Triggering Receptor Expressed on Myeloid Cells 2, a Novel Regulator of Immuneocyte Phenotypes, Confers Neuroprotection by Relieving Neuroinflammation

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ABSTRACT

Background: Microglia can not only detrimentally augment secondary injury but also potentially promote recovery. However, the mechanism underlying the regulation of microglial phenotypes after stroke remains unclear.

Methods: Mice were subjected to middle cerebral artery occlusion for 60 min. At 3 days after reperfusion, the effects of activation and suppression of triggering receptor expressed on myeloid cells 2 on immuneocyte phenotypes (n = 5), neurobehavioral scores (n = 7), infarct volumes (n = 8), and neuronal apoptosis (n = 7) were analyzed. In vitro, cultured microglia were exposed to oxygen–glucose deprivation for 4 h. Inflammatory cytokines, cellular viability (n = 8), neuronal apoptosis (n = 7), and triggering receptor expressed on myeloid cells 2 expression (n = 5) were evaluated in the presence or absence of triggering receptor expressed on myeloid cells 2 overexpression lentivirus.

Results: Triggering receptor expressed on myeloid cells 2 expression in the ischemic penumbra peaked at 3 days after ischemia–reperfusion injury (4.4 ± 0.1-fold, P = 0.0004) and was enhanced in interleukin-4/interleukin-13–treated microglia in vitro (1.7 ± 0.2-fold, P = 0.0119). After oxygen–glucose deprivation, triggering receptor expressed on myeloid cells 2 conferred neuroprotection by regulating the phenotypic conversion of microglia and inflammatory cytokine release. Intraperitoneal administration of triggering receptor expressed on myeloid cells 2 agonist heat shock protein 60 or unilateral delivery of a recombinant triggering receptor expressed on myeloid cells 2 lentivirus into the cerebral ventricle induced a significant neuroprotective effect in mice (apoptotic neurons decreased to 31.3 ± 7.6%; infarct volume decreased to 44.9 ± 5.3%). All values are presented as the mean ± SD.

Conclusions: Activation or up-regulation of triggering receptor expressed on myeloid cells 2 promoted the phenotypic conversion of microglia and decreased the number of apoptotic neurons. Our study suggests that triggering receptor expressed on myeloid cells 2 is a novel regulator of microglial phenotypes and may be a potential therapeutic target for stroke.

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a spectrum of functional phenotypes, including the classical activated phenotype (M1) and the alternative activated phenotype (M2).8,9 M1 microglia acquire a ramified morphology in response to invading pathogens and/or central nervous system damage and also release a wide array of inflammatory cytokines, oxygen-free radicals, and other harmful substances during early inflammation. In contrast, M2 microglia are characterized by an activated, amoeboid morphology and high phagocytic activity. M2 microglia secrete neurotrophic substances, remove necrotic or apoptotic neuronal debris, make dynamic contacts with neurons, and promote the formation of glial scar tissue during late inflammation.

Triggering receptor expressed on myeloid cells 2 (TREM2) is an immunoglobulin-like receptor of the TREM family and is expressed on activated macrophages, immature dendritic cells, osteoclasts, and microglia.10,11 Currently, cells expressing high levels of TREM2 are thought to fulfill important functions in immune surveillance, cell–cell interactions, tissue debris clearance, and the resolution of latent inflammatory reactions.12 By comparison, the absence of TREM2 expression on these cells not only impairs their capacity to phagocytose cellular debris but also increases their production of proinflammatory cytokines.13,14 TREM2 is emerging as an important negative regulator of autoimmunity and associates with an adaptor molecule, DNAX activation protein 12 kDa (DAP12), which contains an immunoreceptor tyrosine-based activation motif that is phosphorylated upon activation of DAP12-linked receptors.14,15 The brain pathology observed in Nasu–Hakola disease patients suggested that disruption of the TREM2/DAP12 pathway leads to neurodegeneration with demyelination and axonal loss.16 Moreover, it has been reported that TREM2 variants strongly increase the risk of developing Alzheimer disease and are involved in the microglial response to Aβ plaque deposition.17,18 Because the role of TREM2 after stroke had not been determined, we hypothesized that modulation of TREM2 by administration of heat shock protein (HSP) 60 or injection of a recombinant TREM2 virus might control poststroke microglial activity and phenotypes.

