



Propagermanium, a CCR2 inhibitor, attenuates cerebral ischemia/reperfusion injury through inhibiting inflammatory response induced by microglia



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ABSTRACT

CCR2 could recruit immune cells migrating into brain after ischemic stroke. It is unclear whether and why Propagermanium (PG, a CCR2 inhibitor) is able to protect against ischemic injury. Middle cerebral artery occlusion (MCAO) and reperfusion injury in C57BL/6J male mice were performed in vivo to mimic ischemic stroke. Cultured BV2 microglia exposed to oxygen and glucose deprivation (OGD)/reoxygenation injury, LPS or IL-4 incubation were served in vitro. TTC staining, neurological score, brain water content, and MRI scan were performed to evaluate stroke outcome. Real time PCR, ELISA, and immunofluorescence were used to estimate inflammatory cytokines expression and releasing. Western blot was utilized to detect pSTAT1/STAT1 expression. Compared with MCAO mice, PG treatment significantly reduced infarction size and brain edema, improved neurological behavior at 72 h after MCAO. For inflammatory response, PG treatment inhibited inflammatory cytokines releasing, such as TNF- α , IFN- γ , IL-1 β , IL-6, IL-12, IL-17, and IL-23. Further studies indicated that PG treatment downregulated mRNA expression of pro-inflammatory iNOS and CD86, and inhibited CD16 expressed in microglia. In vitro, PG incubation inhibited BV2 polarized to pro-inflammatory phenotype through STAT1 downregulation, while had no obvious effect on anti-inflammatory phenotype. Our observations suggest that CCR2 inhibitor PG downregulated pro-inflammatory microglia polarization for decreasing pro-inflammatory microglia phenotype marker, and thereafter inhibited inflammatory responses after MCAO in a STAT1-dependent manner.

1. Introduction

Ischemic stroke is one of the leading causes of death and leads to severe sequelae like dementia, paresis and speech defects. However, there is still a scarcity of therapeutic strategies for ischemic stroke patients (Xu et al., 2016). Postischemic inflammation plays an important role in various stages of cerebral ischemic injury for triggering severe

loss of neurons and exacerbating brain damage.

It was reported that microglia serve as the first defensive line to mediate inflammatory cascades in central nervous system after stroke and also modulate brain repair (C et al., 2015; Mifsud et al., 2014). Microglia are provoked within minutes after ischemia (Jin et al., 2010), and then release a plethora of inflammatory factors. In response to various signals, microglia polarize to two major phenotypes including

Abbreviations: PG, Propagermanium; MCAO, Middle cerebral artery occlusion; OGD, oxygen and glucose deprivation; CCL2, chemokine ligand 2; CCR2, chemokine receptor 2; LPS, lipopolysaccharide; IL-4, interleukin-4; IFN- γ , interferon- γ ; MRI, magnetic resonance image; TTC, 2, 3, 5-triphenyltetrazolium chloride; STAT1, Signal transducer and activator of transcription 1; ANOVA, analysis of variance

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pro-inflammatory and anti-inflammatory phenotype (Sica and Mantovani, 2012). Pro-inflammatory phenotype microglia are associated with the overexpression of multiple pro-inflammatory cytokines such as TNF- α , IL-6, iNOS, and CD86. Anti-inflammatory microglia polarization is associated with the production and release of beneficial mediators, including IL-10, TGF- β , Arg1 and CD206, which will lead to homeostasis, neuroprotection and regeneration (Colton, 2009; Latta et al., 2015; Ma et al., 2017; Mecha et al., 2015). STAT signal plays an important role in microglia polarization (Kacimi et al., 2011). For example, STAT1 primarily mediates the pro-inflammatory phenotype polarization of microglia. Therefore, in IFN- γ -stimulated microglia, inhibits STAT1 phosphorylation reduced expression of pro-inflammatory factors IL-6, IL-1, TNF- α and iNOS (Gan et al., 2017). STAT3 also promotes microglia to pro-inflammatory phenotype polarization (Wei et al., 2017), and STAT6 mediates microglia to anti-inflammatory polarization (Yang et al., 2017). The dual-sword effects of the microglia have been found in various CNS disease such as Alzheimer's and multiple sclerosis (Jimenez et al., 2008; Xiao et al., 2010), and of course it was found in ischemic stroke (Yenari et al., 2010). Therefore, inhibit microglia pro-inflammatory phenotype or promote anti-inflammatory phenotype may be an effective therapeutic strategy for ischemic stroke treatment.

The chemokine receptor 2 (CCR2) is involved in inflammatory reactions following cerebral ischemia, and chemokine ligand 2 (CCL2) binds with high affinity to CCR2 (Schilling et al., 2009). CCR2/CCL2 was found to increase at both 24 h and 72 h after cerebral ischemia and reperfusion (Schilling et al., 2009), and was thought to have the function of recruiting immune cells into the site of inflammation (C and BJ, 2001). It has been demonstrated that microglia express CCR2 (Zhang et al., 2007), and depletion or knockout of CCR2 or CCL2 reduce microglia activation (Muessel et al., 2002; Yao and Tsirka, 2012; Zhang et al., 2007). In addition, CCL2 stimulates microglial activation by inducing microglial pro-inflammatory polarization through its receptor, CCR2 (He et al., 2016).

Propagermanium (PG), a selective CCR2 inhibitor, is an organic germanium compound approving for treating patients with chronic hepatitis type B in Japan (Hirayama et al., 2003). The effect of PG requires glycosylphosphatidylinositol (GPI)-anchored proteins and, as such, inhibits CCR2. Cleavage of GPI anchors by phosphatidylinositol-phospholipase C (PI-PLC) eliminated the inhibitory activity of PG (Yokochi et al., 2001a). The effect of PG is CCR2 specific, and it interrupt CCR2-mediated signaling and chemotaxis without disrupting the receptor itself or its ligands (Yokochi et al., 2001b). Our previous study demonstrated that PG could mimic CCR2 deficiency (Fang et al., 2018). We explored whether PG treatment could be beneficial for ischemic stroke. Our results indicated that PG treatment inhibited microglia transition toward pro-inflammatory phenotype at 72 h after ischemic stroke of mice. Consistently, *in vitro* experiments showed that PG incubation decreased the expression of pro-inflammatory phenotype genes and cytokines through STAT1 signal in BV2 cells. These results revealed a novel pharmacological mechanism of PG in protecting against ischemic stroke.

2. Materials and methods

2.1. Animals

Experiment was conducted on adult C57BL/6 J male mice, weighing 22–24 g which were bought from Nanjing Qinglongshan Animal Breeding Ground. Animals were kept under normal environment of 12 h day-night cycle, temperature of 25 °C and relative humidity of 65%, free water and food. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 80–23, revised 1978). All of the experiments and procedures were performed under the guidelines of the Institutional Animal Care and use committee of China

Pharmaceutical University.

2.2. Middle cerebral artery occlusion (MCAO) and reperfusion injury in mice

Animals were randomly assigned to six groups: 1) sham, 2) model, 3) RS504393 1 mg/kg/day, 4) RS504393 2 mg/kg/day, 5) PG 25 mg/kg/day, 6) PG 50 mg/kg/day. Mice were anesthetized by 3% chloral hydrate (0.1 ml/10 g, ip), and the body temperatures were kept at 37.0 ± 0.5 °C during the surgery. Carefully dissected the mice' right common carotid artery, external carotid artery and internal carotid artery, delivered a 6-0 silicone-coated nylon monofilament into internal carotid through the external carotid stump. As the monofilament was delivered to the internal carotid, it was fixed at about 10 mm. After 45 min of occlusion, the monofilament was removed which caused the reperfusion. The sham-operated mice were just exposure to blood vessels (G et al., 1994). Mice that showed no neurological deficits, and brains with evidence of surgical hemorrhage, or died 1–3 d after MCAO were excluded from the experiment. 180 mice were subjected to MCAO surgery. The injury incidence was around 80%, and the rate of death was about 20%.

