MCPIP1 alleviates depressive-like behaviors in mice by inhibiting the TLR4/TRAF6/NF-κB pathway to suppress neuroinflammation

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Received April 7, 2023; Accepted October 25, 2023

DOI: 10.3892/mmr.2023.13129

Abstract. Lipopolysaccharide-induced (LPS) neuroinflammation serves an important role in the development of depression. Monocyte chemotactic protein-1-induced protein 1 (MCPIP1, also known as ZC3H12A and Regnase-1) possesses endoribonuclease and deubiquitinase activities. In the present study, the effects of MCPIP1 on LPS-induced depression were assessed. A mouse model of hippocampal neuroinflammation was established by intraperitoneal injection of LPS. Microglia were treated with LPS, MCPIP1 overexpression vector, MCPIP1 knockdown vector or TLR4 inhibitor. MCPIP1 alleviated LPS-induced depressive-like behaviors. MCPIP1 facilitated M2 polarization of microglia. MCPIP1 attenuated the inflammatory response in microglia via inhibition of the TLR4/TNF receptor associated factor 6 (TRAF6)/NF-κB signaling pathway. The results indicated that MCPIP1 accelerated M2-polarization of microglia and alleviated depressive-like behaviors of mice via the inhibition of the TLR4/TRAF6/NF-κB signaling pathway.

Introduction

Major depressive disorder (MDD) is a mental illness that is related to suicide and is characterized by cognitive impairment, hopelessness, loss of pleasure, debilitation and low self-esteem (1). The currently available antidepressants for patients with depression include selective serotonin and norepinephrine reuptake inhibitors (2). However, poor treatment adherence and high discontinuation rates result in treatment failure (40 to 60%) (3,4), which indicates the need for a means of preventing MDD. Numerous factors, such as parental depression, chronic disease, sleeplessness, socioeconomic status, stress and interpersonal dysfunction, influence the risk for depression (5). Previous studies have reported that the inflammatory response participates in the pathogenesis of depression (6,7). The protein expression of TNF-α and IL-6 are elevated in patients with depression (8). Reduced cytokine levels within the brain affect neurotrophic support and neurotransmitter metabolism, ultimately leading to inhibition of activated microglia cells (9). SalB significantly decreases expression of pro-inflammatory cytokines IL-1β and TNF-α, thereby inhibiting the activation of microglia in the hippocampus and cortex (10). A previous study reported that lipopolysaccharides (LPS) can induce inflammation-related depression by altering brain-derived neurotrophic factor (BDNF)-TrκB signaling (11). Selanylimidazopyridine alleviates depressive-like behavior by downregulating NFκB, promoting antioxidant activity, and increasing BDNF expression in LPS-induced mice (12).

Cytokines cross the blood-brain barrier and impair microglial function, which leads to depression (13). LPS activates the microglia and induces inflammatory responses (14). Inducible nitric oxide synthase (iNOS) and TNF-α are markers of pro-inflammatory M1 microglia (15). Arginase 1 (Arg-1) and IL-10 are markers of anti-inflammatory M2 microglia (16). Therefore, neuroinflammation may be ameliorated by M2 microglial polarization that further alleviates depression (17).

Monocyte chemotactic protein-1-induced protein 1 (MCPIP1), also termed ZC3H12A and Regnase-1, possesses endoribonuclease and deubiquitinase activities (18). MCPIP1 ameliorates inflammatory responses by destabilizing mRNAs encoding cytokines (19). MCPIP1 induces autophagy and alleviates inflammatory responses in keratitis (20). MCPIP1 depletion aggravates psoriasis-like inflammation in keratinocytes by IL-23/Th17 and STAT3 pathways (21). MCPIP1 promotes TNF-α-induced apoptosis by decreasing NF-κB and cFLIP protein expression (22). MCPIP1 inhibits IL-1β or LPS-induced ubiquitination of TNF receptor associated factor 6 (TRAF6) (23). The present study assessed if the role of MCPIP1 in reducing inflammatory responses was associated with depression progression and whether may represent an effective therapeutic target for depression.

Materials and methods

Animals and treatments. Male C57BL/6J mice (weight, 18-20 g, n=20) were purchased from the Jinan Pengyue Laboratory Animal Breeding Co., Ltd. Mice were housed 2 mice per cage

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Key words: monocyte chemotactic protein-1-induced protein 1, lipopolysaccharide, depression, inflammation, hippocampus, microglia polarization

under a 12 h light/dark cycle at 18-22˚C. The present study complied with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (24) and was approved by the Ethics Committee of Qingdao Mental Health Center (Qingdao, China; approval no. 2022061). The mice were randomly divided into four groups as follows: i) control group (PBS; n=5); ii) LPS group (n=5); iii) LPS + NC group (n=5); and iv) LPS + MCPIP1 group (n=5). An adenovirus-associated vector (AAV) containing MCPIP1 (Shanghai GenePharma Co., Ltd.) was used. After induction using 4% isoflurane and maintenance using 2% isoflurane, 2 μl of negative control (NC) AAV or MCPIP1 AAV were injected into the hippocampus regions (-1.6 mm anteroposterior of bregma, ±1.8 mm lateral and -1.6 mm dorsoventral of bregma) of mice (age, 6 weeks, n=5) using a brain location microinjection pump (200 nl/min; Stoelting Co.). The mice in control and LPS groups were subjected to a sham procedure. At 4 weeks after injection, mice were intraperitoneally injected with PBS or LPS (1 mg/kg) for 2 days (25). If improper injection operation caused the death of the mouse, the mouse was excluded and replaced. All data derived from animal studies were analyzed by an experimenter blind to experimental conditions. The order of the animals was randomized prior to behavioral tests. Sucrose preference test (SPT), open field test (OFT), tail suspension test (TST) and forced swimming test (FST) were performed as described previously (26). After testing depression-like behavior, the mice were sacrificed by anesthesia with isoflurane (4% for induction and 2% for maintenance) and transcardial perfusion using ice-cold PBS and then 4% paraformaldehyde in PBS overnight at 4˚C. Following perfusion, the brains were collected for subsequent analysis. The left hippocampus was frozen and the right hippocampus was treated with paraffin.

SPT. During the adaptation phase, the mice were housed in cages with two bottles of 1% sucrose solution for two days. On the following day, one bottle of 1% sucrose solution and one bottle of tap water were placed in each cage. On day 4 mice were deprived of water and food for 24 h. Mice were allowed access to the 1% sucrose solution and tap water bottles for 3 h, to avoid bottle side preference, the positions of the two bottles were swapped. Sucrose preference during the 3 h test was calculated as: Sucrose consumption/(water consumption + sucrose consumption) x100.

OFT. Briefly, mice were placed in a square wooden box (40x40x40 cm) for 5 min. The mice were gently placed in the center of the square facing in the same direction each time. Anilab software (AVTAS version 5.0, Anilab Scientific Instruments Ltd. Co.) was used to analyze the travel distance of the mice.

TST. Mice were individually suspended from a retort stand with adhesive tape placed 1 cm from the tail tip and placed 50 cm above the floor. The duration of immobility was recorded during a 6 min test and analyzed using Anilab software (ver 6.50, Anilab Scientific Instruments Ltd. Co.).