The present study focused on the role of TREM2 in the phenotypic conversion of microglia after stroke. Here, we report that during ischemia–reperfusion injury, microglia underwent a rapid shift in their effector program, involving morphologic transformation, proliferation, and cytokine release.

Materials and Methods

Animals and Surgical Procedures

All animal-related procedures were approved by the Ethics Committee for Animal Experimentation of Fourth Military Medical University (Xi’an, Shaanxi, China) and proceeded in accordance with the guidelines for Animal Experimentation of the University. Male wild-type C57BL/6 mice were obtained from the Experimental Animal Center of the Fourth Military Medical University. Mice (8 to 12 weeks old) were housed under controlled conditions with a 12-h light/dark cycle, a temperature of 21 ± 2°C, and 60 to 70% humidity for at least 1 week before drug treatment or surgery.

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) using an intraluminal filament technique as described in our previous studies.19 Randomization methods were used to assign individual mice to experimental conditions. All mice were anesthetized with 10% chloral hydrate (350 mg/kg) via intraperitoneal injection. To control MCAO severity, regional cerebral blood flow was monitored using transcranial laser Doppler flowmetry (PeriFlux 5000, Perimed AB, Sweden). The MCAO was considered effective if the regional cerebral blood flow showed a sharp drop to less than 30%, and animals that did not meet this requirement were excluded (see Supplemental Digital Content 1, http://links.lww.com/ALN/B413, which is an analysis report of regional cerebral blood flow in this study). During the surgical procedures, the rectal temperature of the mice was monitored and maintained at approximately 37 ± 0.5°C. After the suture was withdrawn and the mice had recovered from the anesthesia, they were returned to their cages with free access to food and water. For data missing or excluded from the experiments, please see supplemental tables 1 and 2 (see Supplemental Digital Content 1, http://links.lww.com/ALN/B413, which is a table showing missing or excluded data in this study).

Ischemic Penumbra

The ischemic penumbra were microdissected according to established protocols in rodent models of unilateral proximal MCAO. Briefly, a 4-mm-thick coronal brain slice was cut, beginning 6 mm from the anterior tip of the frontal lobe. Next, a longitudinal cut (from top to bottom) was made approximately 1 mm from the midline through the ischemic hemisphere to remove medial tissue. Then a transverse diagonal cut was made at approximately the 2 o’clock position to separate the wedge-shaped penumbra (see Supplemental Digital Content 1 figure 1, http://links.lww.com/ALN/B413), which is a schematic showing of the ischemic penumbra during MCAO).

Intraperitoneal Injection of HSP60

Active mouse HSP60 full-length protein (Abcam, Cambridge, MA) was intraperitoneally administered 1 h before the onset of ischemia. After ischemia, the mice were kept alive for 3 days. The animals were then sacrificed, and their brains were extracted, sectioned, and stained using 2,3,5-triphenyltetrazolium chloride to analyze infarct volume. The mice were divided into four groups as follows: (1) MCAO group treated with intraperitoneal injection of bovine serum albumin (0.2 μg/g) in saline solution; (2) MCAO group treated with a single intraperitoneal injection of HSP60 at 2.5 μg/mouse; (3) MCAO group treated with HSP60 at 3.75 μg/mouse; and (4) MCAO group treated with HSP60 at 5 μg/mouse.
**N9 Cell Culture and Treatment**
The murine N9 microglial cell line was cultured in Iscove's modified Dulbecco's medium (IMDM; HyClone, Logan, UT) containing 4 mM glutamine, HEPES, 5% heat-inactivated fetal bovine serum (FBS; Gibco, Rockville, MD), and 1% penicillin/streptomycin (Gibco) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. The medium was changed every 3 days. Cells were grown to 90% confluence in 6-well plates or 100-mm dishes for experiments involving insult with various cytokines, including lipopolysaccharide (100 ng/ml; Sigma, St. Louis, MO), recombinant murine interferon-γ (10 ng/ml; Peprotech, Rocky Hill, NJ), interleukin-10 (10 ng/ml; Peprotech), interleukin-4 (10 ng/ml; Peprotech), and interleukin-13 (10 ng/ml; Peprotech) for 24 h.

