

Suppression of miR-155 attenuates neuropathic pain by inducing an M1 to M2 switch in microglia

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Abstract

Introduction: The polarization state of microglia affects the progress of neuropathic pain. MiR-155 modulates polarization of microglia, while its role in neuropathic pain has not been well studied.

Material and methods: We separately used lipopolysaccharide (LPS) and interleukin 4 (IL-4) for constructing an M1/M2 polarization model in BV-2 cells. The levels of CD86, iNOS, CD206, Arg and miR-155 were measured by western blot or qRT-PCR, as needed. Subsequently, BV-2 cells were transfected with miR-155 mimics or inhibitor to explore the effects of miR-155 on polarization states. We also constructed a neuropathic pain model by applying spinal nerve ligation (SNL) in Wistar rats with miR-155 agomir or antagomir injection. The withdrawal threshold was measured by Von Frey fibre needle. The levels of interleukin 1 β (IL-1 β), tumour necrosis factor α (TNF- α) and the proportion of M1-polarized microglia in primary microglia from rats were measured by ELISA and flow cytometry.

Results: LPS induced M1 polarization in BV-2 cells with increasing of CD86, iNOS and miR-155, while IL-4 induced M2 polarization in BV-2 cells with increasing of CD206, Arg and decreasing of miR-155. MiR-155 mimics upregulated CD86 and downregulated CD206, whereas miR-155 inhibitor downregulated CD86 and upregulated CD206. MiR-155 antagomir increased the withdrawal threshold, decreased the production of IL-1 β , TNF- α and the proportion of M1-polarized microglia in primary microglia.

Conclusions: Results demonstrate that suppression of miR-155 attenuates neuropathic pain by inducing an M1 to M2 switch in microglia. Our findings provide a new perspective to understand the function of miR-155 in microglia.

Key words: neuropathic pain, microglia, polarization state, miR-155.

Introduction

Neuropathic pain results from damage to the somatosensory system [24]. Microglia is an important neurological specific immune cell, influencing brain

development, maintenance of the neural environment and response to nerve damage and repair. The activation of microglia in response to nerve injury has been associated with the development of neuropathic pain [52]. Multiple components could activate microglia,

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such as neuregulin-1 [44], matrix metalloproteinase [29]. The study on the mechanism of microglia influencing neuropathic pain has been one of the focuses in medical science. Previous studies show that microglia exacerbates neuropathic pain *via* TREM2/DAP12 signal [16]; neuron interacts with microglia in neuropathic pain through purinergic signal [39]; crosstalk between astrocytes and microglia causes neuropathic pain [25]. A recent study has shown that oestrogen alleviates neuropathic pain by inhibiting activation of microglia and astrocytes [19]. Even though a wide variety of cytokines and signal pathways have been found to provide multiple explanations for the roles of microglia in the occurrence and maintenance of neuropathic pain, the precise mechanism of microglia in neuropathic pain has not been clear yet.

Activation of microglia refers to microglia polarization, M1 type and M2 type. Lipopolysaccharide (LPS) or interferon γ (IFN- γ) stimulates activation of microglia into an M1 phenotype, which secretes pro-inflammatory cytokines [18]. Interleukin (IL)-4/IL-13 stimulates activation of microglia to an M2 phenotype, which is responsible for tissue repair and resolution of inflammation [21]. Factors released by activated microglia affect the polarization states by autocrine, such as IL-6, IL-1 β and tumour necrosis factor- α (TNF- α) which cause the M1 polarization in microglia and painful symptoms; IL-10 secreted by activated microglia causes M2 polarization [42]. Increasing evidence suggests that microglia transition from a proinflammatory M1 phenotype to an anti-inflammatory M2 phenotype benefits positive functional outcomes and relieving of neurological disease [37]. Studies demonstrate that antagonizing peroxisome proliferator-activated receptor γ promotes a change of polarization states in microglia [13]. During neuroinflammatory injury, resveratrol affects microglia M1/M2 polarization by regulating PGC-1 α [47]. Cyclic adenosine monophosphate (AMP) promotes M1 to M2 phenotypic conversion of microglia in the presence of Th2 cytokines [9]. In a cuprizone-induced demyelination mouse model, progesterone suppresses NLRP3 inflammasome and induces an M1 to M2 switch in microglia polarization [1], S-citalopram and Fluoxetine promote M2 activation and inhibit M1 activation of microglia *in vitro* [34].

Microglia are derived from CD45⁺ bone marrow precursors of the myeloid lineage, can give rise to dendritic cells, macrophages and granulocytes [33]. It has been reported that the migrate ability of dendritic cells was key for the initiation of protective

pro-inflammatory as well as tolerogenic immune responses [41]. Studies have shown that dendritic cells can play an important role in inflammation-related diseases, the interaction of resting immature dendritic cells with Toll-like receptor (TLR) ligands leads to a cascade of pro-inflammatory cytokines [3,49]. MicroRNAs are short, non-coding RNAs that regulate various biological processes through regulation of mRNAs at a post-transcriptional level. miR-155 can be induced by TLR ligands and some cytokines, such as LPS [32], TNF- α [27] and transforming growth factor β (TGF- β) [17]. During CD4⁺T cell activation, miR-155 drives naïve CD4⁺ T cells to skew toward Th1 and Th17 cells, which are both pro-inflammatory CD4⁺ T cell subsets [38,48]. Ceppi *et al.* found that miR-155 modulated the TLR/IL-1 inflammatory pathway in activated human monocyte-derived dendritic cells [6]. Up-regulation of miR-155 also seems to be a sign of dendritic cells maturation, and it is also essential for their functions [40]. MiR-155-deficient dendritic cells caused less severe GVHD through reduced migration and defective inflammasome activation [7]. Previous studies have reported microRNAs promote neuroinflammation and neuropathic pain, such as miR-32-5P [46] and miR-150 [14]. However, the function of microRNAs in microglia M1/M2 polarization switch has been less reported. miR-155 is considered to be a multiple sclerosis-relevant regulator of polarization states in myeloid cells [26] and also has an important role in promoting M1 polarization of microglia in during bacterial brain infection [51]. One study also shows that suppressing the STAT1-miR-155 signalling axis causes M2 polarization of tumour-promoting macrophage [15]. While the role of miR-155 in regulating microglia M1/M2 polarization switching and neuropathic pain has not been clear yet. Hence, we investigate the effects of miR-155 on microglia polarization states and neuropathic pain.

