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ORIGINAL ARTICLE

Ginkgolide B Protects Against Ischemic Stroke Via Modulating Microglia Polarization in Mice

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SUMMARY

Aim: Ginkgolide B (GB) has shown neuroprotective effect in treating ischemic stroke, related to its property of anti-inflammation. Nevertheless, it is unclear whether GB is able to modulate microglia/macrophage polarization, which has recently been proven to be vital in the pathology of ischemic stroke. **Methods:** We performed transient middle cerebral artery occlusion (tMCAO) on C57BL/6J male mice and induced cultured BV2 microglia and primary bone marrow-derived macrophages to be M1/2 phenotype by LPS+ interferon-γ and IL-4, respectively. Immunofluorescence and flow cytometry were used for detecting the specialized protein expression of M1/2, such as CD206 and CD16/32. qPCR was utilized to detect the signature gene change of M1/2. **Results:** GB significantly reduced cerebral ischemic damage and ameliorated the neurological deficits of mice after tMCAO. More importantly, our experiments proved that GB promoted microglia/macrophage transferring from inflammatory M1 phenotype to a protective, anti-inflammatory M2 phenotype *in vivo* or *vitro*. CV3988 and silencing the platelet activator factor (PAF) receptor by siRNA demonstrated that PAF receptor was involved in the modulation of microglia/macrophage polarization. **Conclusion:** Our results reveal a novel pharmacological effect of GB in modulating microglia/macrophage polarization after tMCAO, thus deepening our understanding of neuroprotective mechanisms of GB in treatment of ischemic stroke. Furthermore, this new mechanism may allow GB to be used in many other microglia/macrophage polarization-related inflammatory diseases.

Introduction

Ischemic stroke which accounts for approximate 85% of the stroke is the second leading cause of death and leads to severe sequelae such as paresis and speech defects with limited effective therapies in the clinical treatment [1]. A cascade of energy failure, excitatory toxicity, and inflammation triggers a severe loss of neurons and massive activation of the resident microglia and infiltrated macrophages following cerebral ischemia [2–4]. Recent studies have demonstrated that these immune cells serve as the first defensive line to modulate central nervous system repair and also mediate inflammatory cascades after ischemia [5,6]. Therefore, the activated microglia/macrophage play an indispensable role in the pathological progress of ischemia and act as an important therapeutic target of ischemic stroke [4,7].

Microglial activation can be classified into two major phenotypes defined as "classical activation" (also termed M1 phenotype or overactivated phenotype) and "alternative activation" (M2 phenotype) [8]. M1 microglia polarization is associated with the production and release of multiple proinflammatory cytokines such as tumor necrosis factor (TNF-α) and interleukin-6 (IL-6). In general, the released factors act in tissue defense and promote the destruction of pathogens [9]. However, the extensive production of inflammatory cytokines by overactivated or dysregulated microglia is constantly involved in the pathogenesis of stroke and induces more widespread damage to ischemic penumbra neurons [10]. In contrast to the M1 phenotype, M2 microglia execute an anti-inflammatory effect and promote wound healing and tissue repair [10]. Several anti-inflammatory cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β), are shown to alleviate proinflammatory responses and enhance the expression of genes that are involved in tissue recovery and repair [11]. In addition, it has been known that microglia/macrophage polarization changes dynamically after the stroke. Microglia/macrophage move quickly to the injury site, displaying M2 phenotype at the initial 3 days following transient middle cerebral artery occlusion (tMCAO), and then the deleterious M1 type will be dominant from poststroke 7 days [12]. Thus, switching microglia from M1 type toward M2 type may be an effective therapeutic strategy for treatment of ischemic stroke. Searching for drugs which are able to modulate microglia phenotypes will broaden our strategies on treating ischemic stroke.

Ginkgolide B (GB), an essential abstract of Ginko biloba, is a potent platelet activator factor (PAF) receptor antagonist [13]. GB has extensive biological and pharmacological effects, such as antiallergy, anti-inflammation, and antioxidation [14,15]. Recent studies have demonstrated that GB exhibits neuroprotection in Parkinson's disease (PD) via anti-inflammation and in ischemic stroke by reducing the degradation of membrane phospholipids [16,17]. In clinical therapeutics, GB is commonly used for improving the functional recovery of patients with ischemic stroke during the recovery phase, but the underlying mechanisms of GB against ischemic damage have not been fully explored yet [18].

In this study, we explored the neuroprotective mechanism of GB in tMCAO model. We demonstrated that GB promoted microglia/macrophage transition toward M2 phenotype. Consistently, *in vitro* experiments showed that GB increased the expression of M2-related signature genes and the cytokines in LPS+ interferon-γ (IFN-γ) or IL-4 stimulated BV2 and BMDM. Moreover, this modulation effect is related to the inhibiting PAF receptor. These results have revealed a novel pharmacological mechanism of GB that protects against ischemic stroke via modulating microglia polarization.

Materials and Methods

Animals

Experiment was conducted on adult C57BL/6J male mice (aged 2–3 months), weighing approximate 25 g which were bought from Comparative Medicine Centre of Yangzhou University. Mice were kept under controlled environment of 22°C, relative humidity of 65%, and 12-h day–night cycle, with free access to water and food. All the experiment and procedures were performed under the guidelines of the Institutional Animal Care and Use committee of Nanjing Medical University. All efforts were made to minimize the use of mice and their suffering.

tMCAO and Drug Treatment

Briefly, the mice were anesthetized with 1.5% isoflurane in 30% oxygen and 70% nitrous oxide, and they were put on the warming pad to keep the body temperatures at 37.0 ± 0.5°C. The right common carotid artery, pterygopalatine artery, and external carotid artery were carefully dissected. A 6-0 silicone-coated nylon monofilament (6023PK; Doccol Corp, Sharon, MA, USA) was then delivered to the right internal carotid through the external carotid stump. Focal cerebral blood flow was measured by laser Doppler flowmetry (Moor VSM-LDF, Wilmington, DE, USA). When the blood flow has reduced by 80%, the thread was fixed. After the 1-h occlusion, the monofilament suture was removed, caused the reperfusion. The sham-operated mice were all treated similarly, except for occlusion.

One hour after surgery, the mice were injected with GB at different dosages (1.75 mg/kg, 3.5 mg/kg, and 7.0 mg/kg, ip, bid) and then sacrificed on 3 and 7 days later, respectively.

Neurobehavioral Evaluation

The neurobehavioral evaluation was performed at 24 h after surgery, according to the published article [19]. The neurological scores were 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.

