# Netrin-1 Promotes M2 Type Activation and Inhibits Pyroptosis of Microglial Cells by Depressing RAC1/Nf-κB Pathway to Alleviate Inflammatory Pain

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**Summary**

Netrin-1 (NTN-1) plays a vital role in the progress of nervous system development and inflammatory diseases. However, the role and underlying mechanism of NTN-1 in inflammatory pain (IP) are unclear. BV2 microglia were treated with LPS to mimic the cell status under IP. Adeno-associated virus carrying the NTN-1 gene (AAV-NTN-1) was used to overexpress NTN-1. Complete Freund's Adjuvant (CFA)-induced mouse was recruited as an *in vivo* model. MTT and commercial kits were utilized to evaluate cell viability and cell death of BV2 cells. The mRNA expressions and secretions of cytokines were measured using the ELISA method. Also, the pyroptosis and activation of BV2 cells were investigated based on western blotting. To verify the role of Rac1/NF-κB signaling, isochamaejasmin (ISO) and AAV-Rac1 were presented. The results showed that NTN-1 expression was decreased in LPS-treated BV2 microglia and spinal cord tissues of CFA-injected mice. Overexpressing NTN-1 dramatically reversed cell viability and decreased cell death rate of BV2 microglia under lipopolysaccharide (LPS) stimulation, while the level of pyroptosis was inhibited. Besides, AAV-NTN-1 rescued the activation of microglia and inflammatory injury induced by LPS, decreasing IBA-1 expression, as well as iNOS, IL-1β and IL-6 secretions. Meanwhile AAV-NTN-1 promoted the anti-inflammation response, including increases in Arg-1, IL-4 and IL-10 levels. In addition, the LPS-induced activation of Rac1/NF-κB signaling was depressed by NTN-1 overexpression. The same results were verified in a CFA-induced mouse model. In conclusion, NTN-1 alleviated IP by suppressing pyroptosis and promoting M2 type activation of microglia via inhibiting Rac1/NF-κB signaling, suggesting the protective role of NTN-1 in IP.

**Keywords**

Netrin-1 • Inflammatory pain • Pyroptosis • Microglia M2 activation • Rac1/NF-κB

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## Introduction

As a kind of chronic pain, inflammatory pain (IP) is mainly caused by the abundant production of inflammatory mediators in damaged tissues and results in allodynia and hyperalgesia [1]. IP seriously damages the physical and mental health of patients. However, the clinical therapy is not satisfactory. Microglia, a kind of macrophage-like cells, are immune cells that in charge of homeostasis of the central nervous system (CNS) and the major mediators of central sensitization [2]. Stimulating microglial cells-released proinflammatory cytokines activates and maintains the pain response [3]. Many studies showed that preventing the inflammation response caused by microglia is an important manner to alleviate IP [4,5].

The status of microglia in the normal brain is quiescent [6]. Under physiological injury or pathological lesions, microglial cells are activated and infiltrate the damaged area [7]. To mark the activated microglia, the special-expressed protein in monocytic lineages (including brain microglia), ionized calcium binding adapter molecule 1 (IBA-1), is found to be upregulated

[8]. Besides, microglial cells in immune system presents two major types of activation: the pro-inflammatory M1 phenotype and the M2 anti-inflammatory type [7]. The pro-inflammatory cytokines, interleukin (IL)-1β, IL-6 and inducible nitric oxide synthase (iNOS), are released by M1 microglia while the anti-inflammatory activity of M2 microglia was based on the secretions of IL-4, IL-10 and arginase 1 (Arg-1) [6,9]. Therefore, the M2 polarization of microglia contributes to inflammatory diseases, IP included.

