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ORIGINAL RESEARCH

Fargesin promotes LPS-alleviated microglial M2 polarization and relieves neuropathic pain

Chenglong Wu[1,*], Ling Zhou[1], Li Wang[1], Lu Liu[2]

[1]Department of Pain Management, Wuhan Fourth Hospital, Puai Hospital, Tongji Medical College, Huazhong University of Science and Technology, 430033 Wuhan, Hubei, China
[2]Department of Anesthesiology, Wuhan Children Hospital, 430016 Wuhan, Hubei, China

***Correspondence**
docll103@163.com
(Chenglong Wu)

Abstract

Neuropathic pain is chronic pain that disrupts a patient's normal life and causes enormous suffering. Fargesin (Far), a product derived from Mulan, has been discovered to exhibit regulatory functions in multifarious diseases. However, the regulatory impacts and related pathways of Far in neuropathic pain progression remain unclear. In this study, following lipopolysaccharide (LPS) stimulation, cell viability decreased, but was reversed by Far treatment (10, 20 and 40 μM). LPS alleviated M2 polarization, but Far addition offset it. Additionally, Far facilitates gene expressions associated with M2 polarization. Lastly, LPS treatment triggered the nuclear factor kappa-B (NF-κB) pathway, but this change was rescued by Far addition. In conclusion, Far promoted LPS-alleviated microglial M2 polarization and relieved neuropathic pain through retarding the NF-κB pathway. This study may provide novel perspectives on Far in neuropathic pain treatment.

Keywords

Fargesin; Neuropathic pain; M2 polarization; The NF-κB pathway

1. Introduction

Neuropathic pain is chronic pain triggered by disease or injury to the somatosensory nervous system and characterized by hyperalgesia, spontaneous pain and allodynia [1]. Several non-neuronal cells in the central nervous system, particularly microglia, simulate neuropathic pain [2]. The long course, easy incidence rate, and diminishing quality of patients' lives have made this disease a significant public health issues [3, 4]. In spite of this, there has not yet been a satisfactory therapeutic method for neuropathic pain. Hence, investigating the mechanism of neuropathic pain and searching effective therapeutic drugs are urgently needed.

Growing numbers of natural products have proven to contain therapeutic properties, and to regulate neuropathic pain progression [5]. Fargesin (Far) is a major component from Mulan, which has been traditionally used to treat sinusitis and rhinitis [6]. Recently, Far has been uncovered to contribute to the regulation of some diseases for amelioration. For instance, Far retards the mitogen-activated protein kinase (MAPK) and NF-κB pathways to modulate macrophage reprogramming, ameliorating osteoarthritis [7]. Also, Far accelerates reverse cholesterol transport and reduces inflammation to improve atherosclerosis [8]. As β_1 adrenergic receptor antagonist, Far also alleviates heart ischemia/reperfusion injury by reducing oxidative stress and apoptosis [9]. In murine swart and immortalized melanocytes, Far can affect the protein kinase A (PKA)/cAMP response element binding (CREB) and p38/MAPK pathways to cut down melanin generation [10]. In addition, Far suppresses epidermal growth factor (EGF)-triggered cell transformation and growth in colon cancer by weakening the cyclin dependent kinase 2 (CDK2)/Cyclin E pathway [11]. Nevertheless, Far's regulatory impacts and correlative pathways in neuropathic pain progression remain unclear.

Far promoted LPS-alleviated microglial M2 polarization and relieved neuropathic pain through retarding the NF-κB pathway. This study suggests that Far may be an effective drug for neuropathic pain.

2. Materials and methods

2.1 Cell culture and treatment

We obtained BV2 cells from the Chinese Academy of Sciences (Shanghai, China). BV2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (11995065, Gbico, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, 10099-141, Gbico, Grand Island, NY, USA) in an incubator (humid; 37 °C; 5% CO_2).