Rotarod Test

The rotarod test is used for testing the motor ability of the mice after tMCAO insult. The mice were placed on rungs of the rotarod to avoid dropping. And the rotarod will accelerate from 4 revolutions/min to 40 rpm in 1 min at a constant rate. A trial ended if the mouse fell off the rungs or gripped and spun around for one complete revolution. Mice were acclimatized to the rotarod for 3 days before surgery until the latency time keep stable. On each day of testing, mice underwent three trials on the rotarod, with an interval of 1 min. The mean latency time was used to show the functional recovery of the mice.

Histological Assessment of the Brain Damage

We evaluated acute infarct volumes 3 days after tMCAO using triphenyl-2,3,4-tetrazolium-chloride (TTC; Sigma, St Louis, MO, USA) histology. The brains were sliced to be 1-mm sections and incubated in 2% TTC solutions for 30 min in the dark. Then, the reaction was stopped by 4% paraformaldehyde and fixed overnight. The infarct area was quantified by IMAGEJ program National Institutes of Health, Bethesda, MD, USA and calculated and exhibited in mm^3.

Brain Tissue Preparation

On 3 and 7 days after tMCAO, the mice were deeply anaesthetized with chloral hydrate (3.5 mg/kg) and then transcardially perfused with saline to wash the blood out of the body, followed by fixation with buffered paraformaldehyde (4%). The brains were collected and dehydrated in 30% sucrose solution for a week then sliced at 30 μm thickness in ice-cold phosphate-buffered saline (PBS) utilizing a vibrating microtome (Leica CM1950, Nussloch, Germany). The sections were stored at −20°C for later use.

Immunofluorescence

Brain slices or cultured coverslips were washed by PBS and incubated with 5% bovine serum albumin (BSA) and then incubated at 4°C overnight with following primary antibody: rat anti-CD16/32 (BD Biosciences Pharmingen, San Jose, CA, USA, 553142,1:50), goat anti-CD206 (R&D Systems, Minneapolis, MN, USA, AF2535, 1:50), rabbit anti-Iba1 (Wako, Tokyo, Japan, 019-19741, 1:1000), goat anti-NeuN (Millipore, Temecula, CA, USA, MAB377). And then the slices were incubated with corresponding secondary antibody for 1 h at room temperature. Finally, they were dyed with Gold antifade reagent with DAPI (Life, Carlsbad, CA, USA, P36930). For *in vivo* experiments, every 6th section was collected for quantification. Every section contained 10 preassigned fields in the striatal penumbra that were adjacent to the ischemic core. The data are expressed as the colocalization rate of CD16/32 versus Iba1 or CD206 versus Iba1. For cell culture experiments, primary cortical neuron and primary microglia were fixed in 4% paraformaldehyde in PBS for 10 min. Cells were then blocked

with 5% BSA containing 0.1% Triton X for 1 h. After that, the cells were incubated with CD16/32(1:200) or MAP2 (CST, Boston, MA, USA, 4542S, 1:500) overnight at 4°C. Afterward, the cells were incubated with corresponding secondary antibody for 1 h at room temperature followed by incubation with 1 μg/mL Hoechst 33342 (Invitrogen, Santiago, CA, USA) for 10 min. Images were captured by fluorescence microscope.

Real-Time PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, 60296010). Reverse transcription of total RNA was performed using TaKaRa Master Mix (TaKaRa, Nojihigashi, Shiga, Japan). The primers were purchased and validated from Gene; real-time qPCR was performed using SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in a Step One Plus instrument (Applied Biosystems). The primers used for qPCR were as follows:

Inducible nitric oxide synthase (iNOS) forward: AATGGCAA CATCAGGTCGGCCATCACT
iNOS reverse: GCTGTGTGTCACAGAAGTCTCGAACTC
Chemokine (C–C motif) ligand 3 (CCL3) forward: ATG CAAGTTCAGCTGCCTGC
Chemokine (C–C motif) ligand 3 (CCL3) reverse: ATGCCGTG GATGAACTGAGG
TNF-α forward: CATCTTCTCAAAATTCGAGTGACAA
TNF-α reverse: TGGGAGTAGACAAGGTACAACCC
Arginase 1 (Arg1) forward: GAACACGGCAGTGGCTTTAAC
Arginase 1 (Arg1) reverse: TGCTTAGTTCTGTCTGCTTTGC
CD206 forward: GCAGGTGGTTTATGGGATGT
CD206 reverse: GGGTTCAGGAGTTGTTGTGG
Ym1 forward: AGAAGGGAGTTTCAAACCTGGT
Ym1 reverse: GTCTTGCTCATGTGTGTAAGTGA

Flow Cytometry

Culture medium was aspirated, and BV2 cells were rinsed with D-Hank's solution, digested, and centrifuged softly. The cells were then incubated with the following antibodies: PE-conjugated anti-mouse MGL1/2 (CD301a/b) (R&D, FAB42979), PE-conjugated anti-mouse CD197 (CCR7) (Ebioscience, San Diego, CA, USA, 12-1971-82), and determined using flow cytometry (Guava Easy Cyte™ 8; Millipore).

Cells Culture and Treatment

The cortical neurons were performed according to our previously described protocol [20]. Then, they were continually cultured for 4 days followed by oxygen glucose deprivation (OGD) or drug treatments.

For cultured microglia, glial cells that were obtained from 1-day-old pups (sex unknown) were cultured for 12–14 days to form a confluent monolayer. Microglia were then shaken off, collected, seeded, and cultured for 1–2 days before drug treatment.

For production of primary macrophages, bone marrow cells were flushed from C57BL/6J male mice (aged 2–3 months, 25 g) femurs and incubated for 7 days with medium containing GM-CSF.

BV2 cell lines were purchased from ATCC and cultured in DMEM supplemented with 10% fetal bovine serum and 4 mM glutamine. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2.

For M1 induction, lipopolysaccharide (LPS) (100 ng/mL) and IFN-γ (20 ng/mL) were added to the cells for 24 h. For M2 induction, interleukin-4 (IL-4) (20 ng/mL) was added to the culture for 24 h. LPS (L4516-1MG) is purchased from Sigma and IFN-γ (AF-315-05) and IL-4 (AF-214-14) are from Pepro-Tech (Suzhou, Jiangsu Province, China).

Conditioned Medium Experiment

The medium was collected from the M1 primary microglia or GB-treated M1 counterparts. Cortical neurons from embryonic mice were cultured for 11 days and then treated with normal medium or the condition medium from the above primary microglia after they were subjected to 60 min OGD. The neurons were incubated with condition medium for 24 h, and then immunofluorescence staining for MAP2 was conducted.