2.3. Drug administration

At the same time of reperfusion, the mice were subjected RS504393, PG at different dosages (RS504393 1, 2 mg/kg/day, ip, once per day; PG 25, 50 mg/kg/day, po, once every 8 h) for 3 days. And then mice were sacrificed at 72 h after MCAO, respectively.

2.4. Neurobehavioral evaluation

The neurobehavioral evaluation was performed at 3 days after MCAO, and the neurological scores were 0–5 according to reference (JB et al., 1986):

- 0: no deficit;
- 1: flexion of the contralateral torso and forelimbs;
- 2: turning to the ipsilateral side when held by tails;
- 3: leaning toward the affected side;
- 4: no spontaneous locomotor activity;
- 5: death.

2.5. Brain edema determination

Brain water content was used to evaluate brain edema. Brain hemispheres were removed and superficially dried. Weighed the brain hemispheres' wet weight, and then dried them overnight at 110 °C for 12 h in a desiccating oven and weighed the dry weight. The total brain water content was calculated according to the following formula: brain water content = $(1 - \text{brain dry weight} / \text{brain wet weight}) \times 100\%$

2.6. Infarct size quantification

2, 3, 5-triphenyltetrazolium chloride (TTC) staining was performed to evaluate infarct volume. The brains were sliced to 1 mm sections and incubated in 2% TTC solutions for 30 min in the dark. Each slice was photographed with a digital camera. The infarction size was measured by ImageJ software and integrated across five slices (Liu et al., 2018).

Infarct size = $(\text{contralateral area} - \text{ipsilateral non-infarct area}) / \text{contralateral area} \times 100\%$

2.7. Magnetic resonance image (MRI) assay

The imaging experiments were performed on PharmaScan small

animal system at 3 days after MCAO. Anesthetized the mice with 2% isoflurane and positioned the mice into the magnet with a laser-controlled system for the animal cradles. Body temperature and respiratory frequency were monitored throughout the experiment and maintained the latter with a water heating pad. The protocol consisted of an ADC-map. The scope of coronal MRI scan was 2 mm from corpus callosum ahead to the ends of the brain. The ADC-map parameters was set as follows: TR: 3000 ms, TE: 30.0 ms, FA: 90.0 deg, SI: 1.00/1.00 mm, FOV: 1.60 cm, MTX: 128 (a), pos: 3.60 mm H, then synthesized ADC images at post-processing workstation, picked the image with the biggest cerebral ischemic lesion, chose 3 regions of interest (ROI) in ischemic lesion (the size was 0.2 cm²) and measured the ipsilateral apparent diffusion coefficient (ADC)(Huang et al., 2013). Measured the ADC of contralateral brain, and calculated relative ADC (rADC), rADC = ipsilateral ADC/contralateral ADC.

2.8. Cell culture and treatment

BV-2, a murine microglia-derived cell line was used in vitro study. BV-2 cells were maintained in growth medium consisting of high glucose Dulbecco's modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% fetal bovine serum (EVERY GREEN, Biotechnology CO, LTD) at 37 °C in an atmosphere of 95% air and 5% CO₂.

For pro-inflammatory phenotype induction, lipopolysaccharide (LPS) (100 ng/ml) (Sigma, USA) was added to the cells for 24 h, or LPS (100 ng/ml) + interferon- γ (IFN- γ) (20 ng/ml) (PEPRO TECH Inc., USA) were added to the cells for 24 h (Park et al., 2014).

For anti-inflammatory phenotype induction, interleukin-4 (IL-4) (20 ng/ml) (PEPRO TECH Inc., USA) was added to the cells for 24 h (Choi et al., 2017).

For oxygen and glucose deprivation (OGD)/reoxygenation injury, BV-2 cells were cultured in Low glucose DMEM (Hyclone, UT) with 50 μ M sodium hydrosulfite for 2 h and then replaced with high glucose DMEM with penicillin, streptomycin, 10% fetal bovine serum for reoxygenation (Fan et al., 2018).

Before pro-inflammatory, anti-inflammatory or OGD stimulates, cells were incubated with RS504393 or PG with different concentration (1, 3, or 10 μ M) for 24 h.

2.9. ELISA assay

Cytokines production in the media collected from supernatant after BV-2 cells stimulation, and brain cortex homogenate samples were assessed by commercial ELISA kit (mlbio, china). IL-10, TGF- β , IL-6, TNF- α , IL-1 β , IL-12, IL-17, IL-23, CCL2, and IFN- γ were quantified following the ELISA kit's protocols.

2.10. Real time PCR

Total RNA was extracted from BV2 cells or brain using TRIzol reagent (Takara, JP). Isolated RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, JP) following standard protocols. Real time PCR was performed using SYBR Premix Ex Taq™ II (Tli RNase HPlus) (Takara, JP) in a Mastercycler (Eppendorf, BRD). The primers used for real time PCR were list as following:

iNOS forward: AATGGCAACATCAGGTGGCCATCACT.
 iNOS reverse: GCTGTGTGTACAGAAGTCTCGAACTC.
 CD86 forward: ACGATGGACCCAGATGCACCA.
 CD86 reverse: GCGTCTCCACGGAACAGCA.
 CD206 forward: GCAGGTGTTTATGGGATGT.
 CD206 reverse: GGGTTCAGGAGTTGTTGTGG.
 Arg1 forward: GAACACGGCAGTGGCTTTAAC.
 Arg1 reverse: TGCTTAGTTCTGCTGCTTTCG.
 β -actin forward: CATCCGTAAAGACCTTTGCCAAC.

β -actin reverse: ATGGTGCCACCGATCCACA.

2.11. Brain tissue preparation

Seventy-two hours after MCAO, the animals were anesthetized with 3% chloral hydrate (0.2 ml/10 g, ip) and then transcardially perfused with saline to wash the blood out of the body. The mice were perfused with 4% paraformaldehyde to fix. The brains were collected and dehydrated in 20% sucrose solution for 24 h and in 30% sucrose solution for 24 h. After that, the brain was embedded by OCT, and was sliced at 15 μ m thickness in using a freezing microtome (Leica, Inc. BRD) in -20 °C. The brain slices were stored at -20 °C for later use.

2.12. Co-immunofluorescence

The brain slices were washed by PBS and blocked with 5% goat serum (PBST) and then incubated at 4 °C overnight with following primary antibody: mice anti-Iba1 (Santa Cruz Biotechnology, USA. 1:50), rabbit anti-CD16 (Abcam, USA. 1:200), rabbit anti-CD206 (Abcam, USA. 1 μ g/ml). After that, the sections were incubated with corresponding fluorescence secondary antibody for 2 h at room temperature. Finally, the brain slices were incubated with DAPI for 30 min. The images were taken using laser scanning confocal microscope (Carl Zeiss. BRD) and analyzed with ImageJ. The cell number calculation was determined by counting of three randomly selected microscopic fields across three slides in the penumbra of ipsilateral cortex.

2.13. assay

The protective effect of RS504393 and PG on BV-2 against OGD was measured using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay. After the experiment, BV-2 cells were seeded in 96-well plates, and 150 μ l/well MTT solution was added. Then incubated the plates at 37 °C for 4 h. Cell viability were detected by the absorbance at 490 nm using microplate reader (Bio-Rad Laboratories, Inc. USA).

