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β-Caryophyllene protects against ischemic stroke by promoting polarization of microglia toward M2 phenotype via the TLR4 pathway

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ABSTRACT

Aims: The objective of the study was to determine whether β-caryophyllene (BCP) exerts a neuroprotective effect in cerebral ischemia-reperfusion (I/R) injury by inhibiting microglial activation and modulating their polarization via the TLR4 pathway.

Main methods: Wild-type (WT) and TLR4 knockout (KO) C57BL/6J mice were subjected to cerebral I/R injury and neurologic dysfunction, cerebral infarct volume, brain edema, microglia activation and polarization, and TLR4 expression were determined. In vitro, primary microglia were stimulated with LPS and IFN-γ or IL-4 to induce polarization of microglia toward M1 or M2 phenotypes.

Key findings: BCP reduced cerebral infarct volume, brain edema, and neurologic deficits in WT mice after I/R. The optimal dose of BCP, 72 mg/kg body weight, inhibited microglial activation and reduced the secretion of proinflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 by microglia of WT mice. BCP inhibited the level of TLR4 in WT mice, and partially reduced neurologic deficits, infarct volume, and brain edema in TLR4 KO mice. Importantly, BCP reduced the number of activated M1-type microglia and increased the number of M2-type microglia in the ipsilateral cortex of both WT and TLR4 KO mice. In vitro, BCP decreased the secretion of proinflammatory cytokines induced by LPS plus IFN-γ, downregulated the level of TLR4 protein, and polarized microglia towards the M2 phenotype.

Significances: The decrease in TLR4 activity mediated, at least in part, the anti-inflammatory effects of BCP and its ability to shift microglia polarization from the M1 to M2 phenotype.

1. Introduction

Ischemic stroke is the third leading cause of death and the most frequent cause of permanent disability worldwide [1]. The pathogenesis of ischemic stroke is multifactorial, but the inflammatory response driven by microglia contributes significantly to brain ischemia [2].

Microglia represent the first line of defense against brain injury, such as the stroke [3]. In response to pathophysiological stimulation, microglia can act as a double-edged sword by switching between "classically activated" M1 and "alternatively activated" M2 phenotypes and modifying their morphology and function. Microglia polarized to the M1 phenotype secrete pro-inflammatory mediators, such as IL-6, IL-1β, and TNF-α, which are involved in tissue damage and prolong the neuroinflammatory response. In contrast, upon polarization to the M2 phenotype, microglia release anti-inflammatory factors, including IL-4, IL-10, arginase-1 (Arg-1), and transforming growth factor-β (TGF- β), which suppress inflammation, promote tissue repair, and provide neuroprotection [2–4]. It is well-documented that following an ischemic stroke, microglia initially polarize to the M2 phenotype, and later transition to the M1 phenotype [5–7]. Therefore, a novel therapeutic agent or approach capable of inhibiting this transition may offer new possibilities for innovative therapeutic strategies.

Toll-like receptor 4 (TLR4) is a key regulator of the activation of microglia and their polarization after brain injury [8–10]. In a mouse model of traumatic brain injury, inhibition of TLR4 signaling attenuates neurological deficits by regulating microglial M1/M2 phenotypic transition [11]. TLR4 also mediates microglial activation and production of inflammatory cytokines in neonatal rat brain following hypoxia

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[12]. Thus, therapeutic agents targeting the TLR4-mediated activation and polarization of microglia may have a central role in the development of new treatments for ischemic stroke.

β-Caryophyllene (BCP) is a natural bicyclic sesquiterpene producing diverse biological and pharmacological effects, such as analgesia [13,14], anti-inflammation [15,16], anti-oxidation [15,17], and prevention of apoptosis [18]. BCP is a small, fat-soluble molecule (204.36 Da) that can cross the blood-brain barrier [19]. It alleviates liver injury induced by D-galactosamine and lipopolysaccharide via suppressing TLR4-mediated inflammatory responses [20]. Additionally, we have previously demonstrated that BCP alleviates ischemic stroke-induced neuroinflammation by interfering with the HMGB1/TLR4 signaling [21]. However, the mechanism underlying the impact of BCP on ischemia-induced inflammation is essentially unknown. Therefore, the current work explores the cellular mechanism of the effect of BCP on inflammation induced by cerebral ischemia/reperfusion (I/R) injury. Specifically, a mouse model of ischemic damage caused by the occlusion of the right middle cerebral artery was used to determine whether BCP exerts its neuroprotective effect by modulating microglial activation and M1/M2 polarization.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Experimental Ethics Committee of Chongqing Medical University and conducted following the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Bethesda, MD, USA). The Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines were followed throughout the current work. All surgeries were performed under anesthesia, and all efforts were made to minimize animal suffering.

Neonatal C57BL/6 mice (1-day old) and adult male wild type (WT) C57BL/6 mice (8–10 weeks) of a specific pathogen-free (SPF) grade were obtained from the Experimental Animal Center, Chongqing Medical University (Chongqing, China). C57BL/6 TLR4 knockout (KO) male mice were a generous gift from Jianhua Peng (Affiliated Hospital of Southwest Medical University, Luzhou, China).

2.2. Transient focal cerebral ischemia model

After the mice were anesthetized with 3.5% pentobarbital, transient focal cerebral ischemia was induced by the right middle cerebral artery occlusion (MCAO) for 60 min [5]. The rectal temperature was maintained at 37.0 ± 0.5 °C throughout the surgery with a temperature-controlled heating pad. Sham-operated animals underwent the same surgical protocol except for MCAO. Neurological function deficit was assessed after the surgery, and only animals that scored from 1 to 4 were used in experiment. Twenty-four mice did not exhibit neurological impairment or died before the end of the study. The remaining animals (n = 237) were all included in subsequent experiments.

2.3. Primary microglia cells

Primary microglia were prepared from cerebral cortices of 1-day old C57BL/6 mice [8,9]. The cortex was dissected and minced in phosphate-buffered saline (PBS) containing 0.25% trypsin (Hyclone, Northbrook, IL, USA). Isolated cells were plated in cell culture flasks at 1.2×10^5 cells/cm^2 in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated for 14 days. Subsequently, microglia were removed from mixed population of glial cells by mild trypsinization method [22]. The primary cells, which commonly contain > 90% microglia (Supplementary Fig. 1), were used in the experiments. For the induction of M1 polarization, microglia were treated with lipopolysaccharide (LPS, 100 ng/mL) and interferon-gamma (IFN-γ, 20 ng/mL) for 48 h. For the induction of the M2 phenotype, microglia were treated with IL-4 (20 ng/mL) for 48 h [5].

Table 1
Sequences of primers used in qRT-PCR.

Gene	Primer	Sequence
CD68	forward	5′ -GACCGTTGTGTGTGTTCTGG-3′
	reverse	5′ -GATGAGCAGCATCACAAGGA-3′
iNOS	forward	5′ -GCTTGTCTCTGGGTCCTCTG-3′
	reverse	5′ -CTCACTGGGACAGCACAGAA-3′
TNF-α	forward	5′ -ACGGCATGGATCTCAAAGAC-3′
	reverse	5′ -AGATAGCAAATCGGCTGACG-3′
CD16/32	forward	5′ -AATCCTGCCGTTCCTACTGATC-3′
	reverse	5′ -GTGTCACCGTGTCTTCCTTGAG-3′
IL-1β	forward	5′ -TGTCTTGGCCGAGGACTAAGG-3′
	reverse	5′ -TGGGCTGGACTGTTTCTAATGC-3′
TGF-β	forward	5′ -TGCGCTTGCAGAGATTAAAA-3′
	reverse	5′ -CGTCAAAAGACAGCCACTCA-3′
Ym-1	forward	5′ -ACCCCTGCCTGTGTACTCACC T-3′
	reverse	5′ -CACTGAACGGGGCAGGTCCAAA-3′
Arg1	forward	5′ -TTAGGCCAAGGTGCTTGCTGCC-3′
	reverse	5′ -TACCATGGCCCTGAGGAGGTTC-3′
CD206	forward	5′ -TCAGCTATTGGACGCGAGGCA-3′
	reverse	5′ -TCCGGGTTGCAAGTTGCCGT; -3′
IL10	forward	5′ -GGCAGAGAACCA TGGCCCAGAA-3′
	reverse	5′ -AATCGATGACAGCGCCTCAGCC-3′
GAPDH	forward	5′ -TGGTGAAGGTCG GTGTGAAC-3′
	reverse	5′ -GCTCCTGGAAGATGGTGATGG-3′

2.4. BCP treatment and experimental groups

For in vivo experiments, BCP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in olive oil and administered intraperitoneally once a day for three consecutive days before the induction of transient ischemia, and 2 h after the surgery [21]. Olive oil was used in sham and I/R groups receiving the vehicle only. Mice were randomly divided into the following groups on the basis of our previous study [21]: sham + vehicle, sham + BCP 72 mg/kg, I/R + vehicle, I/R + BCP 8 mg/kg, I/R + BCP 24 mg/kg, and I/R + BCP 72 mg/kg. In another set of experiments, WT and TLR4 KO mice underwent either the sham surgery or induction of I/R by MCAO and were treated with either BCP (72 mg/kg) or the vehicle.