**Oxygen–glucose Deprivation**
Oxygen–glucose deprivation (OGD) was performed as described previously. In brief, the murine N9 microglial cell line and the HT22 hippocampal cell line were subjected to OGD in experiments 3 and 4. N9 microglia cultured in IMDM without glucose (1% F-12 with 10% FBS; HyClone) were pretreated in a humidified hypoxic chamber (1% O₂, 5% CO₂, 94% N₂) at 37°C for 4 h. Then we replaced the medium with IMDM containing 5% FBS and returned the cells to a 37°C incubator with atmospheric oxygen. The HT22 cells were cultured in Dulbecco's modified Eagle's medium with 10% FBS (v/v), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO₂ and 95% air. Media and cells were collected at 24 h after OGD for protein analysis.

**Lentivirus Transfection**
A recombinant lentivirus containing the gene encoding full-length mouse TREM2 was produced using the GV341 Vector Expression System (GeneChem, Shanghai, China), the Ubi-MCS-3FLAG-SV40-puromycin component sequence, and AgeI and NheI cloning sites. A recombinant lentivirus containing the gene encoding TREM2-RNA interference was produced using the GV112 Vector Expression System (GeneChem), the hU6-MCS-CMV-Puromycin component sequence, and the AgeI and EcoRI cloning sites according to the manufacturer's instructions. A control vector containing the EGFP gene and no transgene was constructed in the same manner. The cells were cultured in IMDM supplemented with 5% FBS. Microglia were infected with the recombinant TREM2 virus or the control virus (multiplicity of infection, 10–20) with 5 μg/ml Polybrene (Santa Cruz Biotechnology, Santa Cruz, CA).

**Western Blot Analysis**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the methods used in this study.

**Immunohistochemistry**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Transfection of Small Interfering RNA into the Mouse Brain**
We performed in vivo transfection of small interfering RNA (siRNA) in C57BL/6 mice according to the method described by Nakajima et al. See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Neurobehavioral Evaluation and Infarct Measurement**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Terminal Transferase-mediated dUTPNick-end Labeling Staining**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Primary Microglia Culture**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Co-culturing HT22 Cells and N9 Cells Using the Transwell System**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Determination of Apoptotic Rate by Flow Cytometry**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Cell Proliferation and Cytotoxicity Assay**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Lactate Dehydrogenase Release**
See Supplemental Digital Content 2 (http://links.lww.com/ALN/B414), which presents the necessary methods used in this study.

**Cytokine Analysis**
Enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN) was used to measure mouse interleukin-1β, interleukin-4, tumor necrosis factor-α, interleukin-6, interleukin-10, and transforming growth factor-β in cell culture supernatant samples according to the manufacturer's instructions.
Statistical Analysis

Brain sections were examined by two independent and blinded investigators (T.J. and F.B.). SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) was used to conduct statistical analyses. All values, except for neurologic scores, are presented as mean ± SD and were analyzed by one-way ANOVA. Between-group differences were detected with Tukey post hoc tests. The neurologic deficit scores are expressed as the median (interquartile range) and were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney U test with Bonferroni correction. Sample size was estimated based on our previous experience.22,23 P < 0.05 was considered statistically significant.