BV2 cells were treated with LPS (1 μg/mL; L6529, Sigma, St. Louis, MO, USA) to mimic a neuropathic pain cell model.

Far (0, 10, 20, 40, 80 and 160 μM; PHL82537, Sigma, St. Louis, MO, USA) were employed to treat BV2 cells.

2.2 Cell counting kit-8 (CCK-8) assay

BV2 cells were plated in a 96-well plate at 1000 cells/well. CCK-8 solution (10 μL, ck04, Dojindo Laboratories, Ku-

mamoto, Japan) was then added to each well. After 4 h, cell viability was evaluated using a spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA).

2.3 Flow cytometry

A 1× binding buffer was used to resuscitate BV2 cells after washing. We mixed BV2 cells with propidium iodide (PI), fluoresceine isothiocyanate (FITC) antibody (ab112511, Abcam, Shanghai, China), cluster of differentiation (CD)32 (M1 macrophage marker) antibody (ab282740) or CD206 (M2 macrophage marker) antibody (ab64693) for 1 h without light exposure. Flow cytometer (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA) was then used for measurement.

2.4 Reverse transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

TRIzol reagent (15596018, Invitrogen, Carlsbad, CA, USA) was used to obtain RNAs from BV2 cells. RNAs were reversed to cDNAs using the PrimeScript® RT reagent kit (RR037B, Takara, Kusatsu, Japan). Next, qPCR was perfomed using the SYBR® Premix Ex Taq™ II kit (RR820A, Takara, Dalian, China). $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expressions.

The primer sequences were:
Interleukin-1 beta (*IL-1*β):
F: 5′-CAGGATGAGGACATGAGCACC-3′,
R: 5′-CTCTGCAGACTCAAACTCCAC-3′;
IL-10:
F: 5′-GCTCTTACTGACTGGCATGAG-3′,
R: 5′-CGCAGCTCTAGGAGCATGTG-3′;
Tumor Necrosis Factor-α (*TNF-*α):
F: 5′-CATCTTCTCAAAATTCGAGTGACAA-3′,
R: 5′-TGGGAGTAGACAAGGTACAACCC-3′;
Arginase 1 (*Arg-1*):
F: 5′-CTCCAAGCCAAAGTCCTTAGAG-3′,
R: 5′-GGAGCTGTCATTAGGGACATCA-3′;
Inducible nitric oxide synthase (*iNOS*):
F: 5′-CGTTCCTGGAGGTGCTTGAA-3′,
R: 5′-TGGAAGCCACTGACACTTCG-3′;
CD206:
F: 5′-CTCTGTTCAGCTATTGGACGC-3′,
R: 5′-TGGCACTCCCAAACATAATTTGA-3′;
IL-6:
F: 5′-GAAACCGCTATGAAGTTCCTCTCTG-3′,
R: 5′-TGTTGGGAGTGGTATCCTCTGTGA-3′;
Glyceraldehyde-3-phosphate dehydrogenase (*GADPH*):
F: 5′-AGGTCGGTGTGAACGGATTTG-3′,
R: 5′-GGGGTCGTTGATGGCAACA-3′.

2.5 Western blot

BV2 cells proteins were isolated using radio immunoprecipitation assay (RIPA) buffer (P0013B, Beyotime, Shanghai, China), then treated to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Migrating proteins to polyvinylidene difluoride (PVDF) membranes (Beyotime, Shanghai, China). After sealing into the membranes, primary antibodies against p-p65 (1:1000; ab76302, Abcam, Shanghai, China), p65 (1:1000; ab32536), p-inhibitor of NF-κB alpha (IκBα, 1:10000; ab133462), IκBα (1:1000; ab32518) and β-actin (1 μg/mL; ab8226) were added for 12 h incubation at 4 °C. Then add the appropriate secondary antibodies (1:5000; ab7090) for another 2 h incubation. Lastly, protein blots were inspected using the chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

2.6 Statistical analysis

Statistical analysis was performed using the GraphPad Prism Software 9 (GraphPad Software, La Jolla, CA, USA). Data were expressed as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) was applied to comparisons between multiple groups. $p < 0.05$ indicates statistically significant differences.