Phagocytosis Experiment

The conditional medium from OGD neuron was collected and administrated to the primary microglia for 24 h. The 2-μm fluorescent microspheres (Invitrogen) were added into microglial cultures for 1 h.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5.0 (San Diego, CA, USA). The data were presented as the means ± SEM. The *in vitro* assays were performed at least three times to confirm the reproducibility. Statistical difference was calculated using ANOVA. A value (P) of <0.05 was considered statistically significant.

Results

Ginkgolide B Significantly Attenuated Infarct Damage and Neurological Deficits in Mice

To investigate the neuroprotective effect of GB in ischemic stroke, mice were treated with saline and different concentration of GB (1.75, 3.5, and 7.0 mg/kg) at 1 h after tMCAO for 3 days, respectively [12]. GB at 3.5 and 7.0 mg/kg significantly ameliorated the infarct volume of tMCAO mice ($P_{(3.5\ mg/kg)} = 0.00325$, $P_{(7.0\ mg/kg)} = 0.000523$; Figure 1A,B). Treatment with GB (≥3.5 mg/kg) also significantly reduced the water content of brain tissue ($P_{(3.5\ mg/kg)} = 0.0316$, $P_{(7.0\ mg/kg)} = 0.00825$; Figure 1C). Furthermore, the neurological deficits based on Bederson scoring were markedly reduced following GB treatment ($P_{(3.5\ mg/kg)} = 0.00599$, $P_{(7.0\ mg/kg)} = 0.00294$; Figure 1D). In addition, we conducted the rotarod test to evaluate the motor ability of the mice at 7 days after tMCAO. As shown in Figure 1E, the rotarod test showed that the latency time was significantly increased with GB (3.5 mg/kg) treatment ($P_{(3.5\ mg/kg)} = 0.000722$, $P_{(7.0\ mg/kg)} = 0.003126$; Figure 1E). Taken together, these results indicated that GB ameliorated ischemic stroke injury.

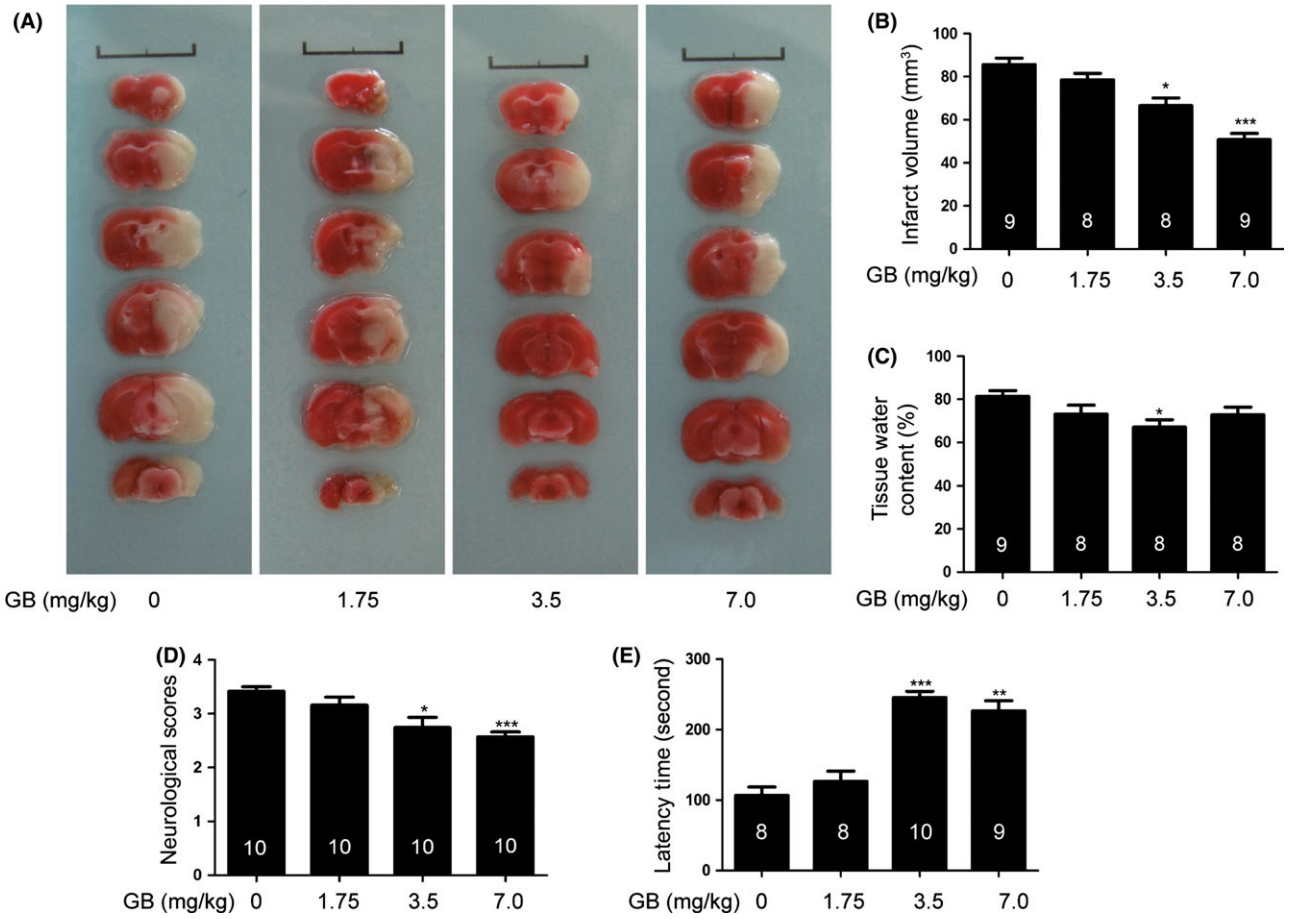

Figure 1 Ginkgolide B (GB) significantly attenuates cerebral ischemia and neurological deficits *in vivo*. (**A, B**) Representative pictures and quantification for analysis of the effect of GB on infarct volume. Scale: 1 cm. The white brain area presents infarcted tissue. (**C**) The effect of GB on the cerebral water content. (**D**) The effect of GB on neurological function. (**E**) Recovery of motor ability was assessed in ischemic mice by rotarod tests during a 7-day follow-up after transient middle cerebral artery occlusion (tMCAO). The brain sections were collected 72 h after I/R. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus corresponding tMCAO group. Data are shown as mean ± SEM, n = 8–10.