For in vitro experiments, BCP was prepared in 0.05% dimethyl sulfoxide (DMSO) [21]. Cells were treated with 1, 5, or 10 μM BCP for 24 h, and then stimulated with LPS (100 ng/mL) together with IFN-γ (20 ng/mL) or IL-4 (20 ng/mL) for additional 48 h [5]. The dose and duration of BCP were selected based on our preliminary experiments and previous studies [15,21]. TAK242, a specific TLR4 antagonist (10 μM, Sigma-Aldrich, St. Louis, MO, USA) was administered 2 h prior to the BCP treatment to block TLR4 signal transduction. Cells were divided into the following groups: (i) control group, incubated in cell culture medium containing 0.05% DMSO alone, (ii) LPS + IFN-γ group, (iii) BCP (10 μM) + LPS + IFN-γ group, (iv) TAK242 + LPS + IFN-γ group, and (v) TAK242 + BCP (10 μM) + LPS + IFN-γ group.

2.5. Neurologic dysfunction

Neurologic dysfunction was assessed by a blinded observer using the modified Longa's method [23].

2.6. Cerebral infarct volume

The volume of the cerebral infarct was determined by 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining of brain sections. The images of the stained specimens were acquired and analyzed with the Image J software. Infarct size was expressed as the percentage of the contralateral hemisphere.

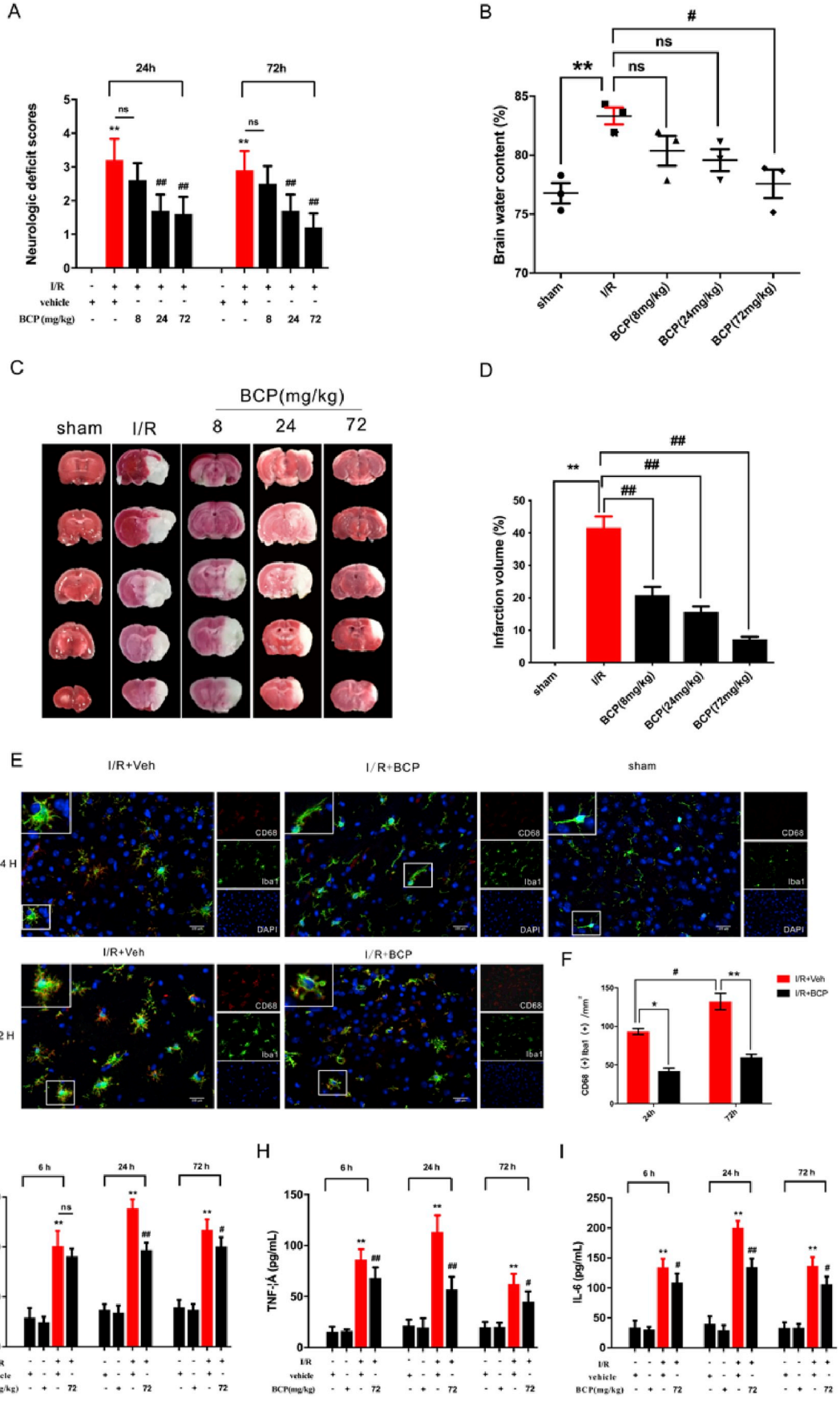

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Fig. 1. BCP protects the ischemic brain, inhibits microglial activation, and decreases the production of proinflammatory cytokines by microglia in WT mice after cerebral I/R injury. (A) Neurological deficit scores in the five groups of WT mice subjected to cerebral I/R. n = 10 mice/group. (B) Brain water content 24 h after cerebral I/R. n = 3 mice/group. (C) TTC staining of the brain of WT mice at 24 h after cerebral I/R. n = 3 mice/group. (D) Relative infarct volume of mice brain. n = 3 mice/group. $^{**}P < 0.01$ vs. sham; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. I/R group; ns indicates no significant difference. (E) A decrease in the number of Iba1 (+)/CD68(+) cells documenting that BCP treatment significantly attenuates the activation of microglia induced by cerebral I/R. (F) Histogram representing the quantitative analysis of Iba1(+)/CD68(+) cells. n = 6 mice/group; $^{\#}P < 0.05$ vs. vehicle-treated group at 24 h; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. the vehicle-treated group at the same time point. Scale bar = 100 μm. BCP decreased microglia-derived pro-inflammatory cytokines IL-1β (G), TNF-α (H), and IL-6 (I) at 6, 24, and 72 h post-cerebral I/R. n = 6 mice/group; $^{**}P < 0.01$ vs. vehicle-treated sham group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. the vehicle-treated I/R group at the same time point.

2.7. Brain water content

Brain water content was measured as a surrogate for the extent of brain edema. This value was calculated as [(wet weight − dry weight)/wet weight] × 100%.

2.8. Immunostaining of brain tissues

For immunostaining, the mouse brain was cut into 8 μm -thick coronal sections as described previously [24]. Sections were fixed in cold acetone for 10 min and blocked with 10% bovine serum albumin (BSA) at room temperature for 1 h. Subsequently, the sections were incubated overnight at 4 °C with the following primary antibodies: rat anti-CD16/32 (diluted 1:200, BD Pharmingen, San Diego, CA, USA), mouse anti-CD206 (1:250, Abcam, Cambridge, UK), rabbit anti-Iba1 (1:1000, Wako, Richmond, USA), mouse anti-CD68 (1:200, Abcam), or mouse anti-TLR4(1:200, Abcam). After washing, the sections were incubated with species-matched secondary antibody (1:1000, Proteintech, Wuhan, China) at 37 °C for 1 h. The nuclei were counterstained with 4' 6-diamidino-2-phenylindole (DAPI) for 5 min. Images of stained sections were acquired with a fluorescence microscope (Nikon, Tokyo, Japan).