Netrin-1 (NTN-1) is a guidance molecule that controls CNS commissural axons and peripheral motor axons. It was reported that NTN-1 acted on nervous and non-nervous system diseases via inhibiting inflammatory responses [10,11]. In the non-nervous system, NTN-1 maintained normal endothelial function by suppressing NF-κB activation and the secretion of TNF-α, IL-1β and IL-6 [12]. NTN-1 alleviated the kidney injury through the inhibitions of cyclooxygenase-2 expression and the contents of inflammatory cytokines in LPS-induced macrophages [13]. On the other hand, NTN-1 plays an important role in autoimmune CNS diseases. A study suggested that NTN-1 knockdown reduced the immunosuppressive effect of Th17/Tregs, and promoted Alzheimer's Disease progression [14]. NTN-1 inhibited the activation of microglia and inflammatory response in the cerebral cortex after subarachnoid hemorrhage (SAH), and thereby alleviated neuroinflammation and nerve injury [15]. Moreover, exogenous NTN-1 treatment decreased SAH-induced neuronal apoptosis and protected against brain injury [16]. Li *et al.* reported that NTN-1 deficiency aggravated the mechanical allodynia and thermal hyperalgesia caused by peripheral nerve injury, and further induced neuropathic pain [17]. However, the role of NTN-1 in IP and its underlying mechanisms are poorly understand.

In this study, we conducted an *in vivo* IP model, CFA-induced mice, along with LP-stimulated BV2 microglia for an *in vitro* IP model. Based on that, the alteration of NIN-1 during IP progression was investigated. A gain-of-function assay was performed to evaluate the role of NTN-1. Moreover, Rac1 and NF-κB signaling were involved in the exploration of the NTN-1 regulatory mechanism on IP. A novel bio-target for IP treatment might be identified during our research.

## Materials and Methods

*Animal models*

Male C57BL/6 mice (8 ~ 9 weeks old, 18 g ~ 22 g) were obtained from the Shaanxi Provincial People's Hospital. All animals were acclimatized for 2 weeks before experiment. They were housed in a standard environment (temperature: 22 °C ~ 24 °C, relative humidity: 55 % ~ 60 %) on a cycle of 12 h light and 12 h dark and fed with free food and water. The establishment of IP model was carried out according to a previous study [18]. Mice were injected with 20 μL CFA in their right hind paw, while control mice were injected with 20 μL of saline. Each group was summed to 10 mice. Animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal protocols were approved by the ethic committee of Jiangxi Provincial People's Hospital.

*Cell culture*

Murine BV2 microglial cells were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). BV2 microglia were incubated in Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific, Massachusetts, USA) which consisted of 10 % foetal bovine serum (FBS, Gibco, Rockville, MD) in a humidified incubator (37 °C, 5 % $CO_2$). When cells reached 70 % ~ 80 % confluence, the cells were treated with 0 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL of LPS (Sigma, St. Louis, MO, USA) for 6 h.

*Transfection*

The full-length complete coding DNA sequences of NTN-1 and Rac1 were amplified and inserted into the plasmid adeno-associated virus (AAV) to construct AAV-NTN-1 and AAV-Rac1 vectors, with the original virus as the control, AAV-empty. BV2 cells were transferred with AAV-empty, AAV-NTN-1 or AAV-Rac1 for 48 h.

*Cell viability assay*

The BV2 microglial cell vitality was detected using the MTT assay kit (Dojindo Laboratories, Kumamoto, Japan). BV2 cells ($1 \times 10^5$/mL) were inoculated in 96-well plates, cultivated to the logarithmic growth phase, and then added the DMEM/F-12 medium supplemented with 5 mg/mL MTT reagent. MTT formazan crystals were mixed with DMSO (Dingguo Changsheng Biotechnology, Beijing, China) and oscillated for 10 min at a low speed. The cell viability was measured using microplate reader (Thermo Fisher Scientific, Massachusetts, USA) by spectrophotometry at 570 nm.

*Cell death assay*

BV2 microglial cell death was detected using the Cell Death Detection Kit (Qiagen, Valencia, CA, USA). Briefly, the LPS-treated microglia were incubated with 10 μL Annexin V-FITC and propidium iodide (Becton, Heidelberg, Germany) at room temperature for 15 min, and then proceed with the annexin V-FITC staining protocol.

*ELISA assay*

The corresponding ELISA kits were used to measure the contents of IL-1β, IL-4, L-6, IL-10, Arg-1 (BioSite, Paris, France) and iNOS (Bio-Techne, Shanghai, China). The concrete experimental steps were performed in accordance with the manufacturer's instructions.

