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IMM-H004 protects against oxygen-glucose deprivation/reperfusion injury to BV2 microglia partly by modulating CKLF1 involved in microglia polarization

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ABSTRACT

Background: IMM-H004 is a novel compound that has been shown to protect against cerebral ischemia/reperfusion injury in our previous works. Chemokine-like factor 1 (CKLF1) is a chemokine that exhibits increased expression in the ischemic brain. Dysregulation of microglia polarization dynamics is a mechanism of injury expansion poststroke.

Purposes: The aim of present study was to investigate the effects of IMM-H004 on cell viability and microglia phenotypes in BV2 microglia suffering from oxygen-glucose deprivation/reperfusion and discussing the involvement of CKLF1 and possible mechanisms.

Results: IMM-H004 protected BV2 microglia from oxygen-glucose deprivation/reperfusion-induced toxicity. We found that the expression of CKLF1 was increased in BV2 microglia with oxygen-glucose deprivation/reperfusion, and IMM-H004 decreased this specially increased expression. Moreover, oxygen-glucose deprivation/reperfusion induced the BV2 microglia to polarize toward an M1 phenotype, and IMM-H004 modulated the polarization shift from the M1 phenotype and skewed toward the M2 phenotype, followed by suppressing the excessive inflammatory response and improving recovery. CKLF1 modulated BV2 microglia toward M1 polarization and induced an inflammatory response. By using receptor inhibitors, we found that OGD/R induced microglia polarization partly through C—C chemokine receptor 4. Furthermore, the Co-IP assay showed that IMM-H004 decreased the amount of CKLF1 binding to C—C chemokine receptor 4 in the BV2 microglia oxygen-glucose deprivation/reperfusion model.

Conclusions: IMM-H004 protects BV2 microglia against oxygen-glucose deprivation/reperfusion injury partly by modulating microglia polarization and further regulating the inflammatory response. The CKLF1/CCR4 axis may be involved in the protective effects of IMM-H004 modulating microglia polarization.

1. Introduction

Ischemic stroke (IS) is a debilitating and devastating disease leading to severe morbidity and mortality worldwide [1]. This disease is caused by occlusion of cerebral blood vessels by embolism or thrombus, resulting in insufficient blood flow to the brain, depriving the brain of oxygen, glucose and other essential nutrients, and further damaging the brain parenchyma [2]. Although rapid reperfusion in ischemic areas has the potential to rescue dying cells in ischemic penumbra, the occurrence of reperfusion also causes an excessive inflammatory response and deteriorating brain injury [3,4]. Multiple mechanisms are involved in ischemia and reperfusion progression, including inflammatory reaction [5], toxicity of excitatory amino acids [6], cellular signal transduction including Ca^{2+} overload and NO damage [7], oxygen free

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radical production [8], and others. To date, inflammatory reaction is considered the major reason for brain damage [9].

As a type of major resident immune cells in the brain, microglia are involved vitally in cerebral immune response when insult occurs [10]. After cerebral ischemia/reperfusion (I/R), the remaining microglia are transformed into activated microglia, producing and releasing a plethora of cytokines and chemokines ranging from cytotoxic mediators to trophic factors, which can exert detrimental or beneficial effects [11]. Currently, microglia polarization dysregulation is found to be a novel mechanism for cerebral ischemia-induced injury [12]. Microglia have two phenotypes: M1 and M2. Microglia with the pro-inflammatory M1 phenotype can release inflammatory cytokines such as IL-1β, TNF-α, and NO, which is often associated with bad outcomes. Microglia showing the anti-inflammatory M2 phenotype can release anti-inflammatory cytokines like TGF-β and IL-10, which exert neuroprotective effects [13]. The polarized microglia have been reported in several central nervous system disorders, such as spinal cord injury [14], multiple sclerosis [15], and intracerebral hemorrhage [16]. In cerebral ischemia, the resident microglia initiate polarization, leading to deleterious pathological responses, and further aggravate the ischemic damage. Modulating microglial polarization via inhibiting M1 or skewing to the M2 phenotype selectively has the potential to be a treatment strategy for cerebral I/R injury.

Chemokine-like factor 1 (CKLF1) is a chemokine with multiple biological activities [17]. Our previous studies showed that the expression of CKLF1 increased significantly as early as 3 h after transient middle cerebral artery occlusion insult in the ischemic brain, peaking on day 2 in rats [18]. Administration of C19, an antagonist peptide of CKLF1 [19], or anti-CKLF1 antibody [20] showed beneficial effects in rats.

IMM-H004 is a novel compound derived from coumarin and screened and modified from a C27/CCR4 system by calcium transient technology. It could block the chemotaxis and calcium mobilization caused by C27 and CCR4 interaction [21]. The C27 peptide is one of the main secreted agonist peptides of CKLF1 that shows similar effects to CKLF1 protein and interacts with C—C chemokine receptor 4 (CCR4) [22]. The chemical structure of IMM-H004 is shown in Fig. 1. IMM-H004 showed protective effects on global cerebral ischemia injury [23] and cerebral I/R-caused injury [24] in rats in our earlier works. It can attenuate the release of inflammatory cytokines in BV2 microglia activated by lipopolysaccharide [25] and suppresses inflammatory response in mice [26,27].

In the present study, we determined the protective effects of IMM-H004 through microglia polarization in a model of BV2 microglia with oxygen-glucose deprivation/reperfusion (OGD/R), and explored the involvement of the CKLF1/CCR4 axis. We demonstrated that IMM-H004 can modulate BV2 microglia toward a lower M1 phenotype polarization and higher M2 phenotype polarization in an OGD/R model, which was further accompanied by attenuating the excessive inflammatory response and shifting to a recovery state, and the CKLF1/CCR4 axis may be involved in the protective effects.

2. Materials and methods

2.1. Materials

The IMM-H004 compound (molecular formula: $C_{16}H_{20}O_4N_2$; molecular weight: 304) was provided by the Department of Chemosynthesis, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College (Beijing, China). The CKLF1 peptide C27 (ALIYRKLLFNPSGPYQKKPVHEKKEVL), with a purity of 97.99%, was provided by GL Biochem (Shanghai, China). All materials under study were endotoxin free.

2.2. BV2 microglial cell culture and IMM-H004 treatment

The BV2 murine microglial cell line was purchased from the Cell Resource Center of Peking Union Medical College. Cells were normally cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 10% fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 at 37 °C. The cells were passaged 2 to 3 times each week, and cells passaged over 3 times could be used for the experiments.