Results

Phenotypic Conversion of Immunocytes after Stroke Was Accompanied by TREM2 Up-regulation

As shown in figure 1, A and B, compared with the sham group, the expression levels of induced nitric-oxide synthase (iNOS, 2.8 ± 0.2-fold, \(P = 0.0015\)) and interleukin-6 (2.9 ± 0.1-fold, \(P = 0.0004\)) were highest at 6 h in the ischemic group. In contrast, brain-derived neurotrophic factor (5.8 ± 0.2-fold, \(P = 0.0002\)) and arginase-1 (3.3 ± 0.1-fold, \(P = 0.0005\)) expression peaked at 7 days. The immunofluorescence assay revealed iNOS and arginase-1 staining in the region of the ischemic penumbra (fig. 1, C and D, respectively), consistent with the model that immunocytes act as a double-edged sword in the process of stroke. Western blotting revealed that the level of TREM2 protein (4.4 ± 0.1-fold, \(P = 0.0004\)) in the ischemic penumbra increased over time and was significantly greater than that in the sham group at 3 days postreperfusion (fig. 1E). This observation was further confirmed by immunostaining for TREM2 at 3 days after reperfusion. A marked increase in TREM2 staining was observed in the MCAO group (fig. 1F). Taken together, these results indicated that TREM2 might be involved in the dynamic changes to immunocytes after ischemia–reperfusion injury.

Level of TREM2 Expression in Different Microglia

Phenotypes Was Altered In Vitro

To verify whether TREM2 expression was related to the detrimental M1 and beneficial M2 phenotypes, we used lipopolysaccharide and interferon-γ as M1 triggers and interleukin-4 and interleukin-13 as M2 triggers to stimulate primary microglia. Lipopolysaccharide interferon-γ treatment enhanced the protein expression of iNOS (1.8 ± 0.1-fold, \(P = 0.0092\)), control vs. lipopolysaccharide interferon-γ in microglia, which displayed the ramified morphology characteristic of the M1 phenotype (fig. 2, A and B). In contrast, the expression of arginase-1 was dramatically increased in interleukin-4/interleukin-13–treated microglia, which showed the activated, amoeboid morphology characteristic of the M2 phenotype (1.6 ± 0.2-fold, \(P = 0.0214\), control vs. interleukin-4/interleukin-13; fig. 2, C and D). We also observed that TREM2 protein expression was induced by interleukin-4/interleukin-13 treatment (1.7 ± 0.2-fold, \(P = 0.0119\)), control vs. interleukin-4/interleukin-13) and inhibited by lipopolysaccharide interferon-γ treatment (0.4 ± 0.1-fold, \(P = 0.0226\), control vs. lipopolysaccharide interferon-γ; fig. 2, E and F). These data indicated that a decrease in the level of TREM2 expression could induce microglia to adopt the detrimental phenotype, whereas an increase in TREM2 expression could lead to the beneficial microglial phenotype.

TREM2 Was Involved in the Modulation of the Microglial Phenotype after OGD In Vitro

To further investigate the role of TREM2 in the regulation of microglial activation states, we transfected N9 microglial cells with recombinant lentiviral vectors containing the gene encoding full-length mouse TREM2 or with TREM2-short hairpin (sh)RNA vectors (transfection efficiency greater than 80%; fig. 3A). In the TREM2-shRNA group, the expression levels of iNOS (2.0 ± 0.3-fold, \(P = 0.0007\)) and interleukin-6 (1.8 ± 0.1-fold, \(P = 0.0001\)) were greatly enhanced by the suppression of TREM2 (fig. 3B). In contrast, in the TREM2-vector group, the expression of arginase-1 (1.5 ± 0.1-fold, \(P = 0.0056\)) and brain-derived neurotrophic factor (6.0 ± 0.1-fold, \(P = 0.0003\)) was increased by the up-regulation of TREM2 (fig. 3C). iNOS and arginase-1 immunofluorescence in the three groups revealed that the microglia displayed a more ramified morphology when TREM2 was up-regulated, whereas microglial morphology was amoeboid when TREM2 was down-regulated (fig. 3, D and E). Taken together, these results suggested that TREM2 might contribute to the switch in microglial phenotypes.