2.9. Immunostaining of primary microglia

Microglia were fixed with 4% paraformaldehyde for 10 min, blocked with 1% BSA for 30 min, and then incubated overnight at 4 °C with primary antibodies against CD16/32 (1:200, BD Pharmingen), CD206 (1:200, Abcam), anti-Iba1 (1:1000, Wako), or TLR4 (1:200, Abcam). Subsequently, cells were incubated with species-matched secondary antibodies (1:1000, Proteintech, Wuhan, China) at 37 °C for 1 h. Cell nuclei were stained with DAPI.

2.10. Isolation of microglia from mouse brain

Microglia were obtained by magnetic-activated cell sorting (MACS) of dissociated brain tissue [25]. Briefly, at 6, 24, and 72 h post-reperfusion, ischemic hemispheres were collected, and minced into 1 mm^3 pieces, and digested with 0.125% trypsin at 37 °C for 20 min. Cells were collected by centrifugation and resuspended in ice-cold PBS. Single-cell suspensions were incubated with APC-conjugated anti-mouse CD11b (eBioscience, San Diego, CA, USA) at 4 °C for 30 min, washed with the MACS buffer, and then incubated with magnetic anti-APC particles (BD Biosciences, USA) at room temperature for 30 min. The cell suspension with bound magnetic particles was applied to the BD Imagnet™ and incubated for 10 min. The supernatant was then discarded, and the isolated microglia were harvested for ELISA and western blotting.

2.11. ELISA

The level of proinflammatory cytokines TNF- α (Boster, Wuhan, China), IL-1 β and IL-6 (Abcam, Cambridge, UK) in the brain tissue or cell culture medium was measured by respective ELISA kits according to the manufacturer's protocol.

2.12. Western blot analysis

The brain samples or cultured cells were sonicated in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Boster, Wuhan, Hubei, China), centrifuged at 12,000 g for 10 min at 4 °C and the supernatants were stored at −80 °C. Protein concentration in the samples was determined with a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Extracted proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in Tris-buffered saline-Tween-20 (TBST) containing 5% skim milk for 1 h and incubated overnight at 4 °C with the following primary antibodies: rabbit anti-INOS (1:1000, Abcam), rabbit anti- Arg1 (1:1000, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-MyD88 (1:1000, Abcam), rabbit anti-TRIF (1:1000, Abcam), rabbit anti-TLR4 (1:1000, Abcam), and mouse anti-GAPDH (1:5000, Proteintech). Membranes were then washed TBST and incubated with species-matched secondary antibodies for 1 h at room temperature. The protein bands were detected using an enhanced chemiluminescence detection system (Bio-Rad, Hercules, CA, USA) and quantified with Image Lab software (Bio-Rad).

2.13. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the primary microglia using Trizol (Vazyme, Nanjing, China) and cDNA was prepared from 2 μg of total RNA with the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Otsu, Shiga, Japan). Primers were synthesized by Shanghai Sheng Gong (Shanghai, China). The quantitative real-time PCR (qRT-PCR) was performed using SYBR Master Mixture Kit (Takara, Otsu, Shiga, Japan) on a Light Cycler 480 II Real-Time PCR instrument (Roche, Indianapolis, IN, USA). The housekeeping gene GAPDH was used to normalize gene expression. The target genes and the corresponding PCR primers are listed in Table 1.

2.14. Statistical analysis

Statistical analyses were performed using SPSS 25.0 software (IBM, Armonk, NY, USA). All data are presented as the mean ± standard deviation (SD). Comparisons of two groups were performed by unpaired two-tailed Student's *t*-test. Comparisons among multiple groups were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's test. The effects of multiple factors were subject to two-way ANOVA analysis. *P*-value < 0.05 was defined as statistically significant.

3. Results

3.1. BCP ameliorates neurologic deficits, infarct volume, and brain edema in WT mice with cerebral I/R injury

I/R injury resulted in severe neurological deficits ($P < 0.01$, n = 10; Fig. 1A), which were significantly alleviated by BCP (24 and 72 mg/kg body weight) at 24 h and 72 h post-reperfusion ($P < 0.01$, n = 10; Fig. 1A). These results indicate that BCP treatment improves the functional outcome after cerebral I/R. At 24 h post-reperfusion, BCP