3. Results

3.1 Fargesin strengthens LPS-triggered BV2 cells cell viability

The molecular structural formula for Far is shown in Fig. 1A. Cell viability was unaffected by Far treatment at 10, 20 and 40 μM, and reduced by Far treatment at 80 and 160 μM (Fig. 1B). Following LPS stimulation, cell viability decreased, but was reversed by Far addition (10, 20 and 40 μM) (Fig. 1C). Cell viability of LPS-triggered BV2 cells was strengthened by Far.

3.2 Fargesin accelerates M2 polarization in LPS-induced BV2 cells

After LPS treatment, CD32 was increased and CD206 was alleviated, but these impacts were offset after Far addition (10, 20 and 40 μM) (Fig. 2). In general, Far accelerates M2 polarization in LPS-induced BV2 cells.

3.3 Fargesin facilitates gene expressions associated with M2 polarization

IL-1β, *TNF-*α, *iNOS*, *IL-6* mRNA expressions were aggrandized and *IL-10*, *Arg-1*, *CD206* mRNA expressions were attenuated after LPS inducement, but these changes were reversed after Far treatment (Fig. 3). Far facilitates gene expressions associated with M2 polarization.

3.4 Fargesin retards the NF-κB pathway

The NF-κB pathway has been confirmed to be a pivotal pathway in neuropathic pain. After LPS stimulation, IκBα is degraded, while p65 is phosphorylated after nuclear translocation [12]. The phosphorylated p65 can activate the NF-κB pathway, thereby promoting the expressions of inflammatory factors [13, 14]. Results from western blot illustrated that the protein expressions of p-p65/p65 and p-IκBα were increased and IκBα protein expression declined after LPS treatment, but these impacts were rescued after Far addition (Fig. 4). In short, Far retards the NF-κB pathway.