Material and methods

Cell culture and polarization

The murine microglial BV-2 cells (CBP60922, Cobioer, China) were purchased from Cobioer Bio-Science Co., Ltd (Nanjing, China) and maintained in Dulbecco's modified Eagle medium (DMEM/F12, 11320082, Gibco, ThermoFisher, USA) with 10% foetal bovine serum (FBS, 16140071, Gibco, USA). Cells

with a density of 4×10^5 /ml were seeded in six well flat-bottom plates and cultured at 37°C with 5% CO₂. Cells were passaged every 2 day with a 1 : 3 split ratio and used at passages 5-10.

According to the previous work [9], M1-polarized BV-2 cells were generated by a 24-hour treatment with LPS at 1 mg/ml (L2880-10MG, Sigma, USA). M2-polarized BV-2 cells were generated by a 24-hour treatment with IL-4 (BMS338, Invitrogen, USA) at 20 µg/ml. BV-2 cells with no polarization (control group) were treated with equivalent phosphate-buffered saline (PBS). In order to verify the association between the expression level of miR-155 and polarization states in microglia, cells were incubated with the LPS for 1 day at 37°C. LPS was set up at a series of concentration gradients (0, 0.1, 1, 5, 10 mg/ml). Cells were incubated with IL-4 for one day at 37°C and IL-4 was set up at a series of concentration gradients (0, 5, 10, 20, 40 µg/ml).

Cell transfection

After a 48-h incubation with LPS (1 mg/ml) or IL-4 (20 µg/ml), miR-155 mimics (2.5 nmol, Biomics Biotechnology Co., Ltd, China), miR-155 inhibitor (2.5 nmol, Biomics Biotechnology Co., Ltd, China) or their respective control (Scramble, 2.5 nmol, Biomics Biotechnology Co., Ltd, China) were separately transfected at a final concentration of 100 nm/ml in Opti-MEM (11058021, Gibco, ThermoFisher, USA) using Lipofectamine® 2000 (11668019, Invitrogen, ThermoFisher, USA) at room temperature. After incubation for one day at 37°C, cells were collected for extracting total RNA or protein.

RNA isolation and qualitative real time PCR analysis

RNA was extracted from mice microglia BV-2 cells according to a standard protocol applying TRIzol® reagent (15596018, Invitrogen, Thermo-Fisher, USA)

according to the manufacturer's instructions. Total RNA was reverse transcribed by using Prime-Script™ II 1st Strand cDNA Synthesis Kit (6210A, Takara, Japan). SYBR® Green PCR Master Mix (4309155, ABI, USA) at 10 µl volumes was used for real time PCR reactions in 96-well optical reaction plates using the Bio-Rad CFX 96 Touch Real-Time PCR Detection System (185-5196, Bio-Rad, China). Parameters: 95°C for 5 min, followed by 40 cycles of 95°C for 3 s and 60°C for 20 s; every cycle was followed by melt curve conditions at 65°C and 95°C with increments of 0.5°C for 5 s, followed by the final plate read. No primer-dimers and non-specific amplification products were identified in this process. GAPDH was used as an endogenous control for data normalization and the comparative threshold cycle method $2^{-\Delta\Delta CT}$ was used to calculate the relative expression. The results were expressed as fold changes relative to the control group. Primers were designed according to the previous study [1].

To determine miRNA expression, TRIzol® reagent was used for extracting total RNA, including miRNA. TaqMan™ MicroRNA Reverse Transcription Kit (4366597, Applied Biosystems, USA) with miRNA-specific primers was used according to the instructions. Reverse transcription reaction products (1 µl) were used for qRT-PCR (Table I). RT-PCR amplification was performed by HaiGene TaqMan miRNA Fluorescence Quantitative PCR Kit: mmu-miR-155 (TAP01115, HaiGene, China) and U6 snRNA (TAP02055, HaiGene, China). Reactions were amplified and quantified using the Bio-Rad CFX 96 Touch Real-Time PCR Detection System (185-5196, Bio-Rad, China). Parameters: 95°C for 5 min, 40 cycles of 95°C for 15 s, 60°C for 30 s, and 70°C for 10 s. U6 small nuclear RNA was used as an endogenous control for data normalization and the comparative threshold cycle method $2^{-\Delta\Delta CT}$ was used to calculate the relative expression.

Table I. Primers used for qRT-PCR

Gene	Forward	Reverse
<i>CD86</i>	CAACGGAATTAGGAAGAC	CTCTGTATGCAAGTTTCC
<i>iNOS</i>	CAAGCACCTTGGAAGAGGAG	AAGGCCAAACACAGCATACC
<i>CD206</i>	CAAGGAAGGTTGGCATTGT	CCTTTCAGTCCTTGGCAAGC
<i>Arg</i>	TCACCTGAGCTTTGATGTCTG	CTGAAAGGAGCCCTGTCTTG
<i>GAPDH</i>	GTGTTTCCTCGTCCCGTAGA	AATCTCCACTTTGCCACTGC

Western blot analysis

BV-2 cells were collected and homogenized in RIPA lysis buffer (Sigma, R0278, USA) supplemented 1× protease inhibitor cocktail (P8340, Sigma, UAS), phosphatase inhibitor cocktail II (P5726, Sigma, USA) and phosphatase inhibitor cocktail III (524631, Sigma, USA) on ice, sonicated and centrifuged at 20,000 g, 4°C for 30 min. The supernatants after centrifugation were collected on ice. Protein concentrations in supernatants were measured by BCA Protein Assay Kit (7780S, CST, USA) at room temperature.