TREM2 Contributed to Neuronal Injury after OGD by Regulating Inflammatory Cytokines In Vitro

We used the Transwell culture system to examine the effects of the factors secreted by N9 microglia on cultured HT22 neurons after OGD (fig. 4A). At 4 h after OGD, the HT22 cells were damaged, and neuronal injury was assessed at 24 h after reoxygenation. We found that TREM2 down-regulation in the microglia in the TREM2-shRNA group increased apoptosis in HT22 cells (fig. 4B) by reducing cell vitality (0.6 ± 0.1, \(P = 0.0101\)) and enhancing lactate dehydrogenase release (343.7 ± 18.0, \(P = 0.0118\); fig. 4C). We next investigated whether the mechanism underlying the role of TREM2 in post-OGD neuronal injury involved the inflammatory response. The enzyme-linked immunosorbent assay results demonstrated that interleukin-6 (13.0 ± 2.2, \(P = 0.0163\)) and interleukin-1β levels (7.6 ± 1.7, \(P = 0.0017\)) in the TREM2-shRNA group were significantly higher than those in the control-vector group, whereas the levels of interleukin-4 (3.7 ± 1.1, \(P = 0.0353\)) and interleukin-10 (7.0 ± 1.9, \(P = 0.0019\)) were increased in the TREM2-vector group (fig. 4D). In summary, these results indicate that TREM2 protected neurons from post-OGD injury.
TREM2 Suppression Enhanced M1 Immunocyte Polarization and Aggravated Neuronal Apoptosis after Stroke

We knocked down TREM2 by using siRNA and found that the protein level of TREM2 (0.4 ± 0.1-fold, \( P = 0.0015 \)) in the TREM2-siRNA group was decreased by almost 70% compared with that in the sham group (fig. 5A). However, whether TREM2 suppression could exacerbate postischemic brain damage remained an important question. As expected, the expression of iNOS (1.6 ± 0.1-fold, \( P = 0.0050 \), TREM2-siRNA vs. MCAO) was markedly enhanced by TREM2 suppression. In contrast, arginase-1 expression was reduced (0.6 ± 0.1-fold, \( P = 0.0385 \)) when TREM2 was suppressed (fig. 5B). Neuronal apoptosis in the ischemic penumbra was also more frequent in the TREM2-siRNA group (43.6 ± 13.6%, \( P = 0.0462 \), TREM2-siRNA vs. MCAO;
The infarct volume and neurologic deficiency of the mice in the TREM2-siRNA group (55.2 ± 11.2%) did not differ from those of the mice in the MCAO group (46.2 ± 8.4%; fig. 5D). These results showed that blockade of TREM2 aggravated neural damage in vivo but did not increase the infarct volume, further demonstrating the protective effects of TREM2 against neuronal apoptosis induced by ischemia–reperfusion injury.

**Up-regulation of TREM2 or TREM2 Activation Alleviated Cerebral Ischemic Injury after Stroke**

The protein level of TREM2 (2.5 ± 0.1-fold, \( P = 0.0158 \), HSP60 + OGD vs. control) was increased in the HSP60 + OGD group at 24 h after OGD/reoxygenation injury, and the opposite phenomenon was observed in the shRNA + OGD group (fig. 6A). When HSP60 was administered at a dose of 2.5 μg, we did not observe any change in the volume of the infarct (46.2 ± 8.4%), but a dose of 3.75 μg induced a statistically significant reduction of the ischemic area (38.5 ± 6.0%, \( P = 0.0013 \), 3.75 μg of HSP60 vs. MCAO). Further increasing the dose to 5 μg had a considerable neuroprotective effect (27.8 ± 2.9%, \( P = 0.0003 \), 5 μg of HSP60 vs. MCAO; fig. 6B). Intriguingly, the number of apoptotic neurons was reduced in the group with 5 μg HSP60 (28.6 ± 4.2%, \( P = 0.0005 \), 5 μg HSP60 vs. MCAO; fig. 6C). For these assays, we unilaterally injected the recombinant TREM2 virus into...
the cerebral ventricle, measured the expression of TREM2 (1.7 ± 0.2-fold, \( P = 0.0377 \)) 10 days after the injection (fig. 6D), and assessed infarct volume and neurologic deficiency at 3 days after reperfusion. The results indicated that TREM2 up-regulation had a neuroprotective effect, as demonstrated by enhanced M2 microglial polarization (iNOS 0.2±0.1-fold, \( P = 0.0011 \); arginase-1 0.5±0.1-fold, \( P = 0.0006 \)), decreased infarct volume (44.9 ± 5.3%), and reduced neuronal apoptosis (31.3 ±7.6%; fig 6, E–G).

