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•Research article•

Mangiferin inhibited neuroinflammation through regulating microglial polarization and suppressing NF-κB, NLRP3 pathway

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[ABSTRACT] Inflammation plays important roles in the progress of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. Microglia is responsible for the homeostasis of the central nervous system (CNS), and involved in the neuroinflammation. Therefore, it could be potential in treatment of neurodegenerative diseases to suppress the microglia-mediated neuroinflammation. Mangiferin, a major glucoside of xanthone in Anemarrhena Rhizome, has anti-inflammatory, anti-diabetes, and anti-oxidative properties. However, the effect of mangiferin on the inflammatary responses of microglia cells are still poorly understand. In this study, we investigated the mechanism by which mangiferin inhibited inflammation in LPS-induced BV_2 microglia cells. BV_2 cells were pretreatment with mangiferin followed by LPS stimulation. *In vitro* assays, NO and cytokines production were quantified. Western blot and immunocytochemistry were used to examine the effect of mangiferin on the polarization of BV2 cells and signaling pathway. The results showed that mangiferin treatment significantly reduced NO, IL-1β, IL-6 and TNF-α production, also reduced the mRNA and protein of iNOS and COX-2, promoted the polarization of inflammatory toward anti-inflammatory, and inhibited activation of NF-κB and NLRP3 inflammasome. These data suggest that mangiferin has an anti-neuroinflammatory property *via* regulating microglia macrophage polarization and suppressing NF-κB and NLRP3 signaling pathway, and may act as a potential natural therapeutic candidate for neuroinflammatory diseases.

[KEY WORDS] Mangiferin; Inflammation; Microglial polarization; NF-κB; NLRP3 inflammasome
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Introduction

Alzheimer's disease and Parkinson's disease are complex process of neurodegenerative disorder. Although the cause remains poorly understood, increasing evidence shows that inflammation plays an important role in the process [1-4]. Microglial are resident innate immune cells in the central nervous system, and play an important role in the damage and repair of the nervous system [5-7]. They respond quickly to harmful stimuli from pathogens such as a virus and bacteria by performing phagocytosis process [8-9]. There are two phenotypes of microglia including pro-inflammation and anti-inflammation [10-12]. In an experimental model, lipopolysaccharide (LPS) binds to LPS-binding protein induces the M1 state of microglia. The complex interacts with toll-like receptor 4 (TLR4) stimulating a transmembrane signaling pathway [13-14]. TLR4 signaling is divided into MyD88-dependent and MyD88-independent style. In the MyD88-dependent pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is activated, which then enters into the nucleus followed by binding to DNA [15-16]. Activation of NF-κB induces transcriptional up-regulation of M1-associated pro-inflammation mediators, such as iNOS, IL-1β, and PGE_2, causing neuroinflammation and inducing nerve damage [17].

Nucleotide binding domain leucine-rich repeat with a pyrindomain containing 3 (NLRP3) inflammasome also plays an important role in the progress of neuroinflammation [18]. Activated NF-κB increases the expression of inactive NLRP3. A subsequent stimulus activates the NLRP3 inflammasome by facilitating the oligomerization of inactive NLRP3, apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and procaspase-1 [19].

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ASC act as an adaptor protein, recruiting procaspase-1 *via* CARD domain. Procaspase-1 produce the two fragments which are a tetrameric form of the active caspase-1 [20]. Then the active caspase-1 enzyme cleaves the proinflammtory cytokines pro-IL-1β and pro-IL-18 into their mature secreted forms. It has been proved that IL-1β plays an noteworthy role in the pathogenesis of neurodegenerative diseases.

Mangiferin (1,3,6,7-tetrahydroxyxanthone-2-β-D glucoside) is a polyphenol found in many plant species. It exhibits potent anti-diabetes, anti-tumour, antibacterial, antiviral, anti-inflammation, anti-oxidant, immunomodulatory and uric acid-lowering activities [21]. It has been reported that mangiferin inhibited COX-2 and PGE_2 in primary rat microglia [22]. However, it is unclear the underlying mechanism of mangiferin in microglia. In this study, we investigated the anti-inflammatory mechanism of mangiferin in mouse microglia (BV_2) cells. First, we determined the effect of mangiferin on the production of neurotoxic (NO) and cytokines (including IL-1β, IL-6, and TNF-α). Then, we estimated the mRNA and protein level of iNOS and COX-2. Finally, the protein level of NF-κB, NLRP3, ASC, and Caspase-1 p20 were determinded. Overall, Our results explored that mangiferin had an anti-inflammatiory effect on BV_2 cells through inhibiting the activity of NF-κB and NLRP3 signaling pathway.