Ginkgolide B Promoted Microglia/Macrophage Polarization Toward M2 Polarization in tMCAO Model

As polarization of microglia/macrophage changes dynamically following the pathological progression of ischemia stroke [12], we chose two time points (3 and 7 days after the injury) to observe whether GB was able to influence the polarization at the penumbra field (Figure S1). As shown in Figure 2A, CD206, a M2 phenotype signature chemokine, was increased in GB-treated group at either 3 or 7 days after tMCAO. At the meantime, CD16/32, which is the M1 phenotype biomarker, was reduced significantly by GB treatment. To confirm this modulation, we counted the colocalization of Iba1 and CD16/32 (or CD206) at these two time points and calculated the colocalization rates, respectively (Figure 2B, C). Apart from the immunofluorescence, we performed additional qPCR and ELISA experiments to detect mRNA expression and release of proinflammatory cytokines in tMCAO model. GB increased M2-related mRNA expression, such as CD206, Arg1, and Ym1 (Figure 2D,E) and promoted the levels M2-related cytokines including IL-10 and TGF-β (Figure 2H,I) either at 3 or 7 days after the insult. Accordingly, GB decreased M1-related mRNA expression, such as CCL3, iNOS, and TNF-α (Figure 2F, G), and the release of M2-related cytokines including IL-6 and TNF-α (Figure 2J,K) at 3 or 7 days after tMCAO. Together, these results suggested that GB promoted microglia/macrophage polarization to M2 phenotype after tMCAO.

Ginkgolide B Facilitated Microglia/Macrophage M2 Polarization *In Vitro*

To further affirm the effect of GB on microglia/macrophage polarization, we cultured BV2 microglia cell line and BMDM (Figure S3). BV2 were treated with LPS (100 ng/mL) + IFN-γ (20 ng/mL) for 24 h to induce M1 phenotype, and IL-4 (20 ng/mL) was used for M2 induction for the same period. As the post-OGD microcircumstance drives the microglia to M1 polarization [12], we examined whether GB promoted polarization of microglia from M1 phenotype toward M2 phenotype (Figure S2). We first investigated the different concentration of GB (0.1, 1 and 10 μM)

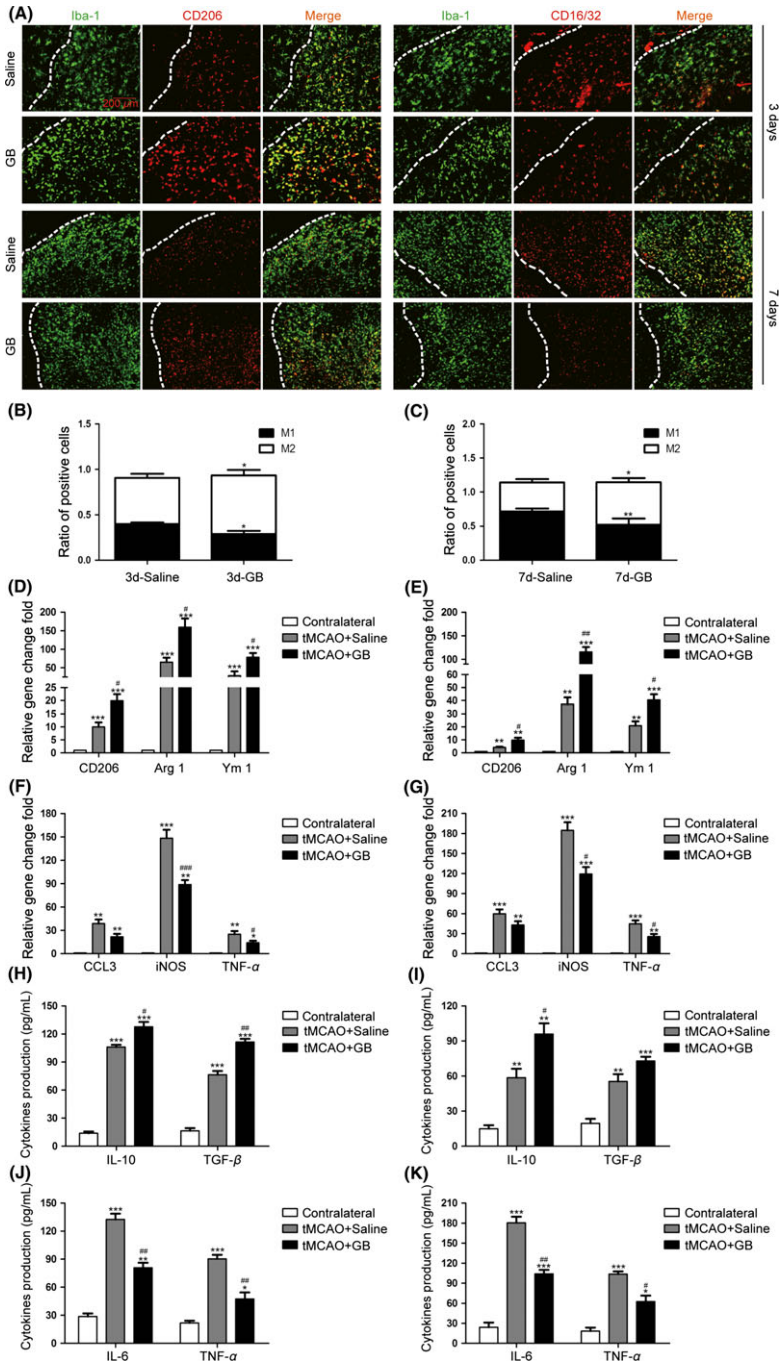

Figure 2 Ginkgolide B (GB) promotes microglia/macrophage polarize toward M2 polarization in transient middle cerebral artery occlusion (tMCAO) model. (**A**) Representative double-staining immunofluorescence of CD206 or CD16/32 and Iba1 and on brain sections obtained from ischemic mice at 3 days and 7 days after tMCAO. The observation area was identified according to Figure S1. (**B**) The immunofluorescence on poststroke 3-day mice brain slice showed the colocalization rate of CD16/32 or CD206 with Iba1. (**C**) Similarly, the colocalization rate of CD16/32 or CD206 with Iba1 in poststroke 7-day slice was shown. (**D, E**) Representative M2-related mRNAs change folds at 3 and 7 days after tMCAO, respectively. (**F, G**) M2-related cytokines (transforming growth factor-β, IL-10) detected by ELISA at 3 and 7 days after tMCAO, respectively. (**H, I**) Representative M1-related mRNAs change folds detected by qPCR at 3 and 7 days after tMCAO, respectively. (**J, K**) M1-related cytokines (TNF-α, IL-6) detected by ELISA at 3 and 7 days after tMCAO, respectively. For immunofluorescences, data are represented as mean ± SEM, n = 6, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus tMCAO + saline group. For ELISA and qPCR experiment, data are represented as mean ± SEM, n = 3–4, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus contralateral tissue; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ versus tMCAO + saline group.