2.14. Western blot

Brain tissues and BV2 cells were lysed in RIPA lysis buffer (PLYGEN, China). The homogenates were centrifuged at 12,000 g for 10 min. Total protein concentration of the supernatants was assessed by BCA kit (Nanjing Jiancheng Bioengineering Institute, China). Equal amounts of the extracted proteins were separated on 8–15% SDS-PAGE gels, and then transferred onto PVDF membranes. Then blocked the membrane with 5% skim milk powder solution, and then incubated with diluted primary antibodies of rabbit anti-STAT1 (Cell Signaling Technology, USA), rabbit anti-p-STAT1 (Cell Signaling Technology, USA) or rabbit anti- β -actin (ABclonal Technology, China) overnight at 4 °C. Membranes were washed with 0.1% Tween-20 in Tris-buffered saline (TBS) and incubated with secondary antibodies for 2 h at room temperature with gentle shaking. The reaction was observed by chemiluminescence reagents provided with the ECL kit (Bioworld, China) and then exposed to a Chemiluminescence Imaging System (P&Q science & Technology, China), and the intensity of bolts was analyzed by ImageJ.

2.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0. The date was presented as the mean \pm SEM. Statistical was performed by SPSS 22.0 software. The neurological scores of mice in administration groups were compared with model group using the Kruskal-Wallis test. For real-time PCR, comparisons between groups were performed by Mann-Whitney *U* test. The other statistical analysis was performed using the one-way analysis of variance (ANOVA). A value (P) of < 0.05

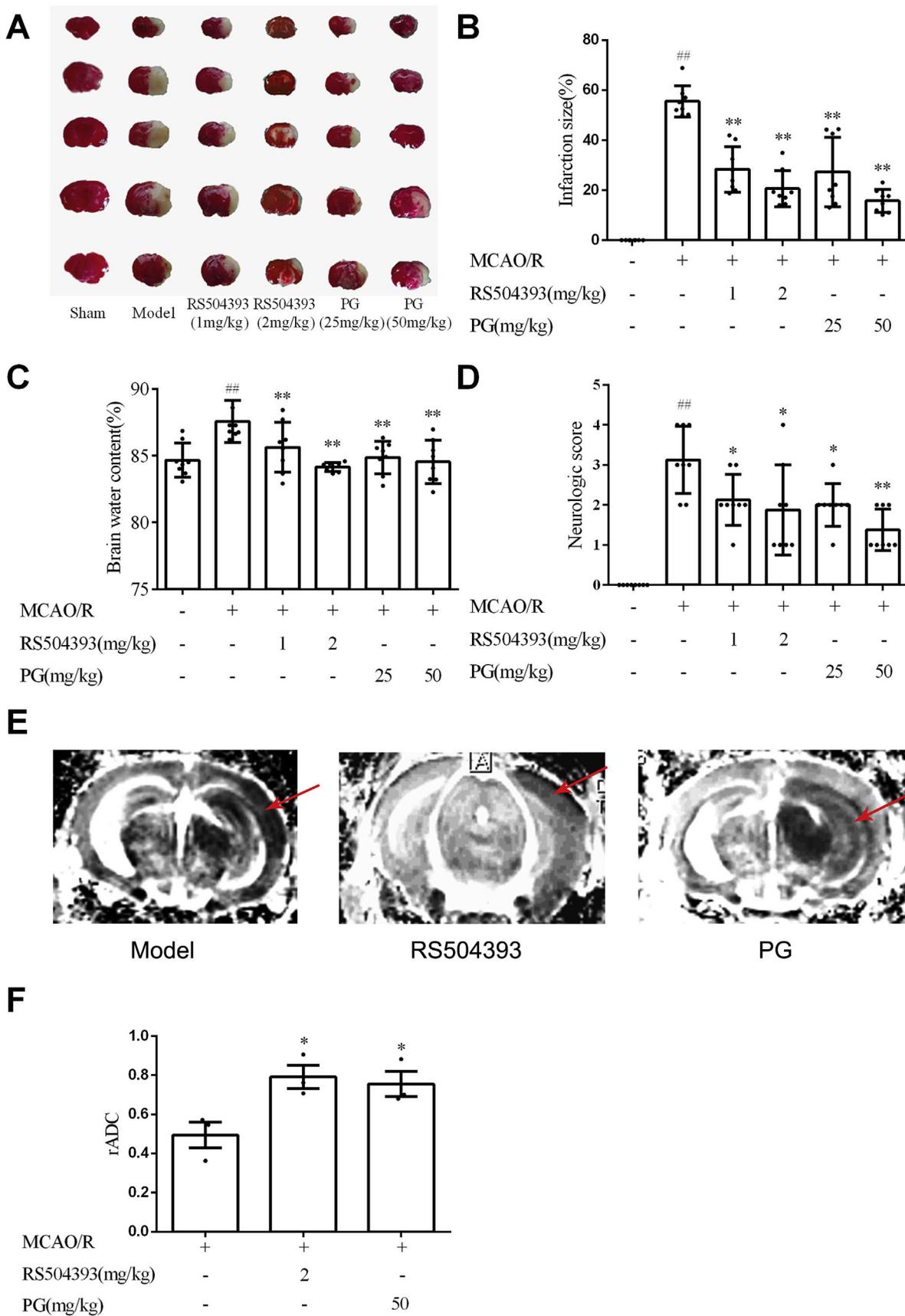


Fig. 1. PG attenuates cerebral ischemia in mice at 72 h after MCAO surgery. (A, B) Representative infarction by TTC staining, and bar graph showing infarct size expressed as percentages (n = 8). The white brain area resents infarcted tissue. (C) Brain edema estimated by brain water content (n = 8). (D) Neurologic score evaluated (n = 8). (E, F) Representative ADC map by MRI, and bar graph showing rADC value (n = 3). ^{##}P < 0.01 versus sham; ^{*}P < 0.05, ^{**}P < 0.01 versus corresponding MCAO model.

was considered statistically significant. Statistical figure was performed with Photoshop cs5, and GraphPad prism Version 6.0.

3. Results

3.1. PG protected against ischemic brain injury at 72 h after MCAO in mice

To investigate the protective effect of PG on cerebral ischemia, the infarct size of mice brains was quantified by TTC staining. Compared with MCAO mice, PG treatment at dosage of 25 and 50 mg/kg significantly reduced infarct size ($P < 0.01$) (Fig. 1A and B), alleviated brain edema ($P < 0.01$) (Fig. 1C), and improved neurologic behavioral impairment ($P < 0.05$, $P < 0.01$) of MCAO/R mice (Fig. 1D). The similar effect was also observed in RS504393 treated mice.

ADC is an indicator which can reflect the diffusion motions of water molecule in the organ. ADC changes after cerebral ischemia (Kassner et al., 2009), and is inversely proportional with the degree of brain injury. As shown in Fig. 1E and F, the rADC of RS504393 or PG treated mice was higher than model group ($P < 0.05$), which demonstrated that PG protected against ischemic brain injury in mice.

3.2. PG inhibited inflammatory cytokines releasing at 72 h after MCAO in ischemic brain

Expression of the inflammatory cytokines were increased at 72 h after stroke. However, in the presence of PG, the dramatic increase of the pro-inflammation factors (TNF- α , IL-1 β , IL-6, IL-12, IL-17, IL-23 and

IFN- γ) was blunted ($P < 0.01$, $P < 0.05$, Fig. 2A–G), and RS504393 had similar effect. Both RS504393 and PG had no effect on TGF- β and IL-10 ($P > 0.05$, Fig. 2H and I).