For western blot, antibodies to the following proteins were used: Anti-CD86 antibody (ab112490, 1 : 5000, Abcam, UK), Anti-iNOS antibody (ab213987, 1 : 5000, Abcam, UK), Anti-CD206 antibody (70-ab32051-050, 1 : 5000, MultiSciences, China), Anti-arginase 1 antibody (70-ab36539-050, 1 : 5000, MultiSciences, China), Anti-GAPDH (1 : 5000, CW0100, Biotech, China). Briefly, equal amounts of protein (50 µg) were separated by 4-20% SDS-PAGE (P0014A, Beyotime, China) and transferred onto polyvinylidene fluoride membranes (P0807-10EA, Sigma, USA). The membranes were blocked with 5% skimmed milk (P0807-10EA, BioMed, China) at room temperature for 10 min, and then incubated with primary antibodies at 4°C overnight. After that, the membranes were incubated with peroxidase-coupled anti-rabbit secondary antibody (R3155, 1 : 5000, Sigma, USA) at room temperature for 2 hours. Membranes were immersed in the enhanced chemiluminescence solution (WBKLS0100, Millipore, USA) at room temperature in dark for one min and exposed under an ImageQuant ECL Imager (28-9605-63, GE Healthcare, USA). All data presented reflect the intensity of one target protein band normalized by GAPDH.

Animals

A total of 105 adult Wistar rats (150-175 g) were obtained from Vitalriver (102, Vitalriver, China). All rats were bred in the Typical SPF Laboratory Animal room with constant temperature (23 ±1°C) and a 12-hour light-dark cycle (light from 9:00 to 21:00) at the Animal Breeding Centre. Rats were acclimated to the environment for 2 weeks before experiments. Animal experiments were performed according to the Yantai Yuhuangding Hospital Animal Ethics Committee. All efforts were made to reduce the number of rats used and their suffering.

Rats were divided into seven groups: rats with surgery but no ligation (Sham group, $n = 15$), rats with L5 spinal nerve ligation (SNL group, $n = 15$), rats with L5 spinal nerve ligation and delivery of scramble (Scramble + SNL group, $n = 15$), rats with L5 spinal nerve ligation and delivery of agomir (Agomir + SNL group, $n = 15$), rats with L5 spinal nerve ligation and delivery of antagomir (Antagomir + SNL group, $n = 15$), rats with delivery of agomir (Agomir group, $n = 15$), and rats with delivery of antagomir (Antagomir group, $n = 15$). Negative control for miR-155 agomir (2.5 nmol, Scramble), negative control for miR-155 antagomir (2.5 nmol, Scramble), miR-155 agomir (5 nmol), miR-155 antagomir (5 nmol) were purchase from Biomart.

Surgical procedure of the neuropathic pain model

L5 spinal nerve ligation (SNL) was operated to induce neuropathic pain in rats [4]. Rats were deeply anesthetized by using 5% chloral hydrate, according to 0.8 ml/100 g body weight peritoneal injection. Briefly, a longitudinal incision was made on the skin of the back, then, the transverse processes of the sixth lumbar vertebra was excised. After that, the right L5 spinal nerves were isolated and firmly ligated with a 6.0 polyamide thread (NW2670, Ethicon, France) and metal skin clips. After complete haemostasis, Polyglactin 910 Suture (CH9113, Ethicon, France) was used to suture the incision. Cages were individually prepared for each rat. After 2 days, the general state, surgical complications or abnormal excessive pain in rats were evaluated and excluded. Metal skin clips were removed after a week under anaesthesia. Sham rats underwent the same operation, with no ligation of L5 spinal nerves. 100 µl of antibiotics (Penicillin Potassium, P102194, Aladdin, China) was injected in the left thigh after surgery. To observe the effect of miR-155 on pain behaviour in rats, scramble (1.0 nmol agomir scramble and 1.0 nmol antagomir scramble), agomir (miR-155, 1.0 nmol) or antagomir (miR-155 inhibitor, 1.0 nmol) was injected into the rats *via* intrathecal catheter synchronizing with surgery.

The mechanical allodynia was measured using the Von Frey fibre needle (YQ1633129456, Stoelting, Wood Dale, IL, USA). The levels of neuroinflammation cytokines (IL-1β and TNF-α) were measured using ELISA assay. The proportion of M1 microglia was analysed by flow cytometry.

Pain threshold assessment

Paw withdrawal threshold (PWT), response to mechanical allodynia, was evaluated in the neuropathic pain model rats. At postoperative days 0, 3, 7 and 14, rats were kept in a transparent plastic box with a metal mesh floor (three rats in each group). The plantar surface of each hind paw was exposed to pressure caused by von Frey fibre needle. It started with the minimum stimulation and gradually increased the pressure until a paw withdrawal reaction occurred. The minimum pressure of paw withdrawal in response to mechanical allodynia was recorded.

Primary microglia isolation and culture

At postoperative days 0, 3, 7 and 14, after PWT assay, rats were treated with 10% chloral hydrate (1.02425, Sigma, USA) at 0.3 ml/100 g. Primary rat's microglia was isolated and purified from surgical lateral spinal cord tissue. In brief, the surgical lateral spinal cord tissue (about 1 mm³) was taken at postoperative days 0, 3, 7, 14 (three rats in each group), and minced into small pieces at room temperature. Microglia was separated by 0.1% trypsin (25300054, Gibco™, USA) for 20 min at 37°C. The primary rats microglia suspension was cultured in 75 cm² tissue culture flasks pre-coated with 5 mg/ml poly-D-lysine (C0312, Beyotime, China) in DMEM/F12 containing 15% FCS (21097, Cayman, USA), 1% penicillin/streptomycin (15140122, Gibco, ThermoFisher, USA), and 1% glutamine (NHA2237550, NovoAB, Sweden) at 37°C. After 3 days in culture, the medium was changed to DMEM/F12 containing 10% FCS and cells were cultured for additional 48 hours at 37°C. Then, supernatants of primary microglia were collected for ELISA. The culture time of primary microglia in each group remained consistent.

ELISA

Primary microglia isolated at postoperative days 0, 3, 7 and 14 was pre-paired for ELISA. Briefly, supernatants of primary microglia separated by 0.1% trypsin were collected by centrifugation at 4°C for 5 min. Concentrations of proinflammatory cytokines (IL-1 and TNF- α) in the supernatants were measured by using IL-1 β ELISA kits (PI303, Beyotime, China) and TNF- α ELISA kits (PT516, Beyotime, China). Assays were performed according to the instructions and a micro-plate reader was used for detecting absor-

bance at 450 nm. Absorbance was then calculated as a concentration using a standard curve.