on BV2 polarization using the flow cytometry to detect the positive percentage of M2 biomarker MGL1/2 and M1 biomarker CCR7, respectively (Figure 3A,E). The data indicated that GB at 1 μM concentration is the most effective for modulating M1/2 gene expression. M2-specialized gene MGL1/2 was upregulated and M1-specialized chemokine CCR7 was downregulated significantly (Figure 3B,F). And 1 μM GB-treated group exhibited high mRNA expression of M2 genes (Arg1, CD206, and Ym1) and secreted higher M2-related cytokines (TGF-β, IL-10), compared with IL-4 group (Figure 3C,D). Meanwhile, GB not only significantly inhibited expression of M1 signature genes including iNOS, TNF-α, and CCL3, but also reduced production of inflammatory cytokines such as IL-6 and TNF-α (Figure 3G,H). Taken together, *in vitro* data demonstrated that GB promoted M2 polarization and inhibited M1 polarization of either BV2 or BMDM.

PAF Receptor was Involved in the Microglia Polarization-Modulating Effect of GB

It has been demonstrated that PAF receptor is a necessary element of inflammatory cascade, for example PAF is indispensable for NF-κB activation in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD model [16]. As GB is the selective antagonist of PAF receptor, we investigated whether PAF receptor was involved in the modulating effect of GB on BV2. By comparing CV3988, another PAF receptor antagonist, we found that GB exerted similar effect with CV3988, such as increasing M2 signature genes expression (Figure 4A–C) and reducing M1 genes expression (Figure 4I–K). In addition, GB and CV3988 increased TGF-β and IL-10 secretion (Figure 4D,E) and decreased IL-6 and TNF-α (Figure 4D,E). This suggested that PAF receptor might be involved in the polarization modulation effect of GB. To verify the modulating role of PAF receptor, we silenced PAF receptor expression on BV2 by siRNA. And as expected, silencing PAF receptor exhibited the M2 promotion effect and M1 inhibition effect, manifested by mRNA expression (Figure 4F–H,N–P). Therefore, we drew the conclusion that PAF receptor was involved in the microglia polarization modulation of GB.

GB Exhibited Protective Effect on OGD Neuron via Modulating Microglia/Macrophage Polarization

To clarify the neuroprotective role of modulating microglia/macrophage polarization, we cultured primary cortical microglia and primary cortical neurons. The post-OGD neurons were incubated with the conditioned medium (CM) collected from different groups of primary microglia. As shown in Figure 5A, the CM from GB-pretreated M1 microglia attenuated OGD-induced neuron damage compared to M1 microglia CM group, revealed by increasing the length of MAP2-positive neuritis (Figure 5C). In addition, we investigated microglial phagocytosis influenced by post-OGD neuronal CM. When the primary microglia were pretreated with GB, it phagocytized more fluorescent microspheres than merely administrated with OGD neuronal CM (Figure 5B,D). In summary, the above data suggested that GB ameliorated neuronal condition by modulating microglial polarization and improved microglial phagocytosis, which might contribute to brain functional recovery after ischemic stroke.

Discussion

With the properties of antioxidation, anti-inflammation, GB has been proven to be an effective neuroprotective agent in various ischemic stroke models [21,22]. In the present study, we have shown that GB exerts neuroprotective effect either during the acute phase (3 days) or recovery phase (7 days) of ischemic stroke. Moreover, this neuroprotection is related to its promoting effects on M2 polarization of resident microglia and infiltrated macrophages. We also have revealed that inhibition of PAF receptor is necessary for GB promoting microglia/macrophage polarization toward M2 phenotype.

As mentioned above, stroke is a severe CNS disease which has limited therapies [23]. Up to date, recombinant tissue plasminogen activator is the only clinical treatment that is approved by the United States Food and Drug Administration. Nevertheless, the extremely narrow therapeutic time window and short half-time confine its application [24,25]. Thus, finding effective drugs for stroke is of emergency. In recent years, increasing evidences suggest that GB plays a neuroprotective role in several CNS disorders, especially in ischemic stroke [16,26]. For example, the infarct volume and the neurological deficits are ameliorated significantly by administrating GB (6 mg/kg) in MCAO mouse model [22]. Consistent with these findings, we have proved that GB (3.5 mg/kg) reduces the infarct volume by approximate 30% and ameliorates the neurological deficits of mice by about 20% following tMCAO. More than that, clinical trials show that GB (50 mg) administration for 2 weeks improves the neurological deficits scores of patients with ischemic stroke in the recovery phase. Therefore, as a promising neuroprotective drug, GB is worthy paying more attention to reveal its therapeutic effects and mechanisms in ischemic stroke.

Numerous studies have shown that GB has considerable anti-inflammatory roles in varied pathological conditions [15]. For example, Liu et al. reported that GB reduces atherogenesis and vascular inflammation via inhibition of PI3K/Akt pathway in activated platelets [27]. And in neuroinflammation induced by ischemic stroke, GB exhibits neuroprotection through anti-inflammatory and antiapoptotic effect by inhibition of TLR4/NF-κB-dependent inflammatory responses [28,29]. Because TLR4/NF-κB signaling pathway is involved in many kinds of physical and pathological progress, such as inflammation and apoptosis, we assume that GB may have other pharmacological effects in disorders. For CNS disorders, the most conspicuous common feature is neuroinflammatory response, which is marked by glia cell activation [30,31]. And it has been noticed recently that activated microglia/macrophage act as double-edged swords, which depend on their phenotypes [32]. For instances, the M2 phenotype improves neuronal survival and tissue repair, while M1 phenotype accelerates the death of neurons and aggravates inflammation [2,33]. And there are reports that microglia/macrophage M1/2 phenotypes balance changes dynamically from several hours to a few weeks after stroke. Consistently, our immunofluorescence results have demonstrated that microglia/macrophage tend to