*Pain behavioural test*

The pain behavioural test was performed using mechanical stimulation as described by Sun et al [19]. The right hind paw of mice was injected with 5 μL AAV-NTN-1 ($1 \times 10^8$ plaque forming units), and then a CFA injection was performed. On one day before CFA injection (Baseline) and the 1st day, 3rd day, 5th day after injection, mice underwent the mechanical hyperalgesia test using the von Frey filaments (Stoelting, Kiel, WI, USA). The back paws of mice were subjected to increase pressures for 3 s ~ 5 s/each filament. The paw withdrawal threshold (PWT) was used as a record of claw withdrawal. The experiment was performed with five replicates.

To conduct the thermal preference testing, mice were placed on a quartz glass, and the soles of the hind paws were stimulated by radiant heat apparatus (390G Plantar Test Apparatus, IITC Life Science Inc.). The baseline latencies of radiant intensity were adjusted to approximately 12 s. The time from the onset of radiant heat to withdrawal was recorded as PWL and an interval of 20 s was used to avoid potential tissue damage.

*Measurement of Nitrite oxide (NO) production*

The content of NO in BV2 microglial cells was detected using the Nitric Oxide assay kit (Thermo Fisher Scientific, Massachusetts, USA.). The concrete experimental steps were carried out according to the manufacturer's instructions. The absorbance of NO at 550 nm was determined.

*RNA extraction and qRT-PCR*

The extraction of total RNA from BV2 cells was performed using TRIzol® reagent (TaKaRa, Dalian, China). The cDNA was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) after the RNA quality was tested. qPCR was carried out using SYBR Premix Ex Taq (Takara) in accordance with the manufacturer's instructions. GAPDH was used as the internal control. Relative expression was calculated via the $2^{-\Delta\Delta Ct}$ method. The primers included in our research were listed as follows:

NTN-1 (forward primer: 5`-ACAACCCGCACAACCTG AC-3`; reverse primer: 3`-GGGACAGTGTGAGCGTG AC-5`);

IBA-1 (forward primer: 5`-ATGAGCCAAACCAGGG ATTTAC-3`; reverse primer: 3`-GGGATCGTCTAGG AATTGCTT GT-5`);

iNOS (forward primer: 5`-AGGGACAAGCCTACCC CTC-3`; reverse primer: 3`-CTCATCTCCCGTCAGT TGGT-5`);

IL-1β (forward primer: 5`-ATGATGGCTTATTACA GTGGCAA-3`; reverse primer: 3`-GTCGGAGATT CGTAGCTGGA-5`);

IL-6 (forward primer: 5`-ACTCACCTCTTCAGAAC GAATTG-3`; reverse primer: 3`-CCATCTTTGGAAGG TTCAGGTTG-5`);

Arg-1 (forward primer: 5`- CCCTGGGGAACACTACA TTTTG-3`; reverse primer: 3`- GCCAATTCCTAGTC TGTCCACTT-5`);

IL-4 (forward primer: 5`-CGGCAACTTTGTCCACGG A-3`; reverse primer: 3`-TCTGTTACGGTCAACTCG GTG-5`);

IL-10 (forward primer: 5`-GACTTTAAGGGTTACCT GGGTTG-3`; reverse primer: 3`-TCACATGCGCCTT GATGTCTG-5`);

GAPDH (forward primer: 5`- GGAGCGAGATCCCTCC AAAAT-3`; reverse primer: 3`- GGCTGTTGTCATAC TTCTCATGG-5`).

*Western blot analysis*

The spinal cord tissues of mice and the cells were quickly collected, rinsed with PBS three times, and then treated with RIPA lysis buffer to extract total protein. The quantitative detection of protein concentration was performed using a bicinchoninic acid kit (Pierce, Rockford, IL, USA). Protein was separated by SDS-PAGE (130 V constant pressure, 2 h), transferred onto PVDF membranes. The membranes were blocked with 5 % skimmed milk at room temperature for 1 h, and then incubated with the primary antibody overnight at

4 °C. After being washed with TBS buffer for 10 minutes, the membranes were incubated with the secondary antibody. The protein bands were detected using an enhanced chemiluminescence kit (Abcam, Cambridge, UK). Antibodies against Rac1 (1:1000, #12282), caspase-1 (1:1000, #24232) and GSDMD (1:1000, #69469) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against NTN-1 (1:1000, ab126729), IBA-1 (1:500, ab178846), p-p65 NF-κB (1:1000, ab76302) and GAPDH (1:1000, ab8245) were purchased from Abcam (Cambridge, MA, USA).