Material and Methods

Reagents

DMEM high glucose medium, penicillin, streptomycin, and Fetal bovine serum were purchased from Gibco (Life Technologies, Grand Island, NY, USA). Mangiferin, LPS (from *E. coli* 0111:B4), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Griess reagent and DMSO were purchased from Sigma Aldrich (St. Louis, MO, USA); UNlQ-10 Column Total RNA Purification Kit was purchased from Sangon Biotech (Shanghai, China). IL-1β, IL-6, TNF-α, IL-10 ELISA kits were purchased from Neobioscience Technology Company (Shenzhen, China). RIPA buffer, protease inhibitors, phosphatase inhibitors, BCA protein assay kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). ECL chemiluminescence substrate was purchased from Thermo Scientific (Waltham, MA, USA). Antibodies were purchased from Cell Signaling (Farmingdale, NY, USA). TransScript First-Strand cDNA Synthesis SuperMix and TransStart Green qPCR SuperMix were obtained from Transgen Biotech (Beijing, China)

Cell culture

Murine BV2 microglial cells were purchased from National Infrastructure of Cell Line Resource (China) and culture in DMEM medium containing 10% FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $mg \cdot L^{-1}$ streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

MTT assay

BV2 cells were seeded into 96-well plates at 1×10^5/well and incubated overnight. Cells were pre-treated with different concentrations of Mangiferin (0 to 150 $\mu g \cdot mL^{-1}$) for 2 h, and then treated with LPS (0.5 $\mu g \cdot mL^{-1}$)) for 24 h. Cell viability was assayed by MTT assay following the manufacture's instruction.

NO assay

Cells were pre-treated with different concentrations of Mangiferin (0 to 150 $\mu g \cdot mL^{-1}$) for 2 h, and then treated with LPS (0.5 $\mu g \cdot mL^{-1}$) for 24 h. The supernatant of culture was collected and the concentration of NO was measured by Griess assay.

Enzyme-linked Immunosorbent assay

Cells were pre-treated with different concentrations of Mangiferin (0 to 150 $\mu g \cdot mL^{-1}$)) for 2 h, and then treated with LPS (0.5 $\mu g \cdot mL^{-1}$) for 24 h. The supernatant of culture was collected and the concentrations of pro-inflammation cytokines were measured by ELISA according to the manufacture's instruction.

RNA extraction and real-time quantitative PCR (qRT-PCR)

Cells were collected and total mRNA was extracted by UNlQ-10 Column Total RNA Purification Kit following the manufacture's instruction. cDNA was synthesized using the TransScript First-Strand cDNA Synthesis SuperMix kit according to the manufacture's instruction. The qPCR reaction was performed in the qTOWER 2.2 System (qTOWER 2.2, Jena, Germany) using the TransStart Green qPCR SuperMix kit. Primers were provided as follows: forward strand for *iNOS*, 5′-CCTGCTTTGTGCGAAGTGTC-3′; reverse strand for *iNOS*, 5′-CCCAAACACCAAGCTCATGC-3′. forward strand for *COX-2*,5′-CCACTTCAAGGGAGTCTGGA-3′; reverse strand for *COX-2*,5′-AGTCATCTGCTACGGGAGGA-3′. β-actin primers were purchased from Sangon Biotech (Shanghai, China, B662302).

Western bloting

Cells were lysed by RIPA buffer with protease inhibitors and phosphatase inhibitors. The concentration of protein was detected by BCA protein assay reagent following the manufacture's instruction. Proteins were seprated using 10% SDS-PAGE and transfereed to PVDF membranes followed by blocking with 5% fat-free milk. The membranes were incubated with primary antibodies at 4 °C overnight and then washed three times with tris-buffer saline tween-20 (TBST). The membranes were incubated with secondary antibody for 1 h at room temperature, then washed with TBST three times, and once with tris-buffer saline (TBS). Immune complexes were detected by ECL chemiluminescence assay with an ECL Western Bloting Kit according to the manufacture's instruction.

