Nrf2 Regulates Microglia-Mediated Phagocytosis and Neuroinflammation Following Intracerebral Hemorrhage

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Research

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Abstract

Background

To explore the effect of microglial functional transformation in hematoma clearance following intracerebral hemorrhage (ICH), and investigate whether Nuclear factor erythroid 2-related factor 2 (Nrf2) -mediated microglial phagocytosis and inflammatory response is beneficial for hematoma clearance and functional recovery in vitro and in vivo experiments after ICH.

Methods

In vitro experiments, BV-2 cells were cultured and randomly divided into 4 groups: normal control, microglia + Nrf2-siRNA (100 nmol/L), microglia + monascin (15 µM), microglia + Xuezhikang (200 µg/mL). In vivo experiments, 42 mice were divided into sham, ICH+vehicle, ICH+Nrf2-/-, ICH+monascin (10mg/kg/day, twice) and ICH+Xuezhikang (0.2g/kg/day, twice). Neurologic scores, hemoglobin levels, microglial phagocytosis, brain expression of CD80/Trem1/TNF-α (pro-inflammatory cytokines) as well as CD206/Trem2/BDNF (anti-inflammatory cytokines) were analyzed at 72 hours after surgery.

Results

The results showed that Nrf2 agonists improved neurological deficits and decreased hemoglobin levels after ICH. The administration of Nrf2 agonist- monascin/ Xuezhikang enhanced microglia-mediated phagocytosis of erythrocytes and bioparticles by up-regulating Nrf2. Alternatively, monascin/Xuezhikang promoted the expressions of Triggering receptor II expressed on myeloid cells (Trem2), CD206 and BDNF, while inhibited the expressions of Trem1,CD80 and TNF-α expressed in microglia. Conversely, Nrf2 inhibition (Nrf2 siRNA or Nrf2-/-) showed the opposite results following ICH.

Conclusions

Microglial functional transformation are involved in hematoma clearance following ICH. Nrf2 activation contributes to microglial functional transformation and phagocytic responses then exerts its neuroprotection after ICH. Nrf2 activator (Monascin/Xuezhikang) enhances hematoma clearance and alleviates neuroinflammation via the regulation of microglial functional alteration following ICH.

1 Background

Intracerebral hemorrhage (ICH) is a catastrophic illness resulting in significant morbidity and mortality[1–3]. Accumulating studies indicate that hematomas and degradation products of hematomas is the primary cause of poor prognosis of intracerebral hemorrhage (ICH)[4]. Recent studies have shown that the effect of our endogenous cerebral scavenging system in hematoma clearance [5] and which
recognized as a new effective therapeutic strategy for ICH, and microglia play an important role in the process of hematoma clearance[4, 6].

Generally, microglia are activated by various pathologic events or changes in brain homeostasis, which are highly diverse and depend on the context and type of stressor or pathology. The complicated functional roles of microglia support the existence of distinct pro-inflammatory and anti-inflammatory functional states following ICH [7, 8]. Pro-inflammatory phenotype is characterized by the production of tumor necrosis factor-α (TNFα), interleukin 1β (IL-1β), CD80, CD86, and CD16/32, etc., and lead to the deterioration of neuroinflammatory and neurons. While the alternative anti-inflammatory phenotype can attenuate inflammation, manipulate phagocytosis, and promote neural repair in the presence of the anti-inflammatory production IL-4, IL-10, brain-derived neurotrophic factor (BDNF), and CD206 etc [9–11]. Triggering receptor I expressed on myeloid cells (Trem1) was identified as an crucial inflammatory amplifier of microglia [12, 13]. Triggering receptor II expressed on myeloid cells (Trem2), which exerts anti-inflammatory and neuroprotective effects, may be a phagocytic receptor that mediates phagocytosis [14, 15].

Nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor γ (PPARγ) have attracted much attention in the regulation of clearance of endogenous hematomas[16, 17], possibly promote the removal of erythrocytes or hematoma remnants via the interaction with Trem1 or Trem2.

In our previous work, we confirmed that Nrf2 agonist -monascin facilitates hematoma clearance and exerts neuroprotection after ICH[18, 19], However, the underlying mechanism of hematoma clearance and its neuroprotection of Nrf2 has not been well-studied.