*Statistical analysis*

Statistical analyses of all data were achieved by using the GraphPad Prism 6 (GraphPad Software, Inc.). Difference analyses were calculated by one-way ANOVA test. Each experiment had at least three biological repetitions. $P$ <0.05 indicated statistical significance. Mean ± standard error of mean (SEM) was the final presented form of data. Statistical analyses were assessed with the SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA).

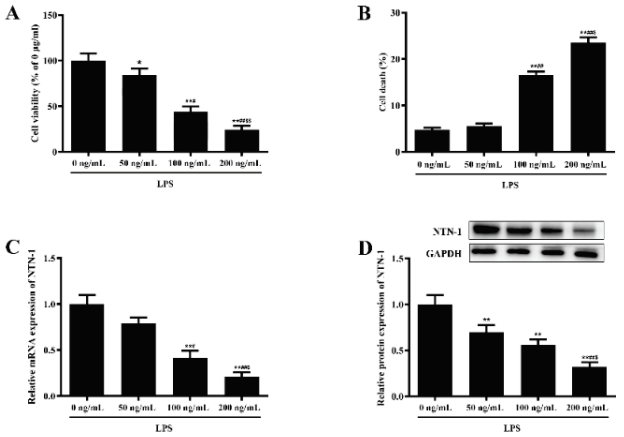


**Fig. 1.** LPS treatment reduced the expression of NTN-1 in BV2 microglial cells. BV2 microglial cells were treated with different concentrations of LPS (0 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) for 6 h. (**A**) The cell viability after treated with different concentrations of LPS. (**B**) Cell death of microglia after LPS treatment. (**C** and **D**) The mRNA and protein expressions of NTN-1 in LPS-treated cells. $^{*}$ and $^{**}$ stands for $P$ <0.05 and $P$ <0.01 vs 0 ng/mL LPS; $^{\#}$ and $^{\#\#}$ stands for $P$ <0.05 and $P$ <0.01 vs 50 ng/mL LPS; $^{\$}$ and $^{\$\$}$ stands for $P$ <0.05 and $P$ <0.01 vs 100 ng/mL LPS. The columns were presented as the mean ± SEM (A-C, n = 5; D, n = 3). One-way ANOVA was utilized to perform data analysis.

## Results

*The expression of NTN-1 was decreased in LPS-treated microglia cells*

The BV2 microglial cells were treated with different concentrations of LPS (0 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL) for 6 h. The result confirmed that LPS significantly suppressed cell viability, and enhanced cell death of microglia in a dose dependent manner (Fig. 1A, B). Moreover, the mRNA and protein expression levels of NTN-1 in cells were dramatically down-regulated by LPS treatment in a dose dependent manner (Fig. 1C,D).

*NTN-1 alleviated LPS-induced pyroptosis and induced M2 type activation of BV2 cells*

To investigate the role of NTN-1 in LPS-induced microglia, the BV2 cells were transfected with the AAV-NTN-1 plasmid. The result suggested that NTN-1 expression was significantly up-regulated by the overexpression of NTN-1 in LPS-treated cells (Fig. 2A). The LPS-induced decrease of BV2 cell viability and pyroptosis promotion, presenting as increases in cell death and caspase-1 and GSDMD protein expressions, was obviously depressed by AAV-NTN-1 transfection (Fig. 2B-D). The expression of IBA-1 was dramatically elevated by LPS, suggesting that LPS induced microglia activation; however, the overexpression of NTN-1 significantly reduced the up-regulation of IBA-1 in LPS-treated cells (Fig. 2E, F). In addition, the mRNA and secretion levels of M1 type markers in microglial cells, iNOS, IL-1β and IL-6, were elevated by LPS which was suppressed by NTN-1 overexpression (Fig. 2H, J, K); and NO production showed a same change trend (Fig. 2G). Meanwhile, NTN-1 overexpression significantly increased the mRNA expressions and concentrations of Arg-1, IL-4 and IL-10, M2 type microglial activation genes, which were decreased under LPS treatment (Fig. 2I, K and L).