To determine whether the effects of IMM-H004 are dose-dependent, various concentrations of IMM-H004 (0.1, 1, 10 μM) were added to the BV2 microglia 2 h before OGD. C 021 dihydrochloride (1, 10, 100 nM)

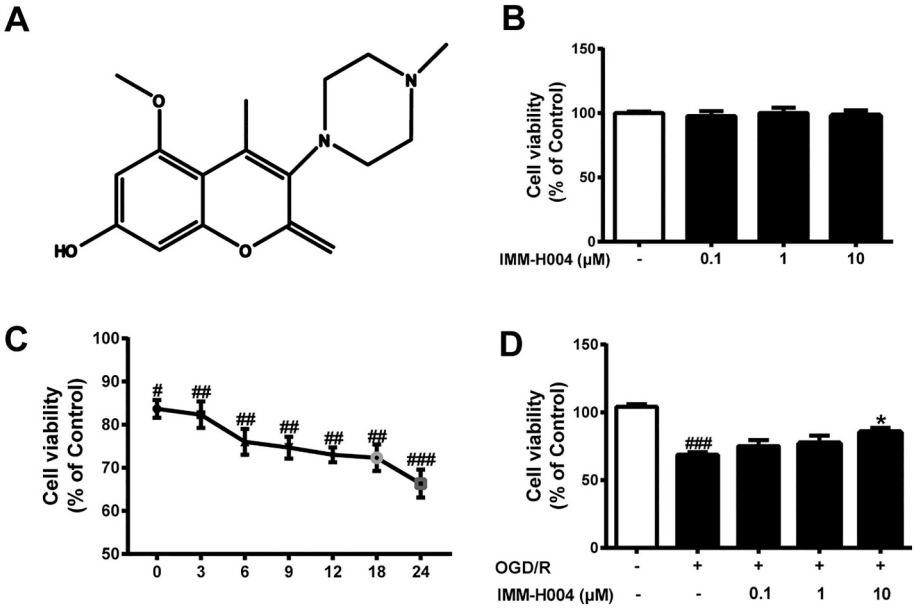

Fig. 1. Protective effects of IMM-H004 against OGD/R-induced toxicity. (A) The chemical structure of IMM—H004. (B) BV2 microglia were incubated with IMM-H004 (0.1, 1, 10 μM) for 24 h, and cell viability was assessed by MTT ($n = 6$). (C) BV2 microglia were exposed to OGD for 6 h followed by reoxygenation for 24 h. Cell viability was detected at 0, 3, 6, 9, 12, 18, and 24 h after reoxygenation by MTT assay ($n = 6$). (D) BV2 microglia were pretreated with IMM-H004 (0.1, 1, 10 μM) 2 h before OGD and reoxygenation for 24 h. Cell viability was detected by MTT assay (n = 6). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. control group. $^{*}P < 0.05$ vs. OGD/R group.

and DAPTA (1, 10, 100 nM) were also applied 2 h before OGD/R treatment in the inhibitor experiments. C27 (1, 10, 100 nM) was added to culture media for 24 h to investigate the effects of CKLF1 on BV2 microglia.

2.3. Oxygen–glucose deprivation and reoxygenation (OGD/R)

BV2 microglia were seeded onto culture plates at a density of 1×10^6/ml. The culture medium was changed to glucose-free Earle's solution, and then a sealed chamber was used to place the plates, followed by expiring oxygen for 20 min via flowing 95% N_2 and 5% CO_2 mixture persistently at a low flow (1.5 l/min). Twenty min later, the chamber was transferred into a 37 °C incubator for 6 h to mimic OGD after clamping the inlet and outlet. OGD was ended by changing the medium to normal feeding medium and restored to a normoxic atmosphere in a 37 °C incubator as reoxygenation. Then, 6 h OGD and 24 h reoxygenation (OGD 6 h/R24 h) was chosen as the optimal condition for BV2 microglia in our experiment. BV2 microglia were incubated with IMM-H004 at various concentrations for 2 h before OGD, and the cells were collected after 24 h reoxygenation for the following assays.

2.4. MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Beyotime Institute of Biotechnology) assay was used to determine cell viability. BV2 microglia were seeded onto 96-well plates at a density of 5×10^4/ml (100 μl/well). After treatment, 10 μl of MTT solution (5 mg/ml) was added per well, and plates were placed in 37 °C for 4 h. DMSO (100 μl/well) was then added and incubated for another 6–8 h at 37 °C to dissolve formazan crystals. The optical density was determined with a microplate reader (Thermo Scientific, MA, USA) at 570 nm.

2.5. Quantitative PCR (qPCR)

Total RNA was isolated from BV2 microglia with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of RNA was calculated at 260 nm in a microplate reader (Thermo Scientific, MA, USA) and a purity of 260 nm/280 nm absorption between 1.8 and 2.1 was qualified for reverse-transcription. Then, 1 μg RNA was used to reverse-transcribe to cDNA by TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) per the manufacturer's instructions. The TransStart Tip Green qPCR Supermix kit (TransGen Biotech, Beijing, China) was used to perform the amplification of cDNA and detection of signals in an Applied Biosystems 7900HT Fast Real-Time PCR System (Foster City, CA, USA). The relative expression of the target gene was normalized to mouse GAPDH as an internal control and calculated by the $2^{-\Delta\Delta CT}$ method. Table 1 shows the primers used.

Table 1
Primers for qPCR analysis.

Gene	Primer
M1	
iNOS	SENS: CAAGCACCTTGGAAGAGGAG
	REVS: AAGGCCAAACACAGCATACC
CD16	SENS: TTTGGACACCCAGATGTTTCAG
	REVS: GTCTTCCTTGAGCACCTGGATC
CD32	SENS: AATCCTGCCGTTCCTACTGATC
	REVS: GTGTCACCGTGTCTTCCTTGAG
M2	
Arg1	SENS: TCACCTGAGCTTTGATGTCG
	REVS: CTGAAAGGAGCCCTGTCTTG
CCL-22	SENS: CTGATGCAGGTCCCTATGGT
	REVS: GCAGGATTTTGAGGTCCAGA
TGF-β	SENS: TGCGCTTGCAGAGATTAAAA
	REVS: CGTCAAAAGACAGCCACTCA
Internal control	SENS: TCATTGACCTCAACTACATGGT
GAPDH	REVS: CTAAGCAGTTGGTGGTGCAG

2.6. ELISA analysis

Commercially available ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) were used to determine the levels of IL-1β and TNF-α in the culture media according to manufacturer's instructions. Quantification of ELISA results was performed at 450 nm in a microplate reader (Thermo Scientific, MA, USA).