FST. Mice were placed in a glass beaker (5,000 ml) containing 4,000 ml of water (25±1˚C) for 6 min, and the struggling time was recorded.

Cells and treatments. Mouse BV2 transformed microglial cells (cat. no. CL-0493; Procell Life Science & Technology Co., Ltd.) were incubated at 37˚C in MEM-non-essential amino acid solution containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. Cells were transfected with 50 nM pcDNA-MCPIP1, empty vector (Shanghai GenePharma Co., Ltd.), short interfering RNA (si)-MCPIP1 or si-NC (Shanghai GenePharma Co., Ltd.) at 37˚C for 24 h using Lipofectamine 3000. LPS (1 μg/ml; cat. no. L8880; Beijing Solarbio Science & Technology Co., Ltd.) was added to the transfected BV2 cells at 37˚C for 12 h. LPS + si-MCPIP1 + TAK-242 group cells were pretreated with TAK-242, a selective TLR4 inhibitor (cat. no. HY-11109; MedChemExpress), at 37˚C for 30 min. The sequences were as follows: si-MCPIP1 Sense: 5'-CCG AGAUCCUCUCCUACAAGU-3', Anti-sense: 5'-UUGUAG GAGAGGAUCUCGGCA-3'. Si-NC Sense: 5'-UUCUCCGAA CGUGUCACGUTT-3'; Anti-sense: 5'-ACGUGACACGUU CGGAGAATT-3'.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RNA (1.0 μg) was reverse transcribed using HiScript® II Q RT SuperMix for qPCR (cat. no. R223-01; Vazyme Biotech Co., Ltd.) according to the manufacturer's protocols. mRNA levels were quantified using a ChamQ SYBR qPCR Master Mix kit (cat. no. Q311-02; Vazyme Biotech Co., Ltd.) according to the manufacturer's protocols. Thermocycling conditions were as follows: Initial denaturation at 94˚C for 30 sec, followed by 35 cycles of denaturation at 94˚C for 5 sec and extension at 60˚C for 30 sec. GAPDH was used as the endogenous control. Relative mRNA expression levels were assessed using the $2^{-\Delta\Delta Cq}$ method (27). Specific primer pairs are listed in Table I.

Western blotting. Proteins were extracted from tissues and cells using RIPA buffer (cat. no. P0013C; Beyotime Institute of Biotechnology). The protein concentration was determined using a BCA protein assay kit (P0012S; Beyotime Institute of Biotechnology). Proteins (30 μg/line) were separated using 10% SDS-PAGE (cat. no. P0012A; Beyotime Institute of Biotechnology) and transferred onto a PVDF membrane (cat. no. FFP24; Beyotime Institute of Biotechnology). After blocking with 5% non-fat dry milk in Tris-buffers saline for 2 h at 25˚C, the membrane was incubated with primary antibodies against MCPIP1 (1:2,000; cat. no. 25009-1-AP; Proteintech Group Inc.), Iba-1 (1:2,000; cat. no. ab178846; Abcam), TLR4 (1:1,000; cat. no. A5258; Abclonal Biotech Co., Ltd.), MyD88 (1:1,000; cat. no. A21905; Abclonal Biotech Co., Ltd.), TRAF6 (1:2,000 cat. no. ab33915; Abcam), phosphorylated (p)-IκBα (1:1,000; cat. no. ab133462; Abcam), IκBα (1:2,000; cat. no. 4814T; CST Biological Reagents Co., Ltd.), p-p65 NF-κB (1:1,000; cat. no. 3033T; CST Biological Reagents Co., Ltd.), p65 NF-κB (1:2,000; cat. no. ab16502; Abcam), IL-6 (1:1,000; cat. no. 21865-1-AP; Proteintech Group Inc.), TNF-α (1:2,000; cat. no. A0277; Abclonal Biotech Co., Ltd.), IL-1β (1:2,000; cat. no. 16806-1-AP; Proteintech Group Inc.), iNOS (1:2,000; cat. no. ab178945; Abcam), Arg-1 (1:2,000; cat. no. 16001-1-AP; Proteintech Group Inc.) and GAPDH (1:10,000, cat. no. 10494-1-AP; Proteintech Group Inc.) at

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
MCPIP1	F: AACTGGTTTCTGGAGCGAGG
	R: CGAAGGATGTGCTGGTCTGT
CD16	F: CAGACAGGCAGAGTGCAGC
	R: ACGTGTAGCTGGATTGGACC
CD32	F: AAGCAGGTTCCAGACAATCCT
	R: TGGCTTGCTTTTCCCAATGC
iNOS	F: GGTGAAGGGACTGAGCTGTT
	R: ACGTTCTCCGTTCTCTTGCAG
IL-4	F: TCACAGCAACGAAGAACACCA
	R: CAGGCATCGAAAAGCCCGAA
IL-10	F: GCATGGCCCAGAAATCAAGG
	R: ACACCTTGGTCTTGGAGCTTATTA
Arg-1	F: GTACATTGGCTTGCGAGACG
	R: ATCGGCCTTTTCTTCCTTCCC
CD206	F: TTCAGCTATTGGACGCGAGG
	R: GAATCTGACACCCAGCGGAA
GAPDH	F: AGGTCGGTGTGAACGGATTT
	R: ACTGTGCCGTTGAATTTGCC

F, forward; R, reverse; MCPIP1, monocyte chemotactic protein-1-induced protein 1; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1.

4˚C overnight. GAPDH was used as an internal reference protein. Following washing with TBS with 0.1% Tween20, membranes were then incubated with the HRP-conjugated Goat Anti-Rabbit IgG (H+L) secondary antibodies (1:10,000; SA00001-2; Proteintech Group Inc.) for 1 h at 25˚C. ECL solution (cat. no. E412-02; Vazyme Biotech Co., Ltd.) was used to detect the bands. Analysis was performed using Image-Pro plus 6.0 software (Media Cybernetics).

Immunofluorescence staining. Frozen brain sections (thickness, 30 μm) were blocked using 10% bovine serum albumin (Thermo Fisher Scientific Inc.) for 1 h at 25˚C. Sections were incubated with antibodies against MCPIP1 (1:400; cat. no. 25009-1-AP; Proteintech Group Inc.) at 4˚C overnight, followed by incubation with Alexa Fluor® 488-conjugated secondary antibody (1:1,000; ab150077, Abcam) at room temperature in the dark for 1 h and DAPI at 25˚C for 5 min. Sections were imaged using a Leica DMI 4000 B fluorescence microscope (Leica Microsystems GmbH). Images were taken from each rat for analysis by Image-Pro plus 6.0 software (Media Cybernetics).

Hematoxylin and eosin (H&E) staining. Paraffin-embedded sections were deparaffinized in xylene at 25˚C for 5 min followed by rehydration using 95, 70 and 50% ethanol solution 3 min in each. The sections were stained with H&E at 25˚C for 8 min and imaged using a Leica DMI 4000 B fluorescence microscope (Leica Microsystems GmbH). Proportion of damaged neuronal cell was calculated by the number of cells exhibiting shrinkage.