*Rac1/NF-κB signaling was involved in NTN-1-mediated IP*

NF-κB and its activator gene, Rac1, were reported to be involved in the pathogenesis of IP [20,21]. To elucidate the potential mechanism of NTN-1 in regulating IP, the relationship between NTN-1 and Rac1/NF-κB signaling was investigated. The result confirmed the reduction of NF-κB phosphorylation and Rac1 expression in NTN-1-overexpressed BV2 cells under LPS stimulation which were reversed by NF-κB activator, ISO, and Rac1 overexpression (Fig. 3A). As expected, the activation of NF-κB and Rac1 upregulation markedly increased the expressions of caspase-1, GSDMD and IBA-1,

NO concentration, the levels of iNOS, IL-1β and IL-6, and decreased cell viability, the levels of Arg-1, IL-4 and IL-10 in LPS and AAV-NTN-1 co-treated microglia (Fig 3B-G). These findings indicated that Rac1/NF-κB signaling was a crucial target in NTN-1 mediated M2 type polarization and pyroptosis of microglial cells.

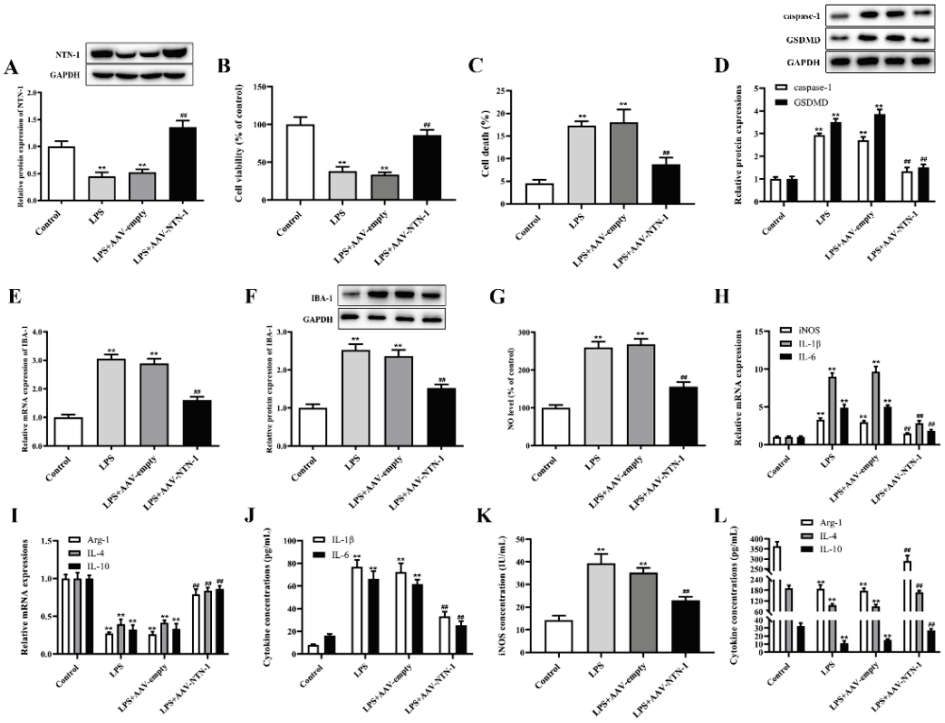

**Fig. 2.** The effect of NTN-1 overexpression on BV2 cells under LPS treatment. The BV2 cells were transfected with AAV-empty and AAV-NTN-1 for 48 h, respectively, and then performed with LPS treatment (100 ng/mL). (**A**, **D** and **F**) The protein expressions of NTN-1, caspase-1, GSDMD and IBA-1 were measured by western blotting. (**B**) The cell viability was evaluated using MTT method. (**C**) Cell death rate of microglia after different treatments. (**E**, **H** and **I**) RT-qPCR assay analysed the mRNA expressions of target genes. (**G**) The content of NO in cells. (**J**, **K** and **L**) The levels of IL-6, IL-1β, iNOS, IL-4, IL-10 and Arg-1 in cells were detected by ELISA. $^{**}$ stands for $P$<0.01 versus control; $^{\#\#}$ stands for $P$ <0.01 versus LPS+AAV-empty group. The columns were presented as the mean ± SEM (A, D and F, n = 3; the others, n=5). Data analysis was based on one-way ANOVA test.