Thus, this research was conducted to further investigate the natural expression of Nrf2 and its relationship with Trem2 and Trem1 after ICH, and explore the mechanism and effects of Nrf2 on phagocytic activity and transformation of microglia-mediated neuroinflammation using Nrf2 agonists monascin/Xuezhikang (monascin, as a new type of Nrf2 agonist, is also an agonist of peroxidase proliferator activated receptor gamma (PPAR-γ)), and Xuezhikang is an extract of cholestilin that has monascin as its main component) and Nrf2-siRNA via vitro experiments or Nrf2-/- via vivo experiments to provide more powerful evidence of Nrf2 as an effective treatment strategies for ICH.

2 Results

2.1 Mortality, Hemoglobin and neurological scores following ICH

All sham-operated mice survived. The total operative mortality of mice was about 7.7% (n=2). The mortality was not significantly different among the experimental groups.

All ICH groups showed a significant decrease in modified Garcia scores and an increase in hemoglobin levels when compared with sham at 72 hours after surgery (p<0.05, Supplemental Fig A, B). Compared
with ICH+ vehicle, Nrf2 agonist, Monascin and Xuezhikang treatment resulted in significant improvement of neurofunctional deficits and a reduced hemoglobin levels at 72 hours after ICH while they were opposite results in Nrf2-/- group (p<0.05, Supplemental Fig. A,B).

### 2.2 Expression of Nrf2

In vitro experiment, western blot results showed that Nrf2 in the monascin and Xuezhikang groups were significantly enhanced, and Nrf2 were decreased in the siRNA group when compared to normal control group (Fig. 1A-C). Furthermore, In vivo experiment, monascin and Xuezhikang promoted the expression of Nrf2, while they were significantly decreased in Nrf2-/- ICH, compared vehicle ICH; Consistently, immunofluorescence detection confirmed the same patterns of Nrf2 expression (Fig. 1D-F).

The red fluorescence is Iba1 expression, the green fluorescence is Nrf2 expression, and the blue fluorescence is nuclear DAPI staining. “Merge” represents the superposition of the first three images. Western blot analysis or immunofluorescence detection indicated that Nrf2 production of microglia was upregulated in the monascin/Xuezhikang groups and downregulated in the siRNA group when compared to normal group (A)-(C) (*, p< 0.05 vs. the control and **, p< 0.01 vs. the control, WB: Western blot; Nrf2: nuclear factor erythroid 2-related factor 2; Iba1: ionized calcium-binding protein 1, microglial marker). Furthermore in vivo experiment, The red fluorescence is Nrf2 expression, the green fluorescence is Iba1 expression. Monascin and Xuezhikang promoted the expression of Nrf2, while they were significantly decreased in Nrf2-/- group, compared to sham or vehicle group. Representative images are shown of immunofluorescence and western blot assays (D)-(F) (*, p< 0.05 vs. sham and #, p< 0.05 vs. ICH + vehicle were statistically significant).

### 2.3 Trem1 and Trem2 Protein Expression

In the siRNA group, western blot analysis revealed the protein levels of Trem2 expression decreased slightly and Trem1 expression increased. While monascin and Xuezhikang reversed the protein expression of Trem1 and Trem2 along with the increase of Nrf2. Immunofluorescence results is similar to the results of WB(Fig. 2A-F).

Compared to sham group, the vivo expression of Trem1 in all the ICH groups was up-regulated to varying degrees. Among them, the expression of Trem1 in the vehicle or Nrf2-/- group remarkably increased. Compared to vehicle group, the expression of Trem1 was enhanced in Nrf2-/- group and that in monacsin and Xuezhikang groups was attenuated. While the expression of Trem2 exhibits roughly the opposite results. The immunofluorescence results were roughly consistent with those of the WB (Fig. 2G-L).