Immunofluorescence staining

BV2 cells were seeded into 6-well plates at 2×10^4/well and incubated overnight. Cells were pre-treated with 150 $\mu g \cdot mL^{-1}$ mangiferin for 2 h prior to treated with 0.5 $\mu g \cdot mL^{-1}$ LPS for 24 h. Cells were fixed with 4% paraformaldehyde followed by permeabilized. The cells were incubated with anti-NF-κB primary antibody overnight. Cells were washed with PBS and incubated with fluorescent secondary antibody.

The nucleus was stained with Hoechst 33342. Fluorescence was analyzed using a fluorescence microscope (BX53, Olympus).

Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA. Differences were considered statistically significant if $P < 0.05$.

Result

Mangiferin protected BV_2 cells and inhibited NO production

The MTT assay was used to assess the effect of mangiferin on survival of BV2 cells. There was no obvious cytotoxicity of mangiferin (50 to 150 μg·mL^{-1}) on BV2 cells, but it significantly inhibited the effect of LPS on the survival rate of BV_2 cells (Fig. 1A). To estimat whether mangiferin inhibited the production of NO in BV_2 cells stimulated by LPS, we pretreated cells with 50 to 150 μg·mL^{-1} mangiferin for 2 h, followed by stimulation with 0.5 μg·mL^{-1} LPS for 24 h. NO production was examined by Griess reagent assay. As shown in Fig. 1B, LPS treatment significantly increased NO production compared to control. Mangiferin inhibited LPS-stimulated NO production in a dose-dependent manner.

Effect of mangiferin on the morphological changes and polarization of the BV2 cells

The effect of mangiferin on the cell morphological changes were observed using microscopy. We have observed the morphology of BV2 cells both under bright-field microscope and dark-field microscopy. Compared with bright-field observations (the results we have not shown), dark-field microscopes showed the details of cell edges more clearly. BV_2 cells were exhibited regular morphology and smooth cell edges in control and mangiferin treatment alone group (Fig. 2A). After LPS treatment, the cell edges are rough and the shape is irregular. It is clear that 100 and 150 μg·mL^{-1} mangiferin pretreatment improved the cellular morphology. There are two well-established phenotype of microglia including pro-inflammation type and anti-inflammation type. As shown in Fig. 2B, the expression of CD16/32 significantly increased by 2.3-fold while CD206 had no obvious increase upon LPS stimulation. Interestingly, 150 μg·mL^{-1} mangiferin treatment significiantly decreased CD16/32 protein but increased the expression of CD206 protein. These results suggest that mangiferin can revert the effect of LPS on the BV_2 microglial polarization, trigger the polarization of inflammatory toward anti-inflammatory.

Mangiferin inhibited iNOS and COX-2 expression induced by LPS

iNOS and COX-2 are major factors in inflammatory responses. To examine whether mangiferin regulates the expression of iNOS and COX-2, we used PCR to measure the mRNA levels of iNOS and COX-2, and western blot was used to determined the expression of protein. As shown in Fig. 3, LPS stimulated the expression of gene and protein of iNOS and COX-2. 50 to 150 μg·mL^{-1} mangiferin treatment suppressed the LPS-induced mRNA and protein levels in a dose-dependent manner. These results suggest that mangiferin suppresses the production of NO by inhibiting mRNA and protein expression of iNOS.

Mangiferin inhibited cytokine secretion induced by LPS

Previous reports have shown that LPS increases the expression of inflammatory cytokines. To examine whether mangiferin effects the production of cytokines in BV_2 cells induced by LPS, ELISA assay was used to assess the protein levels. As shown in Fig. 4, mangiferin suppressed the expression of IL-1β, IL-6, and TNF-α, stimulated the secretion of IL-10. These results suggest that mangiferin modulate the inflammatory response by regulating the production of cytokines in LPS-induced BV_2 cells.

Effect of mangiferin on the activation of NF-κB

NF-κB is a transcription factor which plays an noteworthy role in the inflammatory response. Thus, the effect of mangiferin on activation of NF-κB was measured by immunofluorescent staining and western blot assay. As shown in Fig. 5A, the result observed by immunofluorescent staining showed that the LPS stimulation induced the translocation of NF-κB p65 from the cytosol to the nucleus significantly, whereas 150 μg·mL^{-1} mangiferin treatment noticeably inhibited the nuclear translocation of NF-κB p65. Similar results were observed by western blot assay (Fig. 5B).