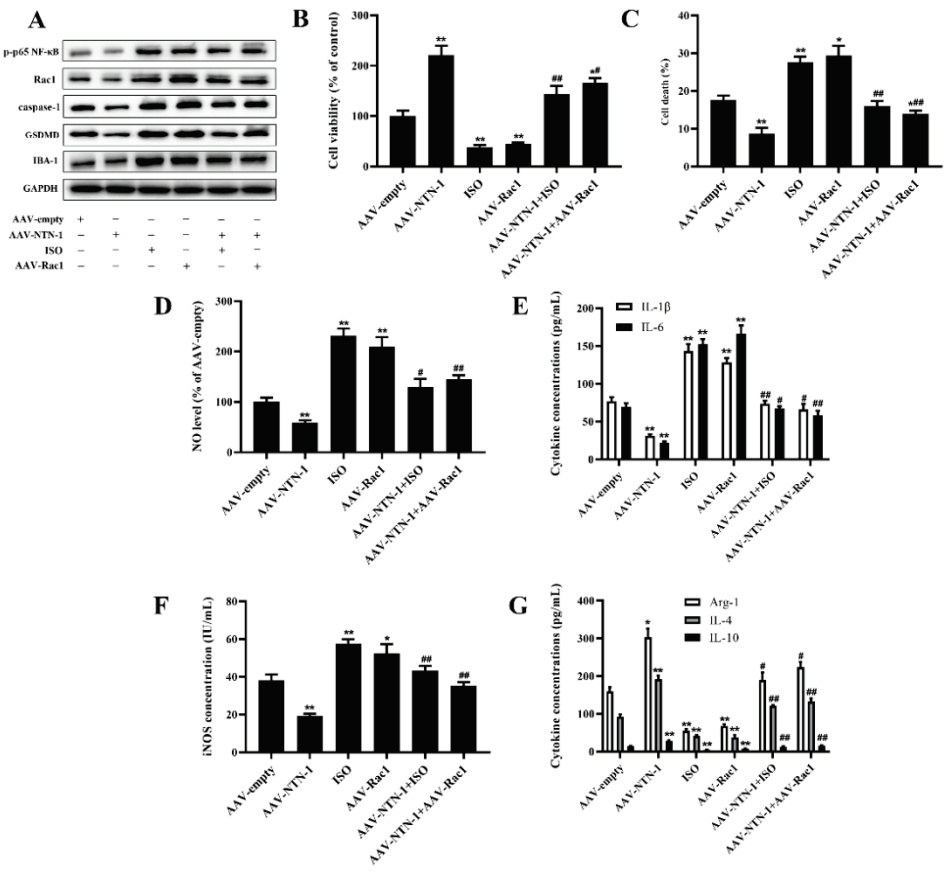

**Fig. 3.** Rac1/NF-κB signaling participated in the regulation of NTN-1 on LPS-induced IP. The BV2 cells were transfected with different plasmids (AAV-empty, AAV-NTN-1 and AAV-Rac1), and co-treated with ISO under LPS stimulation. (**A**) The protein expressions of p-p65 NF-κB, Rac1, caspase-1, GSDMD and IBA-1 in cells were detected by western blot assay. (**B**) The cell viability was evaluated using MTT method. (**C**) Cell death rate of microglia after different treatments. (**D**) The secretion of NO. (**E-G**) The levels of cytokines were detected by using ELISA kits. $^{*}$ $P$ <0.05 and $^{**}$ $P$ <0.01 versus AAV-empty group; $^{\#}$ $P$ <0.05 and $^{\#\#}$ $P$ <0.01 versus AAV-NTN-1 group. The columns were presented as the mean ± SEM (n = 5). One-way ANOVA test was used for data analysis.