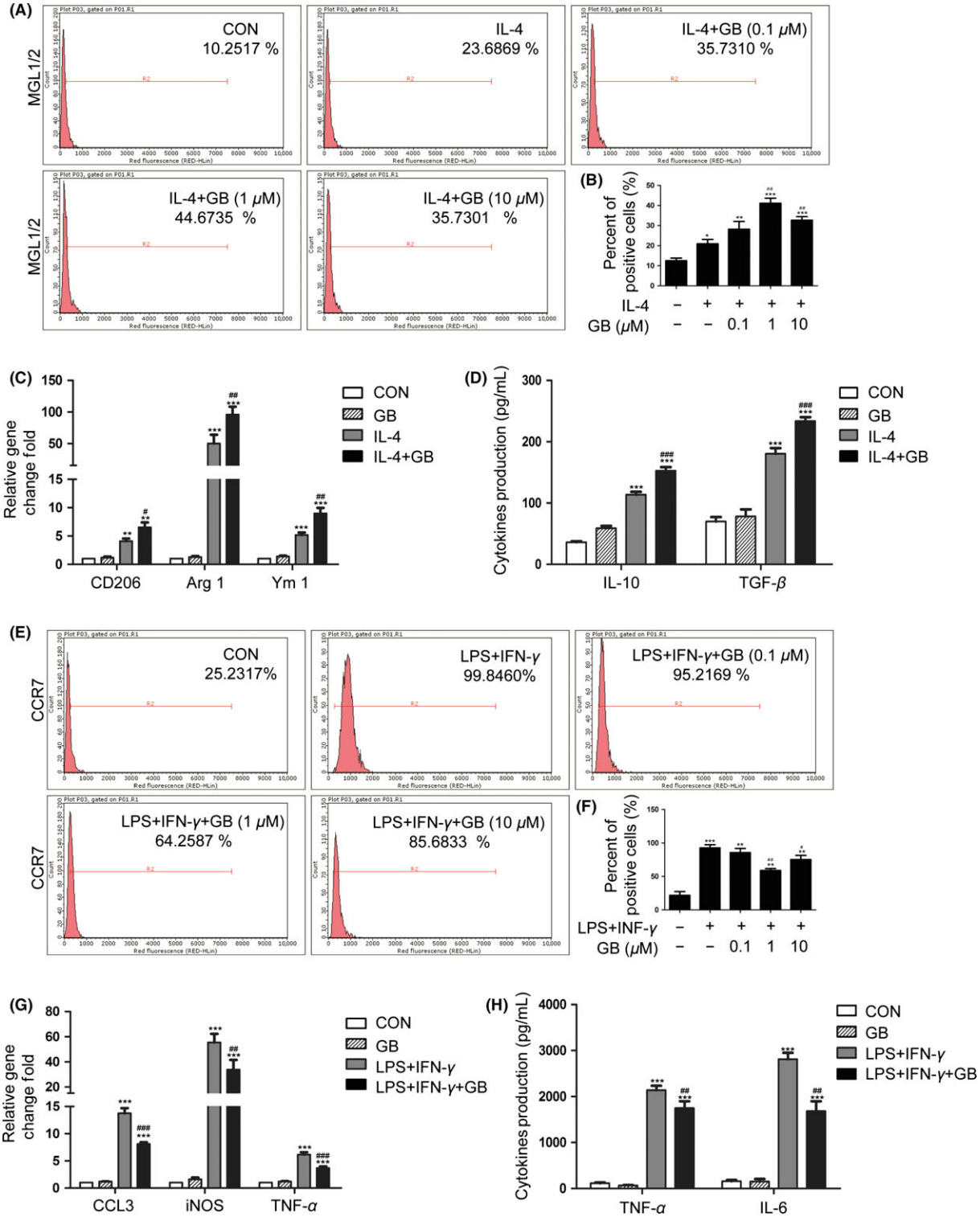

Figure 3 Ginkgolide B (GB) facilities M1-microglia polarizing toward M2 phenotype on BV2 cells. Before M2 induction, BV2 was pretreated by LPS (100 ng/mL) plus IFN-γ (20 ng/mL) for 24 h to become M1 type. GB is pretreated for 2 h before the cells were stimulated by IL-4 (20 ng/mL) for 24 h. (**A**, **E**) The expression of M2-related biomarker MGL1/2 and M1-related biomarker CCR7 detected, respectively, by flow cytometry. (**B, F**) And GB at 1 μM was found to be effective concentration. (**C**) Representative M2-related mRNAs change folds. (**D**) M2-related cytokines (transforming growth factor-β, IL-10) detected by ELISA. (**G**) Representative M1-related mRNAs change folds detected by qPCR. (**H**) M1-related cytokines (TNF-α, IL-6) detected by ELISA. Data are represented as mean ± SEM from three independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control group, P### versus IL-4 or LPS + IFN-γ.