Flow cytometry

Primary microglia was isolated from surgical lateral spinal cord tissue in rats at postoperative day 14 (three rats in each group). According to descriptions from MultiSciences (<http://www.liankebio.com>), cells were isolated and in phosphate-buffered saline (PBS) by gently scraping using a cell lifter and then were incubated on ice with 5 μ l anti-Rat CD11b/c, FITC (70-AR011BC01-100, MultiSciences, China), and 5 μ l anti-Rat CD86, PE (70-AR08604-100, MultiSciences, China). Following a 30-minute incubation at 4°C, cells were fixed in 1% formaldehyde at room temperature for 10 min and acquired by using a FACSCalibur flow cytometer (342973, BD Biosciences, USA). Data were analysed by using BD FACSCanto™ system software v2.4 (646602, BD Biosciences, USA).

Statistical analysis

All data were recorded as the mean \pm standard deviation (SD) of at least three independent experiments. Comparisons between quantitative variables were performed using one-way ANOVA followed by Dunnett's post hoc test. Differences were considered statistically significant when the *p* value was less than 0.05. The SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism v6.0 (GraphPad Software Inc.) were used for statistical analysis.

Results

The effects of LPS or IL-4 stimulation on expression levels of CD86, iNOS, CD206, Arg and miR-155 in BV-2 cells

LPS activates the microglia to M1 type, while IL-4 activates the microglia to M2 type [28]. We measured the expression of CD86 and iNOS, which were considered to be molecular markers in M1 type microglia, CD206 and Arg, which were molecular markers in M2 type microglia [8]. Western blot analysis showed that LPS-induced BV-2 cells tended to be M1 type, with significant upregulating of CD86, iNOS (both *p* < 0.001) and downregulating of CD206, Arg (both *p* < 0.001); IL-4-induced microglia tended to be M2 type, with significant upregulating of CD206, Arg (both *p* < 0.001) and downregulating of CD86, iNOS (both *p* < 0.001) (Fig. 1A, B). Similar to the West-

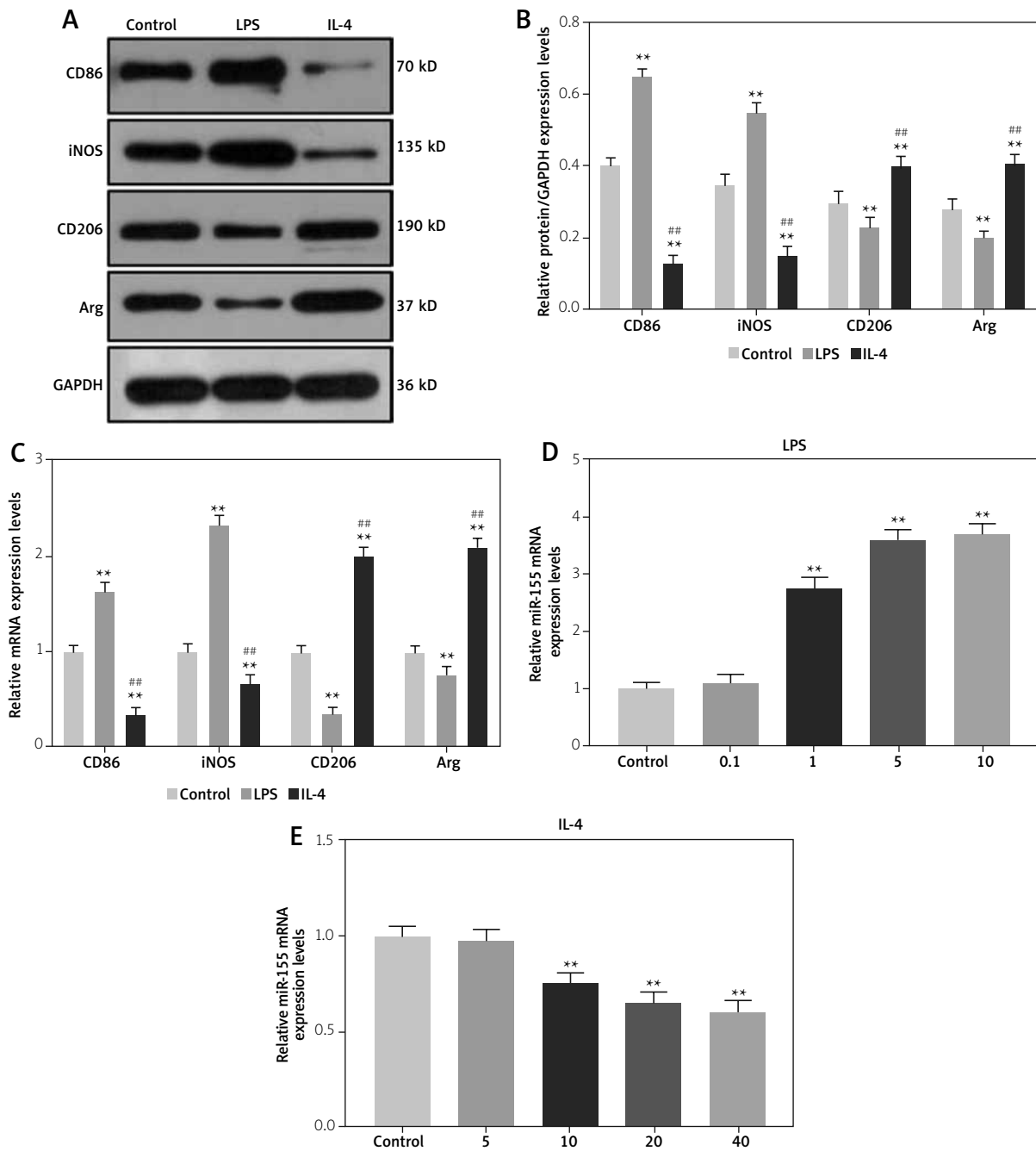


Fig. 1. LPS or IL-4 affects the expression level of CD86, iNOS, CD206, Arg and miR-155. **A, B)** Western blot analysis of protein levels of CD86, iNOS, CD206 and Arg in BV-2 cells stimulated for 24 h with LPS and IL-4, respectively. **C)** RT-PCR result showing mRNA levels of CD86, iNOS, CD206 and Arg in BV-2 cells stimulated for 24 h with LPS and IL-4, respectively. *vs. control, #vs. LPS. ***p* or ##*p* < 0.001. **D)** RT-PCR result showing the mRNA levels of miR-155 in BV-2 cells stimulated 24 h with LPS at 0 mg/ml (control), 0.1 mg/ml, 1 mg/ml, 5 mg/ml and 10 mg/ml, respectively. **E)** RT-PCR result showing the mRNA levels of miR-155 in microglia stimulated with IL-4 at 0 mg/ml (control), 5 mg/ml, 10 mg/ml, 20 mg/ml and 40 mg/ml, respectively. *vs. control. ***p* < 0.001.