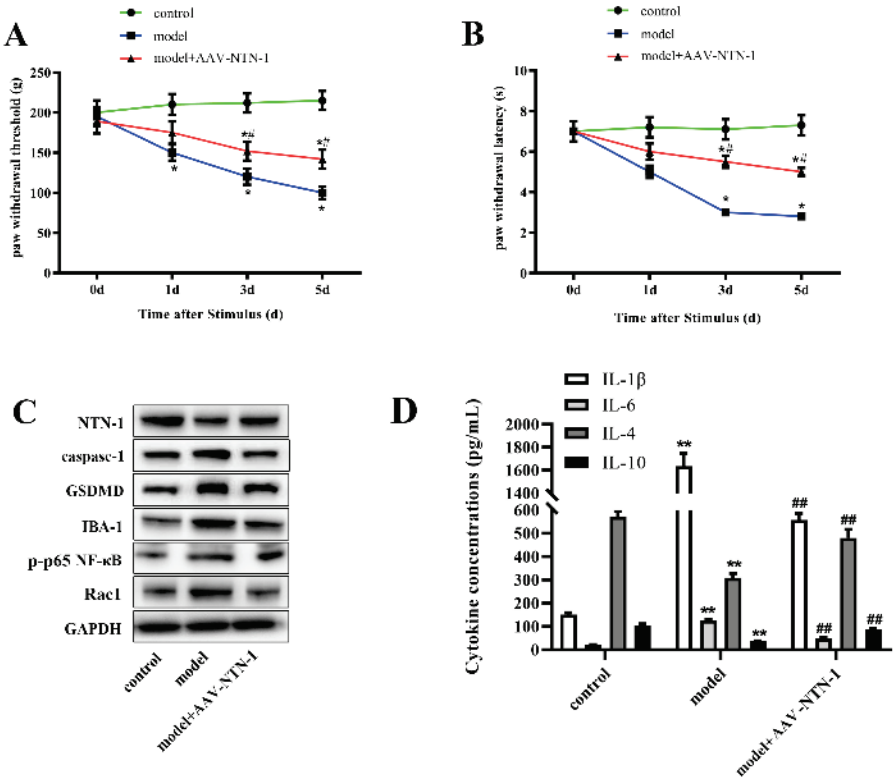


**Fig. 4.** NTN-1 alleviated the CFA-induced IP of mice. Mice were injected with CFA after AAV-NTN-1 injection. Saline injection was used as control. (**A**) The detection of PWT. (**B**) PWL was used to indicate heat hyperalgesia. (**C**) The expressions of target proteins were detected by western blot assay. (**D**) The secretions of cytokines in spinal cord were measured. The columns were presented as the mean ± SEM (A-B, n = 7; C, n = 3; D, n = 7). $^{*}P<0.05$ and $^{**}P<0.01$ versus control; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ versus model. The analysis of this study was based on one-way ANOVA test.

*NTN-1 attenuated CFA-induced IP in mice*

To further verify the role and regulatory mechanism of NTN-1 in IP, a CFA-induced IP mice model was established. As shown in Fig. 4A and B, PWT and PWL were significantly lower than those in the control at day 1, day 3, day 5 and day 7 after CFA injection; when mice were injected with AAV-NTN-1, the CFA-reduced PWT and PWL were increased. The reduction of NTN-1 in CFA-injected mice was up-regulated by NTN-1 overexpression (Fig. 4C). In addition, the NTN-1 up-regulation inhibited the promotion of caspase-1, GSDMD and IBA-1 expressions, as well as Rac1/NF-κB activation, and the levels of IL-6 and IL-1β induced by CFA in mice tissues and serum (Fig. 4C, D). Besides, the secretions of IL-4 and IL-10 were observably declined in CFA mice which was reversed after AAV-NTN-1 treatment (Fig. 4D).

## Discussion

Microglia are distributed in the spinal cord and brain. More importantly, the activated microglia are a crucial factor that associated with inflammatory responses and IP initiating and maintaining. Studies have shown that microglia are activated and aggregated in injured site when local tissue or nerves are damaged, and further secrete pain-related proinflammatory factors and pronociceptive mediators, including IL-6, TNF-α and IL-1β, which can act on neurons to trigger IP [22,23]. In this study, LPS induced the expression of microglial marker, IBA-1, and the secretion levels of NO, iNOS, IL-6 and IL-1β in microglia were increased after LPS treatment, suggesting that the LPS-induced microglia activation triggered inflammation injury. Hence, we can see that the inhibition of microglia activation is an important mean to prevent neuropathic pain.