Enzyme-linked immunosorbent assay (ELISA). The serum levels of IL-6, TNF-α, and IL-1β in mice were assessed using Mouse IL-6 ELISA Kit, Mouse TNF-α ELISA Kit, and Mouse IL-1β ELISA Kit (cat. nos. KE10007, KE10002 and KE10003, respectively; Proteintech Group Inc.).

Flow cytometry assay. BV2 cells were collected using trypsin, washed, and suspended in PBS. The cells were then incubated with FITC-conjugated CD16/CD32 antibodies (1:100; cat. no. 561728; BD Biosciences) and PE-conjugated CD206 antibodies (1:100; cat. no. 568273; BD Biosciences) at 4˚C for 20 min. The expression of CD16/CD32 and CD206 was analyzed using an Accuri C6 Plus flow cytometer (BD Biosciences) and FlowJo v10.9.0 software (Tree Star Inc.).

Ubiquitination assay. pcDNA3.1-Flag-TRAF6, pcDNA3.1-His-MCPIP1, and pcDNA3.1-HA-Ubiquitin plasmids were purchased from Beijing Syngentech Co., Ltd. (Chian). BV2 cells were transfected with Flag-TRAF6, His-MCPIP1 and HA-ubiquitin at 37˚C for 48 h. Then, cells (with the exception of control cells) were stimulated with 1 μg/ml LPS for 12 h and then treated with or without MG132 (20 μM) for an additional 4 h to block proteasomal degradation. The cells were lysed with buffer (50 mM Tris, 140 mM NaCl, 1% SDS), followed by clearing of cell lysates through centrifugation at 10,000 x g for 10 min. For IP assays, the anti-Flag (5 μg, cat. no. 20543-1-AP, Proteintech Group Inc.) were added at 4˚C overnight. After incubation, 50 μl protein A/G Plus-Agarose (Santa Cruz) was added to the protein-antibody complexes and incubated at 4˚C on a rotating device overnight. Immunoprecipitates were washed five times with immunoprecipitation buffer, centrifuged at 10,000 x g for 1 min, and a 2x sample loading buffer was added to the beads before boiling for 5 min. Pull-down samples were subjected to immunoblotting with an HA-tagged antibody (1:5,000; cat. no. 51064-2-AP; Proteintech Group Inc.), Flag-tagged antibody (1:20,000; cat. no. 20543-1-AP; Proteintech Group Inc.), His-tagged antibody (1:2,000; cat. no. 10,001-0-AP; Proteintech Group Inc.), and GAPDH (1:10,000; cat. no. 10494-1-AP; Proteintech Group Inc.). Following washing with TBS with 0.1% Tween20, membranes were then incubated with the HRP-conjugated Goat Anti-Rabbit IgG (H+L) secondary antibodies (1:10,000; SA00001-2; Proteintech Group Inc.) for 1 h at 25˚C. ECL solution (cat. no. E412-02; Vazyme Biotech Co., Ltd.) was used to detect the bands. Analysis was performed using Image-Pro plus 6.0 software (Media Cybernetics).

Statistical analysis. Data are presented as mean ± SD. Statistical significance was evaluated using unpaired Student's t-test and one-way analysis of variance with Tukey's post hoc test using GraphPad Prism version 8 (Dotmatics). $P<0.05$ was considered to indicate a statistically significant difference.

Results

MCPIP1 is enhanced in LPS-treated mice. A significant increase in MCPIP1 mRNA and protein expression levels was demonstrated in the hippocampus of LPS-treated mice compared with the control (Fig. 1A and B). Immunofluorescence results also indicated that the intensity of MCPIP1 staining

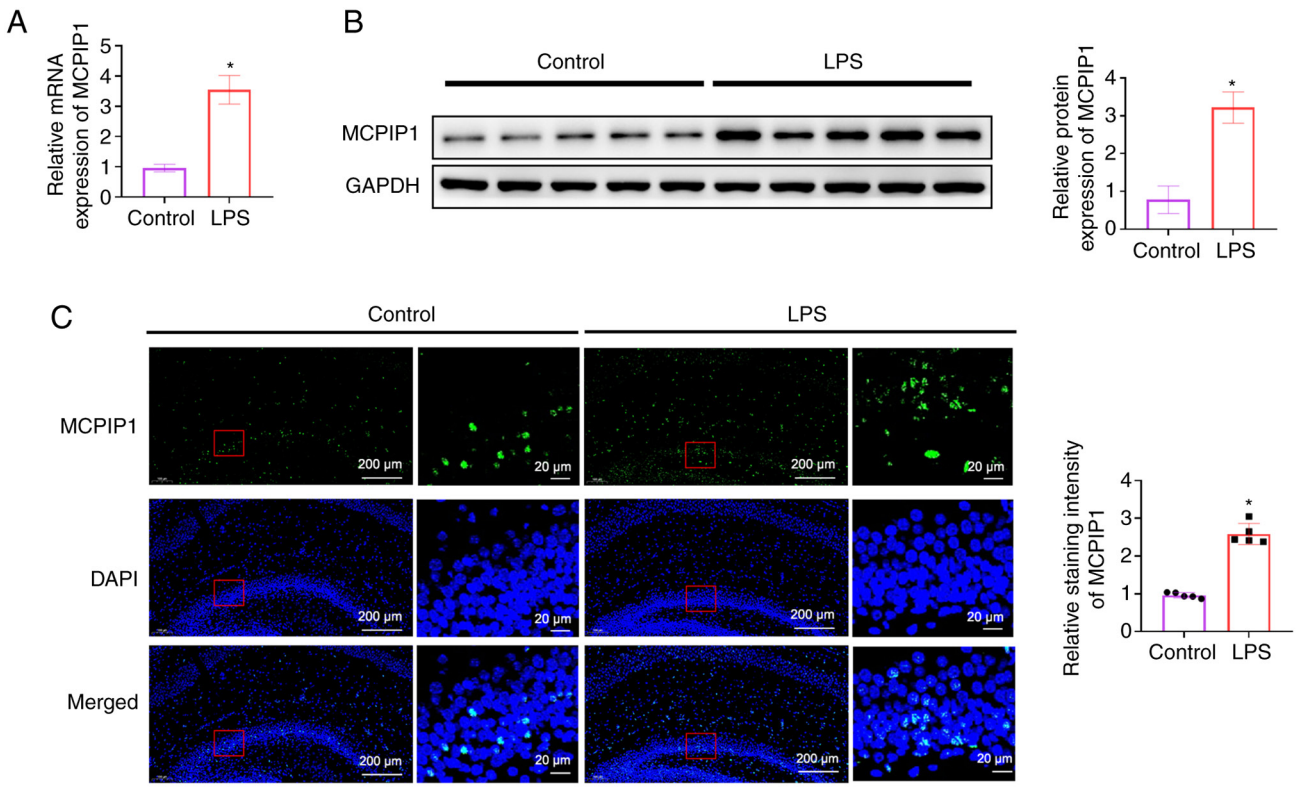

Figure 1. MCPIP1 is enhanced in LPS-treated mice. (A) Reverse transcription-quantitative PCR, (B) western blotting and (C) immunofluorescence staining were used to assess MCPIP1 mRNA and protein expression levels. *P<0.05 vs. control. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide.

increased significantly in LPS-treated mice compared with the control (Fig. 1C).