Effect of mangiferin on the activation of inflammasome

The activation of NLRP3 inflammasome is a key con-

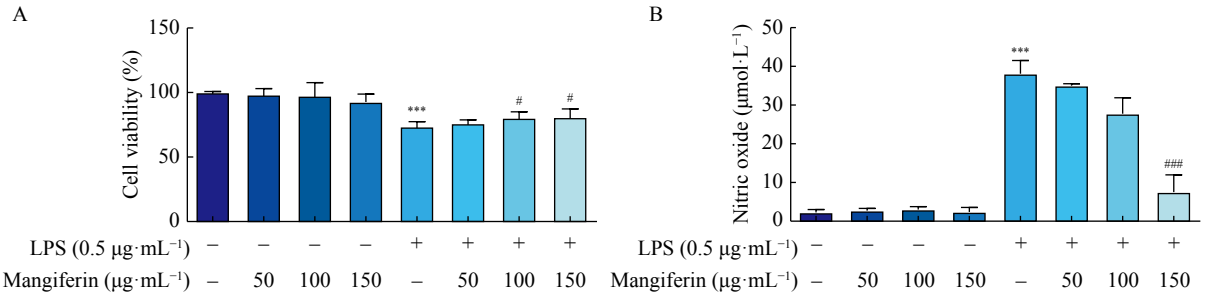

Fig. 1 Effect of mangiferin on the survival rate of BV2 cells and NO production. BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. (A) The viability of BV2 cells were determined by MTT assay. (B) The production of NO was detected by Griess assay. $^{***}P < 0.001$ *vs* control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