2.7. Immunofluorescence analysis

Cells were cultured on Poly-L-lysine (Sigma, St. Louis, MO)-coated cover slips placed in 24-well plates at a concentration of 1×10^5 cells/ml. Cells were fixed with cold 4% PFA for 20 min after washing with cold PBS, followed by washing with PBS for 5 min 3 times. Then, 0.1% (*v*/v) Triton-X-100 (Sigma, St. Louis, MO) was used to permeabilize cells for 10 min, followed by washing for 5 min 3 times again. Then, the cells were blocked with 5% (w/v) bovine serum albumin (BSA) (Sigma, St. Louis, MO) at room temperature for 1 h. After blocking, cells were incubated with the primary antibodies at 4 °C overnight. The primary antibodies were rabbit anti-Iba1 (1:500, Wako Chemical, Osaka, Japan), rat anti-CD16/32 (1:100, BD Biosciences Pharmingen, CA, USA), and goat anti-CD206 (1:200, R&D Systems, MN, USA). The following day, the coverslips were washed with PBS 3 times and incubated with Alexa 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA), Alexa 546-conjugated donkey anti-goat IgG (Invitrogen, Carlsbad, CA, USA) or Cy3-labeled goat anti-rat IgG (Beyotime Biotechnology, Shanghai, China) for 1 h. After washing with PBS, the coverslips were mounted using 90% (v/v) glycerol with a Hoechst 33342 (Beyotime Biotechnology, Shanghai, China). Images were captured with a confocal laser scanning microscope (TCS SP2, Leica, Solms, Germany), and the fluorescence intensity was analyzed by Image-Pro Plus 6.0 software (Media Cybernetics, MD, USA).

2.8. Western blot

BV2 microglia were collected and lysed in RIPA lysis buffer (Beyotime, Shanghai, China) after shaking on ice for 30 min. The cell lysates were centrifuged at 4 °C at 12,000 rpm for 30 min, and then supernatants were isolated, followed by assessment of protein concentrations by a BCA kit (Applygen, Beijing, China). Then, 40 μg of protein per sample was separated on 15% (w/v) SDS-PAGE gels and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% (w/v) BSA at room temperature for 2 h and then incubated with primary antibodies at 4 °C overnight. The primary antibodies were shown as follows: anti-CKLF1 (1:500, Sigma, St. Louis, MO), anti-IL-1β (1:500, Abcam, Cambridge, UK), anti-TNF-α (1:500, Abcam, Cambridge, UK), anti-TGF-β (1:500, Abcam, Cambridge, UK), anti-BDNF (1:1000, Beverly, MA, USA), anti-NF-κB p65 (1:1000, Cell Signaling Technology, Beverly, MA, USA), and anti-Phospho-NF-κB p65 Ser536 (1:1000, Cell Signaling Technology, Beverly, MA, USA). β-actin was used as the endogenous control. The next day, the membrane was washed with TBST buffer 3 times for 10 min, and then incubated with appropriate AffiniPure-conjugated corresponding secondary antibody at room temperature for 2 h. The membranes were washed with TBST buffer 3 times for 10 min, and the expression of each protein was examined with enhanced chemiluminescence plus detection system (Molecular Device, Lmax). Gel-Pro Analyzer (Media Cybernetics, MD, USA) software was used to quantify the optical density of the bands.

2.9. Co-Immunoprecipitation assays (Co-IP)

Protein extraction was performed as described above. Protein-A Sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden) were incubated with 1 μg anti-CKLF1 or anti-CCR4 antibody at 4 °C for 4 h. Then, 500 μg protein of each sample was added and incubated at 4 °C overnight. The beads were washed with lysis buffer 3 times the next day and collected after centrifugation at 4 °C at 3000 rpm for 5 min. The protein was separated in 15% SDS-PAGE, transferred to PVDF membranes and blocked with 5% BSA. Then, the membranes were incubated with primary antibodies anti-CKLF1 (1:500) and anti-CCR4 (1:500) overnight at 4 °C. The other steps were the same as the description in subsection 2.8.

2.10. Statistical analysis

All of the data were expressed as the mean ± standard deviation (SD) and analyzed with GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by a Newman–Keuls post-test was used as statistical analysis in this study. $P < 0.05$ was considered to be statistically significant. All of the data were from at least three separate experiments.

3. Results

3.1. IMM-H004 protects BV2 microglia against OGD/R-caused toxicity

First, the safety of IMM-H004 for BV2 microglia was determined by MTT assay. As shown in Fig. 1B, cell viability was not significantly different between control and IMM-H004-treated groups with different doses (0.1 μM, 1 μM, 10 μM), indicating IMM-H004 has acceptable safety. All data are expressed as the percentage normalized to the control group. To determine whether IMM-H004 could protect BV2 microglia from OGD/R injury, we first tested and chose the reoxygenation conditions that caused further cell toxicity in BV2 microglia. Cells were reoxygenated after 6 h OGD, and cell viability was assessed at 0, 3, 6, 9, 12, 18, and 24 h of reoxygenation. The results showed that cell viability was reduced approximately 16% after 6 h OGD, and reoxygenation caused a further reduction in cell viability in a time-dependent manner. Then, 24 h after reoxygenation, the reduction in cell viability of BV2 microglia came to approximately 34%. We then chose OGD 6 h/R 24 h as the model condition. To investigate the protective effects of IMM-H004 against OGD/R-caused cytotoxicity, IMM-H004 was added to the culture medium at different concentrations 2 h before OGD/R. As shown in Fig. 1D, compared with the control group, cell viability of the OGD/R group dropped markedly, and 10 μM IMM-H004 pretreatment relieved ODG/R-induced cytotoxicity significantly.

3.2. IMM-H004 reduces the increased CKLF1 expression induced by OGD/R in BV2 microglia

To determine whether the expression of CKLF1 was changed in BV2 microglia subjected to OGD/R, qPCR analysis and western blot were used to analyze the mRNA and protein levels of CKLF1. The results showed that the mRNA and protein expression of CKLF1 were all increased markedly after OGD/R (Fig. 2A and B). Moreover, IMM-H004 treatment decreased this elevation in a dose-dependent manner (Fig. 2A and B).

3.3. IMM-H004 modulates the OGD/R-induced microglia polarization in BV2 microglia

To examine the effects of OGD/R insult on microglia polarization, qPCR analysis and immunofluorescent staining were applied for markers of M1 and M2 phenotype. OGD/R significantly increased the mRNA expression of M1 markers, including iNOS, CD16 and CD32, while the expression of M2 markers like Arg1, TGF-β and CCL-22 were insignificantly increased (Fig. 3A). Additionally, the immunofluorescence intensity of CD16/32, a commonly used marker for detecting the M1 phenotype in immunofluorescence analysis, showed enhancement after OGD/R insult, while the immunofluorescence intensity of M2 marker CD206 showed a small but insignificant increase (Fig. 3B). IMM-H004 showed no effects on BV2 microglia polarization under normal conditions. However, it inhibited the OGD/R-induced M1 phenotype polarization and skewed toward M2 phenotype polarization in a dose-dependent manner (Fig. 3A and B).