MCPIP1 overexpression mice exhibit reduced depressive-like behaviors. To further evaluate the potential effect of MCPIP1 on depression, an AAV that overexpressed MCPIP1 was injected into the hippocampus of mice. MCPIP1 mRNA and protein expression levels increased significantly after AAV infusion compared with the AAV-NC (Fig. 2A and B). The SPT provides a measurement of anhedonia, lack of interest in reward stimuli, and depression. The results indicated that LPS treated MCPIP1 overexpression mice exhibited a significantly higher preference for sucrose solution compared with the LPS + NC mice (Fig. 2C), which indicated decreased anhedonia behavior in MCPIP1 overexpression mice. There was no significant difference in the distance moved between mice in the LPS + NC and LPS + MCPIP1 groups in the OFT (Fig. 2D), which indicated that MCPIP1 overexpression did not significantly affect locomotor activity. The TST was then performed as an acute stress assay to measure the immobility time that was associated with the induction of depression using LPS. MCPIP1 overexpression resulted in less despair behavior, as indicated by the shorter time of immobility, compared with that in the LPS + NC mice (Fig. 2E), and this was also confirmed by the FST (Fig. 2F) that serves as an alternative acute stress assay. MCPIP1 overexpression decreased time of immobility, compared with the LPS + NC group (Fig. 2F). H&E staining demonstrated that the hippocampal neurons were normal in the control group and shrunken after LPS treatment. MCPIP1 overexpression also significantly decreased the number of injured neurons compared with the LPS + NC group (Fig. 2G).

MCPIP1 facilitates M2-polarization of microglia and alleviates the inflammatory response via inhibition of the TLR4/TRAF6/NF-κB signaling pathway in vivo. MCPIP1 significantly reduced Iba-1 protein expression levels in LPS-treated mice compared with the LPS + NC group (Fig. 3A). RT-qPCR analysis demonstrated that MCPIP1 overexpression significantly inhibited the LPS-induced elevation of the mRNA expression levels of CD16, CD32 and iNOS, compared with the LPS + NC group (Fig. 3B). MCPIP1 overexpression significantly increased IL-4 and Arg-1 mRNA expression levels compared with the LPS + NC; mRNA expression levels of IL-4 and Arg-1 were significantly reduced by LPS treatment compared with the control. IL-10 and CD206 mRNA expression levels were significantly increased by MCPIP1 overexpression compared with the LPS + NC group; however, LPS treatment did not demonstrate a significant effect on their expression compared with the control (Fig. 3C). ELISA results demonstrated that MCPIP1 overexpression significantly decreased IL-6, TNF-α and IL-1β expression levels in LPS-treated mice compared with the LPS + NC group (Fig. 3D-F). The aforementioned results indicated that MCPIP1 served a role in the M2-polarization of microglia. Western blotting was performed to examine the TLR4/TRAF6/NF-κB signaling pathway. LPS-triggered significantly increased TLR4, MyD88, TRAF6, p-IκBα and p-p65 NF-κB protein expression levels compared with the control, which were significantly reduced by MCPIP1 overexpression compared with the LPS + NC group (Fig. 3G).

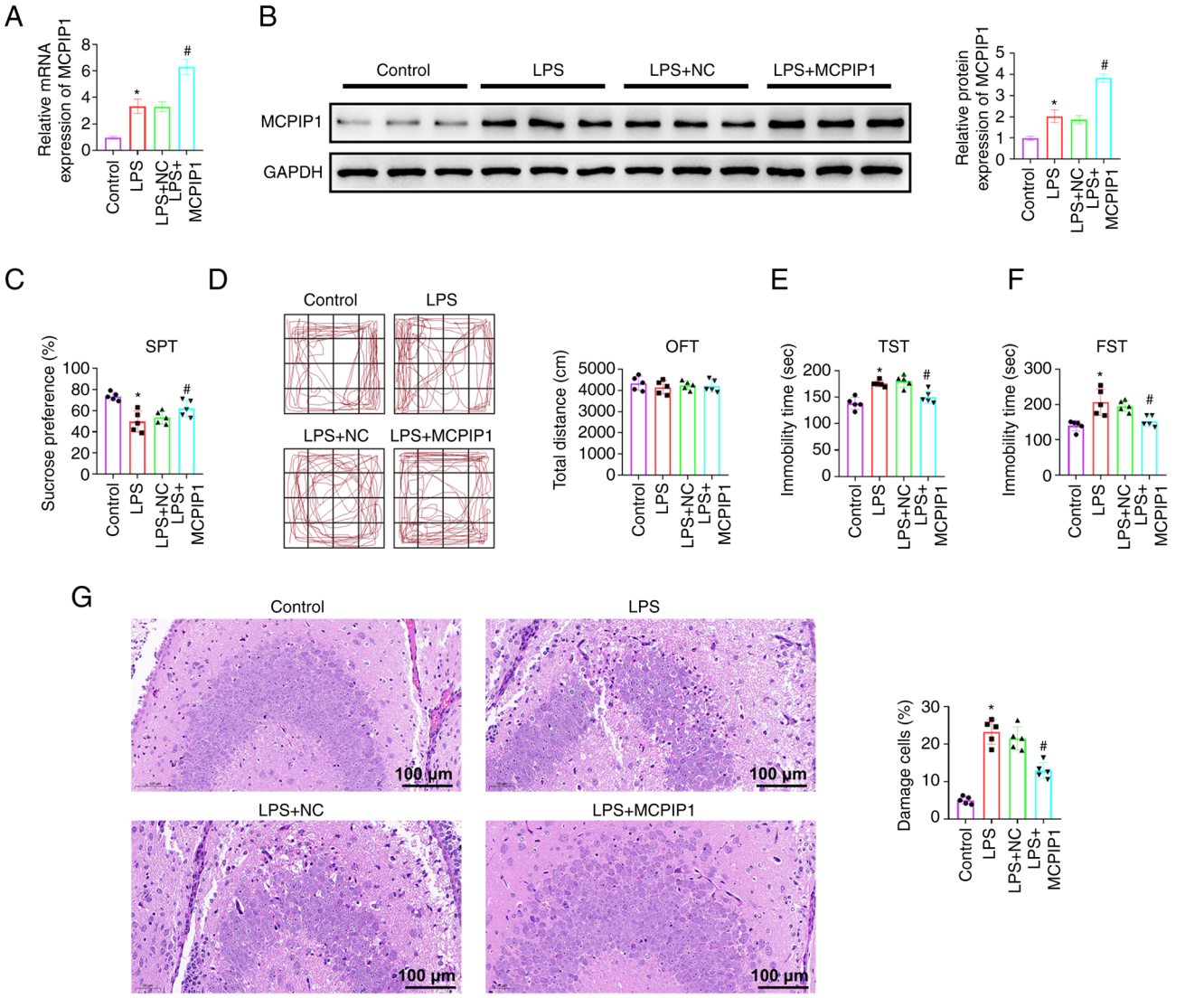

Figure 2. MCPIP1 overexpression mice exhibit reduced depressive-like behaviors. (A) Reverse transcription-quantitative PCR and (B) western blotting were used to assess MCPIP1 expression. Depressive-like behavior was assessed using (C) SPT, (D) OFT, (E) TST and (F) FST. (G) Representative images and quantification of hematoxylin and eosin staining in the hippocampus regions of mice. *P<0.05 vs. control. #P<0.05 vs. LPS + NC group. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide; NC, negative control; SPT, sucrose preference test; OFT, open field test; TST, tail suspension test; FST, forced swimming test.