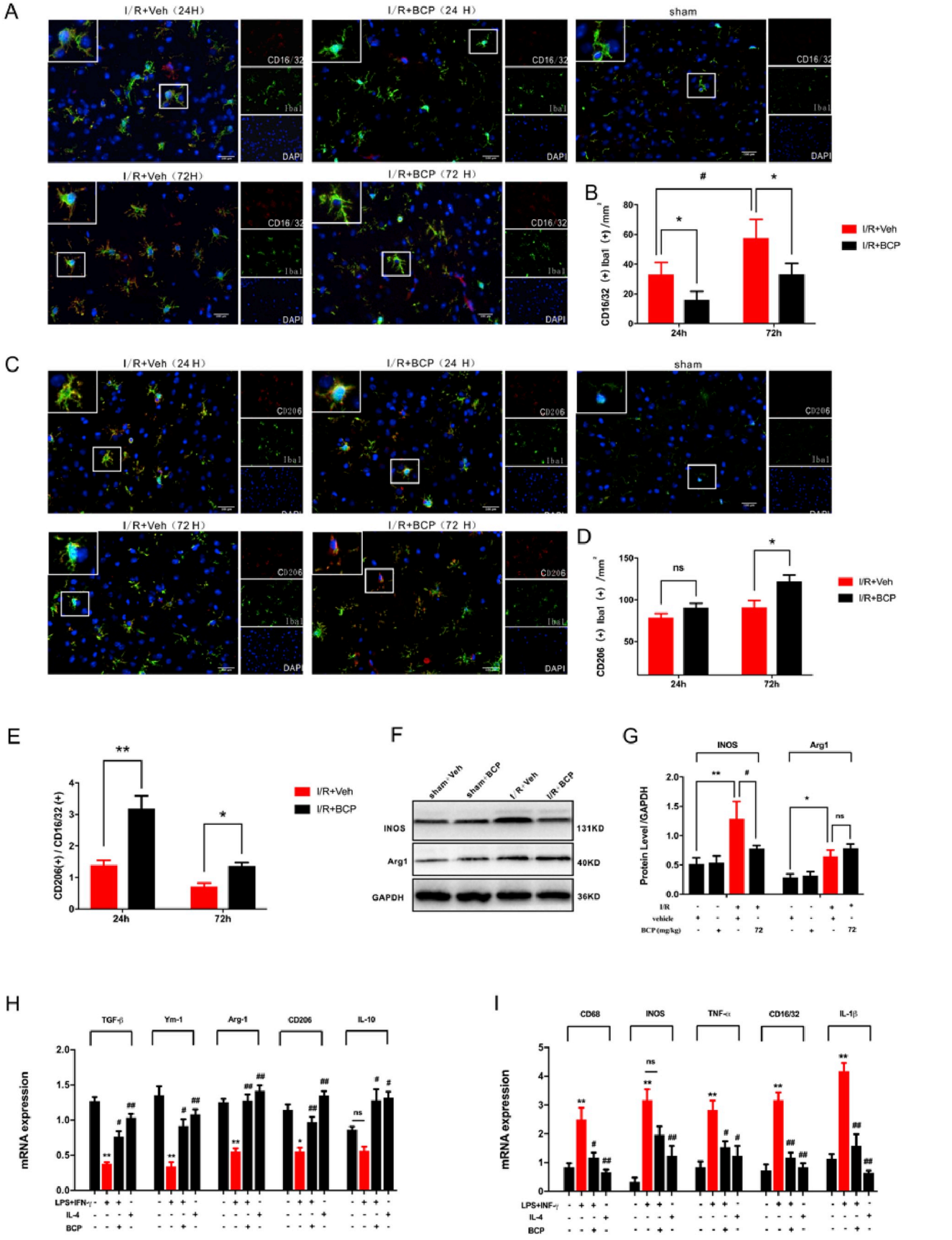

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Fig. 2. BCP mediates microglia activation and polarization in WT mice after cerebral I/R injury. (A and C) BCP reduced the number of Iba1(+)/CD16/32 (+) microglia at 24 and 72 h after cerebral I/R (A) and increased the number of Iba1(+)/CD206 (+) microglia at 72 h post-cerebral I/R(C). Scale bar = 100 μm. (B and D) Histograms showing quantitative analysis of Iba-1(+)/CD16/32(+) microglia (B) and Iba-1(+)/CD206(+) microglia (D). n = 6 mice/group, $^{\#}P < 0.05$ vs. the vehicle-treated I/R group at 24 h, $^{*}P < 0.05$ vs. the vehicle-treated I/R group at the same time points. (E) Ratio of Iba1(+)/CD206(+) microglia to Iba1(+)/CD16/32(+) microglia. (F and G) Determination of INOS and Arg1 protein level by Western blotting. n = 6 mice/group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the vehicle-treated sham group, and $^{\#}P < 0.05$. vs. the vehicle-treated I/R group. (H and I) Expression of pro-inflammatory (CD68, iNOS TNF-α, CD16/32, and IL-1β) and anti-inflammatory (TGF-β, YM-1, Arg-1, CD206 and IL-10) cytokines mRNA in response to different treatments measured by qRT-PCR. n = 6 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS plus IFN-γ stimulation group, ns indicates no significant difference.

(8, 24, and 72 mg/kg) decreased the infarct volume from 41.64 ± 6.03% to 20.80 ± 4.46%, 15.7 ± 2.86, and 7.24 ± 1.34%, respectively ($P < 0.05$, n = 3; Fig. 1C and D). Additionally, BCP at 72 mg/kg reduced water content in the ischemic hemisphere at 24 h post-reperfusion (83.32 ± 1.24% in the BCP-treated group vs. 77.57 ± 2.09% in the vehicle-treated I/R group, $P < 0.05$, n = 3; Fig. 1B). Given the more pronounced effect of the 72 mg/kg dose of BCP on neurologic deficits, infarct volume and brain edema than that of 8 and 24 mg/kg, subsequent experiments were performed using the BCP dose of 72 mg/kg body weight.

3.2. BCP inhibits microglial activation and decreases microglia-derived proinflammatory cytokine production of WT mice after cerebral I/R injury

Brain injury induced by cerebral I/R results in the activation of microglia and enhanced release of inflammatory mediators [26]. Therefore, the effects of BCP on neuroinflammation, microglial activation, and proinflammatory cytokine production after cerebral I/R were determined. Ionized calcium-binding adapter molecule 1 (Iba1) was used to detect all microglia and phagocytic microglia marker CD68 was used to identify reactive cells.

The number of Iba-1(+)/CD68 (+) microglia was markedly elevated in the I/R group at 24 h post-reperfusion, and was further increased at 72 h ($P < 0.05$, n = 6; Fig. 1E and F). The activation of microglia after cerebral ischemia-reperfusion was apparent, as evidenced by their hypertrophic morphology with thickened and retracted processes. The treatment with BCP resulted in fewer Iba-1(+)/CD68 (+) microglia, which had smaller cell bodies and thinner processes ($P < 0.05$, n = 6; Fig. 1E and F). To further determine whether BCP affects the synthesis of pro-inflammatory cytokines by microglia, harvested CD11b-positive cells were isolated from the ischemic hemisphere by MACS and the level of TNF- α, IL-1 β, and IL-6 was measured by ELISA. The production of these cytokines increased markedly at 6, 24, and 72 h post-reperfusion ($P < 0.01$, n = 6; Fig. 1G, H, 1I), but the BCP treatment significantly suppressed these increases ($P < 0.05$, n = 6; Fig. 1G, H, 1I). These results indicate that BCP ameliorates ischemia-induced neuroinflammation, possibly by the inhibition of microglial activation.

3.3. BCP inhibits microglial polarization toward the M1 phenotype and promotes M2 polarization WT mice after cerebral I/R injury

With prolonged reperfusion, from 24 to 72 h, the number of Iba1(+)/CD16/32(+) (M1 microglia) and Iba1(+)/CD206(+) (M2 microglia) cells in the ipsilateral cortex increased markedly ($P < 0.01$, n = 6; Fig. 2A–D). BCP treatment prevented these changes, significantly reducing the number of Iba1(+)/CD16/32 cells and increased the number of Iba1(+)/CD206 cells at 72 h after reperfusion ($P < 0.05$, n = 6; Fig. 2A–D). As a result, the M2-to-M1 ratio was significantly increased in the BCP-treated group compared with the vehicle-treated group ($P < 0.05$, n = 6; Fig. 2E). Western blotting of proteins in CD11b(+) microglia sorted from ischemic hemisphere by MACS demonstrated that the level of both iNOS (M1-like marker) and Arg1 (M2-like marker) was significantly increased at 72 h post-reperfusion compared with the vehicle-treated sham group ($P < 0.05$, $P < 0.01$, n = 6; Fig. 2F and G). BCP treatment significantly decreased the level of iNOS ($P < 0.05$, n = 6; Fig. 2F and G). Additionally, BCP treatment further upregulated the level of Arg1but this change did not reach statistical significance ($P = 0.061$ vs. vehicle-treated I/R group, n = 6; Fig. 2F and G).

To determine whether BCP directly mediates microglial polarization, primary microglia were used to induce M1/M2 phenotype in vitro [5], and the expression of M1 and M2 markers was assessed by qRT-PCR. The expression of mRNA of M1-related genes, including CD68, iNOS, TNF-α, CD16/32, and IL-1β, was distinctly increased by the stimulation with a combination of LPS and IFN-γ ($P < 0.01$, n = 6; Fig. 2I). In contrast, the expression of mRNA of M2-related genes, including TGF-β, CD206, Arg-1, and Ym-1 was markedly suppressed in the presence of LPS and IFN-γ ($P < 0.01$ for TGF-β, Arg-1, and Ym-1; $P < 0.05$ for CD206; n = 6; Fig. 2H); IL-10 was the only M2-related gene that was not upregulated significantly (P = 0.202, n = 6; Fig. 2H). Treatment of the cells with 10 μM BCP significantly reversed these alterations in the expression of M1 and M2 markers, indicating its direct effect on the modulation of microglia polarization ($P < 0.05$, n = 6; Fig. 2H and I). Together, the results indicate that BCP not only prevents the activation of microglia but also inhibits their polarization toward the M1 phenotype and promotes the M2 phenotype, further enhancing its protective effects. The same effect can be expected to be operative in vivo in WT mice affected by cerebral I/R injury.

3.4. BCP protects against focal ischemia-induced brain injury partially by inhibiting TLR4

A growing body of evidence indicates that activation of the TLR4 signaling pathway is required for the activation and polarization of microglia in the central nervous system [11,12,27]. This conclusion is supported by immunohistochemical demonstration of a robust increase the number of Iba1(+)/TLR4(+) cells in the ischemic cortex of the ipsilateral brain at 24 h after cerebral I/R injury ($P < 0.01$, n = 6; Fig. 3A and B). However, BCP treatment significantly reversed this alteration ($P < 0.01$, n = 6; Fig. 3A and B). Western blot analysis further revealed a sharp increase in TLR4 protein and its downstream targets MyD88 and TRIF as early as 6 h after reperfusion ($P < 0.05$ for TLR4 and MyD88, $P < 0.01$ for TRIF, n = 6; Fig. 3C–F). BCP significantly reduced the level of these three proteins at 6 h post-reperfusion ($P < 0.05$ for TLR4 and MyD88, $P < 0.01$ for TRIF, n = 6; Fig. 3C–F). In comparison with the vehicle-treated I/R mice, BCP significantly downregulated the level of TLR4 and MyD88 at 24 h after reperfusion ($P < 0.05$ for TLR4 and MyD88, P = 0.989 for TRIF, n = 6; Fig. 3C–F), and reduced the levels of TLR4 and TRIF at 72 h ($P < 0.05$ for TLR4, $P < 0.01$ for TRIF , P = 0.097 for MyD88, n = 6; Fig. 3C–F).