3.3. PG downregulated mRNA expression of pro-inflammatory cytokines at 72 h after MCAO in ischemic brain

iNOS and CD86 were selected as pro-inflammatory phenotype marker, and CD206 and Arg1 as anti-inflammatory phenotype marker. mRNA level of iNOS, CD86, CD206 and Arg1 were increased after ischemic stroke in MCAO mice brain. Compared to MCAO mice, PG treatment at 25, 50 mg/kg downregulated mRNA expression of iNOS and CD86 ($P < 0.05$, Fig. 3A and B), while had no influence on Arg1 and CD206 ($P > 0.05$, Fig. 3C and D).

3.4. PG inhibited pro-inflammatory microglia polarization at 72 h after MCAO in ischemic brain

We performed additional immunofluorescence experiments to detect microglia phenotype in ischemic area. As shown in Fig. 4A and B, pro-inflammatory marker CD16 was highly presented in Iba1 + cells in MCAO mice brain, and remarkably decreased after PG treatment ($P < 0.01$, Fig. 4C). CD206, one of anti-inflammatory phenotype markers, was increased in MCAO mice brain compared to sham ($P < 0.01$). There was no obvious difference between MCAO and PG treated mice ($P > 0.05$, Fig. 4D).

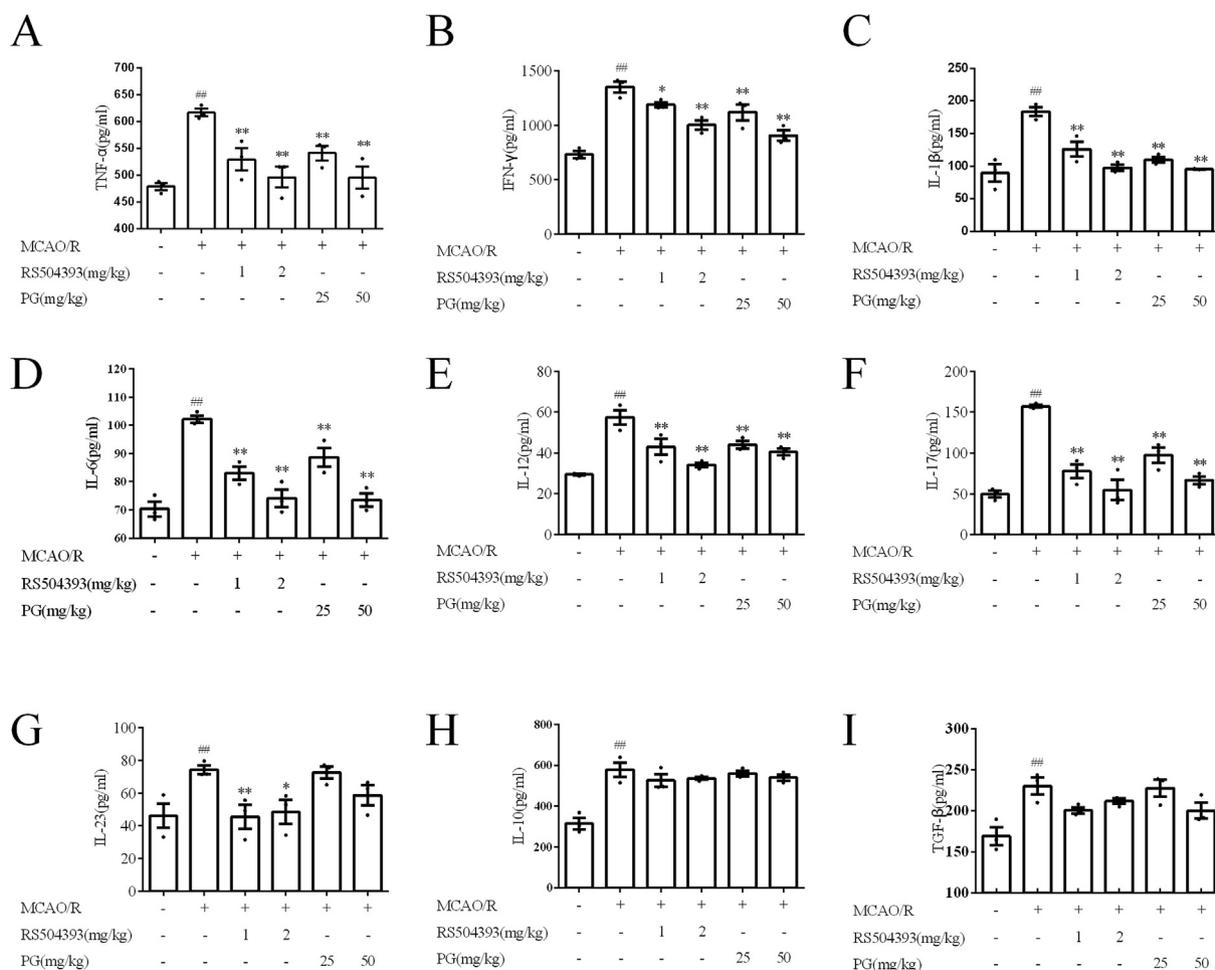


Fig. 2. PG inhibits inflammatory cytokines releasing in ischemic mice brain at 72 h after MCAO surgery. (A) TNF- α ; (B) IFN- γ ; (C) IL-1 β ; (D) IL-6; (E) IL-12; (F) IL-17; (G) IL-23; (H) IL-10; (I) TGF- β ; ## $P < 0.01$, versus sham; * $P < 0.05$, ** $P < 0.01$ versus MCAO model. $n = 3$.

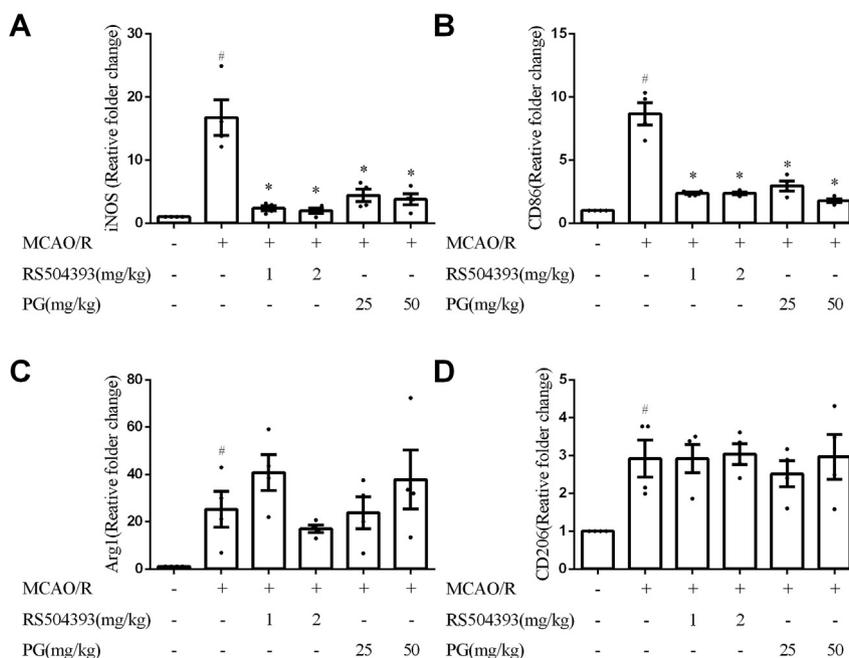


Fig. 3. PG downregulates mRNA expression of pro-inflammatory cytokines in ischemic mice brain at 72 h after MCAO surgery. (A) iNOS. (B) CD86. (C) Arg1. (D) CD206. #P < 0.05, versus sham; *P < 0.05, versus MCAO model. n = 4.