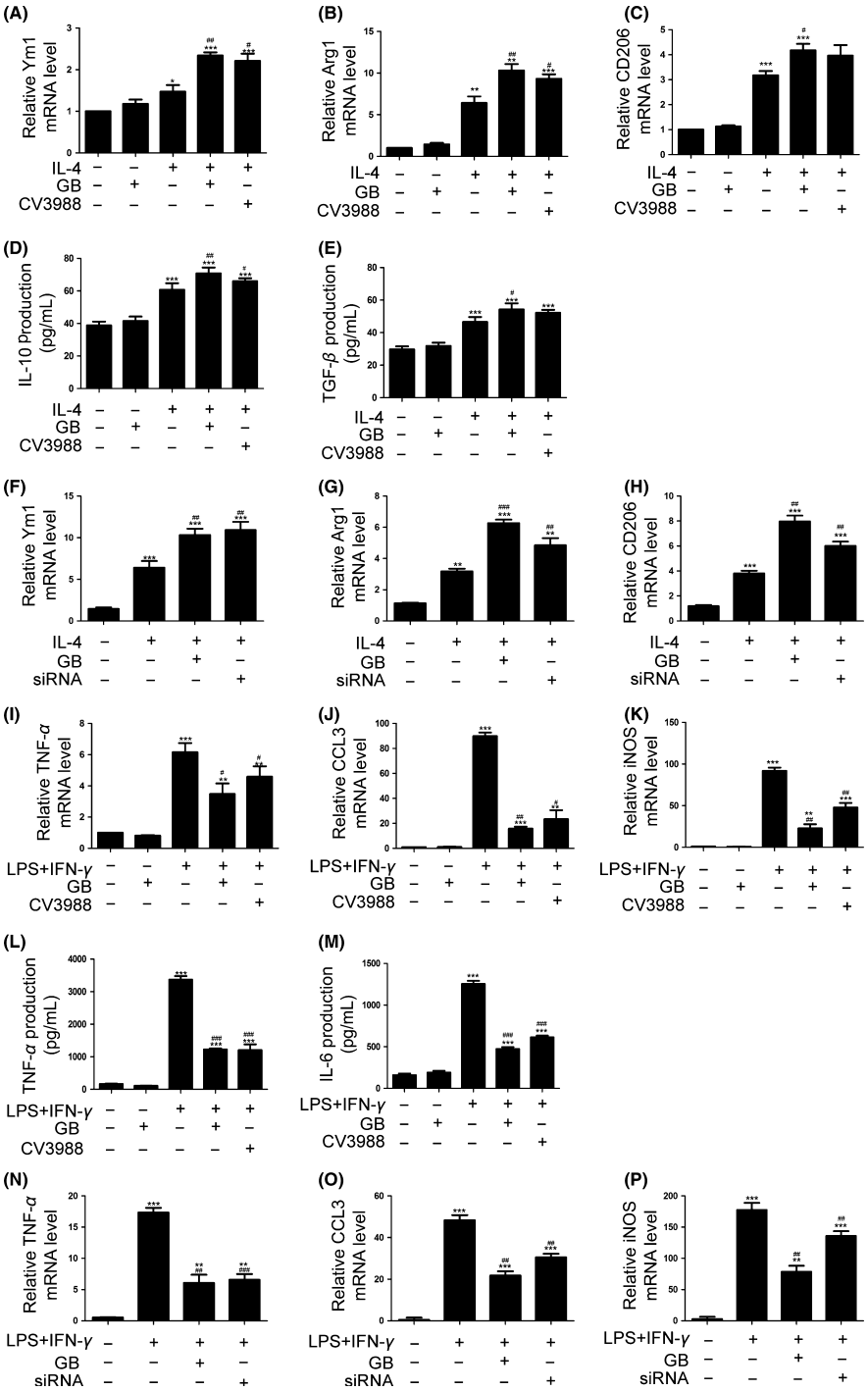

Figure 4 Platelet activator factor (PAF) receptor is involved in the polarization-modulating effect of Ginkgolide B (GB). (**A**–**C**) CV-3988 exhibited similar effect with GB on M1/2 gene modulation. CV-3988(10 μM) is pretreated for 30 min before IL-4(20 ng/mL) was treated. M2-related mRNAs and cytokines were detected when BV2 was treated with IL-4 combined with CV3988. (**D, E**) CV-3988 and GB increased M2 cytokines and reduced the production of M1 cytokines, detected by ELISA. (**F**–**H**) Silencing PAF receptor showed similar polarization-modulating effect with GB. PAF receptor targeting siRNA (100 μM) was used to silence PAF receptor, and the M2-related mRNAs was detected. (**I**–**K**) CV-3988 and GB decreased the expression of M1-related gene on BV2. (**L, M**) ELISA showed the cytokines of M1 phenotype were decreased by treating GB or CV3988. (**N**–**P**) The results of qPCR showed silencing PAF receptor contributed to the downregulation of M1-related gene expression, displaying similar effect with GB. Data are represented as mean ± SEM from three independent experiments, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control group, P### versus IL-4 or LPS + interferon-γ (IFN-γ).

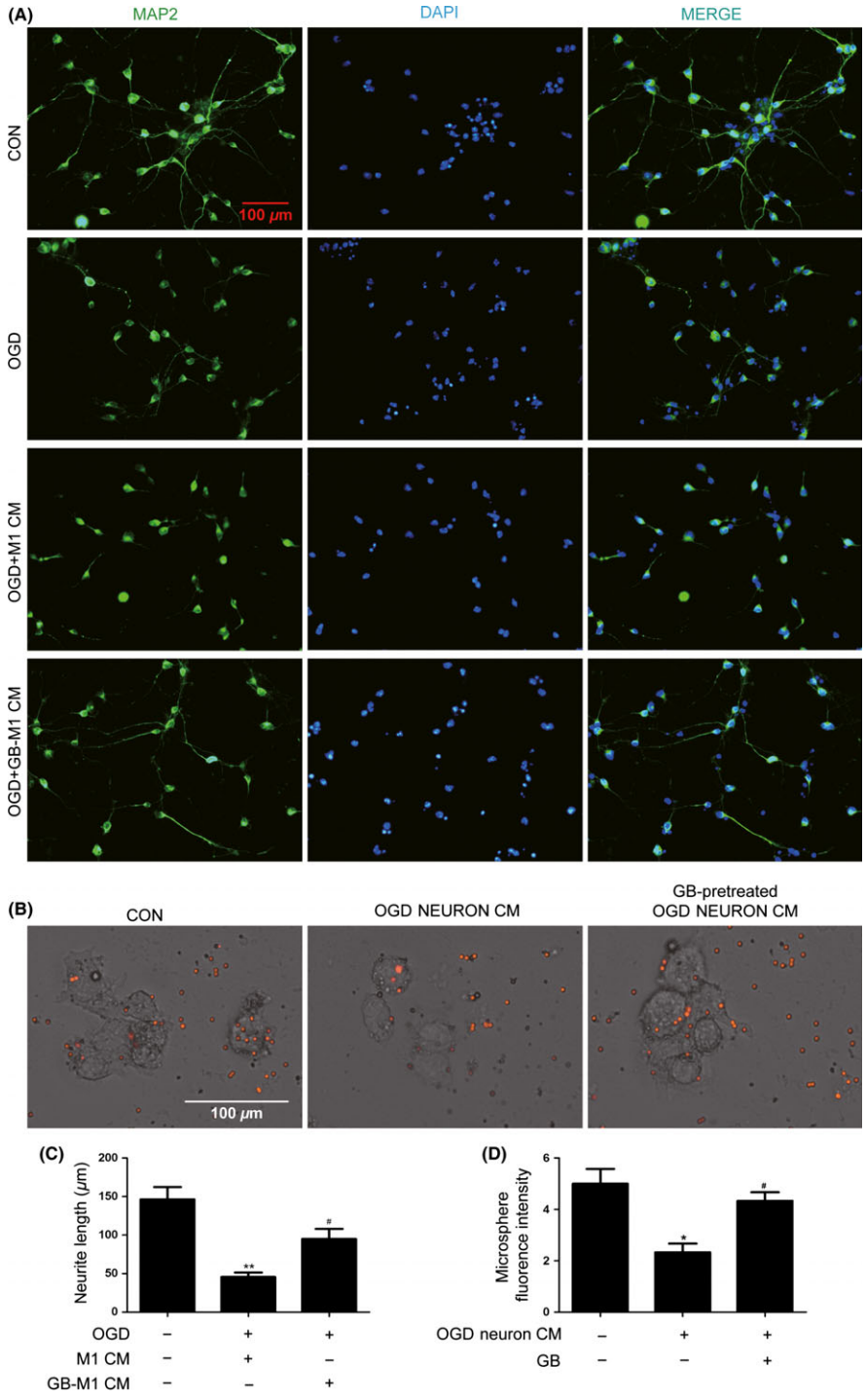

Figure 5 Ginkgolide B (GB) exhibits protective effect on oxygen glucose deprivation (OGD) neuron via pivoting microglia/macrophage polarization. Primary microglia cultures were induced toward M1 phenotype as described in the methods. The conditioned media was collected for the following neuron treatment. (**A**) The CM from GB-pretreated M1 phenotype microglia group improved the MAP2 expression. After 1-h OGD, primary cortical neurons were incubated with different condition medium of microglia (GB-M1 or M1), respectively. The MAP2 assay was conducted to show the condition of neurons. (**C**) The neuritis length was measured by IMAGEJ (National Institutes of Health). (**B, D**) GB improved microglial phagocytosis which was impaired by OGD neuron-conditioned medium. Fluorescent microspheres were added into microglial cultures for 1 h. Images are representative of three independent experiments. Data are represented as mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control group; $\#\#P < 0.01$, $\#\#\#P < 0.001$ versus M1 microglia CM or OGD neuron CM group.