The red fluorescence is Trem1 or Nrf2 expression, the green fluorescence is Trem2 expression, and the blue fluorescence is nuclear DAPI staining. “Merge” represents the superposition of the first three images. In the siRNA group, immunofluorescence or western blot results revealed the protein levels of Trem2 expression decreased slightly and Trem1 expression increased. While monascin and Xuezhikang reversed the protein expression of Trem1 and Trem2 along with the increase of Nrf2(A)-(F). The red fluorescence (in vivo experiment) is Trem1 and Trem2 expression, the green fluorescence is Nrf2 expression. Compared
to sham group, the expression of Trem1 in all the ICH groups was up-regulated to varying degrees. Compared to vehicle group, the expression of Trem1 was enhanced in Nrf2-/- group and that in monacsin and Xuezhikang groups was attenuated. While the expression of Trem2 exhibits roughly the opposite result (G)-(L) (Trem1: triggering receptor I expressed on myeloid cells; Trem2: triggering receptor II expressed on myeloid cells).

### 2.4.1 Phagocytosis of Fluorescent Bioparticles

In the phagocytic test of fluorescent bioparticles, the phagocytosis rate of the normal control group was about 2–3/cell, and that of the siRNA group was about 1–2/cell, while the phagocytosis rate of the monascin and Xuezhikang groups (about 4–5/cell) was significantly higher than that of the siRNA group (Fig. 3A).

The green fluorescence is the Iba1 cells, the red fluorescence is the fluorescent bioparticles, the blue fluorescence is the nuclear DAPI staining. "Merge" represents the superposition of the first three images. The average phagocytosis of the fluorescent bioparticles was about 2–3/cell in the normal control group, about 1–2/cell in the siRNA group, and about 4–5/cell in the monascin/Xuezhikang groups. The phagocytosis rates were significantly increased in the monascin and Xuezhikang groups.

### 2.4.2 Phagocytosis of erythrocytes

In the phagocytic test of erythrocytes, the phagocytosis rate of the normal control group was about 1–2/microglial cell, and that of the siRNA group was about 0–1/cell, while the phagocytosis rate of the monascin and Xuezhikang groups (about 3–6/cell) was significantly higher than that of the siRNA group (Fig. 3B).

The white arrow is microglia, the red arrow is erythrocytes, the blue arrow represents the microglia that swallowed erythrocytes. The average phagocytosis of the erythrocytes was about 1–2/microglial cell in the normal control group, about 0–1/cell in the siRNA group, and about 3–6/cell in the monascin and Xuezhikang groups. The phagocytosis rates were significantly increased in the monascin and Xuezhikang groups.

### 2.5 Expression of CD80/CD206

Compared to normal group, cellular immunofluorescence demonstrated that both CD80 and CD206 in monascin and Xuezhikang groups were enhanced when Nrf2 was elevated, and the expression of CD206 increased more obviously than that of CD80. While the expression of CD80 in the siRNA group slightly increased and CD206 decreased. In this cellular WB, we found that the expression of CD206 in monascin and Xuezhikang groups all statistically increased than that in the siRNA group, while the expression of CD80 was reversed (Fig. 4A-D).

In vivo experiment, compared to sham group, western blot results revealed that the expression of CD80 in the four ICH groups was up-regulated. Among them, more obvious increase of CD80 was observed in
vehicle or Nrf2-/- group, and there is no significant difference between the two groups. Compared to vehicle group, the expressions of CD206 in the monascin and Xuezhikang groups were significantly up-regulated, while CD80 was down-regulated. The results of immunofluorescence detection are roughly consistent with the those of WB (Fig. 4E-I).

The green fluorescence is marked CD80, the red fluorescence is marked CD206, and the blue fluorescence is nuclear DAPI staining. “Merge” represents the superposition of the first three images. Compared to normal group, cellular immunofluorescence demonstrated that both CD80 and CD206 in monascin and Xuezhikang groups were enhanced when Nrf2 was elevated, and the expression of CD206 showed more obviously than that of CD80. In this cellular WB, we found that the expression of CD206 in monascin and Xuezhikang groups all statistically increased than that in the siRNA group, while the expression of CD80 was reversed (A)-(D). In vivo experiment, the red fluorescence is marked CD80, the green fluorescence is marked CD206. Compared to sham group, western blot analysis revealed that the expressions of CD80 in the four groups of ICH all were up-regulated. Among them, more obvious increase of CD80 was observed in vehicle or Nrf2-/- group. Compared to vehicle group, the expressions of CD206 in the monascin and Xuezhikang groups were significantly up-regulated, and CD80 was down-regulated. The results of immunofluorescence detection are roughly consistent with the those of WB (E)-(I).