3.5. PG inhibits the phosphorylation of STAT1 at 72 h after MCAO in ischemic brain

It has been reported that STAT1 could induce macrophage/microglia pro-inflammatory polarization (Platanias, 2005), and STAT1 inhibition was required for reduction of iNOS and pro-inflammatory-related cytokines production (Gan et al., 2017). Western blot was performed to detect the effect of PG on the phosphorylation of STAT1. The results showed that the phosphorylation of STAT1 significantly up-regulated in model group after MCAO/R compared with sham group. After PG treatment, p-STAT1/STAT1 was significantly decreased compared with model group (P < 0.01, P < 0.05, Fig. 5), which indicated that the effects of PG were associated with STAT1 signal.

3.6. PG reduces pro-inflammatory cytokines releasing of BV2 cells when exposed to OGD/R injury

BV2 microglia line was used to mimic microglia in our study. The viability of cells in OGD/R group was markedly reduce (P < 0.01), while treated with PG could increase the BV2 cells survive (P < 0.01, Fig. 6A).

For inflammatory cytokines releasing, IL-6, TNF- α , IL-10 and TGF- β were increased after OGD/R injury (P < 0.01). PG incubation reduced IL-6 and TNF- α level (P < 0.01, P < 0.05, Fig. 6B, C), while had no influence on TGF- β and IL-10 level (P > 0.05, Fig. 6D, E).

For microglia polarization phenotype, mRNA expression of both pro-inflammatory and anti-inflammatory phenotype markers exhibited increase after OGD/R injury. The mRNA of iNOS and CD86 were downregulated by PG incubation (P < 0.05, Fig. 6F, G), while Arg1 and CD206 were not changed significantly (P > 0.05, Fig. 6H, I).

3.7. PG inhibits BV2 cells polarized to pro-inflammatory phenotype

To prove that PG is related to microglia polarization, LPS stimulated BV2 microglia polarized into pro-inflammatory phenotype and IL-4 stimulated BV2 cells polarized into anti-inflammatory phenotype. After stimulated with LPS, BV2 cells secreted more TNF- α and IL-6, which were inhibited by PG (P < 0.01, P < 0.05, Fig. 7A, B). LPS stimulation and PG incubation had no influence on IL-10 and TGF- β releasing

(P > 0.05, Fig. 7C, D).

IL-4 stimulation induced obvious IL-10 and TGF- β releasing, while PG had no effect on them (P > 0.05, Fig. 7G, H). IL-4 stimulation and PG incubation had no influence on TNF- α and IL-6 releasing (P > 0.05, Fig. 7E, F).

Therefore, LPS stimulated BV2 polarized into pro-inflammatory phenotype was studied to discover mechanisms. LPS stimulation induced mRNA overexpression of iNOS and CD86, which were down-regulated by PG at concentration of 3, 10 μ M (P < 0.05, Fig. 8A, B). LPS + IFN- γ stimulation induced much more mRNA expression of iNOS and CD86, and also reversed by PG incubation (P < 0.05, Fig. 8C, D).

As our western blot results, p-STAT1 and STAT1 were over expressed after LPS + IFN- γ stimulation, and inhibited by PG incubation (P < 0.01, P < 0.05, Fig. 8E, F), which were consistent with in vivo results.

4. Discussion

In present study, we found that PG treatment protects against ischemic stroke at 72 h after MCAO surgery by inhibiting pro-inflammatory microglia polarization for suppressing the expression of pro-inflammatory microglia markers and pro-inflammatory cytokines. Firstly, PG reduced infarct size, improved the neurologic score, decreased brain edema, and increased rADC. Secondly, PG inhibited pro-inflammatory cytokines mRNA expression and releasing in ischemic brain. Thirdly, PG inhibited microglia pro-inflammatory marker expression and releasing both in vivo and in vitro, and further research indicated that PG depressed microglia polarizing to pro-inflammatory phenotype through STAT1 pathway.

Previous reports showed that post-ischemic proliferation of microglia peaks at 48–72 h after focal cerebral ischemia and may last for several weeks after initial injury (Kanazawa et al., 2017). Temporal analyses of microglial phenotypes in ischemic animals demonstrated that anti-inflammatory microglia were detectable from 12 h, temporally increased at 1–3 days. On the other hand, pro-inflammatory microglia increased in the first 14 days after ischemic stroke (Hu et al., 2012; Kanazawa et al., 2017; Mabuchi et al., 2000; Perego et al., 2011). According to the reported references and our previous studies, we chose the 72 h after ischemic stroke to investigate the effect of PG on