MCPIP1 accelerates M2-polarization of microglia. LPS treatment significantly upregulated MCPIP1 mRNA and protein expression levels in BV2 cells compared with the control (Fig. 4A and B). MCPIP1 overexpression and knockdown plasmids were transfected into BV2 cells. MCPIP1 protein expression levels significantly increased and Iba-1 protein expression levels significantly decreased after transfection with the MCPIP1 overexpression vector compared with the control (Fig. 4C). MCPIP1 knockdown decreased MCPIP1 protein expression levels and significantly increased Iba-1 protein expression levels (Fig. 5A). LPS treatment significantly increased the protein expression levels of IL-6, TNF-α, IL-1β and iNOS, and significantly decreased the protein expression levels of Arg-1, all compared with the control. MCPIP1 overexpression decreased protein expression levels of IL-6, TNF-α, IL-1β and iNOS, and increased the protein expression levels of Arg-1, all compared with the LPS + NC group (Fig. 4D). MCPIP1 knockdown aggravated PS-induced the protein expression levels of IL-6, TNF-α, IL-1β and iNOS, and further decreased arg-1 expression reduced by LPS (Fig. 5B). MCPIP1 overexpression significantly inhibited the LPS-induced expression of CD16/32 compared with the LPS + NC group. CD206 expression was significantly increased by MCPIP1 overexpression compared with the LPS + NC group; however, LPS treatment did not significantly affect CD206 expression levels compared with the control (Fig. 4E). MCPIP1 knockdown decreased CD206 expression levels compared with the LPS + si-NC group (Fig. 5C).

MCPIP1 alleviates the inflammatory response by inhibiting the TLR4/TRAF6/NF-κB signaling pathway. MCPIP1 overexpression significantly reduced the LPS-induced increase in the protein expression levels of TLR4, MyD88, TRAF6, p-IκBα and p-p65 NF-κB compared with the LPS + NC group. Moreover, MCPIP1 knockdown significantly increased the protein expression levels of TLR4, MyD88, TRAF6, p-IκBα

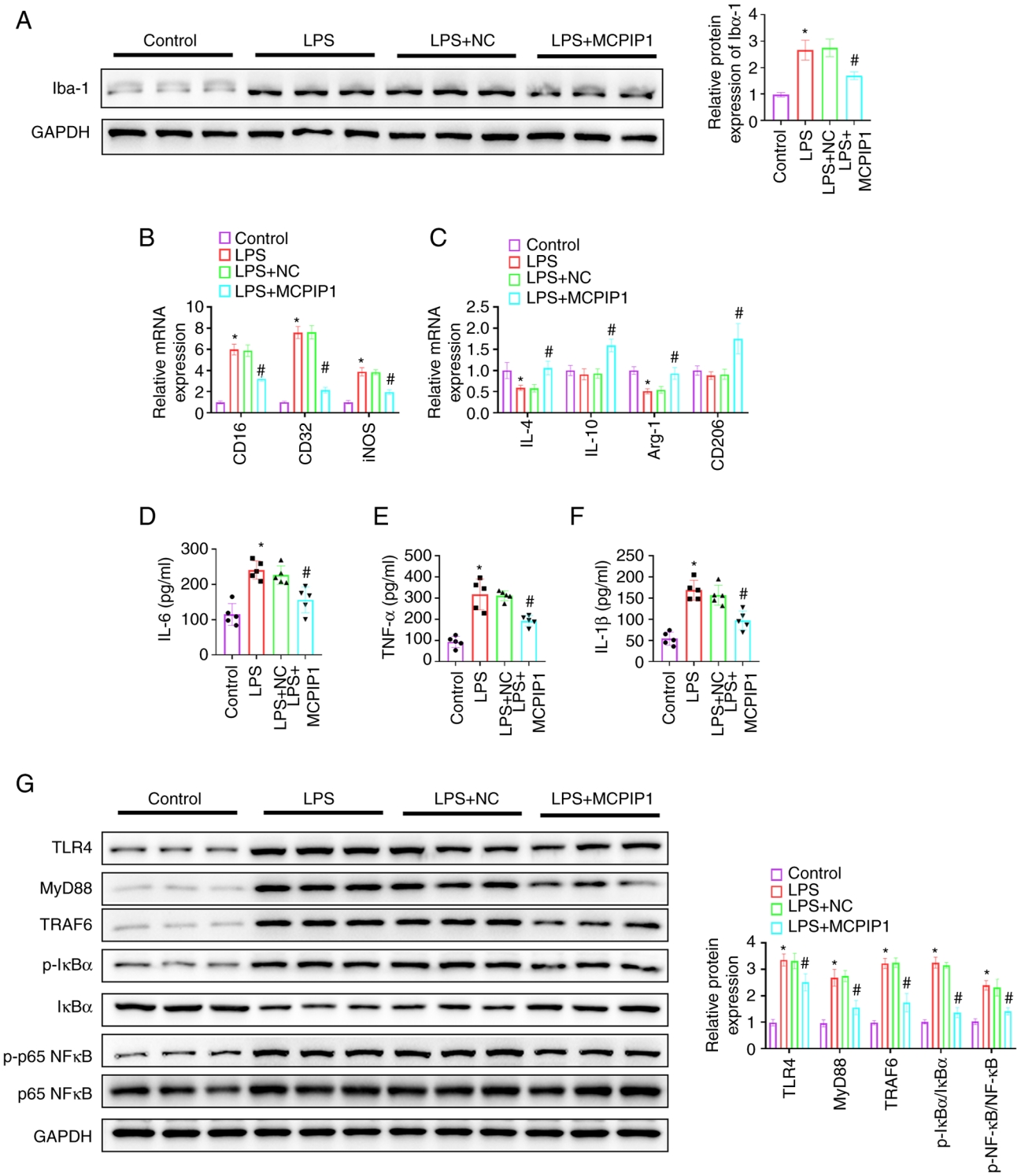

Figure 3. MCPIP1 facilitates M2-polarization of microglia and alleviates the inflammatory response via inhibition of the TLR4/TRAF6/NF-κB signaling pathway *in vivo*. (A) Western blotting was used to semi-quantify Iba-1 protein expression. mRNA expression levels of (B) CD16, CD32 and iNOS, and (C) IL-4, IL-10, Arg-1 and CD206 were quantified using Reverse transcription-quantitative PCR. (D) IL-6, (E) TNF-α and (F) IL-1β levels were assessed using ELISA. (G) Protein expression levels were semi-quantified using western blotting. *P<0.05 vs. control. #P<0.05 vs. LPS + NC group. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide; NC, negative control; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; TRAF6, TNF receptor associated factor 6; p, phosphorylated.