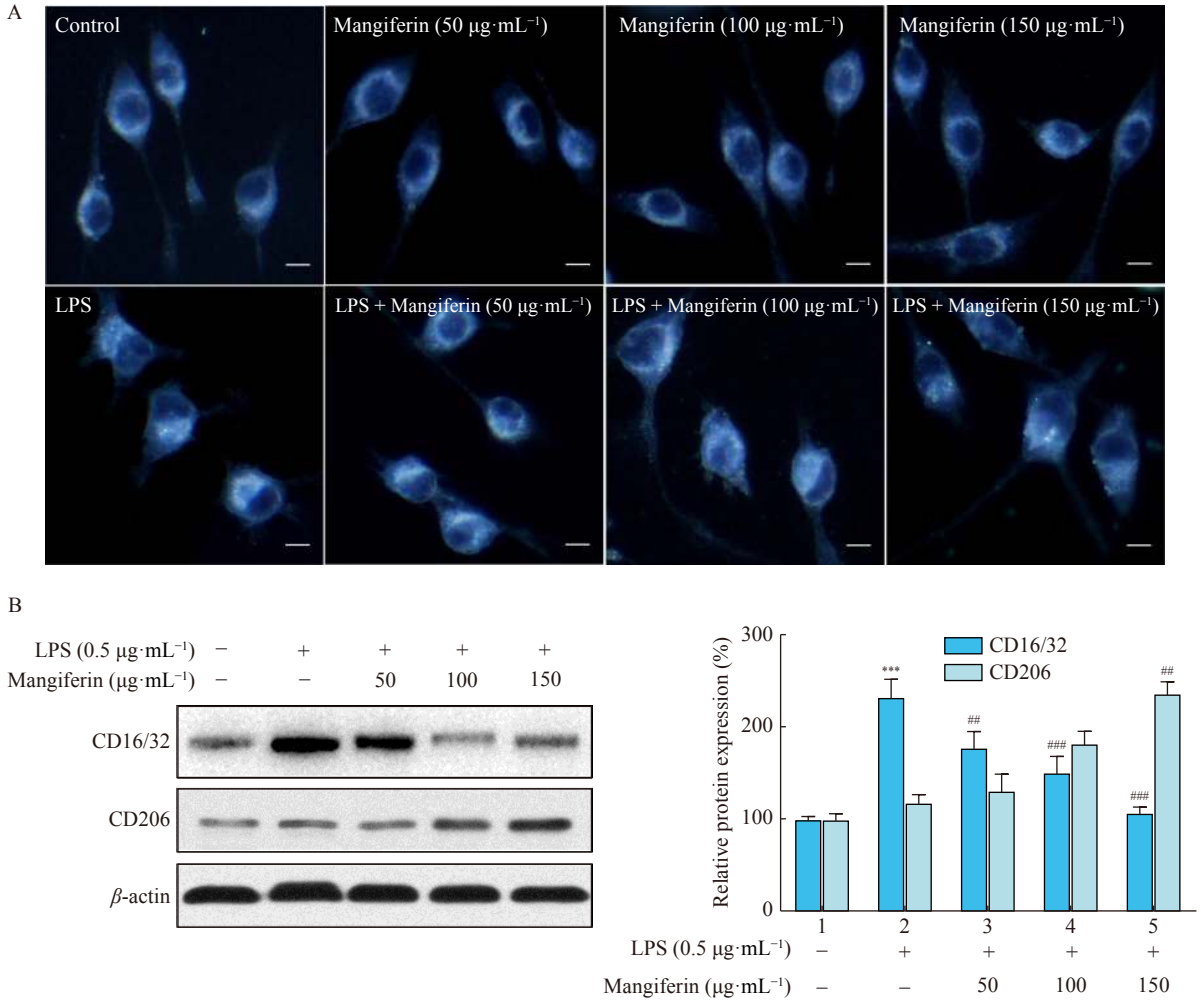

Fig. 2 Effect of mangiferin on the morphological changes and polarization of the BV2 cells. BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. (A) The cellular morphology was observed using dark-field microscopy (40 ×), and the images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Scale bar = 20 μm. (B) Expression of CD16/32 and CD206 was detected by Western blot (means ± SD, *n* = 3). The expression of CD16/32 and CD206 in LPS only group was compared with which expressed in control, respectively. The expression of CD16/32 and CD206 in mangiferin treatment group was compared with which in LPS only group, respectively. $^{***}P < 0.001$ *vs* control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

tributor to the development of neuroinflammation. To examine whether mangiferin affects the activation of NLRP3 inflammasome induced by LPS in BV_2 cells, the levels of NLRP3, ASC, and Caspase-1 p20 protein were determined by western blot. As shown in Fig. 6, the results indicated that mangferin reduced the expression of NLRP3, ASC and mature caspase-1 protein in LPS-activated BV_2 cells.

Discussion

Inflammation is closely related to a variety of neurodegenerative diseases. Nerve microglia are often used as an important cellular model for studying neuritis. This study used LPS to induce a model of microglia BV2 inflammation [23]. Microglia have different polarization patterns. Studies have shown that after stimulation of macrophages by factors such as LPS and IFN-γ, cells exhibit a stronger pro-inflammation polarization pattern. This study showed that the expression of CD16/32 was significantly increased after 24 hours of LPS stimulation. After treatment with different concentrations of mangiferin, CD16/32 showed a downward trend in a concentration-dependent manner. Therefore, mangiferin inhibits LPS-stimulated BV2 cell polarization, and triggers the polarization of inflammatory toward anti-inflammatory.

Studies have shown that LPS binds to receptors on the cell surface followed by activating the transmembrane signals, which leads to the phosphorylation of IKB in the cytoplasm and releasing NF-κB. Activated NF-κB enters into the nucleus and promotes the expression of downstream genes [24]. Studies have shown that IL-1β, TNF-α, iNOS and other protein expression levels are significantly up-regulated after LPS stimulation [25-26]. The results of this study showed that LPS treatment of BV2 cells for 24 h, the cell survival