NTN-1, a neuronal guidance factor that regulates nervous system development, has been discovered to be involved in the pathogenesis of various disorders including cancer, depression and inflammatory diseases [24-26]. Previous studies demonstrated that the expression of NTN-1 in inflammatory-injured tissues were negatively correlated with inflammatory response, and NTN-1 played an anti-inflammatory effect by inhibiting the secretion of pro-inflammatory factors [27-29]. Furthermore, scientists found that exogenous recombinant human NTN-1 could suppress microglia activation, thereby alleviating neuroinflammation and brain injury [15]. These findings suggested that NTN-1 played a crucial role in diseases related to inflammation. Our study consistently indicated that the decreased NTN-1 expression in LPS-treated microglia and CFA-induced tissues, and NTN-1 overexpression attenuated LPS-induced inflammatory responses which

implied that M1 type activation of microglia was depressed. Nevertheless, the findings in this study also presented that NTN-1 elevated the levels of cytokines related to M2 type microglial activation, the anti-inflammatory phenotype. Yang and colleagues illustrated that the therapeutic value of resveratrol to neuroinflammation was achieved by mediating microglia polarization [30]. A recent study indicated that transferring microglia from M1 to M2 type by icariin was contributed to attenuate intraocular inflammation and alleviate uveitis [31]. Supporting our conclusions, the promotive effect of oleanolic acid administration on Arg-1 and IL-10 secretions dramatically enhanced its intervention to M2 microglial polarization in neuropathic pain [32].

As a recently discovered type of programmed cell death, pyroptosis is associated with various inflammatory diseases due to its correspondence with non-canonical and canonical inflammasomes [33]. Under inflammatory stimulus, the accumulated inflammasomes activated caspase protein, especially caspase-1, leading to the cleaved GSDMD-induced pore formation and membrane disruption, finally pyroptotic cell death [34,35]. A recent study indicated GSDMD-contributed pyroptosis as a crucial modulator in the genesis of hyperalgesia, and that inhibiting pyroptosis could depress the IP caused by CFA in rats [33]. Li and colleagues demonstrated that targeting NLRP3 to restrain pyroptosis and inflammation was an effective manner for neuropathic pain therapy [36]. Consistently, our research also validated the beneficial role of overexpressing NTN-1 to alleviate IP injury which was achieved by depressing inflammation-related pyroptosis.

Multiple molecular mechanisms have been found to be involved in IP, the Rac1/NF-κB signaling pathway included [37]. Activation of NF-κB pathway in microglia promoted IP via enhancing the secretion of pro-inflammatory cytokines [21]. Lin *et al.* demonstrated the inhibitory effect of nodakenetin on inflammatory responses during IP pathogenesis exerted by depressing NF-κB activation [38]. Consistent with previous findings, our study proved that the activation of NF-κB increased pro-inflammatory factors levels in LPS-induced microglia and reversed the inhibition of NTN-1 overexpression; moreover, promoting Rac1 also presented the consistent functions. According to previous evidence, Rac1 was capable of upstream activating NF-κB in a plenty of cells [39]. A substrate of Rac1, PAK1, had the ability to trigger the translocation of p65 NF-κB into the nucleus [40]. Based on that, the modulation role of Rac1 on inflammatory diseases is emerging and has been validated. Wang and colleagues indicated that Rac1 participated in maintaining the acute IP induced by bee venom injection and might be utilized as a developing target for clinical pain therapy [20]. By activating Rac1-dependent NADPH oxidase, caveolin-1 dramatically promoted ROS aggravation in microglia which led to mechanical neuropathic pain [41]. Consistent with our findings, the crucial part of Rac1/NF-κB signaling in IP has been illustrated in the research of Sun *et al.*: suppressing Rac1/NF-κB signaling by TIPE2 upregulation was an effective manner to attenuate the inflammation stimulated by activated microglia and IP [37].

In summary, this study proved the important role of NTN-1 in IP. NTN-1 overexpression alleviated M1 type inflammatory responses in microglia induced by LPS and CFA, both *in vitro* and *in vivo*. Furthermore, NTN-1 promoted M2 microglia activation by regulating the Rac1/NF-κB signaling. These analyses suggest NTN-1 as a potential target for therapy in IP.

## Abbreviations

NTN-1, Netrin-1; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa-B; CFA, complete Freund's adjuvant; AAV, adeno-associated virus; PWT, paw withdrawal threshold; PWL, paw withdrawal latency; NO, nitrite oxide.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements - Authors' contributions

Yanyan Yin designed the study and wrote the paper; Material preparation and Data collection were performed by Yan Yan and Xia Jin; Data analysis and Software were performed by Yuanyuan Fu; Ying Chen designed the study and commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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