and p-p65 NF-κB compared with the LPS + NC group, indicating that LPS-induced NF-κB activation was increased by MCPIP1 knockdown (Fig. 6A). LPS treatment markedly promoted the ubiquitination of TRAF6 and MCPIP1 notably inhibited LPS-induced TRAF6 ubiquitination in BV2 cells with or without MG132 treatment (Fig. 6B). TAK-242 was used to evaluate the function of MCPIP1 in the LPS-induced inflammatory response via the TLR4//TRAF6/NF-κB signaling pathway in BV2 cells. Western blotting demonstrated that TAK-242 treatment significantly reversed the upregulated

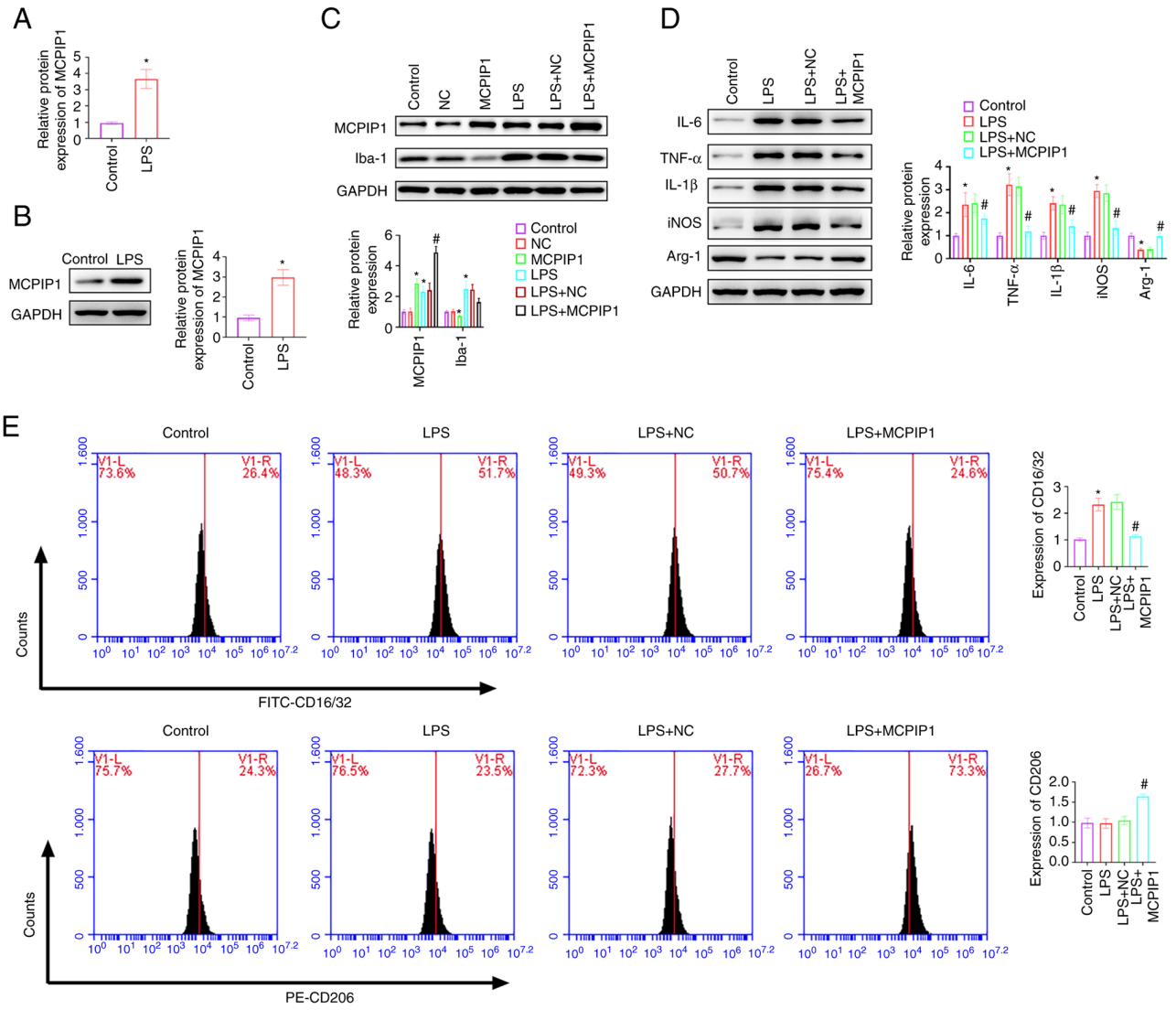

Figure 4. MCPIP1 accelerates M2-polarization of microglia. (A) Reverse transcription-quantitative PCR and (B) western blotting were used to assess MCPIP1 mRNA and protein expression levels. Protein expression levels were assessed using (C and D) western blotting and (E) flow cytometry. *P<0.05 vs. control. #P<0.05 vs. LPS + NC group.

protein expression levels of IL-6, TNF-α, IL-1β and iNOS and the downregulated protein expression levels of Arg-1 induced by MCPIP1 knockdown in BV2 cells, compared with the LPS + si-MCPIP1 group (Fig. 6C). Treatment with TAK-242 significantly reversed the upregulated protein expression levels of TLR4, MyD88, TRAF6, p-IκBα and p-NF-κB p65 which were induced by MCPIP1 knockdown (Fig. 6D). These data indicated that MCPIP1 knockdown promoted the LPS-induced inflammatory response via activation of the TLR4/TRAF6/NF-kB signaling pathway in BV2 cells.

Discussion

MDD is a mental health condition associated with numerous symptoms that include physical, cognitive, emotional and social aspects (28). Although advances have been made in the anti-depressant treatment of MDD, it still presents a high mortality rate (29). Therefore, new therapeutic biomarkers must be identified. previous studies have reported that MCPIP1 serves important biological roles in lipid homeostasis (30), insulin secretion (31) and the inflammatory response (32), and also regulates the development of certain diseases, such as hidradenitis suppurativa (33), primary biliary cholangitis (34), skin inflammation (35) and clear cell renal cell carcinoma (36). Here, LPS could induce MCPIP1 expression and overexpression of MCPIP1 decreased the LPS-induced inflammatory response. Overexpression of MCPIP1 in macrophages partially protects mice from LPS-induced septic shock (37){Huang, 2013 #74}. Han *et al* (38) reported that the level of MCPIP1 increased and the level of SIRT1 decreased in LPS induced Kupffer cells or RAW 264.7 macrophages. Overexpression of MCPIP1 alleviated cytokine secretion and p65 nuclear translocation. MCPIP1 overexpression induced by MG132 has been reported to alleviate sepsis-induced pathologic changes, water content and protein leakage in the lungs, the induction of systemic inflammatory mediators and to improve the 7-day mortality rate in a rat model (39). A previous study reported that MCPIP1 expression was upregulated in LPS-treated microglia and the mouse brain, and that levels of pro-inflammatory cytokines were increased in MCPIP1 deficient mouse