**Discussion**

Although substantial advances have been made in the treatment of stroke and in understanding the diverse mechanisms of neuronal death induced by ischemic stroke, the currently available neuroprotective therapies for acute ischemic stroke are limited and only moderately effective. Thus, improving neurologic outcomes and searching for reliable predictors within the pathophysiologic mechanisms of stroke is a major societal priority. In this study, we provide several pieces of evidence to support the hypothesis that microglial TREM2 is highly involved in the modulation of microglial activation states after experimental stroke (fig. 7).

Neuroinflammation, a specialized immune response that occurs in the central nervous system, is recognized as a pivotal hallmark of many pathologic conditions.6,24 The process of neuroinflammation after ischemic stroke is characterized by the activation of microglia and astrocytes, increased concentrations of various cytokines and chemokines, disruption

![Image of TREM2 Confers Neuroprotection as a Novel Regulator](http://anesthesiology.pubs.asahq.org Zahl et al.)
of the blood–brain barrier, and the subsequent invasion of cells from the hematopoietic system to the site of cerebral infarction.25,26 Studies of neuroinflammation in diverse brain diseases may contribute to the development of effective neuroprotective therapies.27,28 One approach to such investigations is to characterize the mechanisms underlying the effects of currently established neuroprotective strategies.

Microglia, the major components of the intrinsic brain immune system and the key cellular mediators of neuroinflammatory processes, contribute to the regulation of neuronal death, neurogenesis, synapse elimination, and neuronal surveillance.25,29 These microglia are key cellular mediators of neuroinflammatory processes, produce a variety of factors, and subserve both neurotoxic and neuroprotective functions.9,30 As a result, microglial activation states have become a primary focus in studies of cellular neuroimmunology and neuroinflammation.31 Specific receptors expressed on the microglial surface and the downstream signaling pathways that are involved in microglial activation have been extensively studied.30,32 However, activated microglia and recruited macrophages (especially bone marrow-derived macrophages) present some common features and some distinct characteristics in the inflamed central nervous system, preventing the use of immunologic methods to distinguish between activated microglia and recruited macrophages in vivo.

Receptors belonging to the TREM family have been detected exclusively on myeloid cells and have been implicated in innate immune responses.33 The TREM2 gene is located on chromosome 17C in mice, and in the brain, TREM2 is highly expressed by microglia.34,35 It is well known that in humans, rare mutations causing the loss of TREM2 function lead to Nasu–Hakola disease. TREM2 is also a newly identified risk gene for Alzheimer disease, and it regulates inflammatory processes in peripheral tissues by modulating the release of inflammatory cytokines.36 TREM2 deficiency led to uncontrolled inflammation in a model of experimental autoimmune encephalomyelitis.13

Fig. 4. Effect of triggering receptor expressed on myeloid cells 2 (TREM2) on neuronal damage and inflammatory cytokine release after oxygen–glucose deprivation in Transwell system. (A) Mimetic diagram of the Transwell co-culture system. (B) Neuronal apoptosis was reduced in the TREM2-vector group, as shown by flow cytometry analysis (n = 5). (C) The TREM2-short hairpin RNA (shRNA) group showed reduced cell viability (n = 8) and an increase in the lactate dehydrogenase (LDH) level in the media (n = 6). *P < 0.05 versus the control-vector group. (D) Enzyme-linked immunosorbent assay–based comparisons of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), interleukin-4 (IL-4), and interleukin-10 (IL-10) levels in the control-vector, TREM2-vector, and TREM2-shRNA groups (n = 6). *P < 0.05 versus the control-vector group. **P < 0.01 versus the control-vector group. FITC = fluorescein isothiocyanate; PI = phosphatidylinositol.
Therefore, TREM2 plays a key role in the negative regulation of autoimmunity and mediates microglial phagocytosis of apoptotic neurons in various neurologic diseases.37