3.4. OGD/R induces microglia polarization partly through CCR4 on BV2 microglia

CCR4 and C—C chemokine receptor 5 (CCR5) are two receptors of CKLF1, and we assumed that increased CKLF1 in OGD/R-insulted BV2 microglia may exert effects on microglia polarization through these two receptors. To verify our hypothesis, experiments using C 021 dihydrochloride (CCR4 selective inhibitor) and DAPTA (CCR5 selective inhibitor) were conducted. C 021 dihydrochloride blocked the increased mRNA expression of M1 markers iNOS, CD16 and CD32 and increased mRNA expression of M2 markers Arg1, TGF-β and CCL-22 compared with the OGD/R group (Fig. 4A). Furthermore, C 021 dihydrochloride attenuated the increased immunofluorescence intensity of M1 marker CD16/32 and increased the immunofluorescence intensity of M2 marker CD206 compared with the OGD/R group (Fig. 4B). However, DAPTA showed no significant function in OGD/R-induced polarization as determined by both qPCR analysis and immunofluorescence staining (Fig. 4A and B).

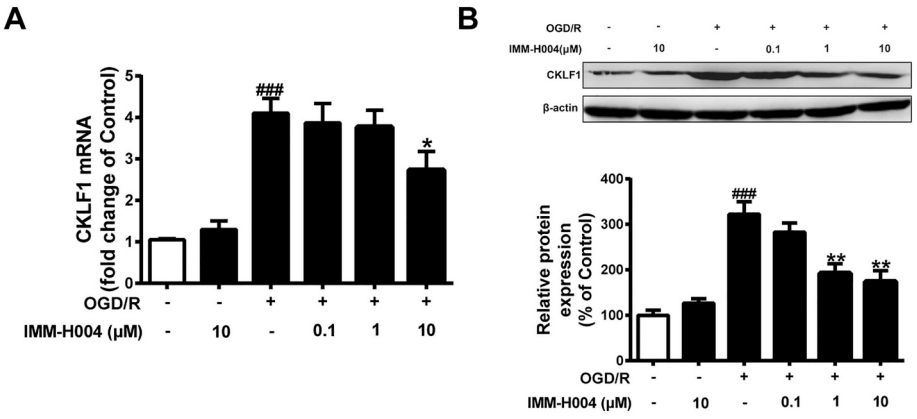

Fig. 2. IMM-H004 lowers the increased CKLF1 expression induced by OGD/R in BV2 microglia. (A) qPCR analysis of mRNA expression of CKLF1 in BV2 microglia ($n = 3$). (B) Representative blots of protein expression in CKLF1 in BV2 microglia and corresponding quantitative analysis (n = 3). $^{###}P < 0.001$ vs. control group. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. OGD/R group.

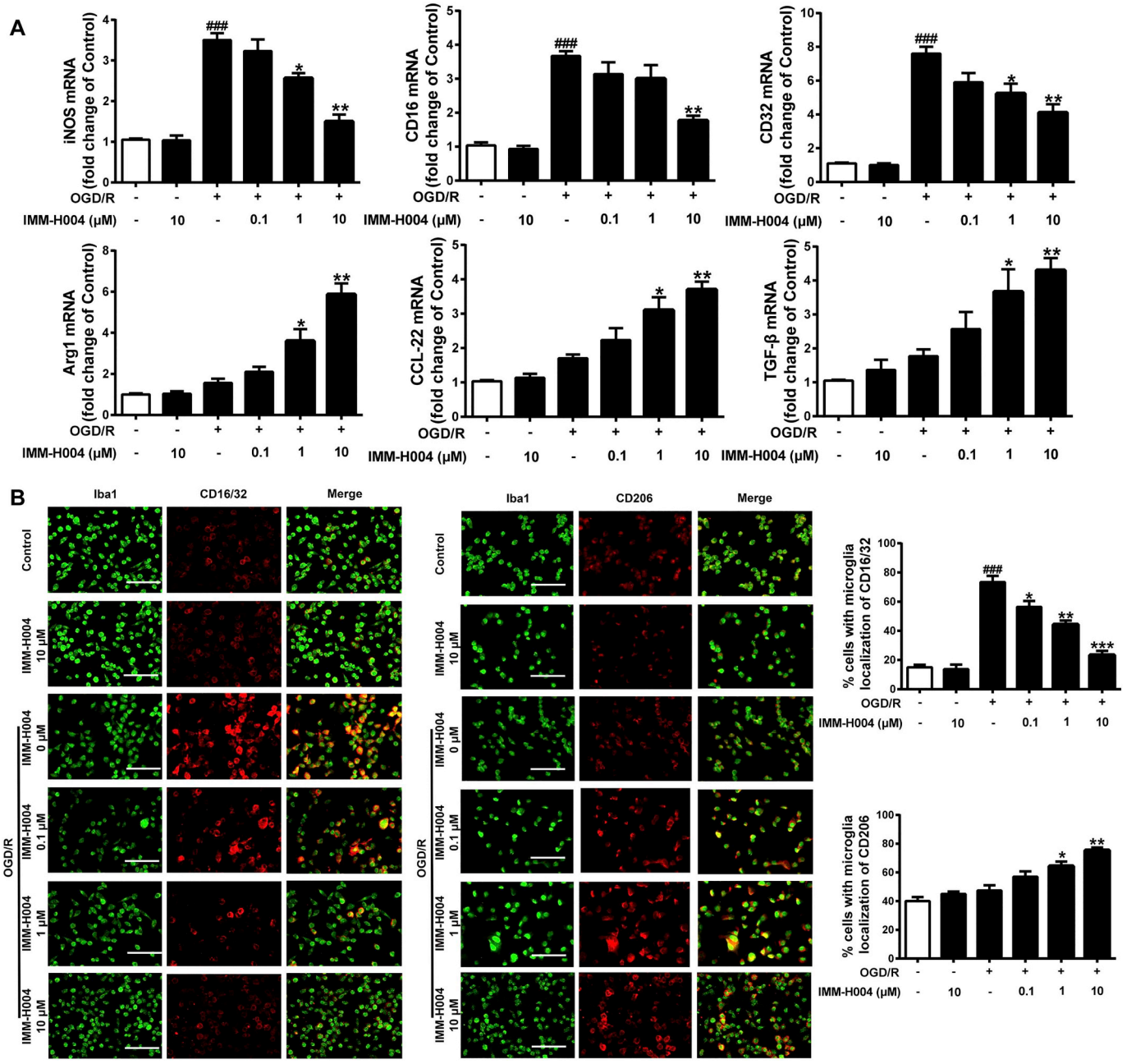

Fig. 3. IMM-H004 modulates the OGD/R-induced polarization in BV2 microglia. (A) qPCR analysis of mRNA expression of M1 markers (iNOS, CD16, CD32) and M2 markers (Arg1, CCL-22, TGF-β) in BV2 microglia ($n = 3$). (B) Representative photomicrographs of double-staining immunofuorescence of CD16/32 with Iba1 and CD206 with Iba1 in OGD/R-injured BV2 microglia. Quantitative analysis of CD16/32-positive and CD206-positive BV2 microglia (n = 3). Scale bars, 100 μm. $^{###}P < 0.001$ vs. control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. OGD/R group.