ern blot results, RT-PCR analysis showed that LPS stimulation caused an increase in CD86, iNOS, and a decrease in CD206 and Arg, while IL-4 stimulation caused a decrease in CD86, iNOS, and an increase in CD206 and Arg (both $p < 0.001$) (Fig. 1C). Then, the expression level of miR-155 was increased with LPS stimulation at a series of concentration gradients (Fig. 1D), and decreased with IL-4 stimulation at a series of concentration gradients (Fig. 1E).

The effects of miR-155 on M1/M2 polarization switch in BV-2 cells

To explore the effects of miR-155 on microglia polarization, scramble, miR-155 mimics or miR-155 inhibitor was transfected into BV-2 cells with LPS treatment or IL-4, respectively. We found that LPS caused significant upregulation of miR-155, and miR-155 mimics aggravated the effect ($p < 0.001$), while the miR-155 inhibitor blocked this effect ($p < 0.001$) (Fig. 2A). In IL-4 induced BV-2 cells, the expression level of miR-155 was significantly decreased ($p < 0.001$), miR-155 inhibitor aggravated the effect ($p < 0.05$), while miR-155 mimics blocked this effect ($p < 0.001$) (Fig. 2B). Then, we measured the expression level of CD86 and CD206 in BV-2 cells by western blot and RT-PCR. Western blot showed that LPS significantly increased the protein level of CD86 and decreased the protein level of CD206 ($p < 0.001$), miR-155 mimics aggravated the effect on CD86 ($p < 0.001$) and CD206 ($p < 0.05$), while miR-155 inhibitor restrained the changes ($p < 0.001$) (Fig. 2C, D). Similar to the western blot results, RT-PCR analysis showed that miR-155 mimics caused significant increasing of CD86, significant decreasing of CD206 (both $p < 0.001$), while inhibition of miR-155 caused decreasing of CD86 and increasing of CD206 (both $p < 0.001$) (Fig. 2E). In IL-4 induced BV-2 cells, western blot and RT-PCR analysis collectively showed that IL-4 significantly decreased the protein level of CD86 and increased the protein level of CD206 ($p < 0.001$), miR-155 inhibitor aggravated the effect on CD86 ($p < 0.05$) and CD206 ($p < 0.001$), while miR-155 mimics restrained the changes ($p < 0.001$) (Fig. 2F-H).

The effects of miR-155 on neuropathic pain in rats

To examine the effect of miR-155 on neuropathic pain, L5 SNL was operated and the pain withdraw threshold was assessed. Data showed that SNL

strikingly caused decreasing of withdraw threshold ($p < 0.001$), while miR-155 antagomir restrained the effect ($p < 0.001$) (Fig. 3A). IL-1 β and TNF- α , which are neuropathic pain-related molecules, promote activation and M1 polarization in microglia [22,43]. Hence, we tested whether miR-155 could regulate the level of IL-1 β and TNF- α in microglia. We collected the supernatants of primary microglia from surgical lateral spinal cord tissue and measured the concentration of IL-1 β and TNF- α by ELISA. Data showed that SNL caused significant increasing of IL-1 β and TNF- α ($p < 0.001$), and miR-155 agomir aggravated this effect ($p < 0.001$), while miR-155 antagomir could blocking the effect of SNL ($p < 0.001$) (Fig. 3B, C).

The effects of miR-155 on the production of inflammatory cytokines and the proportion of M1 microglia in primary microglia from rats

We evaluated the M1 microglia proportion in primary microglia from surgical lateral spinal cord tissue by flow cytometry. CD11b is the marker gene for activated microglia [50], and CD86 is the marker gene for M1 microglia. Thus, we considered CD11b⁺ CD86⁺ as M1-polarized microglia in this study. Data showed that the proportions of CD86⁺ in CD11b⁺ microglia in the sham group, SNL group, scramble + SNL group, antagomir + SNL group, agomir + SNL group, antagomir group, and agomir group were 2.11%, 7.74%, 8.24%, 3.59%, 10.45%, 2.68%, 4.41%, respectively (Fig. 4A). SNL caused increasing of CD86⁺ in CD11b⁺ in the scramble + SNL group, while antagomir decreased the proportion of CD86⁺ in CD11b⁺ microglia in the antagomir + SNL group ($p < 0.001$) (Fig. 4B).

Discussion

Neuropathic pain refers to pain as a result of damage to the nervous system including nerves, spinal cord and certain central nervous system (CNS) regions. Neuropathic pain often causes spontaneous pain, allodynia and hyperalgesia. Microglia, the immune cell of the nervous system, has two polarization states, M1 and M2 type. M1 microglia is classical activated microglia, which is known to play a pro-inflammatory role, while M2 microglia is involved in anti-inflammatory/immunoregulatory processes [28]. The imbalance between M1 and M2 microglia causes a variety of diseases [28], including