**2.6 Transformation of Microglial neuroinflammation**

Flow cytometry showed that the total expression of CD80 in the siRNA group was increased, the CD206/CD80 ratio was decreased, and the phenotype was transformed to the Pro-inflammatory phenotype. In the monascin and Xuezhikang groups, both the total expression of CD206 and the CD206/CD80 ratio were increased, and the microglia were polarized toward the Anti-inflammatory phenotype (Fig. 5).

The ratio of CD206/CD80 and the total expression of CD206 increased significantly in the monascin and Xuezhikang groups, and the microglial phenotype transformed to the Anti-inflammatory phenotype (green fluorescent FITC: CD80; red fluorescent APC: CD206).

**2.7 TNF-α and BDNF Protein Expression**

In this study, we found that Nrf2-siRNA promoted the protein expression of TNF-α, and monascin and Xuezhikang reversed the TNF-α inflammatory factor production instead of BDNF, especially in the Xuezhikang group (Fig. 6A-D).

Compared with the sham or vehicle group, the protein expression of TNF-α in Nrf2-/- group went up, and monascin and Xuezhikang reversed the TNF-α inflammatory factor production instead of BDNF ($p< 0.05$) (Fig. 6E-H).

The protein expression of BDNF was significantly increased in the monascin and Xuezhikang groups and decreased in the Nrf2-siRNA group(A)-(D) or in the Nrf2-/- group(E)-(H), while TNF-α protein expression
decreased in the monascin and Xuezhikang groups and increased in the Nrf2-siRNA or Nrf2-/- group (TNF-α: tumor necrosis factor α; BDNF: brain-derived growth factor).

3 Discussion

In the present study, we have made the following observations: (1) The monascin and Xuezhikang groups can promote the up-regulation of Nrf2 in BV-2 microglia cells or in C57BL/6 mice, and Nrf2 was down-regulated in the siRNA-Nrf2 group or Nrf2-/-group. (2) The monascin and Xuezhikang groups had a stronger ability to swallow fluorescent bioparticles or erythrocytes, and the expression of Trem2, CD206, BDNF was increased. (3) Nrf2 inhibition showed the opposite results, the expression of Trem2, CD206, BDNF was decreased and the ability to swallow fluorescent bioparticles or erythrocytes became weaker. (4) Compared to vehicle group, the expression of Trem1, TNF-α was increased in the Nrf2-/- group, while monasin and Xuezhikang groups reversed these expressions to varying degrees.

Microglia have historically dominated the field of neuroimmunology, and not only are they classified as surveillant for potential insults in the brain, they also initiate the immune response, release inflammatory mediators, and have been described as vital regulators of the cascade of neuroinflammatory [20, 21].

In the process of neuroinflammation, the activated microglia undergo dynamic neuroinflammation changes to their surface receptors, inflammatory factors, and phagocytic function [22], and the local extracellular and intracellular signals determine the characteristics of the microglia [23]. Pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) activate microglia to a pro-inflammation states [24]. In neuroinflammatory conditions such as ICH and neurodegenerative diseases in which pathogenic amyloid-β or hematoma residues accumulate, activation of microglia is further promoted mainly via myeloid differentiation factor 88/TIR-domain-containing adaptor inducing interferon-β(MyD88/TRIF) and up-regulation of nuclear factor kappa light-chain enhancer of activated B cells(NF-κB), varying amounts of inflammatory mediators are released, and neuroinflammatory are aggravated, affecting the survival of neuronal cells in the central nervous system (CNS)[25, 26]. Primary mediators such as TNF, IL-1β, and IFNγ are released and promote the production of secondary mediators such as matrix metalloproteases (MMP), nitric oxide (NO), and arachidonic acid [27, 28]. In addition, TNF-α promotes the production of high-mobility group protein box-1 (HMGB1: a ligand of neuroinflammatory amplifier Trem1), which, in turn, stimulates microglia to release large amounts of TNF-α to activate more microglia [12, 29, 30]. There seems to be a self-feedback cascade loop in the process of microglial activation, and these inflammatory signals can be amplified by the self-feedback loop of microglial activation and build an immune cascade inflammation network [20].