TLR4 KO mice were used to determine whether TLR4 is a crucial target of BCP. At 24 h post-reperfusion, the absence of TLR4 resulted in a significantly improved infarct volume and brain water content (n = 3, $P < 0.05$ vs. WT), although the neurologic deficit did not change ($P = 0.512$ at 24 h, $P = 1.000$ at 72 h) (Supplementary Figs. 2A–2C). Moreover, at 24 h post-reperfusion, BCP-treated TLR4 KO mice had smaller infarct volume (4.14 ± 0.81% vs. 7.73 ± 1.58%; $P < 0.05$, n = 3; Fig. 4A and B), lower brain water (75.08 ± 1.00% vs.78.75 ± 0.42%; $P < 0.05$, n = 3; Fig. 5C) than the vehicle-treated TLR4 KO mice. In addition to pairwise comparisons by two-tailed Student's t-test, the four groups (WT + vehicle, WT + BCP, TLR4

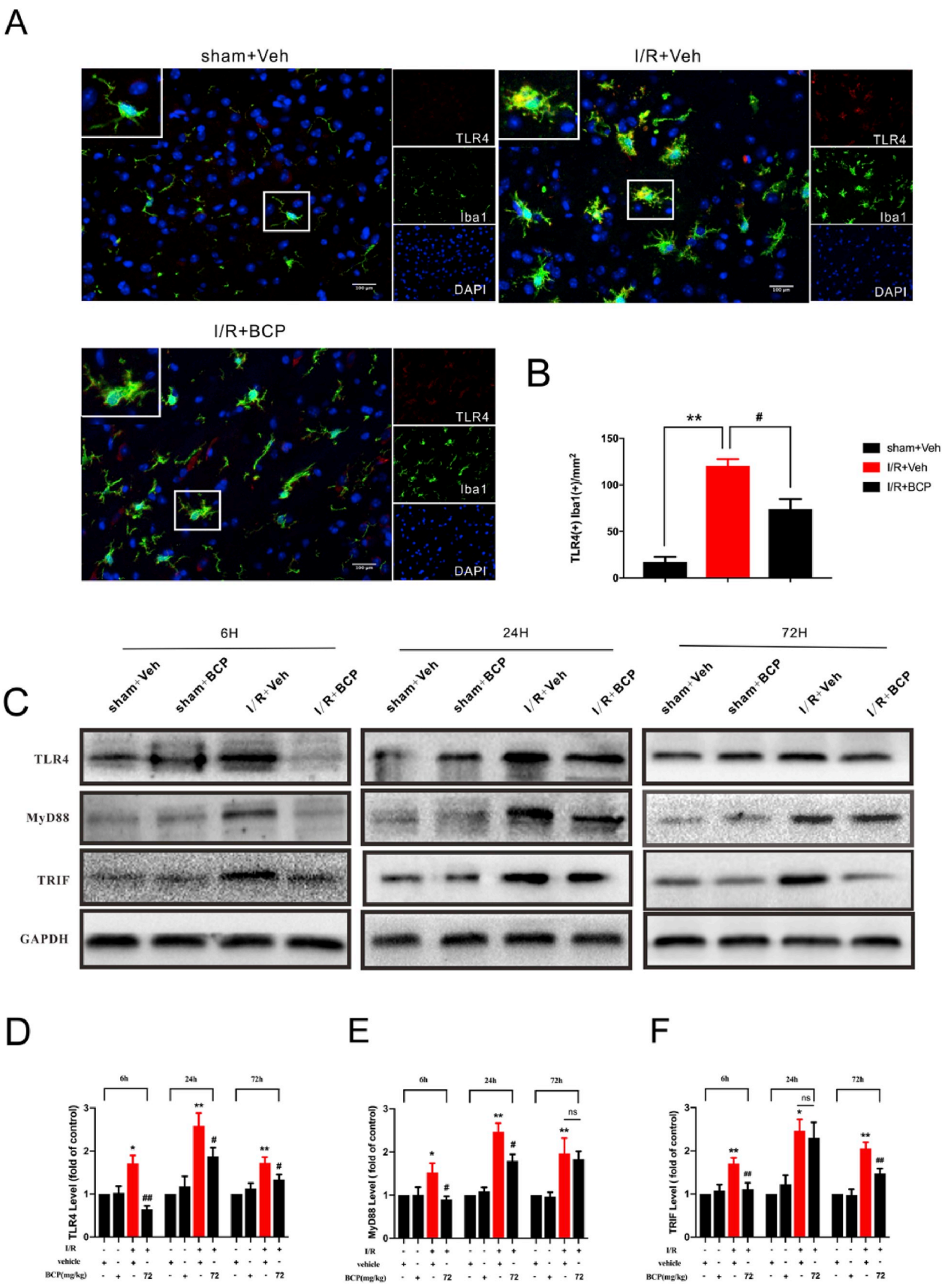

Fig. 3. BCP decreases the level of TLR4 and its downstream targets MyD88 and TRIF in WT mice after cerebral I/R injury. (A) Representative images of coronal sections stained with antibodies against Iba-1 and TLR4 at 24 h post-cerebral I/R. Scale bar = 100 μm. (B) Histogram showing the quantitative analysis of Iba-1(+)/TLR4(+). n = 6 mice/group, $^{**}P < 0.01$ vs. the vehicle-treated sham group, $^{\#}P < 0.05$ vs. the vehicle-treated I/R group. (C) Western blotting of TLR4, MyD88, and TRIF proteins. (D–F) Histogram showing the quantitative analysis of the level of TLR4, MyD88, and TRIF proteins. n = 6 mice/group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated sham group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the vehicle-treated I/R group at the same time points, ns indicates no significant difference.