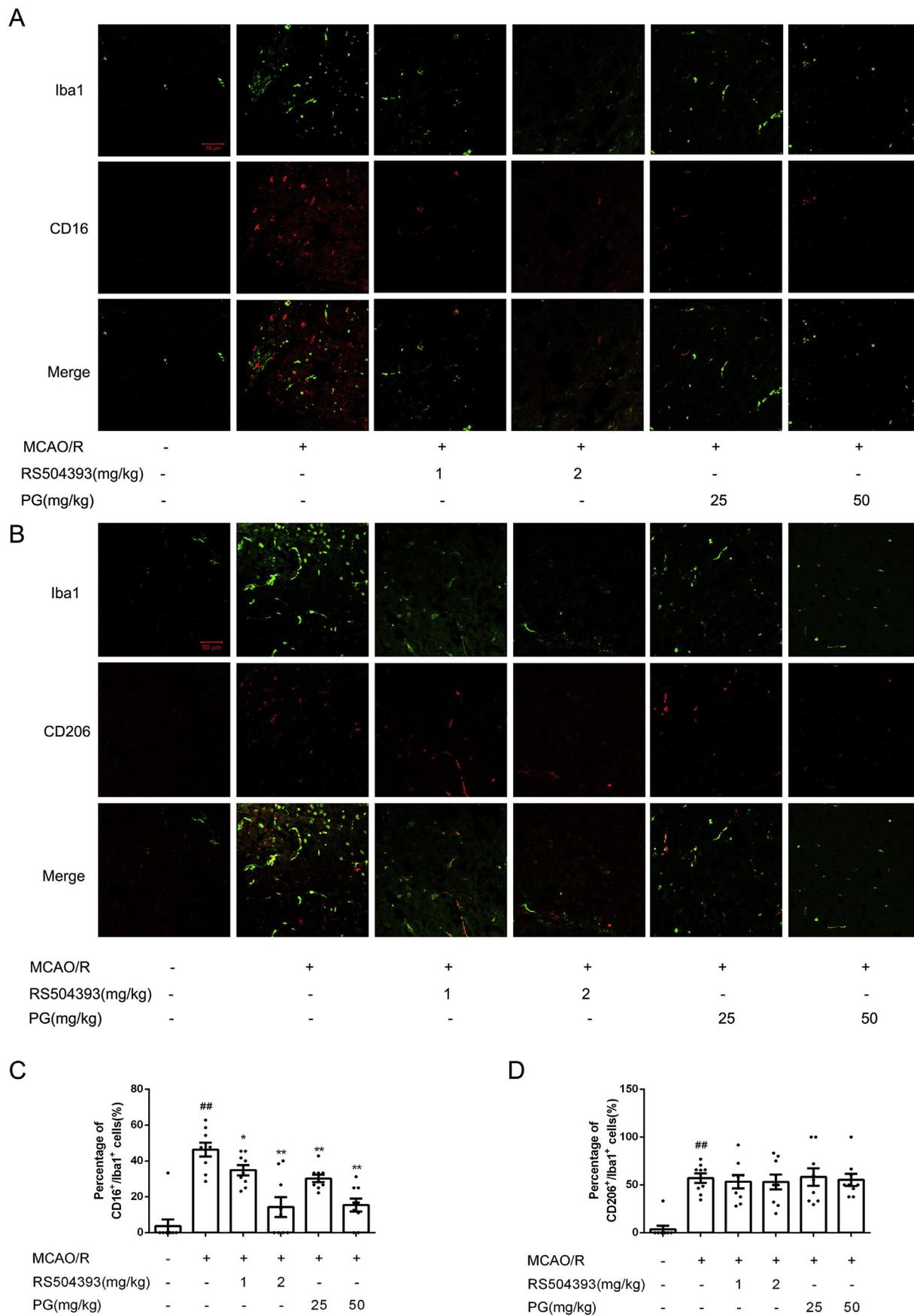


Fig. 4. PG inhibits microglia pro-inflammatory polarization in ischemic mice brain at 72 h after MCAO surgery. (A, C) Representative double-staining immunofluorescence, and bar graph showing colocalization rate of CD16 with Iba1. (B, D) Representative double-staining immunofluorescence, and bar graph showing colocalization rate of CD206 with Iba1. ##P < 0.01, versus sham; *P < 0.05, **P < 0.01, versus MCAO model. n = 3.

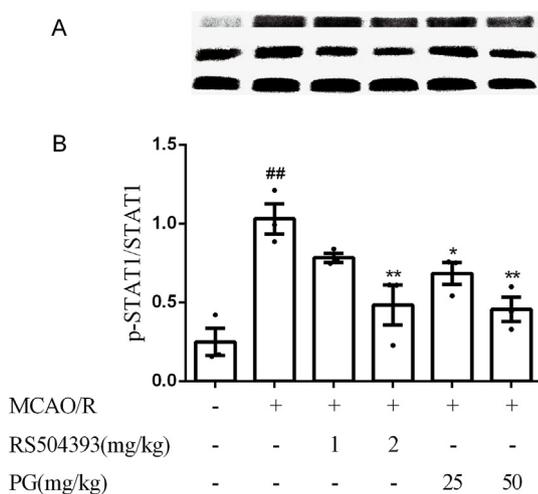


Fig. 5. PG inhibits the phosphorylation of STAT1 at 72 h after MCAO/R. (A) Representative western blot of p-STAT1, STAT1 and β -actin. (B) Bar graph showing quantification of p-STAT1 level compared to STAT1. ## $P < 0.01$, versus sham. * $P < 0.05$, ** $P < 0.01$, versus model. $n = 3$.

microglia polarization and inflammatory response.

Increasing evidences demonstrate that CCR2/CCL2 plays an important role in several central nervous system (CNS) disorders including ischemic stroke (Chu et al., 2014). Compared with WT mice, CCR2 deficient mice are reported to have smaller infarcts size, lower blood-brain barrier permeability and reduced expression of inflammatory cytokines after ischemic stroke (OB et al., 2007). In addition, in a combined radiation/traumatic brain injury model, CCR2 deficiency prevents hippocampal-dependent cognitive impairment (Hammond et al., 2014). Therefore, CCR2 inhibition may be beneficial for treatment of ischemic stroke. Consistent with this hypothesis, we have proved that PG reduces the infarct size, ameliorates the neurologic injury and brain water content at 3 days after MCAO. CCR2 inhibiting may be worthy paying more attention to reveal the therapeutic effects and mechanisms in cerebral ischemia.

Inflammation is recognized as a vital contributor to the pathophysiology after ischemic stroke (MA et al., 2010). Ameliorating inflammatory damage is an accepted therapeutic for treatment of stroke. CCL2/CCR2 mediates the migration and activation of various inflammatory cells including macrophages, microglia and astrocytes, and CCL2/CCR2 also affects the expression of various inflammatory factors in the brain. It was reported that the overexpression of inflammatory factors, like IL-1 α , IL-1 β , IL-6, IL-18, IL-17 and TNF- α , were increased in CCR2 $-/-$ mice (OB et al., 2007). Therefore, CCR2 inhibition might ameliorates CNS inflammation following stroke. Our results indicated that PG reduced the releasing of inflammatory cytokines, including IL-1 β , IL-6, IL-12, IL-17, IL-23, IFN- γ , and TNF- α .

For CNS disorder, neuroinflammatory response is marked by glia cell activation (Kim et al., 2015; Pinheiro et al., 2016). Microglia activation is the first step of inflammatory response in the brain, which was activated within minutes after ischemic brain injury, followed by infiltration of other immune cells (Jin et al., 2010; Nakajima and Kohsaka, 2004). Activated microglia produce a variety of pro-inflammatory cytokines such as TNF- α and anti-inflammatory cytokines such as TGF- β (C and J, 2012; Lambertsen et al., 2009). Therefore, activated microglia act as double-edged swords in inflammation. Whether the microglia act as a pro-inflammatory cell or an anti-inflammatory one depend on its polarization phenotype (Loane and Kumar, 2016). Searching for ways that modulate microglia phenotypes will be an effective therapeutic strategy for treatment of ischemia stroke. Inhibiting CCR2 or CCL2 would reduce the activated of microglia (Muessel et al., 2002). CCL2 could modulate microglia migrate in vitro, and the mechanism was associated with MEK, ERK1/2, and Akt

pathway (Bose et al., 2016).

There is no clear immunohistochemical antibody being able to distinguish macrophage with microglia (Ma et al., 2017). The number of macrophage in CCR2 deficient mice brain dramatically decrease compared to the WT mice (Schilling et al., 2009), and using anti-CCR2 antibody also block the macrophage infiltrate into the brain (Wattananit et al., 2016). After CCR2 inhibited by PG, an influx of blood-derived macrophages was significantly diminished in brain of mice. Therefore, marked cells by Iba1 were mainly microglia. We found that PG reduced percentage of CD16+/Iba1+ cells which mark pro-inflammatory microglia, and has no effect on CD206+/Iba1+ cells which mark anti-inflammatory microglia. It has been reported that the cells expressing CD16 other than Iba-1+ microglia include monocytes (Badrnya et al., 2014), NK cells (Grudzien and Rapak, 2018), neutrophil (Liverani et al., 2013), etc. Therefore, we speculated that the CD16+ cells that are not Iba-1 positive in immunofluorescence data may be monocytes, neutrophil, or NK cells. Real-time PCR experiment also suggested that PG decreased mRNA expression of pro-inflammatory markers iNOS and CD86, while no obvious effect on anti-inflammatory markers CD206 and Arg1. Consistently, in vitro real-time PCR results have demonstrated that PG reduced mRNA expression of pro-inflammatory phenotype markers iNOS and CD86, while had no influence on anti-inflammatory phenotype markers CD206 and Arg1 after OGD/R injury.

Our results in vivo showed that PG inhibited pro-inflammatory cytokines expression, suppressed pro-inflammatory microglia polarization and ameliorated inflammatory response after ischemic stroke. Therefore, we focused on inflammatory response of microglia in vitro to demonstrated the effect of PG on inflammatory mediators releasing and mRNA expression. LPS activation of immortalized murine BV2 microglia has been widely used as an in vitro model to study the pro-inflammatory and neurotoxic factors released from activated microglia (Zheng et al., 2018). Therefore, we used LPS stimulated BV2 cells to investigated the effect of PG on pro-inflammatory cytokines release and expression of BV2 cells.