Our study indicated that activation or up-regulation of TREM2 might be involved in the switch of microglial phenotypes and in promoting neurologic recovery after ischemic stroke. First, in primary microglia, the level of TREM2 expression was decreased in cells with the M1 phenotype and increased in those with the M2 phenotype. Interestingly, in vivo, we found that TREM2 expression increased as early as 6h after MCAO but peaked at approximately 3 days. Nevertheless, the mechanisms underlying these changes remain incompletely understood. To further explore and understand the relationship between TREM2 regulation and the transformation of microglial activation states, we showed in vitro that TREM2 was involved in the modulation of microglial phenotypes and participated in neuronal injury after OGD by regulating inflammatory cytokines. We then knocked down brain TREM2 expression using RNA interference lentiviral vectors and up-regulated TREM2 expression using recombinant lentiviral vectors containing the gene encoding full-length mouse TREM2 to investigate its role in poststroke neuroinflammation and neuropathology. We confirmed that suppression of TREM2 enhanced M1 microglial polarization and increased neuronal apoptosis, whereas up-regulation of TREM2 or TREM2 activation alleviated cerebral ischemic injury after stroke. Sieber et al.38 found no such effect on the lesion size and supported a contradictory model in which the subacute inflammatory reaction after stroke is attenuated in TREM2 knockout mice. Nevertheless, other groups have reported a role for TREM2 in the microglial phagocytosis in the infarcted brain and in the worsening of neurologic
outcomes after stroke. Although the role of TREM2 after stroke has been controversial, our findings support the scenario that TREM2 confers neuroprotection by relieving neuroinflammation. Many signaling pathways may be involved in the interactions between neurons and microglia, and many receptors expressed on microglia participate in the processes of microglial activation; therefore, suppression of TREM2 alone cannot increase the area of cerebral infarction, but it can increase the number of apoptotic neurons.

HSP60 is the only known agonist of TREM2, and whether HSP60 could exert a neuroprotective effect by binding with TREM2 remained unknown. Here, we provide the first demonstration that exogenous HSP60 activates TREM2 and reduces the detrimental effects of brain injury.

Fig. 6. Up-regulation of triggering receptor expressed on myeloid cells 2 (TREM2) induced microglia to adopt the alternative activated phenotype (M2) and conferred neuroprotection against stroke. (A) The heat shock protein 60 (HSP60)-induced effect on TREM2 protein expression in the oxygen–glucose deprivation model (n = 5). *P < 0.05 versus control group. (B) Comparison of the percentage of infarct volume among the groups that received different doses (n = 7). The effects of HSP60 on neurobehavioral measures. Each symbol represents the score of a single mouse. The horizontal bar indicates the mean value of each group. **P < 0.01 versus the middle cerebral artery occlusion (MCAO) group, ***P < 0.001 versus the MCAO group. (C) Apoptosis in the ischemic penumbra was assessed at 3 days after reperfusion using terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) staining. Scale bar = 20 μm. (Lower) quantification and statistical analysis of the TUNEL staining in each group. *P < 0.05 versus MCAO group. (D) Unilateral transformation of the recombinant TREM2 lentivirus into the cerebral ventricle up-regulated the expression of TREM2. *P < 0.05 versus control-lentivirus group. (E) Western blot analysis showed that TREM2 up-regulation induced microglia to adopt the M2 phenotype (n = 5). (F) Comparisons of percentages of infarct volume among the three groups (n = 7). *P < 0.05 versus sham group. (G) The effect of recombinant TREM2 lentivirus on cellular apoptosis and neurobehavioral measures. Each symbol represents the score of a single mouse. Arg-1 = arginase-1; LV = lentivirus; OGD = oxygen–glucose deprivation; shRNA = short hairpin RNA.
TREM2 Confers Neuroprotection as a Novel Regulator