Nrf2 is a pleiotropic transcription factor widely present in microglia. It is a steady-state regulator of cellular oxidative stress and has a strong antioxidant capacity. The antioxidants produced, such as heme oxygenase (HO-1), superoxide dismutase, catalase, glutathione sulphydryl transferase, and haptoglobin (Hp), can be activated [31]. Nrf2 is also an important target for regulating microglia in stroke and the
neurodegenerative diseases discovered in recent years. Up-regulating Nrf2 can down-regulate NF-κB and promote microglial transformation to the Anti-inflammatory phenotype [32, 33].

Monascin has been confirmed as a new type of Nrf2 agonist[34], is also an agonist of peroxidase proliferator activated receptor gamma (PPAR-γ), which can inhibit oxidative stress in pathological conditions and promote the absorption of hematomata and reduce intracerebral edema in our animal models of ICH[18, 19]. Xuezhikang is an extract of cholestin that has monascin as its main component, and is used mainly as traditional Chinese medicine (TCM) to fight atherosclerosis and exert anti-inflammatory effects by inhibiting toll-like receptor 4 (TLR4)/NF-κB[35, 36].

Our research found that monascin/Xuezhikang can promote the up-regulation of Nrf2 of Iba1-marked microglia, and the expressions of Trem2 were significantly increased correspondingly, and their ability to phagocytose erythrocytes or fluorescent bioparticles was enhanced following the administration of Nrf2 agonist. In contrast, the expression of Trem2 in Nrf2-knocked-down microglia or Nrf2-knocked-out mice were significantly decreased, and their phagocytic ability was significantly reduced.

In addition, Monascin/Xuezhikang can also resist effectively the neuroinflammation cascade of Trem1 via up-regulating Nrf2. In all the ICH groups, Trem1 was significantly up-regulated, suggesting that Trem1 is highly related to the neuroinflammation of ICH. The Trem1 expression in the Nrf2-/- group was the most, and the Nrf2 agonists monascin and Xuezhikang reversed the expression of Trem1, which indicated that the change of Nrf2 directly affected the expression of Trem1. The microglial cellular experiment further verified that Monascin/Xuezhikang can effectively suppress Trem1 expression via the up-regulated Nrf2. while Trem2 had the opposite effects. The mechanism remains vague, and further investigations between Trem1 and Trem2 are needed to fully evaluate the phenomenon.

Monascin and Xuezhikang promoted the expression of the anti-inflammatory phenotype marker CD206, and the proportion of CD206/80 increased significantly, while the expression of the pro-inflammatory phenotype marker CD80 increased and the CD206 decreased following the Nrf2 knocked-down in microglia or knocked-out in mice. These events show that the microglia were transformed into the Anti-inflammatory phenotype following administration of monascin/Xuezhikang. Intriguingly, we found that CD80 also increased in the monascin and Xuezhikang groups in the cellular experiment, but the increase in CD206 was significantly greater than that of CD80, and the ratio of CD206/CD80 is higher. This suggests that monascin and Xuezhikang may strengthen the immunity of microglia and are generally more beneficial to the survival of neurons. Further studies are needed to decipher the perplexity. With the transformation of the microglial neuroinflammation to the anti-inflammatory phenotype, the pro-inflammatory factor TNF-α was significantly decreased instead of the nerve repair factor BDNF, which greatly inhibited neuroinflammation. This result is consistent with those of previous studies [37].

4 Conclusions

Our results showed that administration of the Nrf2 agonist monascin/Xuezhikang can enhance the phagocytic capacity of microglia, and improved anti-inflammation by promoting the expressions of
Trem2, BDNF and CD206 while inhibiting the pro-inflammatory cascade of Trem1, TNF-α and CD80 in microglia. Nrf2 inhibition reversed the effects of Nrf2 agonists. Therefore, Nrf2 activation plays a critical role in hematoma clearance and neuroprotection following ICH. This may provide a new microglia-based strategy for the treatment of ICH.