Microglia polarization could be affected by the time and severity of injury, aging and microenvironment (Ma et al., 2017). The signal transducer and activator of transcription (STAT) family members, such as STAT1, STAT3 and STAT6, have crucial roles in regulating pro- or anti-inflammatory gene expression and the phenotypic switching of microglia (Hu et al., 2014). In IFN- γ -stimulate pro-inflammation polarization microglia/macrophage, the decrease of phosphorylation of STAT1 would result in dramatically decreasing of pro-inflammatory cytokine IL-6, IL-1 β , and TNF- α , which suggests that STAT1 is related to microglia pro-inflammation polarization (Gan et al., 2017). In our study, PG downregulated expression of p-STAT1 and STAT1 in MCAO/R mice and in BV-2 cells stimulated with LPS (100 ng/ml) + IFN- γ (20 ng/ml), which indicated that PG decreased pro-inflammatory microglia polarization through STAT1 pathway.

It should be emphasized that CCR2 inhibition has been proved to have potential in treatment of ischemia stroke (Chu et al., 2014). Apart from the anti-inflammatory effect of CCR2 inhibition, it also improves blood brain barrier permeability. However, the study revealed that CCR2 inhibitor INCB3344 may be poisonous to stroke recovery, which exhibiting larger infarct volumes and worse functional outcomes at 24 h after stroke (Chu et al., 2014). The mechanism may be relative to inhibition of Ly6Chi monocytes infiltrate to brain, which would polarize to anti-inflammatory phenotype. Therefore, the effects and mechanisms of CCR2 inhibition against ischemic injury is complex, and further study is necessary for us to address.

5. Conclusion

According to the present study, CCR2 inhibitor PG inhibited pro-inflammatory microglia polarization and improved tissue repair and recovery after MCAO through STAT1 pathway. This finding broadens

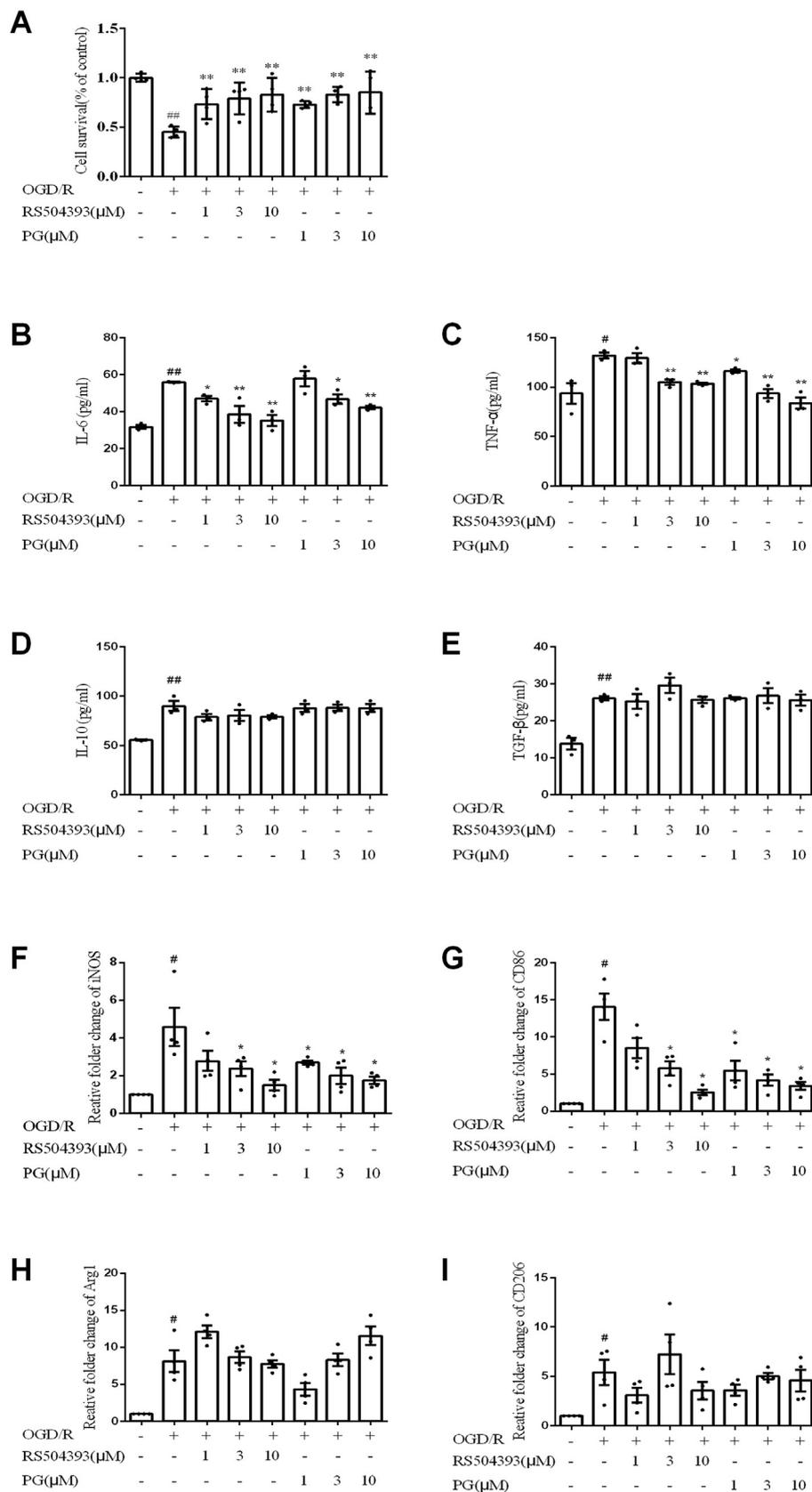


Fig. 6. PG protects against BV2 cells from OGD/R injury. (A) MTT assay. (B) IL-6 releasing (n = 3). (C) TNF- α releasing (n = 3). (D) IL-10 releasing (n = 3). (E) TGF- β releasing (n = 3). (F) mRNAs expression of iNOS (n = 4). (G) mRNAs expression of CD86 (n = 4). (H) mRNAs expression of Arg1 (n = 4). (I) mRNAs expression of CD206 (n = 4). ##P < 0.01, #P < 0.05, versus sham; *P < 0.05, **P < 0.01, versus model.