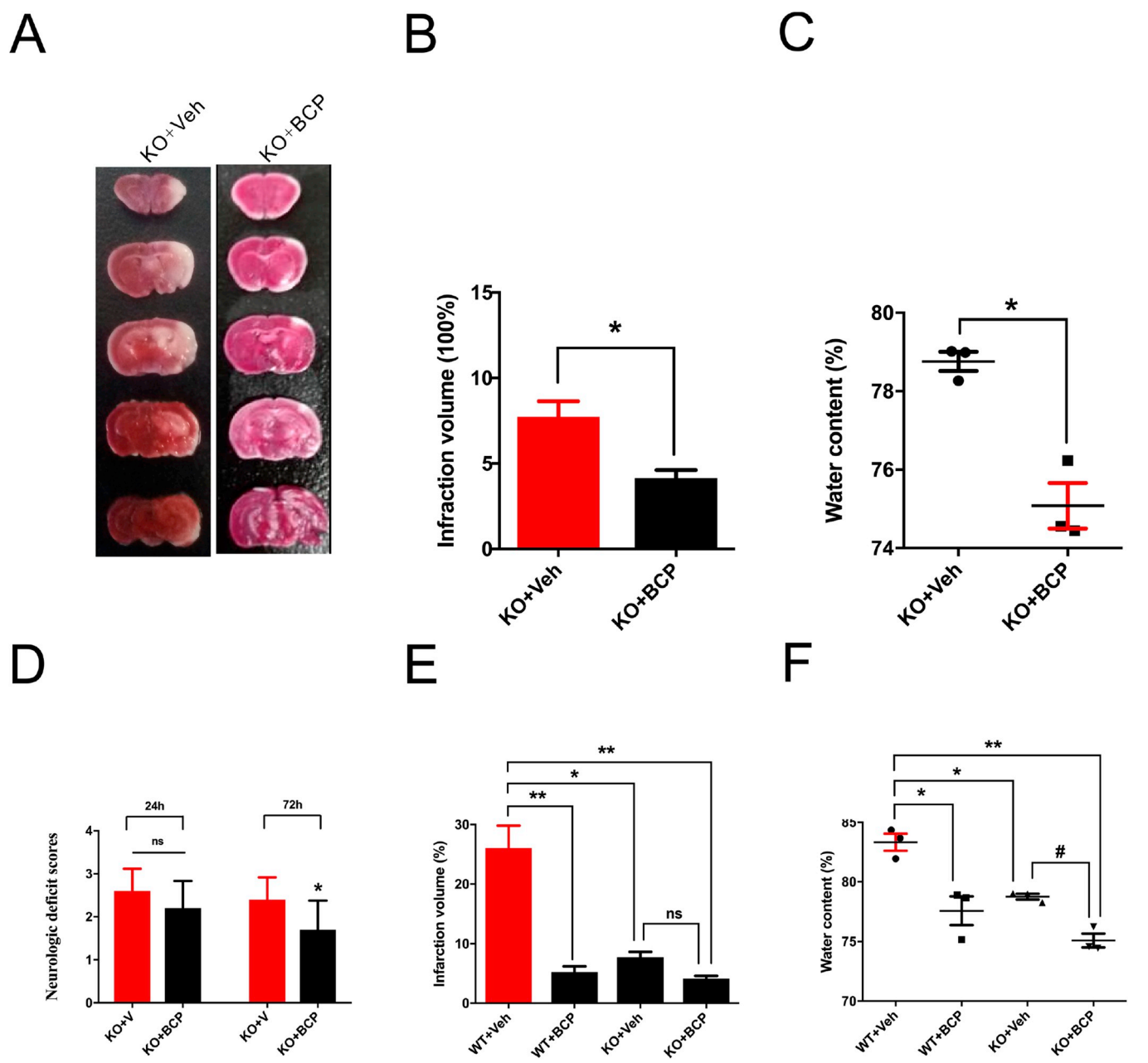

Fig. 4. BCP has limited protection in TLR4 KO mice after cerebral I/R injury. Infarct volume (A, B) and brain water content (C) in TLR4 KO mice treated with vehicle or BCP at 24 h post-reperfusion. $^{*}P < 0.05$ vs. vehicle-treated KO mice, n = 3 mice/group. (D) Neurologic deficits in TLR4 KO mice treated with vehicle or BCP measured at 24 and 72 h after reperfusion. n = 10 mice/group; $^{*}P < 0.05$ vs. vehicle-treated KO mice. (E) Infarct volume and (F) brain water content in WT and KO mice treated with vehicle or BCP. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated WT mice, $^{\#}P < 0.05$ vs. vehicle-treated TLR4 KO mice, ns indicates no significant difference.

KO + vehicle, and TLR4 KO + BCP) were compared by the two-way ANOVA post hoc test. In this analysis, data for WT mice presented in Fig. 1D were used for comparison with KO mice. These statistical calculations indicated that the reduction in brain water content was significantly different between BCP- and vehicle-treated TLR4 KO mice ($P < 0.05$, n = 3; Fig. 4F), while the reduction in infarct volume was comparable in these two groups ($P = 0.606$, n = 3; Fig. 4E). BCP also significantly reduced neurologic deficits at 72 h post-reperfusion ($P < 0.05$, n = 10; Fig. 4D). Together, the results document that while protection afforded by BCP against cerebral I/R may not be exclusively dependent on TLR4, TLR4 is a significant target of the beneficial effect of BCP.

3.5. BCP inhibits activation of microglia and mediates their polarization in TLR4 KO mice after cerebral I/R injury

The effect of BCP on microglia activation and polarization in TLR4 KO mice was assessed by immunohistochemistry. The staining pattern of CD68(+), CD16/32(+), and CD206(+)/Iba1(+) cells was comparable in sham-operated TLR4 KO and WT mice. The BCP treatment resulted in a significant reduction of CD68(+)/Iba1(+) microglia in TLR4 KO mice ($P < 0.05$ vs. vehicle-treated, n = 6; Fig. 5A and B). Additionally, BCP significantly decreased the number of CD16/32(+)/Iba1(+) microglia and increased the number of CD206(+)/Iba1(+) microglia in TLR4 KO mice at 72 h post-reperfusion ($P < 0.05$ vs. vehicle-treated, n = 6; Fig. 5C–F). Interestingly, at 72 h post-

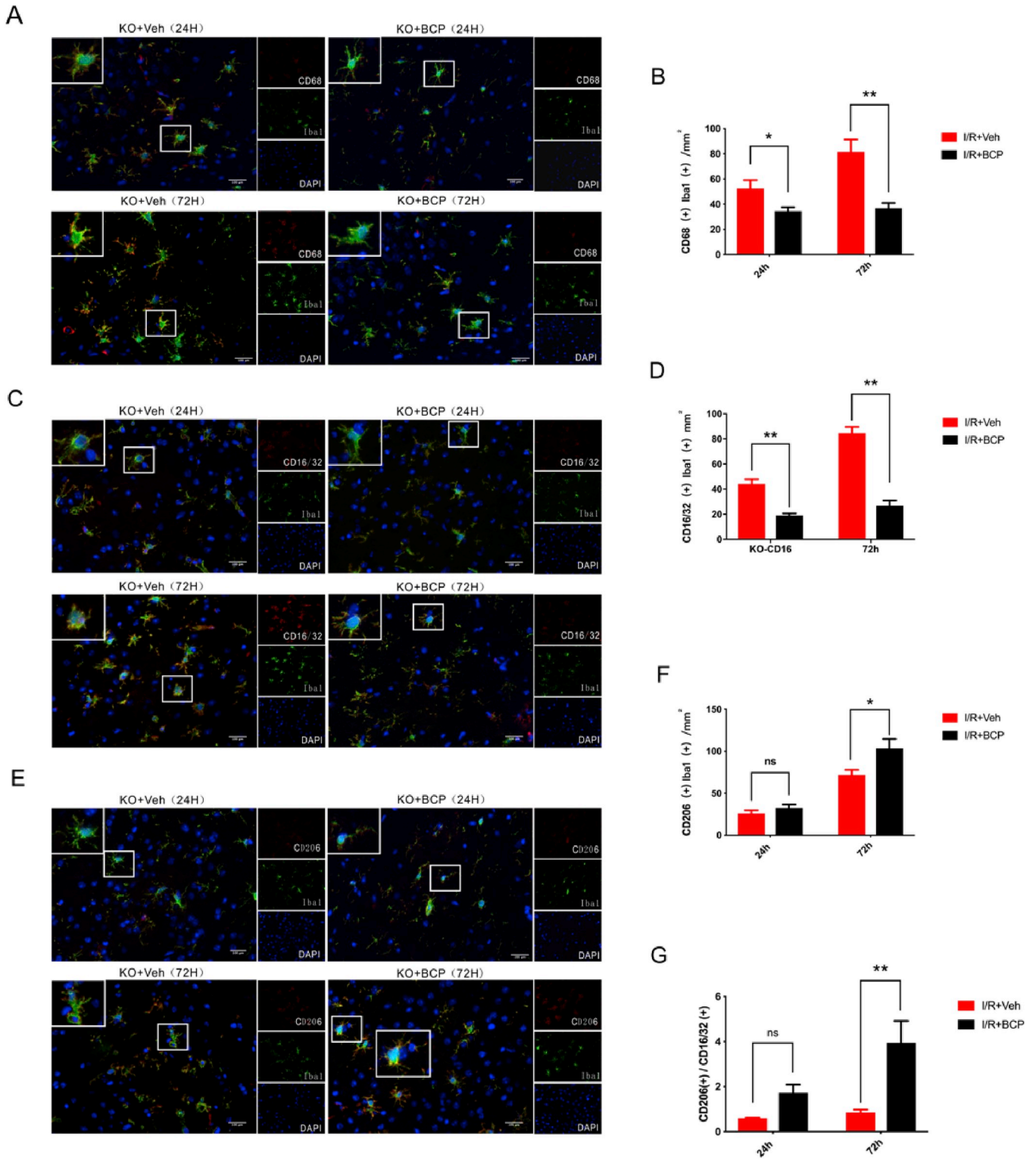

Fig. 5. BCP inhibits activation of microglia activation and mediates their polarization in TLR4 KO mice after cerebral I/R injury. (A) Immunostaining of CD68 (red) and Iba1 (green) illustrating microglial activation. (C) Immunostaining of CD16/32 (red) and Iba1 (green) documenting the M1 phenotype microglia. (E) Immunostaining of CD206 (red) and Iba1 (green) to identifying the M2 phenotype microglia. (A, C, E) Scale bars = 100 μm. Histograms showing the quantitative analysis the number of cells double-positive for Iba-1 and CD68 (B), CD16/32(D), and CD206 (F). (G) Ratio of Iba1(+)/CD206 (+)-to- Iba1(+)/CD16/32 (+) cells. n = 6 mice/group, $*P < 0.05$, $**P < 0.01$ vs. the vehicle-treated I/R group at the same time point, ns indicates no significant difference. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reperfusion, BCP treatment increased the M2:M1 ratio in TLR4 KO mice ($P < 0.01$ vs. vehicle-treated, n = 6; Fig. 5G). These results further demonstrate that TLR4 may represent a critical factor in BCP-mediated neuroprotection after cerebral I/R.