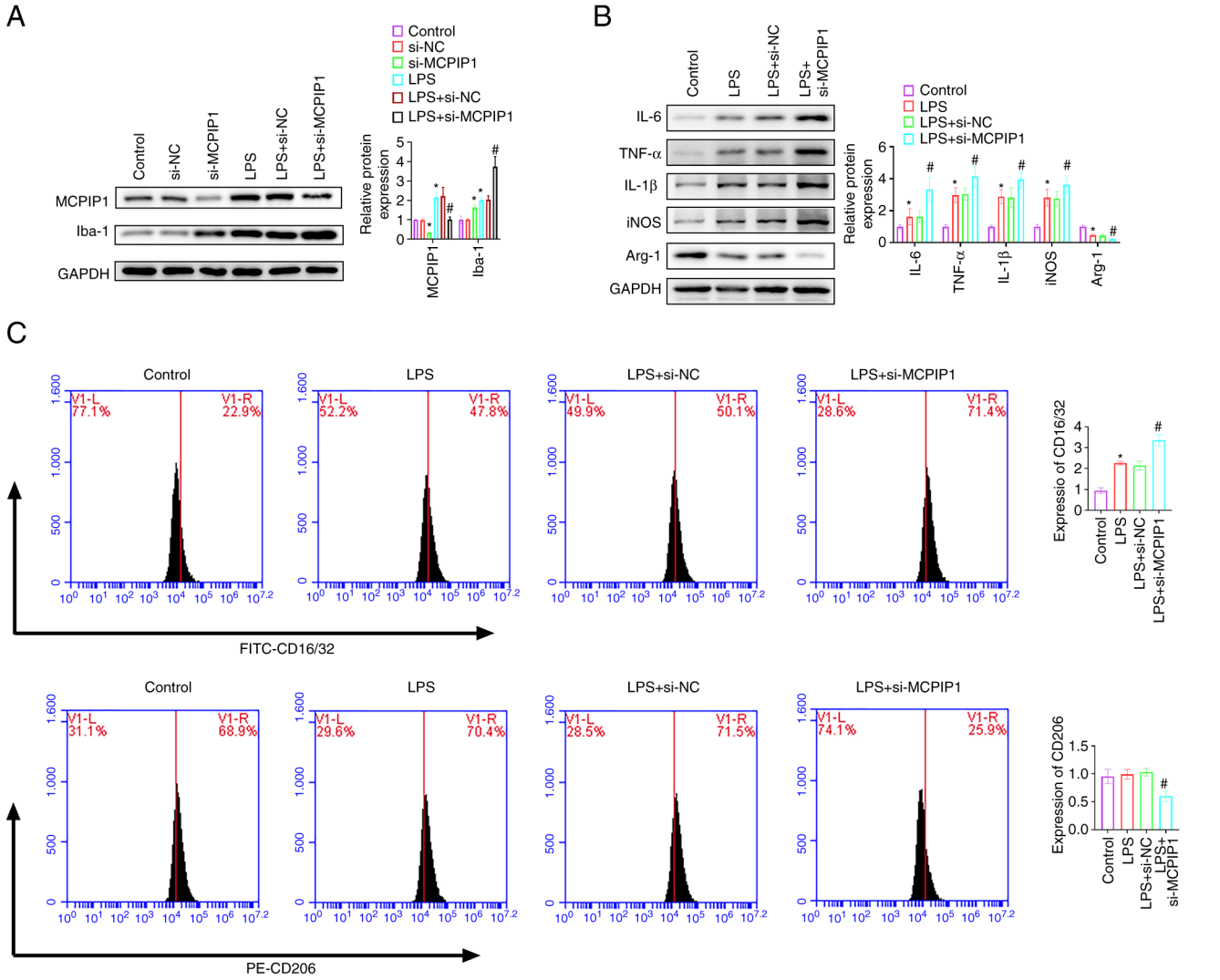

Figure 5. MCPIP1 knockdown inhibits M2-polarization of microglia. Protein expression levels were assessed using (A and B) western blotting and (C) flow cytometry. *P<0.05 vs. control. #P<0.05 vs. LPS + NC group. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide; NC, negative control; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; si, short interfering RNA.

brains (40). Similarly, in the present study, LPS-treated mice presented injured neurons, microglial activation and depression-like behaviors. Overexpression of MCPIP1 in mice alleviated the pathological symptoms, and this was consistent with the observation that melatonin alleviated LPS-induced depressive-like behaviors (41). Collectively, these results indicated that MCPIP1 may represent a novel therapeutic target for MDD and that MCPIP1 overexpression may relieve depressive-like behaviors in LPS-induced mice.

Previous studies have reported that neuroinflammation is a risk factor for depression (42-44). LPS increases cytokine levels and strengthens depressive-like behaviors in mice (45). It has been reported that ibrutinib inhibits LPS-induced depressive-like behaviors and pro-inflammatory cytokine levels by inhibiting NF-κB activation (46). Wang *et al* (47) reported that palmatine relieved depressive like behaviors, inhibited pro-inflammatory cytokines (TNF-α, IL-6, CD68 and iNOS) and enhanced anti-inflammatory cytokines (IL-4, IL-10, CD206, Arg1 and Ym1) in LPS-induced mice and BV2 cells. Zhang *et al* (14) reported that curcumin converted M1 phenotype to M2 phenotype by reducing iNOS, IL-1β, IL-6 and CD16/32 expression and inducing Arg-1, IL-4, IL-10 and CD206 expression in LPS-stimulated BV2 cells. Similarly, the present study demonstrated that MCPIP1 overexpression alleviated depressive-like behaviors in LPS-induced mice, significantly inhibited the expression of pro-inflammatory cytokines and markers of M1 microglia, such as IL-6, TNF-α, IL-1β, CD16/32 and iNOS, and significantly increased the expression of anti-inflammatory cytokines and markers of M2 microglia, such as IL-4, IL-10, Arg-1 and CD206. These results demonstrated that MCPIP1 inhibited inflammation and depressive-like behaviors during LPS treatment.

TLR4 is an important component of the inflammatory response, and its upregulation is related to depression and the activation of microglia (48). A previous study reported that baicalin relieved LPS-induced depressive-like behavior through a decrease in TLR4 expression and activation of the PI3K/AKT/FOXO1 signaling pathway (49). TLR4 inhibition has been reported to decrease MCPIP1 expression in LPS/ischemia-induced microglia (50). In the present study, MCPIP1 overexpression significantly decreased TLR4 expression in LPS-stimulated mice and BV-2 cells. TLR4 elevated