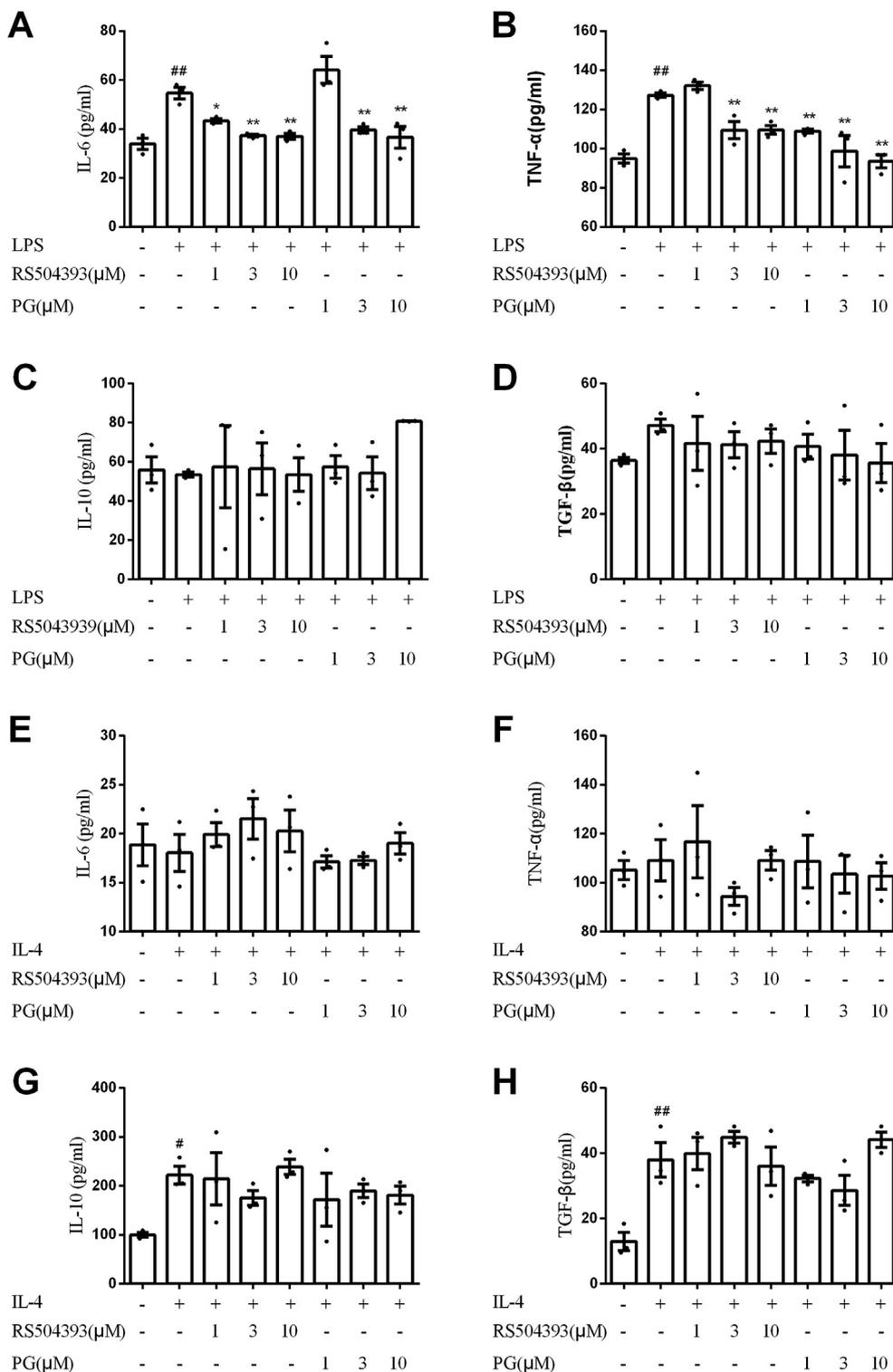


Fig. 7. PG inhibits BV2 cells polarized to pro-inflammatory phenotype. (A) IL-6 releasing after LPS stimulation. (B) TNF-α releasing after LPS stimulation. (C) IL-10 releasing after LPS stimulation. (D) TGF-β releasing after LPS stimulation. (E) IL-6 releasing after IL-4 stimulation. (F) TNF-α releasing after IL-4 stimulation. (G) IL-10 releasing after IL-4 stimulation. (D) TGF-β releasing after IL-4 stimulation. ##P < 0.01, #P < 0.05, versus sham; *P < 0.05, **P < 0.01, versus model. n = 3.

our understanding on the mechanisms and the effects of CCL2/CCR2 on cerebral ischemic and CNS neuroinflammation, which indicated that CCL2/CCR2 could be a target for stroke treatment.

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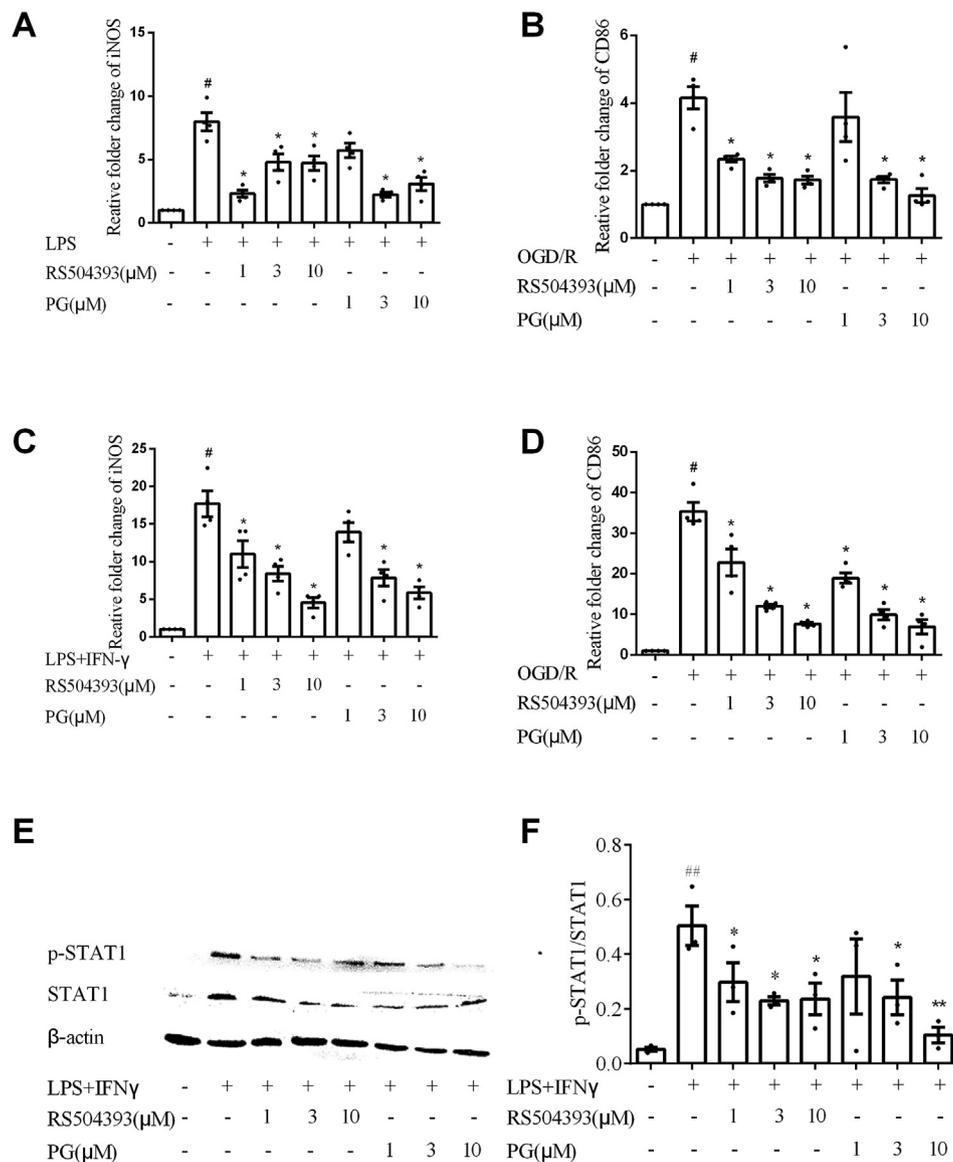


Fig. 8. PG inhibits pro-inflammatory BV2 cells polarization through downregulating p-STAT1/STAT1. (A) mRNA expression of iNOS after LPS stimulation. (B) mRNA expression of CD86 after LPS stimulation. (C) mRNA expression of iNOS after LPS + IFN- γ stimulation. (D) mRNA expression of CD86 after LPS + IFN- γ stimulation. (E, F) Representative western blot of p-STAT1 and STAT1, and bar graph showing quantification of p-STAT1 level compared to STAT1. #P < 0.05, ##P < 0.01, versus sham. *P < 0.05, **P < 0.01, versus model. n = 3.

Disclosures

The authors have no conflicts of interest to declare.

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