3.5. IMM-H004 regulates inflammatory response after OGD/R in BV2 microglia

To evaluate the effects of IMM-H004 on inflammatory response, pro-inflammatory and anti-inflammatory cytokines were investigated. Secreted IL-1β and TNF-α in culture medium were assessed by ELISA, and the two cytokines were significantly increased in the OGD/R group, while IMM-H004 treatment reduced these levels significantly (Fig. 5A). We also performed western blot analysis to quantify the protein levels of IL-1β and TNF-α, and the results showed that IMM-H004 reduced the levels of these two pro-inflammatory factors which sharply increased with OGD/R insult (Fig. 5B). Moreover, the protein levels of anti-inflammatory cytokine TGF-β, and neurotrophic factor BDNF, which is helpful for neuronal survival and brain repair, all increased markedly with IMM-H004 treatment (Fig. 5B). These results suggested that IMM-H004 could modulate the deleterious inflammatory response after OGD/R injury toward a more beneficial orientation.

To determine the involvement of nuclear factor-κB (NF-κB) pathway in OGD/R-mediated microglia polarization, expression of p-NF-κB and NF-κB were determined by western blot. An activated NF-κB pathway was observed in the OGD/R group, and IMM-H004 inhibited this activation (Fig. 5C). Moreover, immunofluorescence staining showed that IMM-H004 inhibited OGD/R-induced translocation of the NF-κB p65 subunit into the nucleus (Fig. 5D).

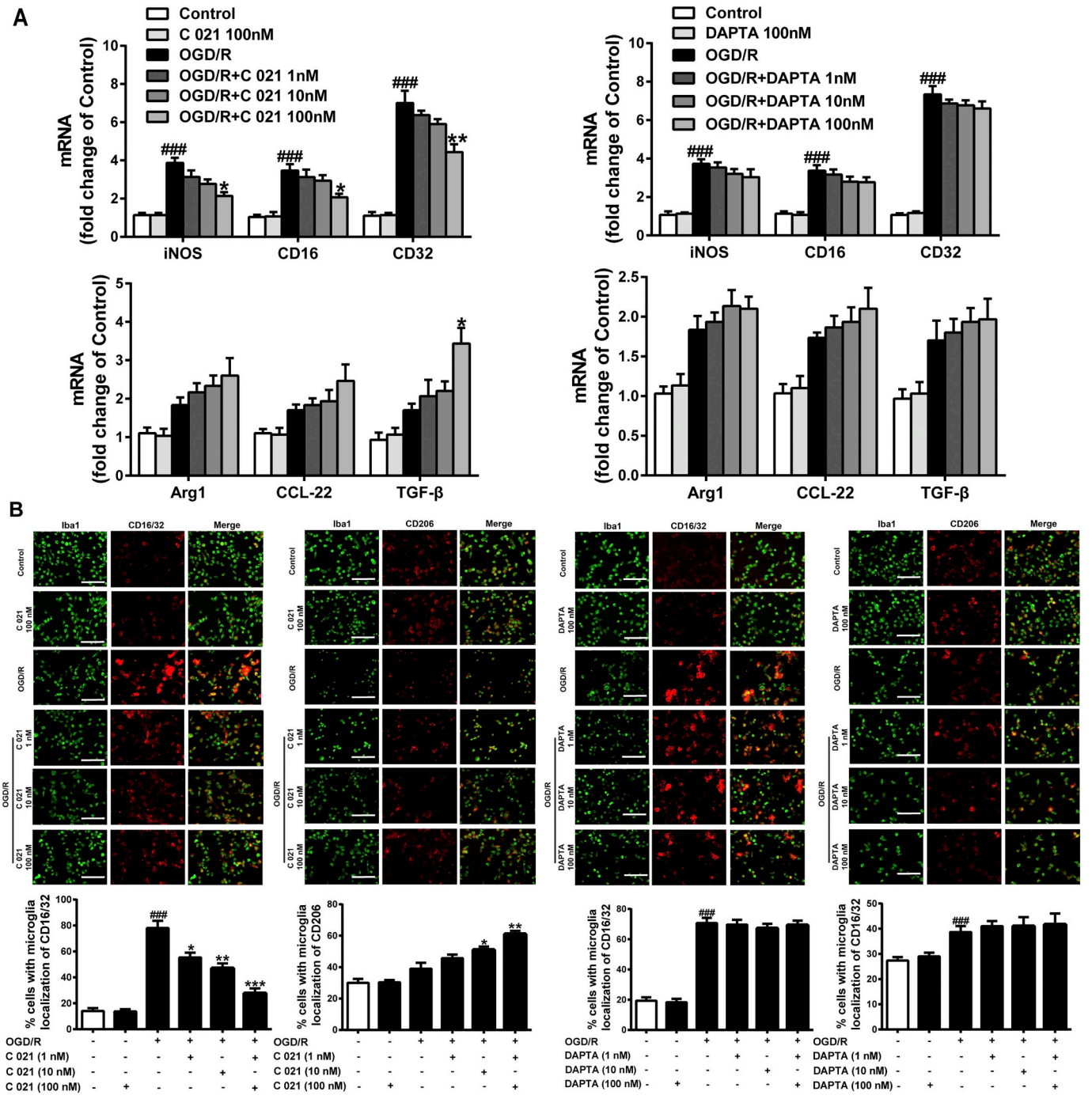

Fig. 4. OGD/R-induced BV2 microglia polarization is partly dependent on CCR4. (A) qPCR analysis of mRNA expression of M1 markers (iNOS, CD16, CD32) and M2 markers (Arg1, CCL-22, TGF-β) in BV2 microglia (n = 3). (B) Representative photomicrographs of double-staining immunofluorescence of CD16/32 with Iba1 and CD206 with Iba1 in BV2 microglia. Quantitative analysis of CD16/32-positive and CD206-positive BV2 microglia (n = 3). Scale bars, 100 μm. $^{###}P < 0.001$ vs. control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. OGD/R group.

3.6. IMM-H004 decreases amount of CKLF1 binding to CCR4

To investigate whether IMM-H004 influences the amount of CKLF1 interacting with CCR4, a Co-IP assay was used. When CCR4 protein was immunoprecipitated and then blotted using CKLF1 antibody, OGD/R insult increased CKLF1 expression in CCR4, and IMM-H004 decreased CKLF1 expression in CCR4 (Fig. 6). Increased levels of CCR4 co-immunoprecipitated with CKLF1 was observed in the OGD/R group, and IMM-H004 decreased these levels of CCR4 co-immunoprecipitated with CKLF1 (Fig. 6). Immunoprecipitation examination results demonstrated that IMM-H004 decreased CKLF1 binding to CCR4 in BV2 microglia exposed to OGD/R.