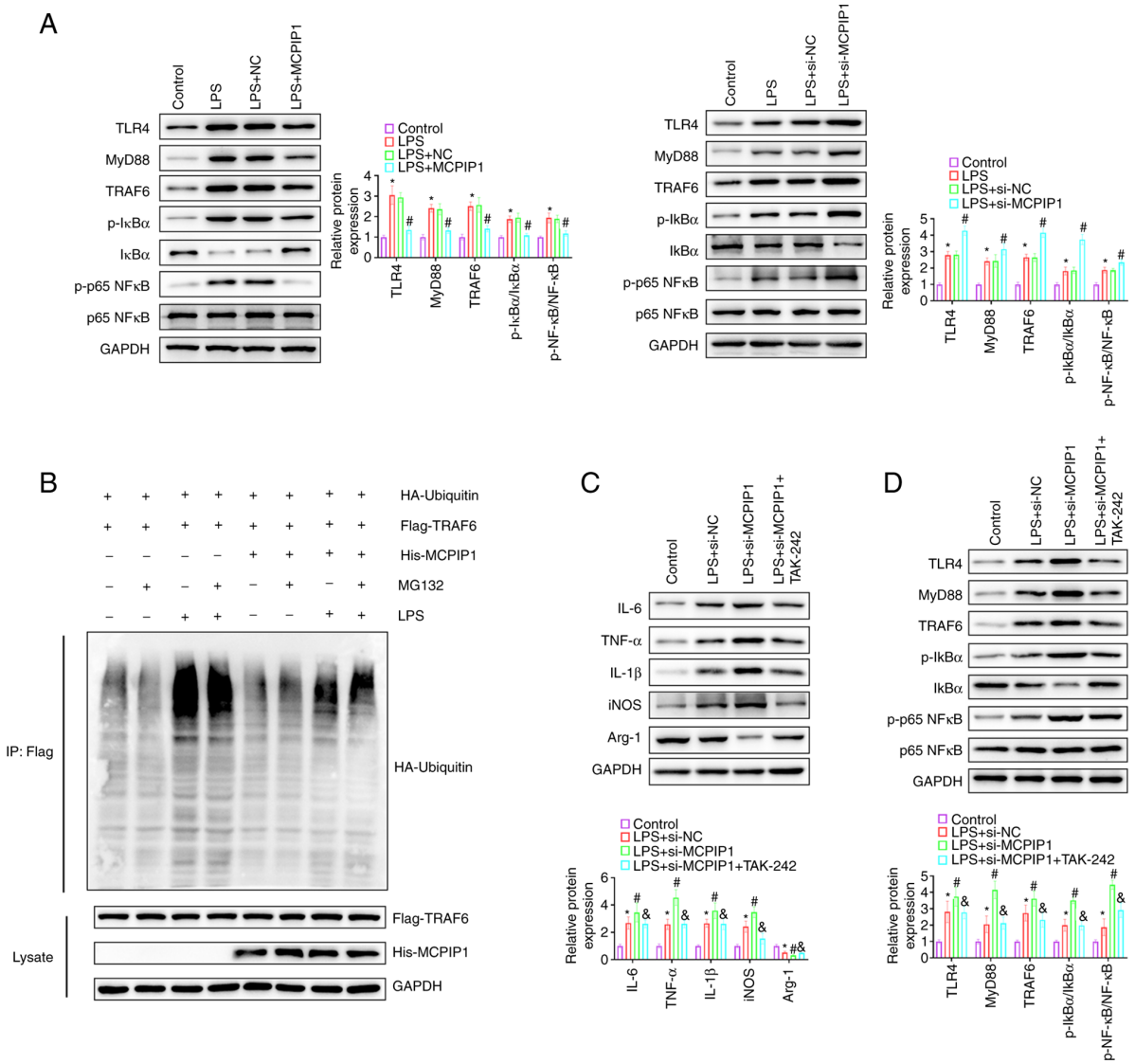

Figure 6. MCPIP1 alleviates the inflammatory response via inhibition of the TLR4/TRAF6/NF-κB signaling pathway. (A) Protein expression levels were semi-quantified using western blotting. (B) Flag-TRAF6 was isolated by immunoprecipitation followed by immunoblotting with anti-HA specific polyubiquitin antibody to assess ubiquitin conjugation. BV2 cells were treated with TAK-242, a selective TLR4 inhibitor. (C and D) Protein expression levels were semi-quantified using western blotting. *P<0.05 vs. control. #P<0.05 vs. LPS + NC group. &P<0.05 vs. LPS + si-MCPIP1. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide; NC, negative control; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; TRAF6, TNF receptor associated factor 6; p, phosphorylated; si, short interfering RNA.

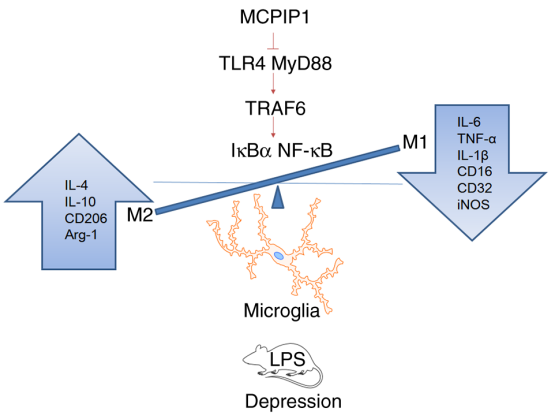

Figure 7. Schematic illustration of the role MCPIP1 in LPS-induced mice and microglia. MCPIP1, monocyte chemotactic protein-1-induced protein 1; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; Arg-1, arginase 1; TRAF6, TNF receptor associated factor 6.

TRAF6 expression through the MyD88-dependent pathway, thereby promoting cytokine release (51). A previous study reported that repression of TLR4/MyD88/TRAF6 alleviated pro-inflammatory microglial polarization (52). In agreement with these studies, the present study demonstrated that MCPIP1 reduced LPS-induced neuroinflammation via deactivation of the TLR4/TRAF6/NF-κB signaling pathway. NF-κB has been reported to be a crucial downstream component of the TLR4 signaling pathway in the context of LPS induced neuroinflammation (53). It has been previously reported that hesperetin, a citrus Flavonoid, inhibited the inflammatory response by inhibiting the TLR4/NF-kB signaling pathway in LPS-induced mice and microglia (54). Similarly, curcumin ameliorated LPS-induced neuroinflammation by inducing M2 polarization of microglia though the TREM2/TLR4/NF-κB signaling pathway (14). In the present study, MCPIP1 promoted M2-polarization of microglia and alleviated

neuroinflammation by suppressing the TLR4/TRAF6/NF-κB signaling pathway. These results were consistent with those of a previous study, which reported that loganin, an iridoid glycoside obtained from traditional Chinese medicine Cornus officinalis, attenuated Aβ-induced inflammatory response in BV-2 cells by suppressing the TLR4/TRAF6/NF-κB signaling pathway (55). These results demonstrated that MCPIP1 attenuated LPS-induced inflammation via inhibition of the TLR4/TRAF6/NF-κB signaling pathway.

There are limitations of the present study. First, the inflammatory mechanism mediated by MCPIP1 is complicated, and we only targeted the TLR4/TRAF6/NF-κB pathway. Thus, other pathways involved in the treatment of depressive-like behaviors need to be further study. Second, we studied the role of MCPIP1 only in animal models of LPS-induced depressive-like behaviors, and its role in other animal models of depression needs to be further studied.

The aforementioned results indicated that MCPIP1 alleviated LPS-induced depressive-like behaviors and that MCPIP1 promoted the M2-polarization of microglia via the inhibition of the TLR4/TRAF6/NF-κB signaling pathway (Fig. 7).

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (81872605).

Availability of data and material

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QA was responsible for conception and design. QA and JX performed the experiments. FP and SS performed data analysis and interpretation. QA and JX confirm the authenticity of all the raw data. QA and SS wrote the manuscript. All authors reviewed the final manuscript.

Ethics approval and consent to participate

The experimental protocol of our study was performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Ethics Committee of Qingdao Mental Health Center (Qingdao, China; approval no. 2022061).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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