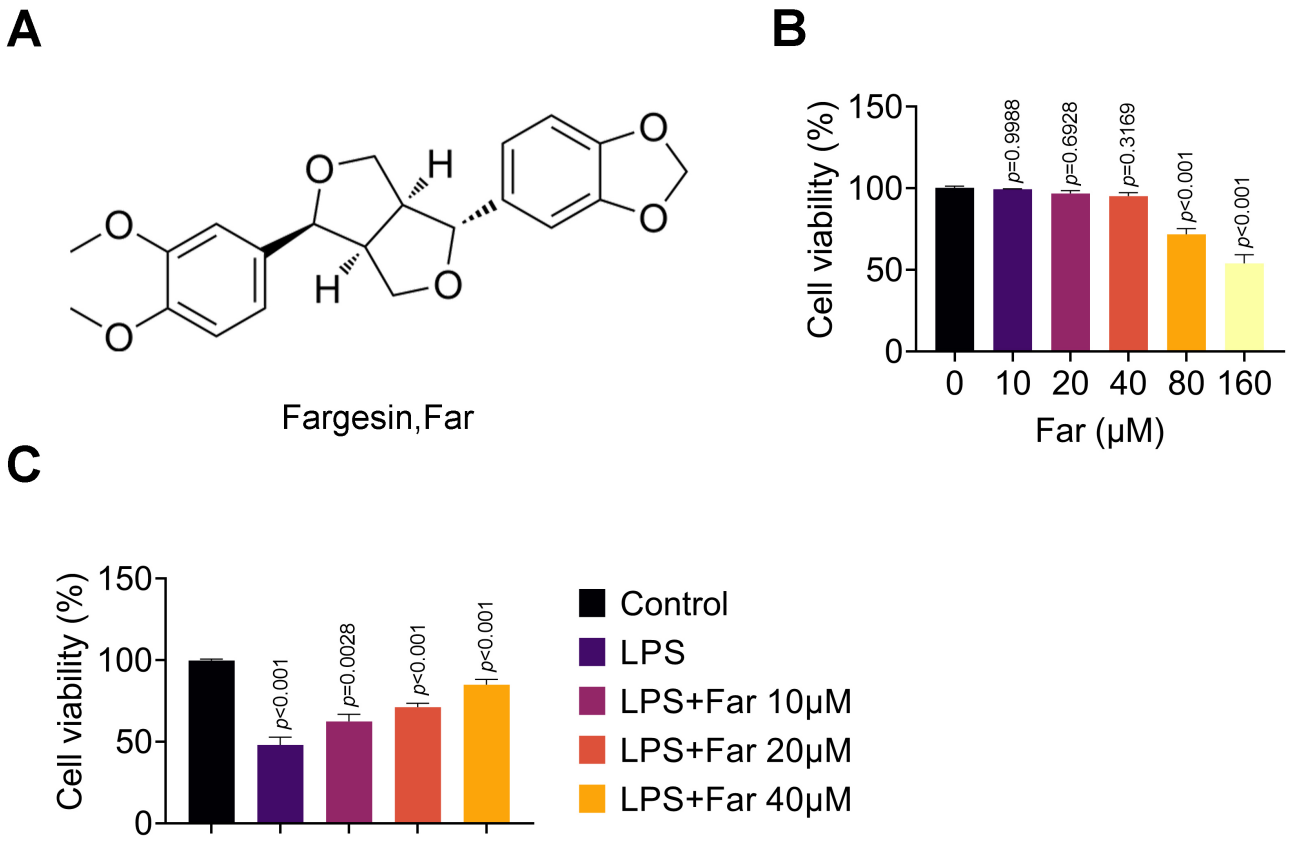

FIGURE 1. Fargesin strengthens LPS-triggered BV2 cells cell viability. (A) Molecular structural formula for Far was displayed. (B) Cell viability was confirmed with different Far concentrations (0, 10, 20, 40, 80 and 160 μM) through CCK-8 assay. (C) Cell viability was examined in the Control, LPS, LPS + Far 10 μM, LPS + Far 20 μM and LPS + Far 40 μM group through CCK-8 assay. Far: Fargesin; LPS: lipopolysaccharide.