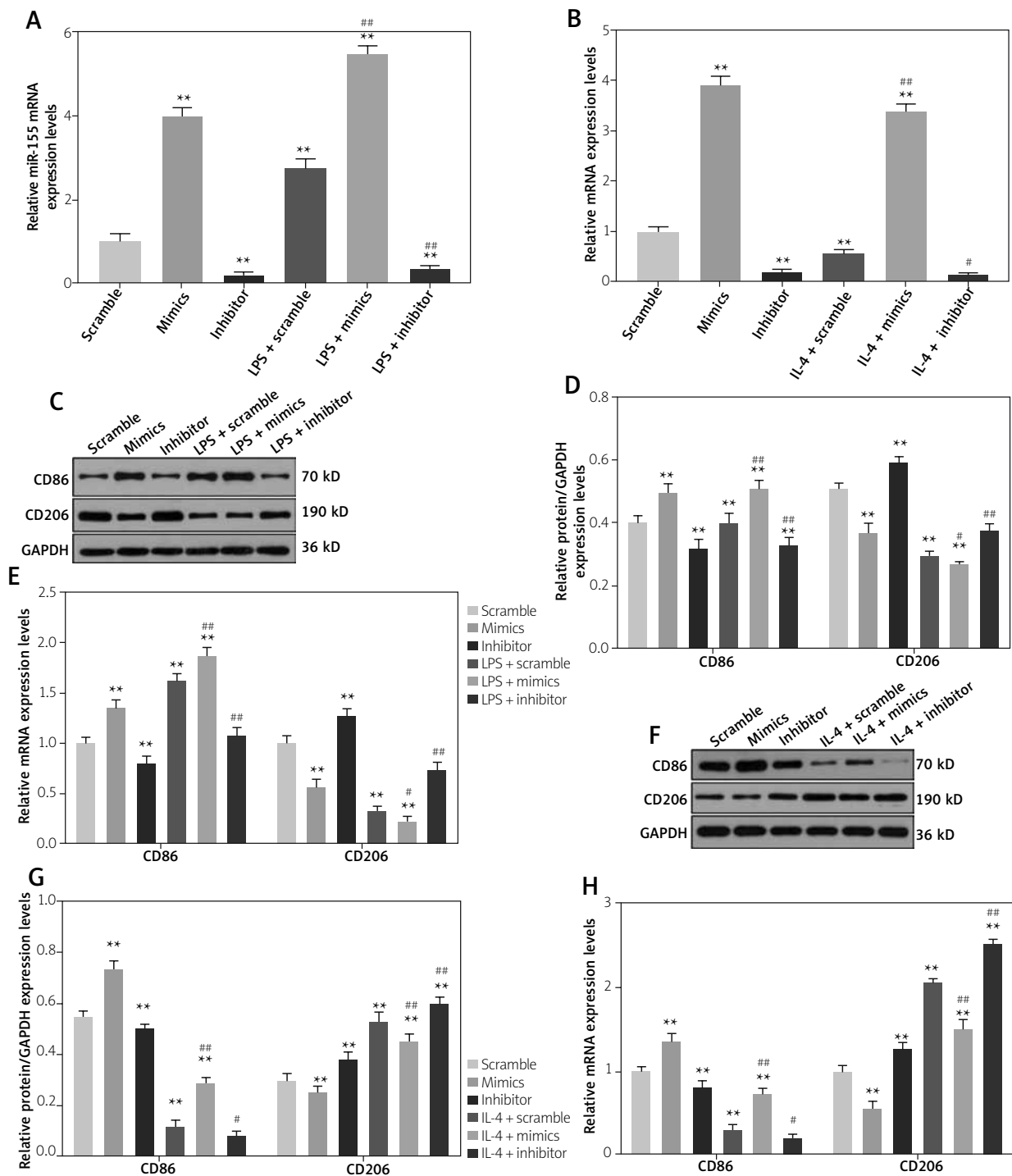


Fig. 2. miR-155 alters the polarization states in BV-2 cells. **A)** The expression level of miR-155 in BV-2 cells analysed by RT-PCR. *vs. scramble, #vs. LPS + scramble. **p* or #*p* < 0.05, ***p* or ##*p* < 0.001. **B)** The expression level of miR-155 in BV-2 cells analysed by RT-PCR. *vs. scramble, #vs. IL-4 + scramble. **p* or #*p* < 0.05, ***p* or ##*p* < 0.001. **C, D)** Western blot analysis of protein levels of CD86 and CD206 in BV-2 cells treated with LPS or not. **E)** RT-PCR analysis showing the mRNA levels of CD86 and CD206 in BV-2 cells treated with LPS or not. *vs. scramble, #vs. LPS + scramble. **p* or #*p* < 0.05, ***p* or ##*p* < 0.001. **F, G)** Western blot analysis of protein levels of CD86 and CD206 in BV-2 cells treated with IL-4 or not. **H)** RT-PCR data showing the expression of CD86 and CD206 in BV-2 cells treated with IL-4 or not. *vs. scramble, #vs. IL-4 + scramble. **p* or #*p* < 0.05, ***p* or ##*p* < 0.001.