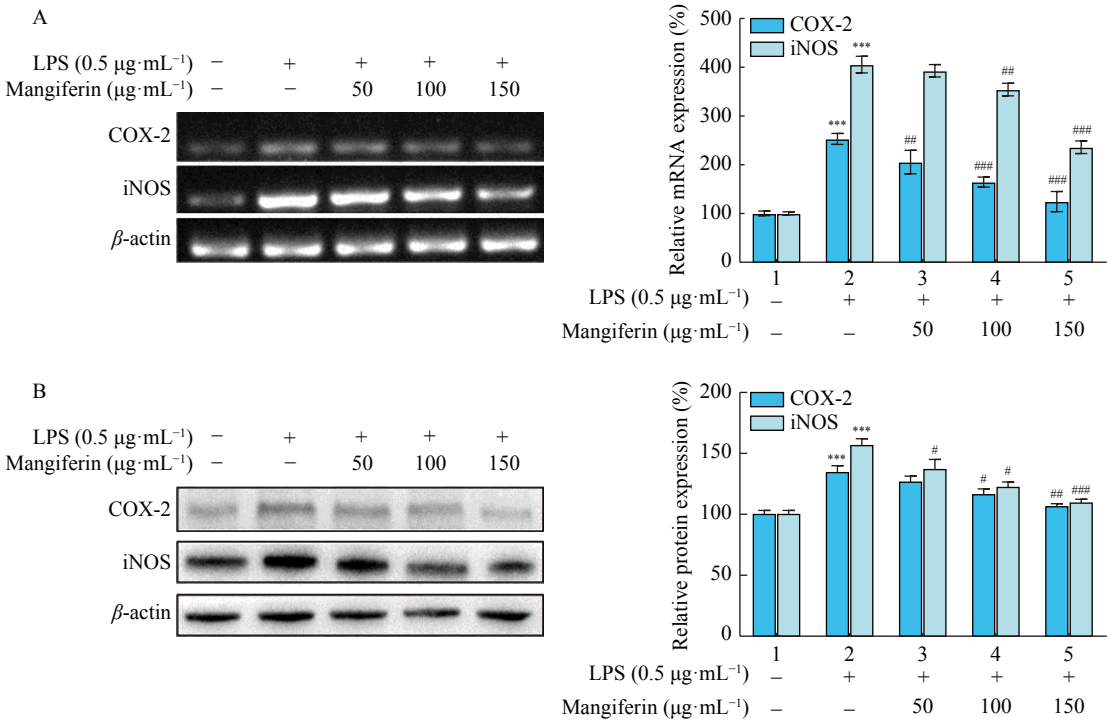

Fig. 3 Effect of mangiferin on iNOS and COX-2 expression. BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. (A) The mRNA levels of iNOS and COX-2 were determined by PCR. (B) Protein levels of iNOS and COX-2 were measured by Western blot assay (means ± SD, n = 3). The mRNA levels of iNOS and COX-2 in LPS only group was compared with which expressed in control, respectively. The mRNA levels of iNOS and COX-2 in mangiferin treatment group was compared with which in LPS only group, respectively. The protein data statistics used the same method. $^{***}P < 0.001$ *vs* control; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

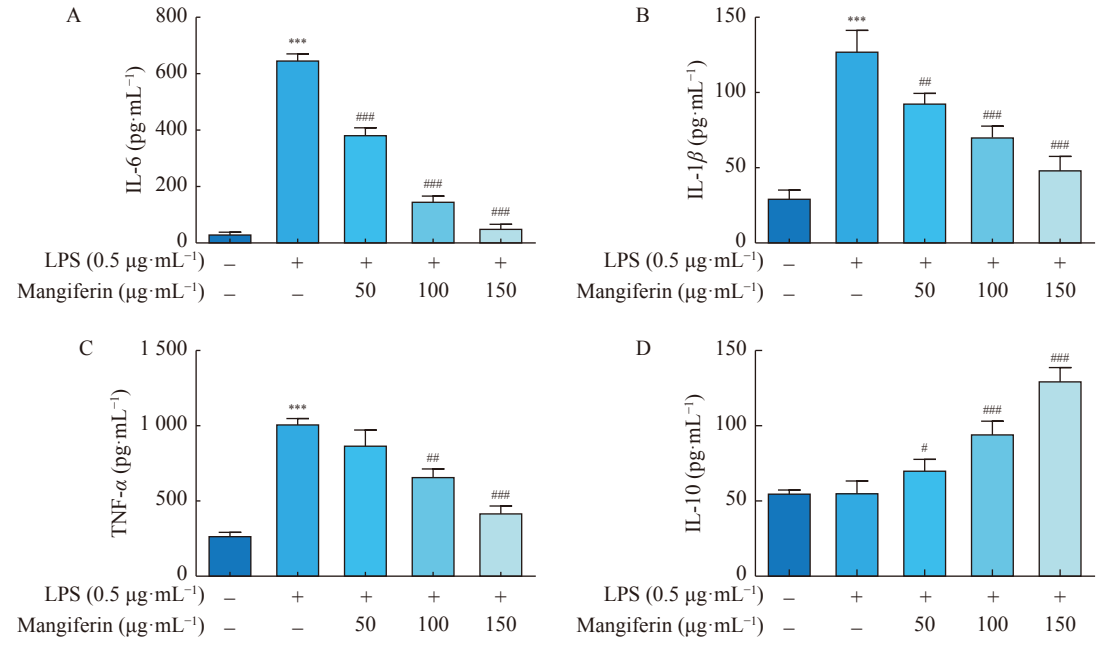

Fig. 4 Effect of mangiferin on cytokine secretion (means ± SD, n = 3). BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. The supernatants were examined for cytokine production. IL-1β, IL-6, TNF-α, and IL-10 were measured by ELISA analysis. (A) IL-1β; (B) IL-6; (C) TNF-α; (D) IL-10. $^{***}P < 0.001$ *vs* control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