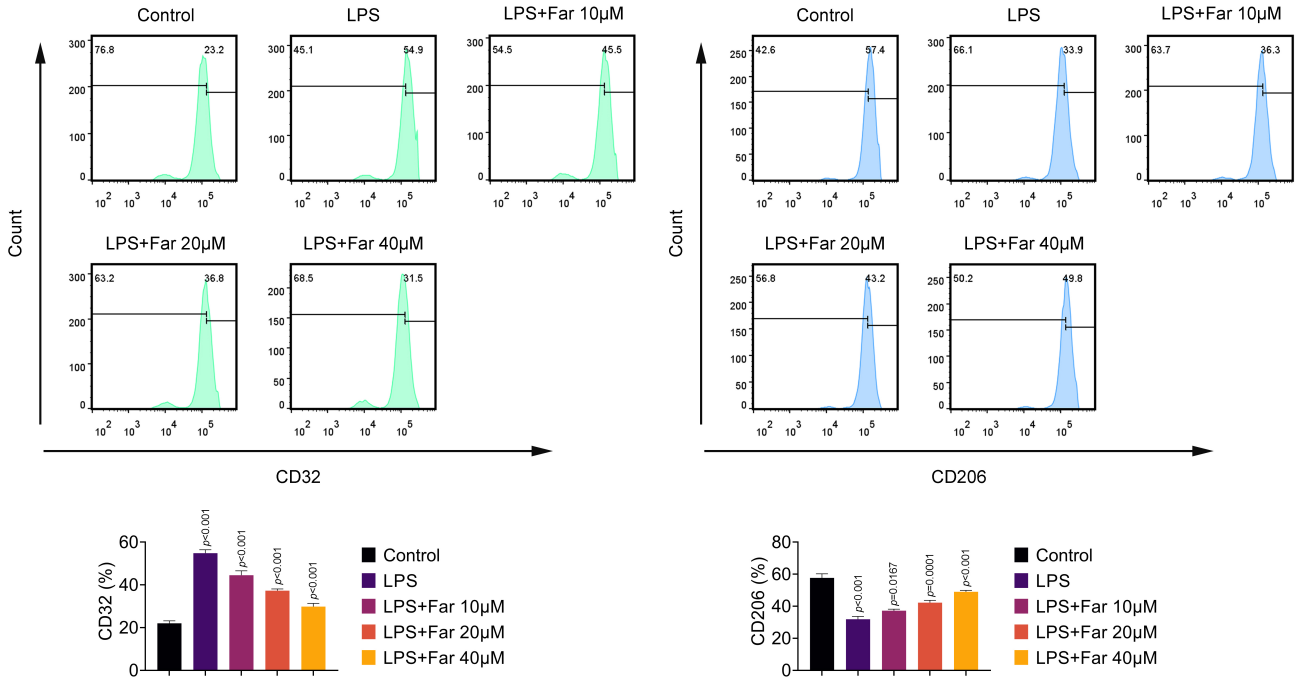

FIGURE 2. Fargesin accelerates M2 polarization in LPS-induced BV2 cells. Groups were separated into Control, LPS, LPS + Far 10 μM, LPS + Far 20 μM and LPS + Far 40 μM group. The CD32 and CD206 percentages were verified through flow cytometry. LPS: lipopolysaccharide; CD: cluster of differentiation; Far: Fargesin.