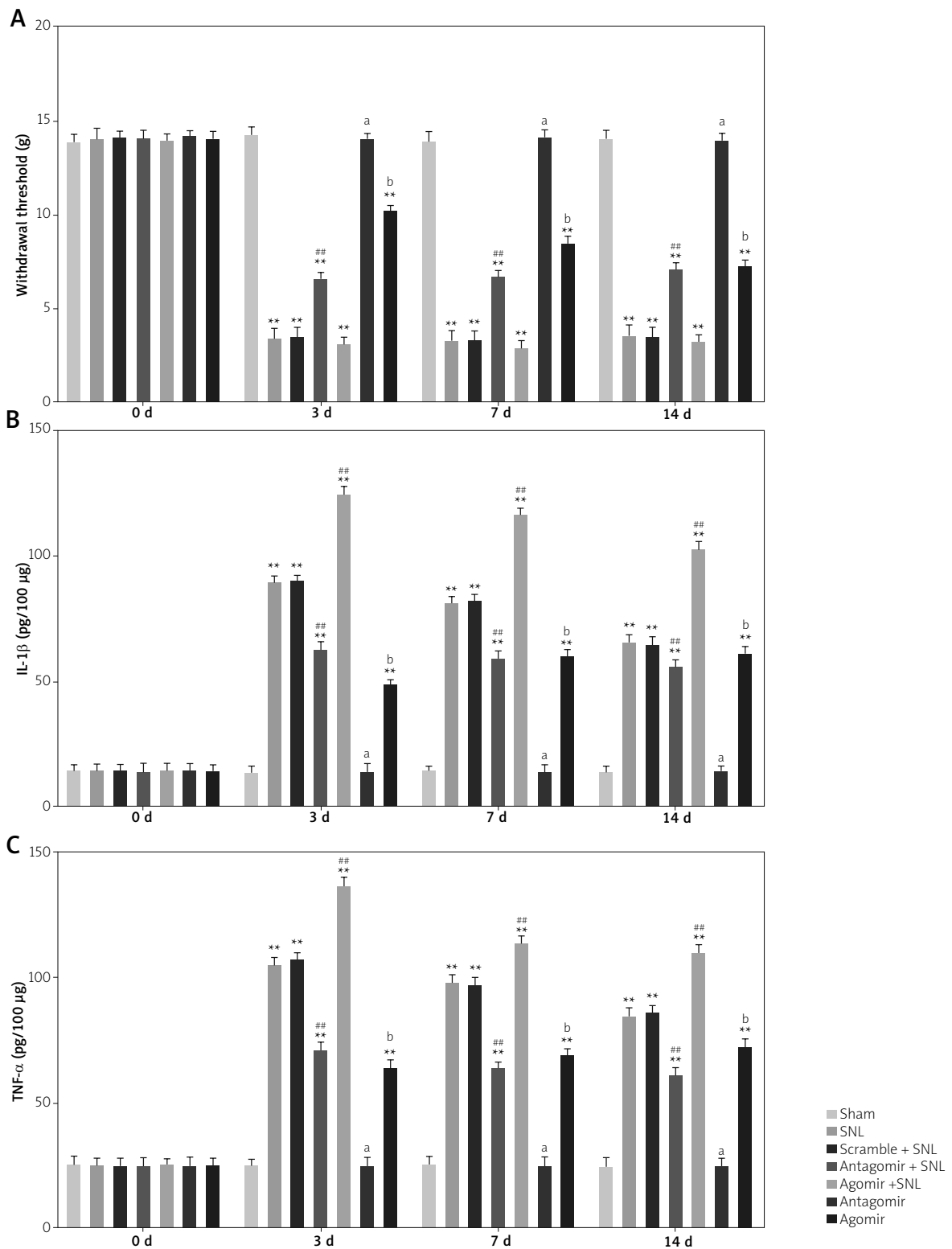


Fig. 3. MiR-155 affects the levels of neuropathic pain in rats and the production of inflammatory factors in primary microglia. **A)** Bar chart showing withdrawal threshold levels in rats at postoperative days 0, 3, 7 and 14 ($n = 3$). **B, C)** Bar charts showing the levels of IL-1 β (**B**) and TNF- α (**C**) in primary microglia measured by ELISA at postoperative days 0, 3, 7 and 14 ($n = 3$). *vs. sham, #vs. scramble + SNL, ^avs. antagomir + SNL, ^bvs. agomir + SNL. * p or # p or ^a p or ^b p < 0.05, ** p or ## p or ^{aa} p or ^{bb} p < 0.001.