3.7. CKLF1 modulates BV2 microglia polarization toward the M1 phenotype, aggravating the inflammatory response

The effects of CKLF1 on BV2 microglia were investigated. First, MTT assay was used to determine the effects of CKLF1 on BV2 microglia. As shown in Fig. 7A, cell viability significantly decreased in the C27-treated group at a dose of 100 nM, indicating C27 is toxic to BV2

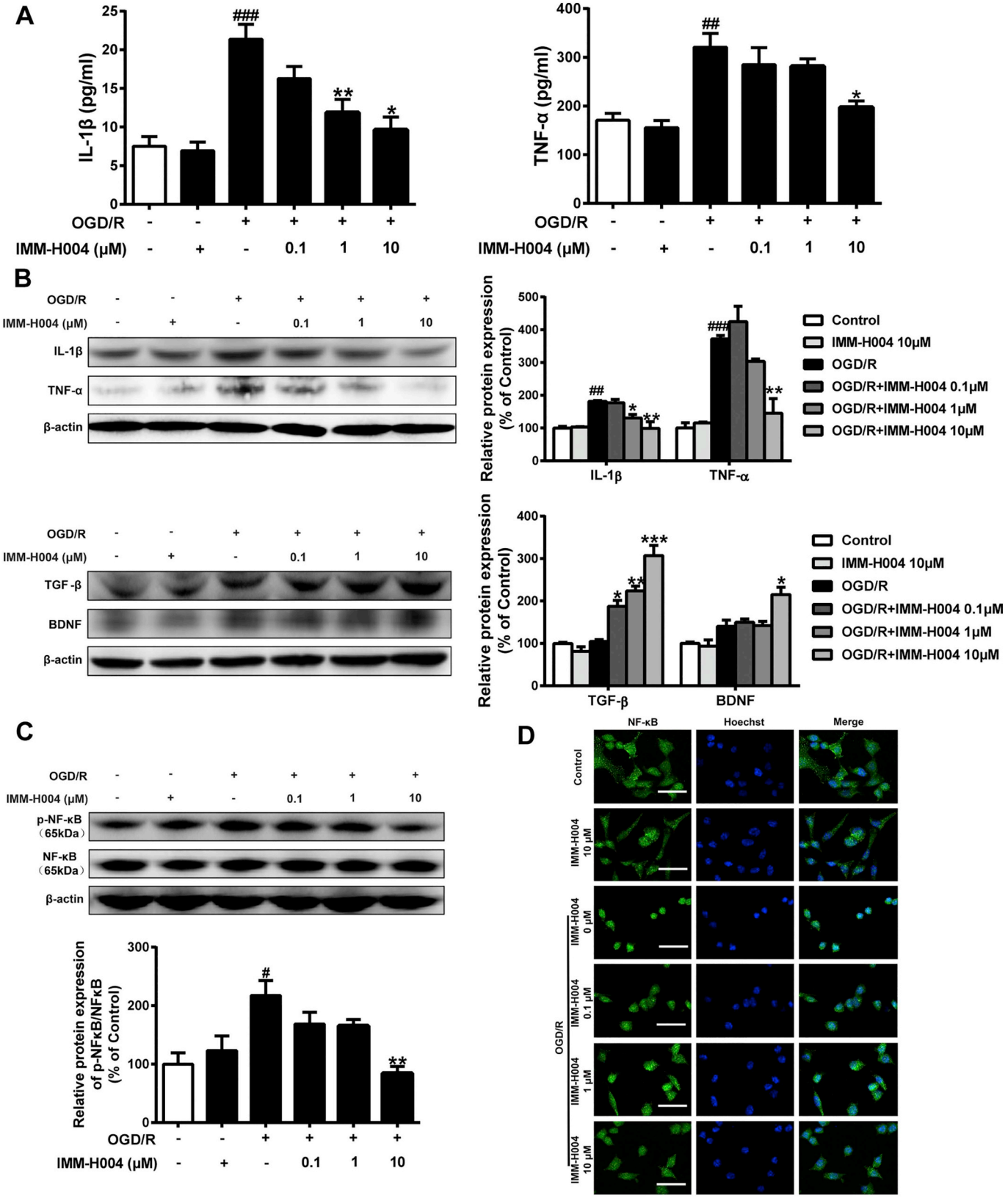

Fig. 5. IMM-H004 modulates inflammatory response in OGD/R-injured BV2 cells. (A) Expression of IL-1β and TNF-α in culture media were determined using ELISA ($n = 3$). (B) Representative blots and densitometry data for IL-1β, TNF-α, TGF-β and BDNF (n = 3). (C) Representative blots and densitometry data for p-NF-κB/NF-κB ratio in BV2 microglia (n = 3). (D) Translocation of NF-κB toward the nucleus determined by Immunofluorescence staining (n = 3). Scale bars, 50 μm. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. OGD/R group.

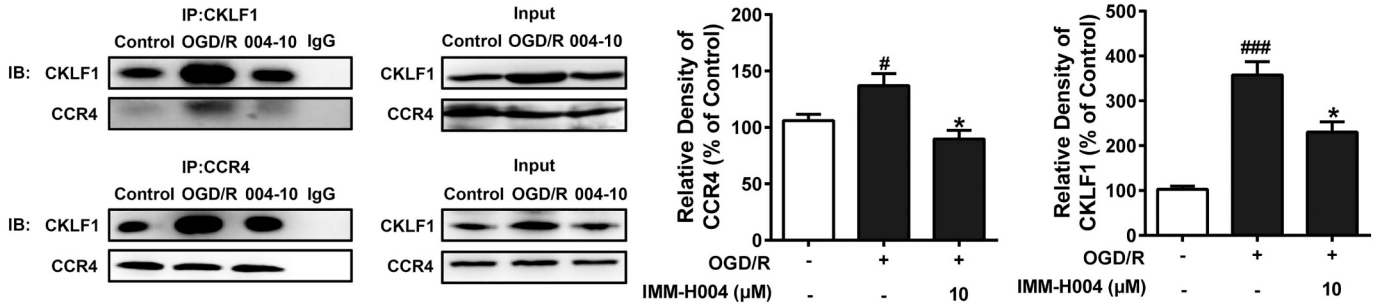

Fig. 6. IMM-H004 decreases amount of CKLF1 binding to CCR4. Representative blots of CO-IP assay for CKLF1 in CCR4 and CCR4 in CKLF1 in BV2 microglia. Quantitative analysis of the CCR4 co-immunoprecipitated with CKLF1 and CKLF1 co-immunoprecipitated with CCR4 ($n = 3$). $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ vs. control group. $^{*}P < 0.05$ vs. OGD/R group.

microglia. To examine the effects of CKLF1 on microglia polarization, qPCR analysis and immunofluorescence staining were applied for detecting M1/M2 markers. C27 increased the mRNA expression of iNOS, CD16 and CD32, characterized as M1 markers, in a dose-dependent manner in BV2 microglia. The expression of Arg1, TGF-β and CCL-22, characterized as M2 markers, showed decreased expression when stimulated by C27 (Fig. 7B). Moreover, the immunofluorescence intensity of CD16/32 showed enhancement after C27 treatment, and the immunofluorescence intensity of M2 marker CD206 showed attenuation with C27 stimulation (Fig. 7C). IL-1β and TNF-α in culture medium were assessed by ELISA, and the two cytokines were increased after C27 treatment (Fig. 7D).