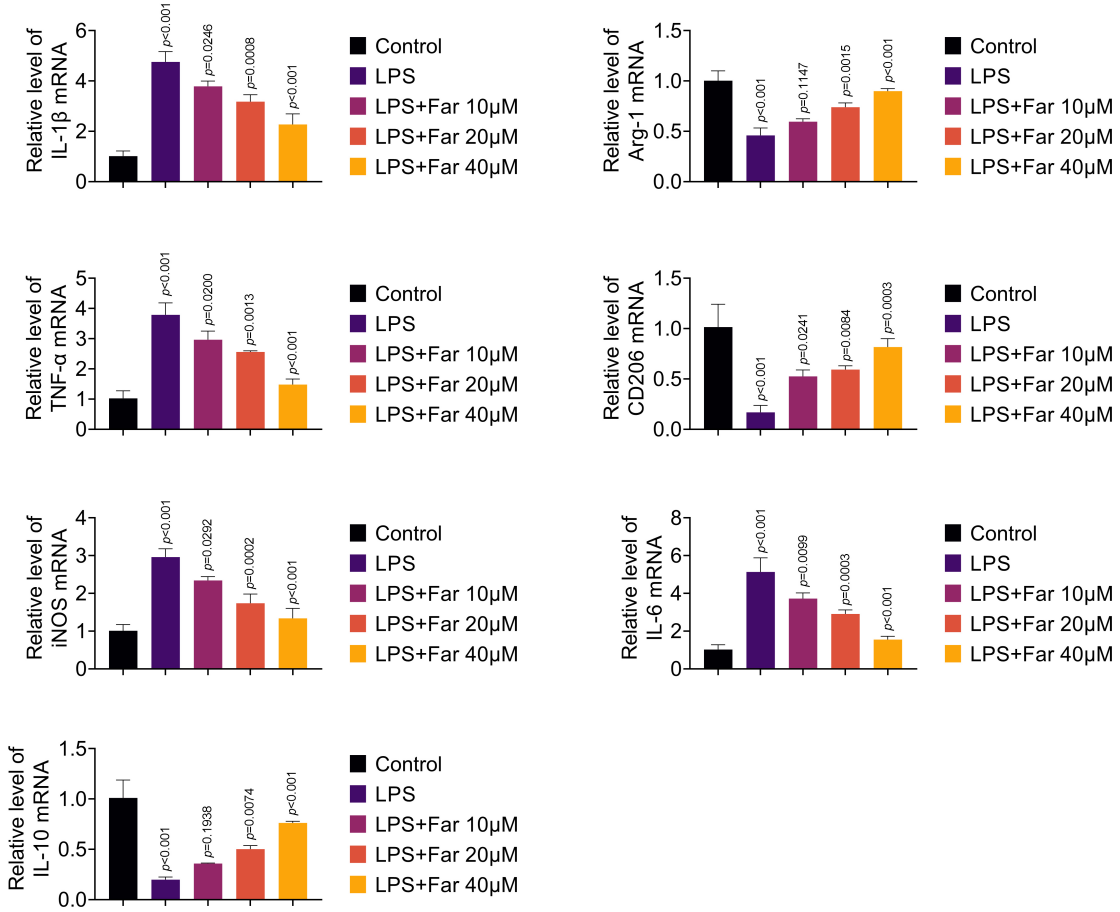

FIGURE 3. Fargesin facilitates gene expressions associated with M2 polarization. Groups were separated into Control, LPS, LPS + Far 10 μM, LPS + Far 20 μM and LPS + Far 40 μM group. The mRNA expressions of *IL-1β*, *IL-10*, *TNF-α*, *IL-6*, *Arg-1*, *iNOS* and *CD206* were examined through RT-qPCR. IL: Interleukin; LPS: lipopolysaccharide; Far: Fargesin; Arg-1: Arginase 1; TNF-α: Tumor Necrosis Factor-α; CD206: cluster of differentiation 206; iNOS: Inducible nitric oxide synthase.