rate was reduced, NO production and inflammatory cytokine expression were significantly up-regulated. Mangiferin is a natural polyphenolic compound with various physiological activities [27-30]. Previous studies have shown that mangiferin

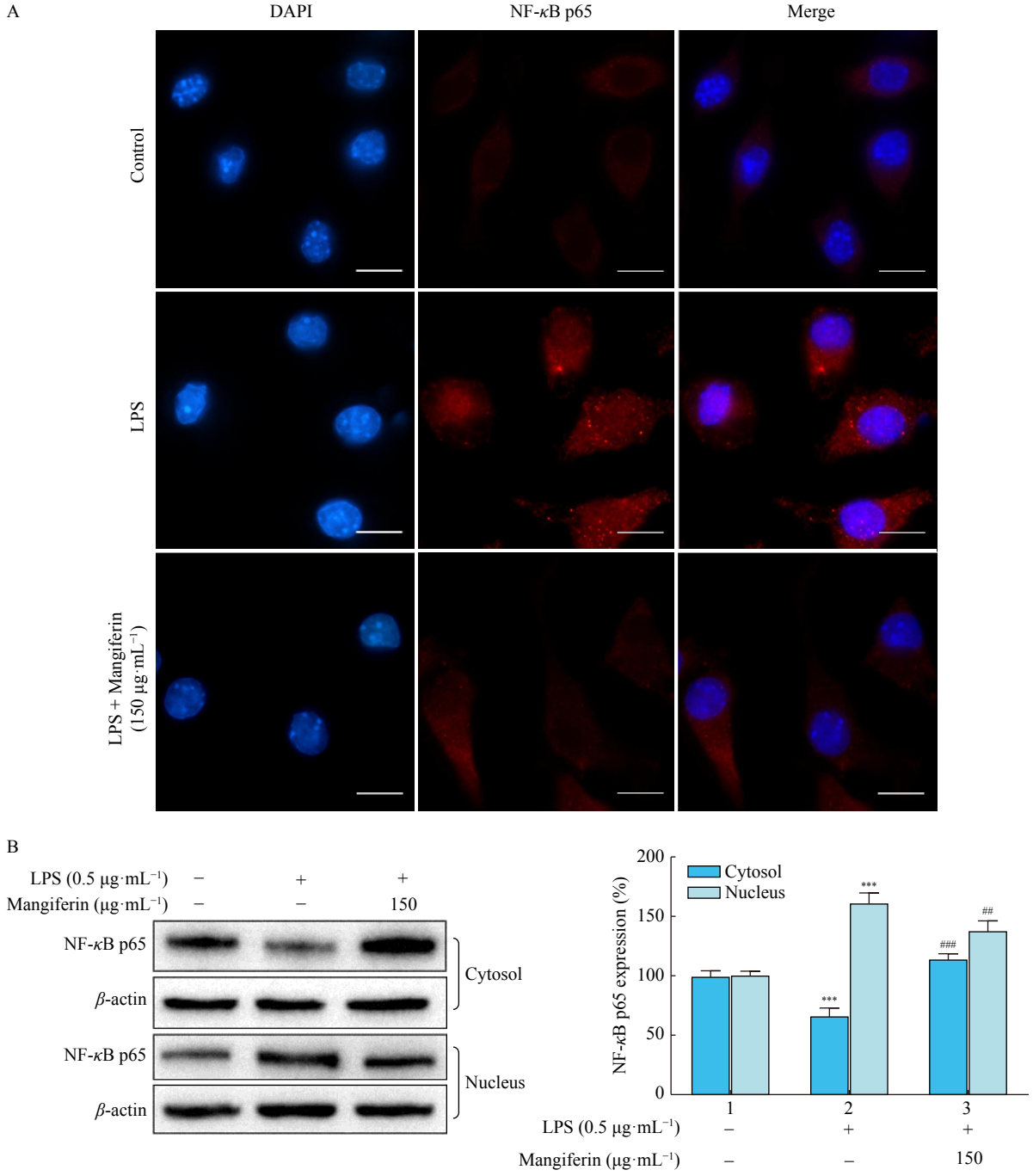

Fig. 5 Effect of mangiferin on NF-κB activation. BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. (A) Cells were stained with anti-NF-κB (Red) antibody, and nuclear was stained with Hoechst 33342 (Blue). Fluorescence was visualized by a fluorescence microscope (40 ×). The images shown are representative of three independent experiments. (B) NF-κB protein in cytosol and nucleus were determined by Western blot. Quantitation was performed on three independent experiments and presented as the means ± SD. For NF-κB protein in cytosol, the expression of NF-κB in LPS only group was compared with which expressed in control. The expression of NF-κB in mangiferin treatment group was compared with which in LPS only group. The NF-κB protein data in nucleus statistics used the same method. $^{***}P < 0.001$ *vs* control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

can inhibit the expression of iNOS and COX-2 in BV2 cells induced by LPS, but the mechanism is unclear. Cells were pretreated with different concentrations of mangiferin. The results showed that mangiferin inhibited LPS-induced NO and inflammatory cytokine production in a concentration-dependent manner, probably because mangiferin significantly inhibited NF-κB activation.