4. Discussion

Despite the fact that IMM-H004 shows beneficial effects in various ischemia stroke models, the mechanism of IMM-H004 exerting its protective effects remains unclear. The fast and marked increase in CKLF1 in the ischemic brain suggests that CKLF1 may play an important role in ischemic stroke. In the present study, we demonstrated that IMM-H004 protected BV2 microglia against OGD/R-induced toxicity. We further showed that IMM-H004 shifted OGD/R-injured BV2 microglia from the more pro-inflammatory M1 phenotype to the more anti-inflammatory M2 phenotype. Partly through skewing microglia polarization, IMM-H004 modulated the inflammatory response after OGD/R to a beneficial state. IMM-H004 decreased the amount of CKLF1 binding to CCR4 after OGD/R. We also found that CKLF1 modulated BV2 microglia toward the M1 phenotype and showed inflammation-related toxicity effects. Therefore, we suggested that IMM-H004 provided protective effects for OGD-R-injured BV2 microglia by regulating microglia polarization and further inflammatory response, at least in part, which may be related to the CKLF1/CCR4 axis.

Post-ischemic neuroinflammation is a vital pathophysiological process that determines the development and outcome of ischemic stroke [28]. Excessive inflammation post stroke leads to aggravated neuronal death and neurological deficiency. Microglia are primary mediators of insult and injury in the cerebral innate immune response [12,29]. Multiple researchers have provided strong evidence that microglia polarization and the subsequent neuroinflammatory responses contribute to post-stroke secondary brain injury. Studies have shown that M1-polarized microglia are involved in the onset and aggravation of neuroinflammation, contributing to cell death by releasing pro-inflammatory mediators [30–32], and M2 polarized microglia are related to the resolution of neuroinflammation, removing cellular debris and assisting in restoring tissue regeneration [33,34]. Although both phenotypes of microglia are activated in cerebral ischemia, the vulnerable brain environment favors M1 microglia, leading to inflammatory deterioration and injury [12,35]. Thus, modulating microglia polarization may be a potential target for stroke therapy. Therefore, we investigated the effects of IMM-H004 on microglia-mediated neuroinflammation and focused on microglia polarization in the current study. We established BV2 microglia with OGD/R model to mimic cerebral I/R injury in vitro and assessed the protective effects of IMM−H004.

In this study, we found that the M1 phenotypic markers iNOS, CD16 and CD32 were markedly increased after OGD/R insult, indicating that microglia were polarized toward the M1 phenotype under hypoxic conditions. Importantly, this M1 polarization induced by OGD/R was clearly attenuated by IMM-H004 pretreatment, and IMM-H004 also skewed the microglia polarization to the M2 phenotype revealed by increasing M2 phenotypic markers Arg1, CCL-22, TGF-β, and CD206. Moreover, pro-inflammatory cytokines including IL-1β and TNF-α in culture medium were increased significantly with OGD/R insult, and IMM-H004 reduced release of these proinflammatory mediators. TGF-β is an anti-inflammatory cytokine, and BDNF is a factor that can protect damaged neurons from death, improve the pathological morphology of the neurons, and help to restore neurogenesis. IMM-H004 enhanced the expression of TGF-β and BDNF in BV2 microglia exposed to OGD/R, which is beneficial for attenuating the excessive inflammatory response and improving recovery. NF-κB is considered a central transcription factor for inflammatory mediators and is important in microglial activation [36,37]. Numerous studies have shown that cerebral ischemia leads to NF-κB activation [38,39]. In the present study, we found that OGD/R insult caused NF-κB activation in BV2 microglia, which can be attenuated by IMM-H004 administration.

IMM-H004 is a compound first screened in a C27/CCR4 interaction system, and it blocks calcium mobilization and chemotaxis, indicating it may disturb the coordination between CKLF1 and CCR4. In this study, we found increased expression of CKLF1 in OGD/R-injured BV2 microglia, and IMM-H004 treatment decreased this elevation. Moreover, we investigated the functions of two receptors of CKLF1, CCR4 and CCR5 in the OGD/R-induced microglia polarization. CCR4 inhibitor C 021 dihydrochloride, but not the CCR5 inhibitor DAPTA, could modulate the OGD/R-induced microglia polarization, suggesting the microglia polarization may partly dependent on CCR4. We investigated the effects of CKLF1 on BV2 microglia, and found that CKLF1 modulates BV2 microglia polarization toward the M1 phenotype. Moreover, we found that CKLF1 binding to CCR4 was increased in BV2 microglia exposed to OGD/R, and IMM-H004 decreased the amount of CKLF1 binding to CCR4, indicating that the protective effects of IMM-H004 may involve the CKLF1/CCR4 axis.