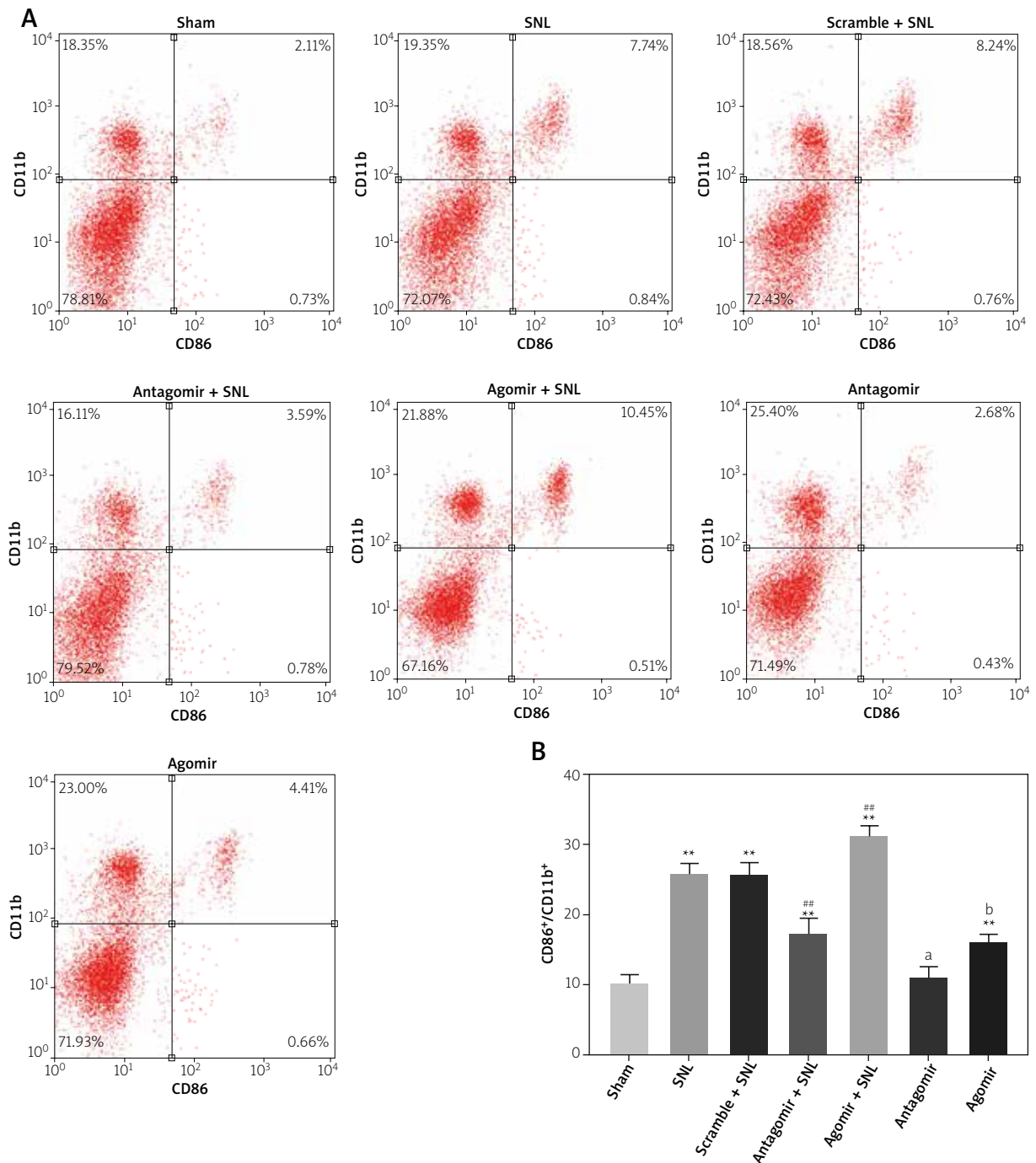


Fig. 4. MiR-155 regulates the proportion of M1 microglia *in vivo*. **A**) Flow cytometry analysis showing the proportion of CD86⁺ CD11b⁺ microglia in primary microglia (*n* = 3). Cells were isolated from surgical lateral spinal cord tissue in rats, then microglia were selected based on the levels of CD86 (x-axis) and CD11b (y-axis). CD86⁺ CD11b⁺ positively stained cells were gated on the upper right corner. **B**) A bar chart showing the proportion of CD86⁺ cells in CD11b⁺ microglia (*n* = 3). **vs.* sham, #*vs.* scramble + SNL, ^a*vs.* antagomir + SNL, ^b*vs.* agomir + SNL. **p* or #*p* or ^a*p* or ^b*p* < 0.05, ***p* or ##*p* or ^{aa}*p* or ^{bb}*p* < 0.001.

neuropathic pain [10]. Manipulating the proportion of M1 and M2 type is considered to be a promising way for relieving neuropathic pain [37]. Our report implicates that miR-155 is an important regulator in switching of polarization states in microglia, and inhibition of miR-155 promoting M2- polarization, which is beneficial to relieving neuropathic pain. We suggest that inhibition of miR-155 should be expected to attenuate neuropathic pain *via* promoting the switch from M1 to M2 polarization in microglia.

As the prototypical endotoxin, LPS binds the CD14/TLR4/MD2 receptor complex, which promotes the secretion of pro-inflammatory cytokines, eicosanoids and nitric oxide. Microglia can be activated into M1 type by LPS stimulation [47]. Cytokine IL-4 induces differentiation of naive helper T cells. IL-4 in extravascular tissues promotes activation of macrophages into M2 polarization and inhibits classical activation of macrophages into M1 polarization [5]. In our research, LPS and IL-4 were separately used to construct M1 or M2 polarization model in BV-2 cells. We found that the expression level of miR-155 was increased in M1 microglia and decreased in M2 microglia. A previous study shows that miR-155 is highly expressed during M1-polarization and promotes inflammation in microglia [51]. Our research also demonstrated that miR-155 could regulate the polarization states of microglia by showing that miR-155 mimics promoted M1 polarization and miR-155 inhibitor promoted M2 polarization in BV-2 cells, consistent with the research in tumour-associated macrophages [30]. Nuclear factor- κ B (NF- κ B) is a key redox-signalling mechanism regulating the balance of M1/M2 polarization in microglia [35]. MiR-155 promotes M1 macrophage polarization and inflammatory responses *via* the SOCS1/NF- κ B pathway during myocardial infarction [12]. Inhibition of miR-155 caused downregulation of NF- κ B and p38 mitogen-activated protein kinase activation by mediating SOCS1 [36]. Thus, we infer that microglia-secreted miR-155 regulate M1/M2 balance *via* the SOCS1/NF- κ B pathway.

As miR-155 is a regulator of microglia polarization states, inhibition of miR-155 is potentially a promising way to reverse the M1 polarization caused by nerve injury into M2 polarization. In this study, we constructed a neuropathic pain model by using SNL and injected miR-155 agomir or antagomir into the rats *via* intrathecal catheter. SNL of the neighbouring (L5) spinal nerve is the most popular periph-

eral neuropathy model [45]. In the SNL, L5 spinal nerves are tightly ligated at a location distal to the dorsal root ganglia. Allodynia and hyperalgesia are induced quickly after ligation, and last for more than 4 months. Our result shows that SNL caused neuropathic pain in rats, while inhibition of miR-155 could significantly weaken the neuropathic pain caused by SNL. The relation between miR-155 and neuropathic pain has been previously studied, for example, miR-155 regulates differentiation of regulatory T cells in patients with neuropathic pain [11], and microglia miR-155 also exacerbates neuropathic pain through targeting SGK3 [23] and SOCS1 [36]. Thus, we suppose that inhibition of miR-155 can relieve neuropathic pain.

Activation of M1 microglia caused increasing of production of pro-inflammatory cytokines, such as TNF- α and IL-1 β [37]. Excessive secretion of pro-inflammatory cytokines and inflammatory responses causes neuronal damage and apoptosis [31]. We found that SNL treatment caused increasing of IL-1 β and TNF- α , while miR-155 antagomir significantly reduced the effect of SNL. SGK3 (serum- and glucocorticoid-inducible kinases 3) is a target for miR-155 [23] and inhibition of SGK1 and SGK3 enhanced production of inflammatory substances in microglia [2]. MiR-155 can also enhance NF- κ B signalling activation by targeting LXR α and facilitate immunoinflammatory reactions in heat stress-treated BV-2 cells [20]. Thus, we infer miR-155 affects the levels of pro-inflammatory cytokines IL-1 β and TNF- α by targeting SGK3 or LXR α . The production of these cytokines, such as IL-1 β and TNF- α , are necessary for the M1 polarization of microglia [53]. Thus, miR-155 possibly affects M1 polarization of microglia through indirectly regulating the production of IL-1 β and TNF- α .

We also found that SNL induces increasing of M1 microglia in primary microglia, while miR-155 antagomir could significantly attenuate this effect in rats, consistently with the study on macrophage showing that inhibition of miR-155 decreases M1 macrophage polarization [12]. An excess of pro-inflammatory cytokines plays an important role in the development of neuropathic pain. Therefore, modulation of inflammation by inhibition of M1 polarization and promoting M2 polarization could be a great way for treatment of neuropathic pain.

In conclusion, this study demonstrates that miR-155 are highly expressed in M1-polarized microglia

and inhibition of miR-155 decreases the production of IL-1 β and TNF- α , the proportion of M1-polarized microglia and relieve neuropathic pain. This study contributes to a new understanding of miR-155 in microglia and benefits to alleviating neuropathic pain.

Disclosure

The authors declare no conflict of interest.

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