Other studies have shown that LPS-activated NF-κB stimulates the expression of NLRP3 [31-32]. The active NLRP3 cleaves Caspase-1 into an active form of Caspase p20 by

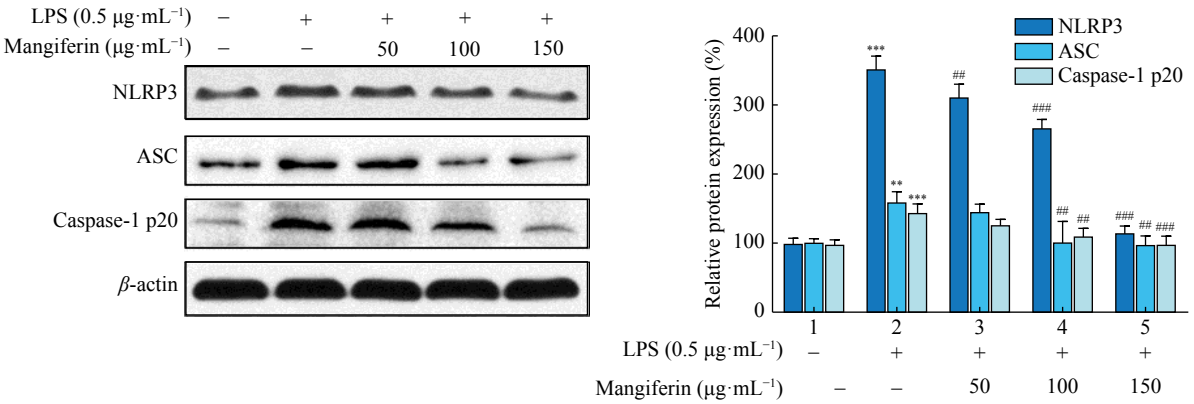

Fig. 6 Effect of mangiferin on NLRP3 inflammasome (means ± SD, n = 3). BV2 microglial cells were pretreated by 50 to 150 μg·mL^{-1} mangiferin for 2 h, and then stimulated by 0.5 μg·mL^{-1} LPS for 24 h. Expression of NLRP3, ASC and active caspase-1 was detected by Western blot. The expression of NLRP3, ASC and active caspase-1 in LPS only group was compared with which expressed in control, respectively. The expression of NLRP3, ASC and active caspase-1 in mangiferin treatment group was compared with which in LPS only group, respectively. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs* control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs* LPS only group

binding to ASC. The activated inflammasome promotes the transduction of inflammatory signals. In this study, the expression of NLRP3, ASC, and caspase p20 protein were significantly up-regulated by LPS treatment of BV2 cells for 24 h. With pretreatment by different concentrations of mangiferin, the expression of NLRP3, ASC, and caspase p20 protein were significantly reduced.

In summary, this study explored the anti-inflammatory mechanism of mangiferin in microglial. The results showed that mangiferin inhibited LPS-induced inflammation by inhibiting NF-κB and NLRP3 inflammasome.

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