been shown to decrease neuronal inflammation both *in vitro* and *in vivo* [14]; however, research indicates that the inflammatory reaction is not that different between wild-type mice and TREM2-knockout mice in the early stage of middle cerebral artery occlusion/reperfusion, which may be due to the state of Mi/MΦ [45]. The neuroinflammatory process in stroke is complex, and we found in the present study that TREM2 also has an effect on cell death (apoptosis and pyroptosis). Therefore, the interplay of neuroinflammation and cell death needs further investigation.

5. Conclusions

In conclusion, by constructing *in-vivo* and *in-vitro* SAH models, the neuroprotective mechanisms of TREM2 after SAH were investigated. The knockdown of TREM2 in the mouse brain aggravated cognitive impairment, BBB permeability, and cell death (apoptosis and pyroptosis). Moreover, in cocultured microglia and neurons, the overexpression of TREM2 in microglia decreased the cell apoptosis and pyroptosis of neurons after SAH. Thus, TREM2 alleviates secondary brain injury through attenuating cell death in both mice and cultured neurons with SAH, making TREM2 a promising therapeutic target for SAH.

Availability of Data and Materials

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

Author Contributions

JL and ZZ performed the experiments and drafted the manuscript. JL, ZZ, MZ, SL, XZho, and ZL participated in the experimental design and conceived. MQ and YH designed the feeding protocol, helped to feed the mice, and coordinated the study. BY and FQ performed the neurobehavioral studies as well as participated in the sample collection and staining experiments. SL, XZho, XZha, and ZL participated in data analysis and reviewed the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Animal handling and all of the related experimental procedures were carried out according to the National Institutes of Health guidelines and approved by the Animal Ethics Review Committee of Wannan Medical College (approval number: WNMC-AWE-2023293).

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Conflict of Interest

The authors declare no conflict of interest.

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