polarize toward M2 phenotype at 3 days after tMCAO and M1 phenotype begins to increase at 7 days. We have also found that GB treatment modulates this pathological course, increasing their polarization toward M2 phenotype and inhibiting M1 expression. Consistently with the immunofluorescence, GB promoted the M2-related mRNA expression and inhibited M1-related counterparts. These results together suggested that GB promoted microglia/macrophage polarization to M2 phenotype after tMCAO. In addition, R. Tanaka et al. have reported that the macrophages infiltrate into the CNS after tMCAO in a few hours and BMDM/macrophages migrate to both infarct area and peri-infarct area and become dominant after 3 days of injury [34]. Because there is no immunohistochemical method being able to distinguish macrophage with microglia, the immunofluorescence actually indicates the phenotypes of both two kinds of cells [35]. To confirm the promoting effect of GB on microglia/macrophage polarization, therefore, *in vitro* experiments, we treated microglia cell line BV2 and BMDM/macrophages with LPS + IFN-γ and IL-4, respectively. GB increases the M2 phenotype and inhibits the M1 polarization of BV2 and BMDM, manifested by changes in expression of M1/2-related mRNA and cytokines. Furthermore, our results have showed that the gene expression change is more apparent in BMDM than that in BV2, indicating that the primary cells are more sensitive to stimuli than cell lines. Ischemic stroke is not only a CNS disease but also causing robust immune responses in periphery. After ischemia, the infiltrated BMDM is like the bridge between CNS and peripheral immune system. Therefore, GB has an effect on BMDM, which indicates it may take protective effect over the whole body, either in CNS or periphery.

It has been shown that promoting the immune cells to a favorable M2 phenotype is apparently of profound significance in neuroprotection [36]. To emphasize the significance of microglia polarization by GB, we conducted the conditioned medium experiment in cortical primary neurons and cortical primary microglia. The results have shown that post-OGD neurons treated with the conditioned medium of GB-pretreated M1 microglia expressed more MAP2 than those treated by M1 microglia conditioned medium only. Microglia treated with GB exhibit better phagocytosis, which would benefit brain homeostasis and neuronal survival after stroke. These evidences further support the protective contribution of GB to modulating microglia/macrophage polarization. Taken together, our results have revealed that GB protects mice against ischemic stroke injury by promoting microglia/macrophage transition toward M2 phenotype.

Moreover, in the present study, we have investigated the mechanisms of GB regulating microglia/macrophage polarization. Generally, microglia/macrophage phenotypes are decided by microcircumstances and modulated by many transcription factors, such as STATs, PPAR-γ, and KLFs [37]. Recently, several receptors, for example the class A scavenger receptor and neuron-derived orphan receptor 1, have been also implicated to be involved in the microglia/macrophage polarization [38,39]. PAF is a bioactive lipid mediator that causes platelet aggregation, vascular constriction, and inflammation [40]. It indicates that PAF plays a critical role in many CNS diseases. For example, in a MPTP-induced PD model, PAF and its receptor are indispensable for inflammation via NF-κB signaling pathway, which is a pivoting pathway modulating both M1/2 polarization [16]. Similarly, PAF and PAF receptor are closely linked to the pathological progress of ischemic stroke and their expression will drastically increase after ischemia [41]. Thus it is presumable that PAF receptor is involved in microglia/macrophage polarization under ischemic inflammatory conditions. CV3988, another PAF receptor antagonist, exhibits similar modulation with GB. Downregulation of PAF receptor by utilizing siRNA also results in similar M2 promotion effect on BV2, further indicating that PAF receptor is involved in the modulation effect. Therefore, we have revealed the novel pharmacological effect of PAF receptor, participating in the microglia/macrophage polarization.

Apart from PAF receptor pathway, GB might modulate microglia/macrophage polarization through other signaling pathways, such as influencing microRNA expression. This presumption is based on our recent result that GB regulates expression of several microRNAs, such as microRNA-155 and microRNA-206 in the acute stage after tMCAO (X. Lan, L. Cao, J. Zhu, Y. Bian, J. Ding, Y. Fan, G. Hu, unpublished data). As mircroRNA155 is proved to be a key microRNA modulating microglia/macrophage polarization [42], microRNA modulation might be also involved in the influence of GB on microglia/macrophage polarization. Therefore, the systematic mechanisms GB modulating microglia/macrophage phenotype and their interactions need to be further elucidated.

It should be emphasized that GB has been proved to be safe and efficient in clinical stroke therapy [43]. Compared with other microglia/macrophage polarization-modulating drugs, such as malibatol A [44] or Metformin [45], GB possesses its unique advantages. Contrast to metformin which only takes effect during recovery period (>14 days) after stroke, GB exhibits significant neuroprotection for the whole period following the insult. Nevertheless, further study is necessary for us to address whether GB could directly promote M0 microglia/macrophage to M2 phenotype under the stroke pathology.

Conclusion

In this research, we have demonstrated that GB treatment enhances the postischemic M2 polarization of microglia/macrophage and improves tissue repair and recovery after MCAO. Furthermore, our results have suggested that inhibiting PAF receptor accounts for GB modulating microglia/macrophage polarization. This finding broadens our understanding on the pharmacological effects of GB, indicating that GB could be utilized on microglia/macrophage polarization-related inflammatory diseases.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

The following supplementary material is available for this article:

Figure S1. The identification of the observation windows.
Figure S2. The conditioned medium from post-OGD neuron enhances microglia polarize towards M1 phenotype.
Figure S3. Ginkgolide B facilities M1-microglia polarizing towards M2 phenotype on BMDM.
Figure S4. The efficiency of PAF receptor targeting siRNA inference.