3.6. BCP reduces microglial activation and polarization induced by LPS and IFN-γ via the TLR4 signaling

The mechanism underlying the impact of BCP on microglial

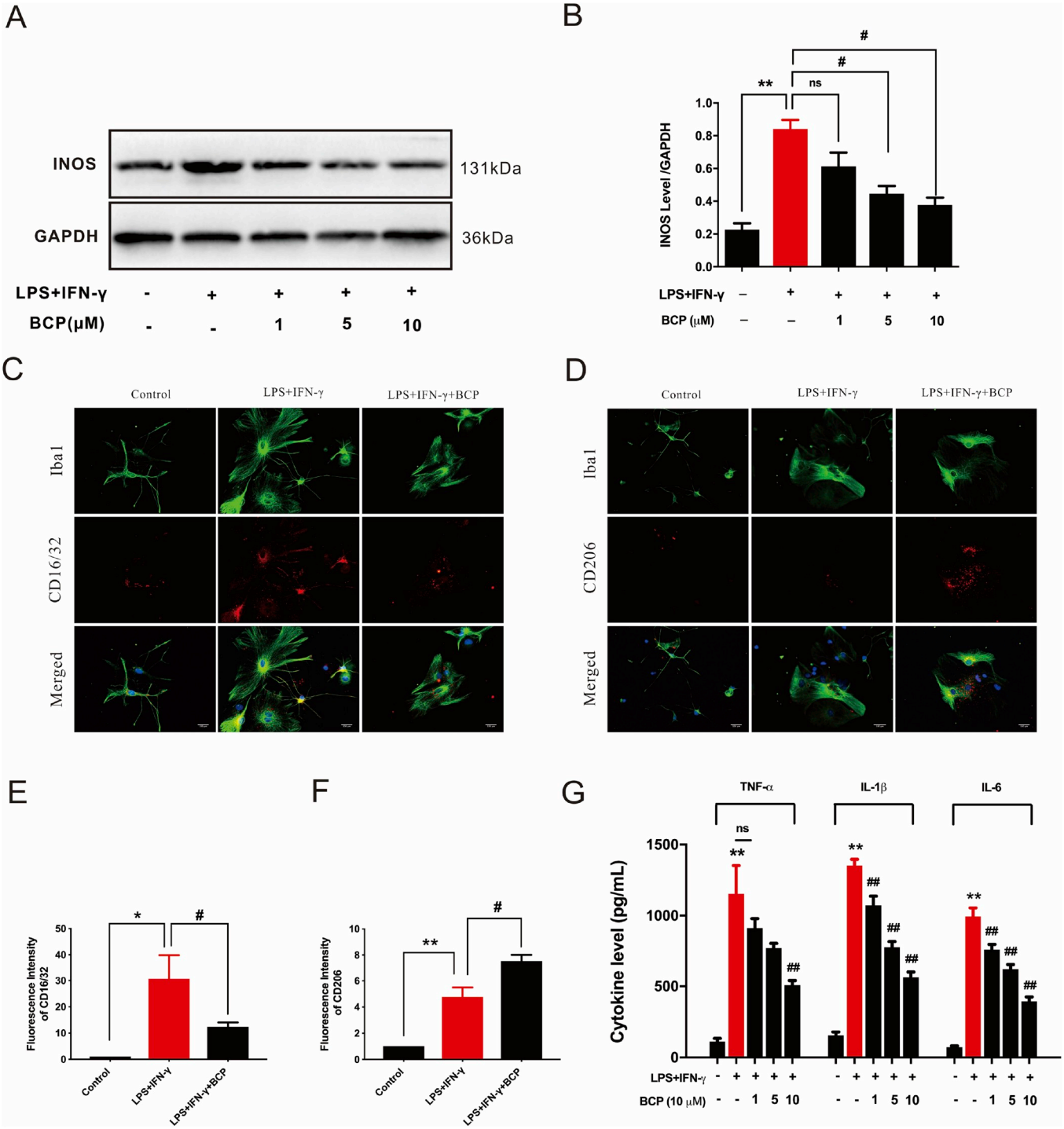

Fig. 6. BCP decreased microglial activation and polarization of microglia induced by LPS and IFN-γ. (A, B) iNOS level was determined by Western blotting. (C) Immunostaining of CD16/32 (red) and Iba1 (green), documenting the M1 phenotype microglia. Nuclei are stained by DAPI (blue). (D) Immunostaining of CD206 (red) and Iba1 (green) documenting the M2 phenotype microglia. Scale bar = 100 μm. (E, F) Histogram showing quantitative analysis of cells double-positive for Iba-1 and CD16/32 (E) and double-positive for Iba-1 and CD206 (F). (G) Measurement of TNF-α, IL-1β, and IL-6 levels by ELISA. n = 6 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the LPS plus IFN-γ group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

activation and polarization was studied in vitro. ELISA assays demonstrated that BCP decreased the level of TNF- α, IL-1 β, and IL-6 ($P < 0.01$, n = 6; Fig. 6G) and reduced the level of iNOS in primary microglia stimulated by the combination of LPS and IFN-γ ($P < 0.05$, n = 6; Fig. 6A and B). Additionally, BCP significantly downregulated CD16/32 and upregulated CD206 ($P < 0.05$, n = 6; Fig. 6C-F). These effects of BCP were dose-dependent.

Next, the possibility that TLR4 is a target of BCP was tested. Western blotting has shown that the exposure of LPS/IFN-γ-stimulated cells to BCP significantly decreased the level of TLR4 ($P < 0.05$, n = 6; Fig. 7C and D). This finding was confirmed by immunofluorescence, which also demonstrated marked downregulation of TLR4 by BCP ($P < 0.05$, n = 6; Fig. 7A and B). To strengthen the evidence of the role of TLR4 mediating the effects of BCP, the activity of TLR4 was inhibited by its a