5 Materials And Methods

In our previous study, we have showed that TREM-1 inhibition improves neurological deficits and brain edema in subarachnoid hemorrhage [38].

5.1a Cellular Materials

Experimental cells: BV-2 cells (Solebo)

5.2a Cellular Experimental Methods

5.2.1 Experimental Cell Groups: Normal Group, siRNA Group, Monascin Group, Xuezhikang Group

5.2.2 Cell Culture

BV-2 cells were cultured in a humidified incubator with 5% CO₂ at 37°C. Every 2–3 days, Dulbecco’s Modified Eagle Medium (DMEM) was replaced, and non-adherent cells were removed. When the primary cultures reached 80% confluence, cells were harvested using 0.25% trypsin–EDTA solution and subcultured, and the third passage of cells were selected for the subsequent experiments.

5.2.3 Screening of Monascin / Xuezhikang Concentration and the Transfection Sequence of Nrf2-siRNA by qPCR

In order to screen the best sequence of Nrf2-siRNA, and to screen the best drugs concentration of monascin or Xuezhikang, RT-PCR was used to detect the Nrf2-mRNA levels. 1 ml aliquots of 5, 15, 30 µmol/L monascin or 100, 200, 500µg/ml Xuezhikang were added to a 12-well plate with three multiple holes in each group, and Nrf2 interference vector(siNrf2) were synthesized (Gene Pharma, Shanghai, China), and transfected by incubation in DMEM containing GP-transfect-Mate (Gene Pharma, Shanghai, China).

Total RNA was extracted with Trizol reagent. First-strand cDNA was synthesized using the Prime Script RT Master Mix Kit (ABI-Invitrogen, Thermo Fisher Scientific, Grand Island, NY, USA), and β-Actin was used as the internal control. The quantification of endogenous control mRNA levels was performed using TaqMan assays. The data were analyzed using the 2⁻ΔΔCt method.

The Nrf2-siRNA (100 nM) primer sequences screened by RT-PCR had sense chain GCAGGACAUGGAUUUGAUUTT and antisense chain AAUCAAAUCCAGGUCUGCCTG, and 15µmol/L
monascin or 200ug/ml Xuezhikang were selected for the subsequent experiments.

5.2.4 Cell Immunofluorescence

The immunofluorescence was performed as previously described[39]. The BV2 microglial cells are treated by Nrf2-siRNA, monascin, and Xuezhikang, respectively. The cells were collected and fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100, and then blocked with goat serum for 60 min. The samples were incubated with primary antibody (anti-Nrf2, anti-Trem1, anti-Trem2, anti-CD80, anti-CD206, Abcam) at 4°C overnight, incubated with a fluorescent dye-conjugated secondary antibody in the dark for 1 h, and then stained with 4',6-diamidino-2-phenylindole (DAPI). Three different visual fields were randomly selected for each sample to be photographed under a fluorescence microscope.

5.2.5 Phenotype of BV-2 Cells Detected by Flow Cytometry

The BV-2 cells were inoculated and the 100 µL cell suspension with a final concentration of 10 x 10^6 cells/mL was pipetted into a round-bottom Eppendorf tube. Following incubation with the primary antibody CD80/CD206 (anti-CD80 1:50, anti-CD206 1:50 Abcam) for 2h, the microglial cells were washed and then were incubated fluorescent-labeled secondary antibody for 1h. The percentage of CD206 in the total and the relative ratio of CD206 to CD80 were detected by flow cytometry. CD206 is shown as APC in the Q1 quadrant; CD80 is displayed as fluorescein isothiocyanate (FITC) in the Q4 quadrant. The common mark is in the Q2 quadrant. The percentage of CD206 and CD80 (%) = 100 × (Q1+Q2)/(Q2+Q4) is the phenotype of the microglia. The phenotype of the cells was evaluated by quantitative percentage.