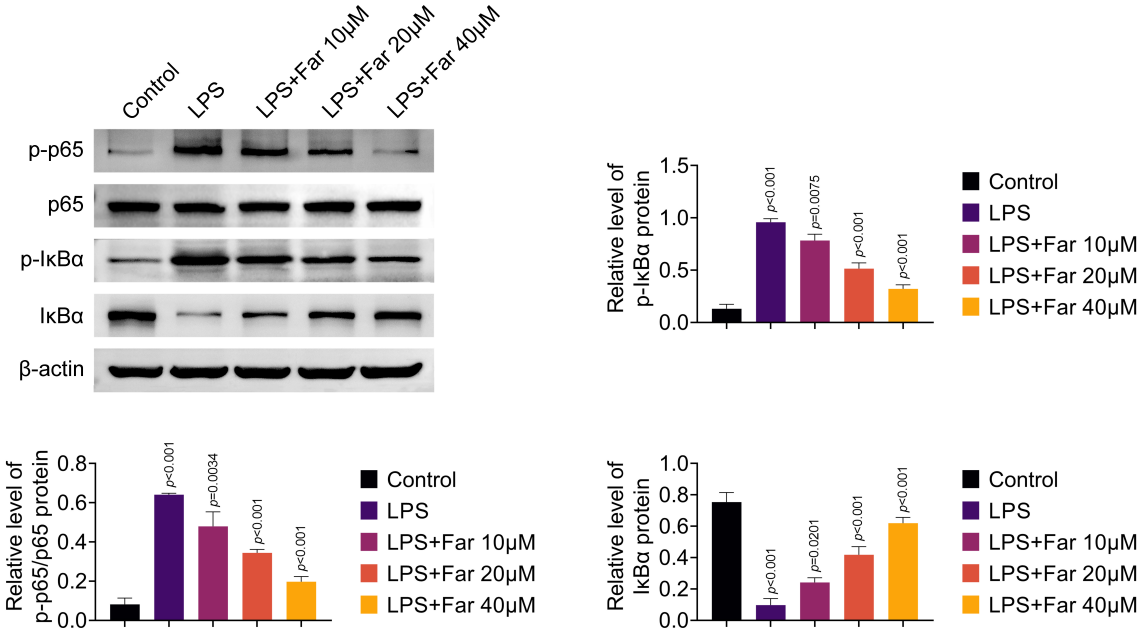

FIGURE 4. Fargesin retards the NF-κB pathway. Groups were separated into Control, LPS, LPS + Far 10 μM, LPS + Far 20 μM and LPS + Far 40 μM group. Protein expressions of p-p65, p65, p-IκBα and IκBα were determined through western blot. LPS: lipopolysaccharide; Far: Fargesin; IκBα: inhibitor of NF-κB alpha.

4. Discussion

Far has been demonstrated to have regulatory functions in multiple diseases [7–11]. However, the regulatory effects and related pathways of Far in neuropathic pain progression remain unclear. This study showed that BV2 cells' declining viability caused by LPS can be restored by Far treatment.

Evidence suggests that neuroinflammation is a key contributor to neuropathic pain [15]. Known as major mediators of neuroinflammation, microglia are macrophage-like immune cells in the central nervous system [16]. Microglia can be polarized into a classical phenotype (pro-inflammatory, M1) or an alternative phenotype (anti-inflammatory, M2). M1 phenotype aggravates neurotoxicity while M2 phenotype exerts neural protection role [17, 18]. Therefore, polarizing microglia from M1 to M2 polarization may be a promising strategy to improve neuroinflammatory diseases. Researchers have increasingly focused on modulating M1/M2 polarization in neuropathic pain progression. Paeonol regulates microglial M1 and M2 polarization to relieve neuropathic pain through affecting the RhoA/p38MAPK signaling pathway [19]. Stigmasterol targets the toll-like receptor 4 (TLR4)/NF-κB pathway to affect microglial M1/M2 polarization, improving neuropathic pain [20]. Furthermore, lidocaine affects M1/M2 microglia polarization to alleviate chronic constriction injury-triggered neuropathic pain [21]. A similar finding was found in this work: M2 polarization was alleviated by LPS treatment, but this impact was offset by Far addition. In addition, Far modulated gene expressions associated with M2 polarization by attenuating IL-1β, TNF-α and iNOS as well as aggrandizing IL-10, Arg-1 and CD206. In general, Far treatment accelerated M2 polarization.

NF-κB is a multifunctional transcriptional regulator essential for regulating microglial activation and neuroinflammation [22, 23]. Cells that undergo oxidative stress activate NF-κB enters the nucleus, which leads to the generation of proinflammatory cytokines (such as TNF-α, IL-1β and IL-6), thereby aggravating neuropathic pain [24, 25]. Neuropathic pain progression is well documented to be regulated by the NF-κB pathway. For example, Diosmetin modulates the nuclear factor erythroid 2-related factor 2 (Nrf2)/NF-κB pathway to ameliorate neuropathic pain [12]. Moreover, Fn14 modulates the NF-κB pathway to contribute to neuropathic pain development [26]. Additionally, oxyntomodulin retards the NF-κB pathway to alleviate TNF-α-stimulated neuropathic pain [27]. Importantly, Far retards the NF-κB pathway [7, 8, 28]. A similar result was shown in this study, in which the NF-κB pathway was triggered after LPS treatment, but it was rescued by Far addition.

5. Conclusions

In conclusion, Far promoted LPS-alleviated microglial M2 polarization and relieved neuropathic pain through retarding the NF-κB pathway. The limitations of this study will lead to further exploration of Far's role in neuropathic pain progression in the future.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

CLW and LL—designed the study and carried them out; prepare the manuscript for publication and reviewed the draft of the manuscript. CLW, LZ and LW—supervised the data collection, analyzed the data, interpreted the data. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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