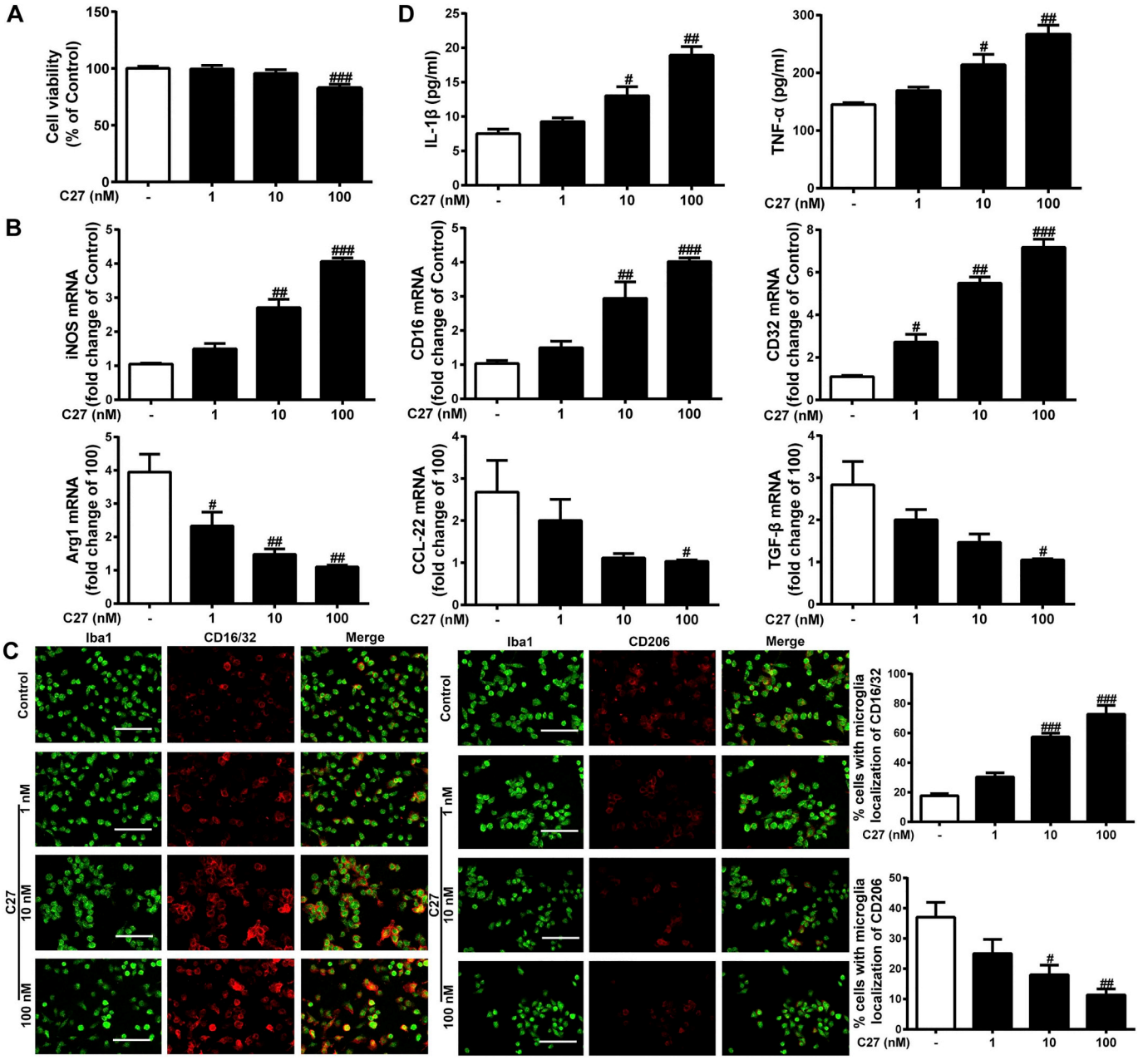

Fig. 7. Effects of CKLF1 on BV2 microglia. (A) BV2 microglia were incubated with C27 (1, 10, 100 nM) for 24 h, and cell viability was assessed by MTT ($n = 6$). (B) qPCR analysis of mRNA expression levels of M1 markers (iNOS, CD16, CD32) and M2 markers (Arg1, CCL-22, TGF-β) in C27-stimulated BV2 microglia ($n = 3$). (C) Representative photomicrographs of double-stained immunofluorescence of CD16/32 with Iba1 and CD206 with Iba1 in C27-stimulated BV2 microglia. Quantitative analysis of CD16/32-positive and CD206-positive BV2 microglia (n = 3). (D) Expressions of IL-1β and TNF-α in culture media of C27-stimulated BV2 microglia were determined using ELISA (n = 3). Scale bars, 100 μm. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. control group.

5. Conclusions

In conclusion, our current work showed that IMM-H004 protected against OGD/R-caused cytotoxicity in BV2 microglia, and the neuroprotective function may be related with microglia polarization modulation. The mechanism of protection against OGD/R insult may be the attenuation of pro-inflammatory M1 phenotypic polarization and skewing toward anti-inflammatory M2 phenotypic polarization. The OGD/R-induced microglia polarization may by partly through CCR4, and CKLF1 can modulate BV2 microglia polarization toward the M1 phenotype. IMM-H004 provides protective effects via modulating microglia polarization, which may involve the CKLF1/CCR4 axis. Moreover, IMM-H004 blocked NF-κB activation, which is closely related to M1 microglia polarization, alleviating the expression of downstream pro-inflammatory cytokines. Additionally, IMM-H004 increased the expression of anti-inflammatory cytokine TGF-β, which is directly related to M2 phenotypic microglia. Thus, IMM-H004 exerts significant protective effects against cerebral ischemic injury, partly through regulating the polarization of microglia, and this effect is possibly through inhibiting the CKLF1/CCR4 axis. These findings provide new evidence for IMM-H004 as a potential medicine for ischemic stroke (Fig. 8).

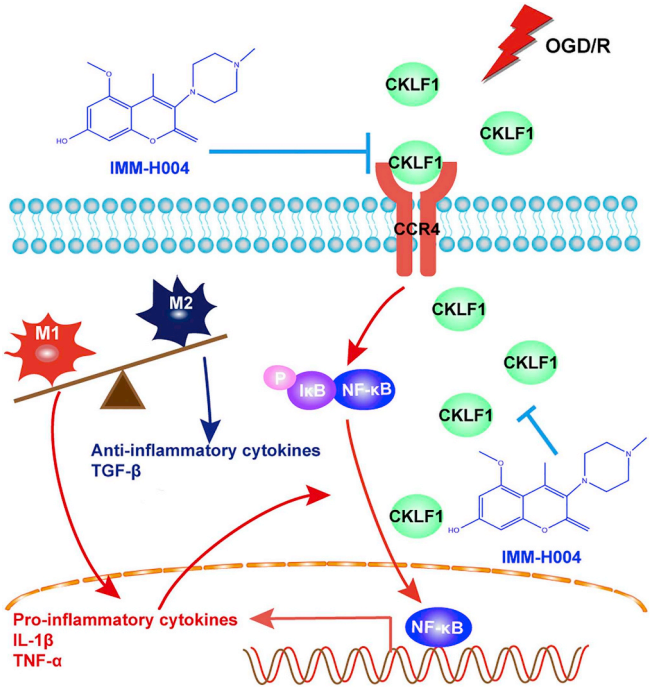

Fig. 8. IMM-H004 protects BV2 microglia from OGD/R-induced toxicity on BV2 microglia partly through modulating microglia polarization. CKLF1 expression is increased due to the OGD/R insult in BV2 microglia and leads BV2 microglia to polarize toward the pro-inflammatory M1 phenotype, followed by an excessive inflammatory response. IMM-H004 decreases the amount of CKLF1 binding to CCR4 in OGD/R and inhibits M1 polarization and promotes M2 polarization, leading to an attenuated inflammatory reaction and improved recovery.

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Chen Chen, Qidi Ai, Shifeng Chu, Zhao Zhang, and Naihong Chen conceptualized of and designed the study. Chen Chen, Qidi Ai and Naihong Chen acquired and analyzed the data. Chen Chen, Qidi Ai, and Naihong Chen drafted the text and prepared the figures.

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Disclosures

The authors have no conflicts of interest to disclose.

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