5.2.6 Detection of Expression of Nrf2, Trem1, Trem2, CD80, CD206, TNF-α, and BDNF protein by Western Blot (WB)

The BV2 cells were collected and treated with radioimmunoprecipitation (RIPA) lysis buffer, and measured using a bicinechonic acid (BCA) kit as previously described[8]. Equal amounts of protein were loaded on a SDS polyacrylamide gel, electrophoresed, and then transferred to a polyvinylidene fluoride (PVDF) membranes, which were then incubated with primary antibodies ((anti-Nrf2, anti-Trem1, anti-Trem2, anti-CD80, anti-CD206, anti-TNF-α, and anti-BDNF, Abcam) overnight at 4°C. The membranes were incubated with a horseradish peroxidase-conjugated secondary antibody and visualized by means of chemiluminescence. Densitometry was performed in order to quantify the signal intensity using ImageJ software (https://imagej.net).

5.2.7.1 Observation of Phagocytosis of Fluorescent Bioparticles by Immunofluorescence

The BV2 cells were treated with 10 mg/mL zymosan fluorescent bioparticles (Alexa Fluor594 conjugate; zymosan: BV-2 = 40:1) for 1 h and fixed with 4% paraformaldehyde, before being permeabilized with Triton X-100. The samples were then placed in 10% goat serum for 2 h, incubated with anti-lba1 antibody
(1:100; Abcam) overnight at 4°C, and then incubated with a FITC-conjugated secondary antibody. The phagocytosed bioparticles were observed using a fluorescence microscope.

### 5.2.7.2 Observation of Phagocytosis of erythrocytes

After the erythrocytes are separated and purified, they are counted and co-cultured with microglia at a ratio of 1:40 (microglia: erythrocytes). That microglia swallowed erythrocytes were observed using a microscope.

5b Materials and Methods

In our previous study, we have showed that Nrf2 agonist -monascin facilitates hematoma clearance, alleviates cerebral edema and exerts neuroprotection after ICH[18, 19].

5.1b Animal Materials

Experimental male Nrf2-/-C57BL/6 mice (4–5 weeks old) were purchased from Cyagen Model Biological Research Center(Taicang) Co.,Ltd. (Suzhou, China), the other male C57BL/6 mice (4–5 weeks old) were purchased from Animal Experimental Center of Shanxi Medical University. All animal experiments were approved and conducted in accordance with to the guidelines of the Ethics Committee of Shanxi Medical University, Shanxi, China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering.

5.2b Animal Experimental Methods

**5.2.1 Experimental Design

42 mice were randomized to the following groups: sham (n = 8), ICH+ vehicle (n = 9), Nrf2-/- +ICH (n = 8), ICH+ monascin (10mg/kg/day, twice, n = 8), Xuezhikang+ ICH (0.2g/kg/day, twice, n = 9). Dead animals were replaced before final assessment. All gavages were administered by gastric perfusion 6 h after ICH for 72 hours.

**5.2.2 ICH Model

Experimental ICH model was induced by injecting collagenase type IV (0.5 units in 2 µl saline) into the basal ganglia areas using stereotaxic instruments. They were fixed on a stereotaxic apparatus under chloral hydrate anesthesia (10ml/kg, intraperitoneally), exposed the skull and reveal bregma. A1-mm cranial bur hole was drilled in the skull (coordinates: 0.9 mm posterior to the bregma, 1.5 mm lateral to the midline), and next, collagenase was infused into the right basal ganglia (4 mm deep from the dura mater) with a micro injector. The needle was remained in place for an additional 15 min to prevent "back-leakage" before the injector was withdrawn. The Sham-operated mice were syringed with equivalent dosages physiological saline. After the surgery, the skull hole was sealed with bone wax and the incision was sutured. Animals were allowed to recover after successful ICH induction that was confirmed by Rosenberg’s neurological score [40].
5.2.3 Neurobehavioral tests

As previously described [6, 18–19], before being scarified for tissue collection, all animals were subjected to neurofunctional assessments using the modified Garcia tests. The modified Garcia test involves a 18-point sensorimotor assessment that includes six individual tests. Each test has a score ranging from 0 to 3, with a maximum score of 18. The individual tests evaluate spontaneous activity, response to side stroking, vibrissae touch, climbing, lateral turning, and forelimb walking.