This result appears inconsistent with the findings of Kawabori et al., who did not observe changes in HSP60 expression after MCAO or hypothermia. Another previous study showed that HSP60 may be associated with the delayed death of CA1 pyramidal neurons after transient ischemia and that the induction of HSP60 protected the neurons from ischemic damage. The contradictory results can be explained as follows: Recombinant 70-kDa HSP70 is an antiapoptotic protein that protects cells against stress and thus may be a useful therapeutic agent in the management of patients with acute ischemic stroke. To date, several in vitro studies have clearly demonstrated the feasibility and efficacy of HSP70-mediated neuroprotection. Emerging evidence indicates that HSPs are critical regulators of normal neural physiological functions, as well as of cell stress responses. HSP60 is a member of multiple HSP superfamilies, and its role in ischemic stroke remains unclear. In the brain, HSPD1/HSP60 is endogenously expressed in astrocytes, neurons, microglia, oligodendrocytes, and ependymal cells. We did not observe endogenous HSP60 expression after MCAO. Interestingly, the recombinant 60-kDa HSP used in our study had a neuroprotective effect against MCAO in mice. The binding of HSP60 with TREM2 is one of the possible neuroprotective mechanisms. Moreover, the specificity of the binding between TREM2 and...

Fig. 7. Modulatory effect of triggering receptor expressed on myeloid cells 2 (TREM2) on the phenotypic conversion of microglia after stroke. Subjecting resting (M0) microglia to a proinflammatory stimulus, lipopolysaccharide (LPS), and recombinant murine interferon-γ (IFN-γ) in vitro drives the cells toward the classical activated (M1) phenotype and is accompanied by a decrease in TREM2 expression. The M1 microglia displayed high expression of cytokines and chemokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as of induced nitric-oxide synthase (iNOS); these factors are key in cytotoxicity and tissue injury. In addition, the production of enzymes involved in tissue repair and remodeling, such as arginase-1 (Arg-1), was suppressed. In contrast, the treatment of M0 microglia with interleukin-4 and interleukin-13 (IL-4 and IL-13) drove M0 phenotypic conversion toward the augmented phagocytic M2 form and was accompanied by an increase in TREM2 expression. The M2 microglia exhibited strong expression of Arg-1 and brain-derived neurotrophic factor (BDNF) and weak expression of iNOS. Activating or up-regulating TREM2 in vivo had a neuroprotective effect by inducing phenotypic conversion of immunocytes and by reducing neuronal apoptosis. Down-regulation of TREM2 aggravated neuronal injury by increasing proinflammatory cytokine release, although this might be one of many pathways involved in stroke. OGD = oxygen–glucose deprivation; TGF-β = transforming growth factor-β.
HSP60 was confirmed in an experiment using mouse thymoma BWZ_36 cells, which are negative for surface HSP60 but became positive after transfection with TREM2 followed by incubation with the chaperone protein. Nonetheless, the specificity of HSP60 and how these mechanisms function in a variety of conditions remain incompletely understood, thus requiring further study. The limitation in our experiments was that most of the mice after MCAO did not survive more than 2 weeks. Therefore, the function of microglia in the chronic phase of ischemic stroke deserves further exploration. As noted above, the main role of microglia in the chronic phase of ischemic stroke is still ambiguous. It is essential to further examine the long-term dynamics of M1/M2 polarization in a chronic inflammatory animal model and to monitor the balance of microglial polarization under microglia-specific conditions.

Conclusions
The current work provides the first demonstration of the importance of TREM2 as a novel regulator of microglia phenotypes that may hold great clinical promise as a therapeutic for stroke by altering the microglial response from one of tissue injury to one of repair. The data we have presented provide new information and tools that can be used in further studies focused on the roles of TREM2 under physiologic conditions and in the pathogenesis of stroke.

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Competing Interests
The authors declare no competing interests.

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References