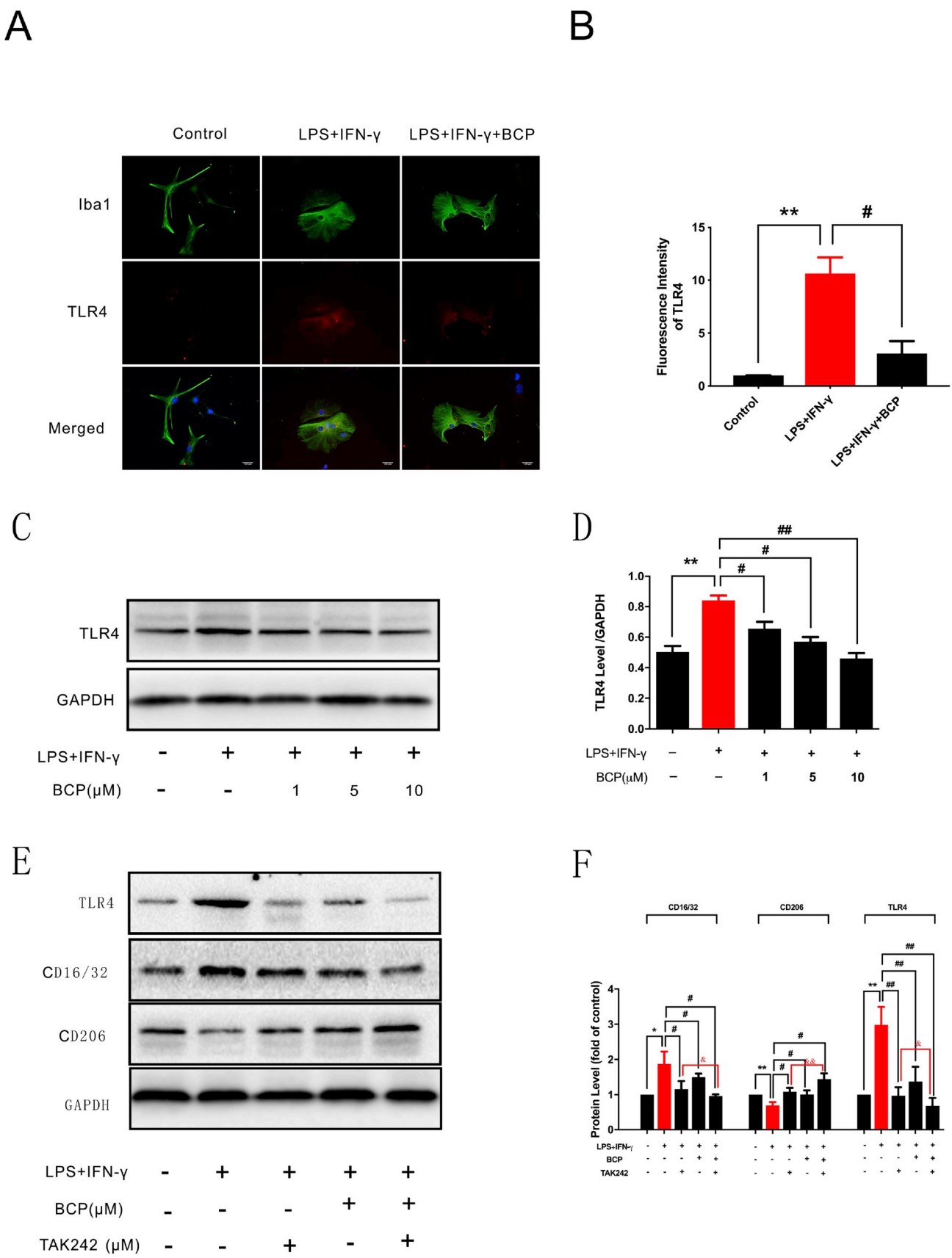

Fig. 7. BCP modulated microglia toward M2 polarization via TLR4 in vitroRepresentative images of the detection of TLR4 by immunohistochemistry (A, B; scale bar = 100 μm) and Western blotting (C, D). (E, F) Protein level of M1 markers (CD16/32), M2 markers (CD206), and TLR4 in primary microglia detected by Western blotting. n = 6 per group, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the LPS + IFN-γ group. $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ vs. the TAK242 + LPS + IFN-γ group.

highly specific blocker TAK242 (10 μM). Inhibition of TLR4 further intensified the impact of BCP effect on microglia M2 polarization ($P < 0.05$, n = 6; Fig. 7E and F). Together, these findings document that TLR4 is a crucial target of BCP; however, it is not an exclusive regulator of the effects of BCP on microglia.

4. Discussion

Inflammation is increasingly recognized to be the critical regulator of the pathological progression of ischemic stroke [28,29]. The present study demonstrates that BCP at the dose of 72 mg/kg body weight improved the neurologic deficits, infarct volume, and brain water content in WT mice with cerebral I/R injury. In addition, BCP inhibited the level of TLR4 in WT mice, but only partly reduced neurologic deficits, infarct volume, and brain edema in TLR4 KO mice with cerebral I/R. Furthermore, BCP inhibited microglial activation and decreased the formation of proinflammatory cytokine by microglia at 6, 24, and 72 h after I/R injury in WT mice. Importantly, BCP inhibited microglial polarization toward the M1 phenotype while promoting polarization toward the M2 phenotype in both WT and TLR4 KO mice. In vitro, BCP mediated activation and polarization of primary microglia induced by the combination of LPS and IFN-γ. This effect of BCP was accompanied by downregulation of TLR4 and CD16/32 and upregulation of CD206, and was intensified by blocking the activity of TLR4. These findings indicate that TLR4 is a significant target of BCP and its protective effect are exerted, at least in part, through the TLR4-mediated activation of microglia activation and promoting polarization of microglia toward the beneficial M2 phenotype.

Activated microglia can assume two phenotypes in the injured brain. M1 microglia secrete pro-inflammatory cytokines, aggravating brain damage, while M2 microglia mitigate inflammation and release trophic factors, promoting brain repair [3,30]. Xiao and coworkers [5] have reported that microglia respond dynamically to the ischemic injury, experiencing a transition from the early "healthy" M2 phenotype to the “unhealthy” M1 phenotype. Thus, therapeutic strategies targeting cerebral inflammation should be redirected from the general suppression of microglia to shifting the balance from the detrimental to the beneficial response of microglia after brain injury. Our previous work documented that the benefits of BCP in cerebral I/R mouse model are linked to its anti-inflammation effect [15,16,21]. However, it remained unknown whether the impact of BCP on the inflammation after cerebral I/R depends on the modulation of the activation and polarization of microglia. Recently, Askari [31] demonstrated that BCP alleviated LPS-induced microglia imbalance by promoting the beneficial M2 phenotype of microglia, characterized by the production of anti-inflammatory and reduction of pro-inflammatory factors. These findings are partially consistent with the present results. Here, we have documented that cerebral I/R strongly activated polarization of microglia toward the M1 phenotype. The treatment of mice with BCP resulted in a switch from M1 to M2 polarization after cerebral I/R injury. Moreover, the in vitro experiments demonstrated that the BCP treatment significantly suppresses the inflammatory microenvironment and directly modulates microglia polarization. Thus, the findings indicate that BCP acts by direct modulation of microglia polarization, rather than by general suppression of microglia activation. This effect constitutes the crucial mechanism by which BCP ameliorates the impact of ischemic brain injury.

To date, how BCP modulates microglia polarization in the cerebral I/R model is not fully understood. It has been reported that TLR4 plays a key role in microglia polarization after ischemic injury of the brain [10]. TLR4-deficient mice had smaller infarct volume and decreased inflammatory response after ischemic brain injury [32]. The data obtained in the current study demonstrate that BCP significantly downregulates the level of TLR4 in WT mice with cerebral I/R. Although Student's t-test indicated a significant difference in infarct volume, brain water content, and neurologic deficits between BCP-treated WT and vehicle-treated TLR4 KO mice, the differences were no longer statistically significant when two-way ANOVA post-hoc test among the four experimental groups (WT + vehicle, WT + BCP, TLR4 KO + vehicle, and TLR4 KO + BCP) was performed. The obtained data also documented that BCP decreased the M1-phenotype microglia in WT mice with cerebral I/R, as evidenced by the decrease in the number of the CD16/32(+)/Iba1(+) cells at both 24 h and 72 h post-reperfusion. This effect of BCP was also observed in TLR4 KO mice with cerebral I/R. Additionally, BCP increased the number of M2-phenotype (CD206-positive) microglia at 72 h post-reperfusion in TLR4 KO mice. In vitro, the effect of BCP on microglia M2 polarization was further intensified when TLR4 activity was inhibited. Interestingly, BCP treatment increased the CD206-to-CD16/32 ratio at both 24 h and 72 h post-reperfusion. Together, these findings indicate that BCP partially targets TLR4, although the protection of the brain against I/R injury by BCP might be not uniquely dependent on TLR4 inhibition.

Some limitations of the present study should be acknowledged. The experiments did not prove unequivocally whether the effects of BCP on microglia are critical for neuroprotection after cerebral I/R. The physiological function of the brain involves different cerebral cellular components, such as neurons, astrocytes, vascular cells, microglia, and pericytes that work together as a functional unit [33]. Therefore, the possibility that peripheral immune cells and various types of brain cells cooperate to mediate the protective effect of BCP following cerebral I/R. Moreover, the effect of BCP on microglial phagocytosis was not examined. Further pharmacological investigation of the impact of BCP on microglia function is necessary. Nevertheless, our results demonstrated that BCP can, at least in part, modulate microglia polarization and microglia-mediated inflammatory processes.

5. Conclusion

BCP protects ischemia brain partially by inhibiting the TLR4-mediated activation of microglia and promoting their polarization toward the beneficial M2 phenotype. The results of the current work point to the BCP treatment as a novel therapeutic strategy for patients with cerebral ischemia and other types of acute brain injury. Thus, the protective effects of BCP warrant further studies.

Declaration of competing interest

We declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116915.

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