5.2.4 Immunofluorescence Detection

After intraperitoneal anesthesia, heart perfusion were performed by ice Phosphate-buffered saline (PBS) and 4% paraformaldehyde. The brain tissues of mice were removed on ice and placed in 4% paraformaldehyde overnight at 4°C. Dehydration was made using sucrose PBS buffer (20% and 30%) at the same temperature. After fixed embedding of OCT (optimal cutting temperature compound), frozen sections were cut coronally into 4-µm slices. The immunofluorescence methods were based on previously described methods.

5.2.5 Western blot

Total protein from brain tissue was collected, and protein concentrations were determined with a BCA kit, the following procedure is similar to the foregoing cellular experiment methods.

5.3 Statistical Methods

All statistical and graphical analyses and were carried out using SPSS 22.0 (IBM, Armonk, NY, USA) and GraphPad Prism 7.0 (https://www.graphpad.com/scientific-software/prism) software. All data are presented as the mean ± SEM (standard error of the mean). One-way analysis of variance (ANOVA) was performed for comparisons among multiple groups, and the SNK-q test was used for pairwise comparison between groups; a value of $p < 0.05$ was taken as being statistically significant.

6 Innovation

Intracerebral hemorrhage (ICH) is a catastrophic illness causing significant morbidity and mortality. Removing hematomas and degradation components and inhibiting neuroinflammation may be an extremely effective treatment strategy. Trem1 was identified as a crucial inflammatory amplifier of microglia. Trem2, which exerts anti-inflammatory and neuroprotective effects, may be a phagocytic receptor that mediates phagocytosis. This research was conducted to investigate the natural expression of Nrf2 and its relationship with Trem2 and Trem1 after ICH, and further explore the mechanism and effects of Nrf2 on phagocytic activity and transformation of microglia-mediated neuroinflammation.

Abbreviations

BDNF brain-derived neurotrophic factor
CNS  central nervous system
DAMPs  danger-associated molecular patterns
HMGB1  high-mobility group protein box-1
HO-1  heme oxygenase-1
Hp  haptoglobin
ICH  intracerebral hemorrhage
IFNγ  interferon-gamma
IL-1β  interleukin 1β
MMP  matrix metalloproteases
Myd88  myeloid differentiation factor 88
NF-κB  nuclear factor kappa light-chain enhancer of activated B cells
NO  nitric oxide
Nrf2  nuclear factor erythroid 2-related factor 2
PAMPs  pathogen-associated molecular patterns
PPAR-γ  peroxisome proliferator activated receptor gamma
TCM  traditional Chinese medicine
TLR4  toll-like receptor 4
TNFα  tumor necrosis factor-α
Trem1  triggering receptors I expressed on myeloid cells
Trem2  triggering receptors II expressed on myeloid cells
TRIF  TIR-domain-containing adaptor inducing interferon-β

Declarations
Ethics, consent and permissions
All animal experiments were approved and conducted in accordance with the guidelines of the Ethics Committee of Shanxi Medical University, Shanxi, China. All surgical procedures were performed under anesthesia, and every effort was made to minimize suffering.

Consent for publication

All authors have read the manuscript and have agreed to submit it in its current form for consideration for publication in Journal of Neuroinflammation.

Availability of data and materials

All data and materials are available on request.

Competing interests

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Author Contributions

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Authors' information

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Figures
Figure 1

Immunofluorescence (vitro×400 and vivo×200) and Western blot protein expression of Nrf2.
Figure 2

Protein expression of Trem1/Trem2 is determined by immunofluorescence (vitro×400 and vivo×200) and Western blot.
Figure 3

A. Phagocytosis test of fluorescent bioparticles (immunofluorescence ×100). B. Phagocytosis test of erythrocytes (×400).
Figure 4

Expression of CD80/CD206 detected by immunofluorescence (vitro×400 and vivo×200) and Western blot.
Figure 5

Transformation of microglial neuroinflammation as shown by flow cytometry.
Figure 6

Protein expression of inflammatory factor TNF-α and BDNF by Western blot.

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