Dimethyl itaconate attenuates CFA-induced inflammatory pain via NLRP3/ IL-1β signaling pathway

Jiaqi Lin
Zhejiang University School of Medicine Second Affiliated Hospital
Jinxuan Ren
Zhejiang University School of Medicine Second Affiliated Hospital
Bin Zhu
Zhejiang University School of Medicine Second Affiliated Hospital
Yi Dai
Zhejiang University School of Medicine Second Affiliated Hospital
Dave Schwinn Gao
Zhejiang University School of Medicine Second Affiliated Hospital
Suyun Xia
Zhejiang University School of Medicine Second Affiliated Hospital
Zhenzhen Cheng
Zhejiang University School of Medicine Second Affiliated Hospital
Yangyuxin Huang
Zhejiang University School of Medicine Second Affiliated Hospital
Lina Yu (zryulina@zju.edu.cn)
The Second Affiliated Hospital of Zhengzhou University https://orcid.org/0000-0003-1342-2969

Research

Keywords: Dimethyl itaconate, inflammatory pain, macrophages, microglia, NLRP3 inflammasome complex, IL-1β

Posted Date: November 23rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1050454/v1

Abstract

Background

Itaconate plays potent anti-inflammatory effects and has gradually been discovered as a promising drug candidate for treating inflammatory diseases. However, its roles and underlying mechanism on pain remain unknown.

Methods

In the current work, we investigated the effects and mechanisms of dimethyl itaconate (DI, a derivative of itaconate) in a mouse model of complete Freund's adjuvant (CFA)-induced inflammatory pain. Male/Female C57 BL/6 mice were randomly divided into five groups: a vehicle group, an CFA group ,an CFA+PBS group and an CFA + DI(10mg /d and 20 mg/d) group.DI was performed for 11 consecutive days after CFA models were established.Paw withdrawal frequencies and paw withdrawal latencies were used to Behavioral Tests. The activation of macrophages and microglia, the level of proinflammatory cytokine production, the number of M1/M2 macrophages were evaluated .The possible involvement of the NLRP3/ IL-1β signaling pathway was also investigated.

Results

DI significantly reduced mechanical allodynia and thermal hyperalgesia, decreased peripheral inflammatory cell infiltration and the expression of pro-inflammatory factors IL-1β and TNF-α, and upregulated anti-inflammatory factor IL-10. Interestingly, DI promoted macrophages at the inflammatory site polarization from M1 into M2 type. Additionally, DI inhibited activation of macrophages in dorsal root ganglion (DRG) and microglia in the spinal cord, exhibiting reduced expressions of pro-inflammatory cytokines. Mechanismly, DI exerts the analgesic action primarily via inhibiting the activation of NLRP3 inflammasome complex and the release of IL-1β in derived and resident macrophages in the hind paw, DRG and spinal cord.

Conclusion

DI could alleviate the pain-like behavior of CFA mice by inhibiting the infiltration of plantar inflammatory cells and macrophages activation in DRG and microglia in the spinal cord. The analgesic behavior of itaconate was related to the inhibition of NLRP3 inflammasome. This study suggested possible evidence for prospective itaconate utilization in the management of inflammatory pain for the first time.

Introduction

Chronic inflammatory pain is associated with a number of clinical diseases such as osteoarthritis, rheumatoid arthritis, and fibromyalgia [1]. The torture of long-term inflammatory pain causes deterioration in the patients' health-related quality of life and even increases depressive symptoms and suicidal tendencies[2, 3]. Inflammatory pain originates from chemical stimuli, tissue damage, or autoimmune activation that causes nociceptors to respond to various stimuli and elicits pain hypersensitivity. Peripheral stimulation directly promotes the local release of inflammatory mediators, including prostaglandins, histamine and neurogenic factors, and which subsequently trigger a series of chain reactions including neurogenic inflammation, peripheral and central sensitization, and causing pain eventually [4, 5] At present, non-steroidal anti-inflammatory drugs (NSAIDs) are recommended as the first-line pharmacotherapy to relieve pain and inflammation [6]. However, the long-term gastrointestinal side effects of these drugs greatly limit their clinical application. Thus, there is an urgent need to find new, safe and effective anti-inflammatory pain drugs.

More and more evidence show that the accumulation, proliferation and activation of peripheral macrophages and central glial cells play key roles in peripheral and central pain sensitization [7, 8]. Macrophages produce various inflammatory mediators, such as inflammatory cytokines, growth factors, and lipids after tissue injury and infection, directly leading to primary hyperalgesia at sites of injury or inflammation [9, 10]. Microglial cells, the resident immune cells in the central nervous system, exhibit many immunological characteristics with peripheral macrophages[11]. More and more studies have found that many types of inflammatory mediators derived from activated microglia actively participate in the occurrence and development of pain[12, 13]. And microglial inhibitors and inhibitors of activated microglia-derived cytokines could help controlling and managing not only acute painful behaviors but, more importantly, their transition to persistent pain hypersensitivity conditions[14, 15].

The typical pro-inflammatory cytokine IL-1β has been shown to be produced by peripheral macrophages and activated microglia in the dorsal horn and is known to be upregulated in the various acute and chronic pain disorders[16-19]. Moreover,intrathecal injection of IL-1β has a profound and long-lasting effect on pain hypersensitivity[19]. IL-1β was originally produced as a cytosolic preprotein, which requires proteolysis at specific sites to activate its biological functions and make it released outside the cell. The cleavage and subsequent secretion of IL-1β is mediated by the nod-like receptor protein 3 (NLRP3) inflammasome[20, 21]. After activation of NLRP3, it couples with ASC and Caspase-1thus form NLRP3 inflammasome, thereby regulating pro-IL-1β cleaved into a mature form and quickly released into the extracellular environment to cause inflammation and pain [22]. Therefore, inhibiting the inflammasome complex and reducing the release of IL-1β are particularly important for the treatment of pain.

Itaconate is an α, β-unsaturated dicarboxylic acid ($C_5H_6O_4$), an essential intermediate metabolite isolated from the tricarboxylic acid cycle in immune cells, especially macrophages. It is derived from cis-aconitate decarboxylation mediated by immune response gene 1 in the mitochondrial matrix [23]. In recent years, the anti-inflammatory effect of itaconate has gradually been discovered and has attracted great attention[24, 25]. It can activate nuclear factor E2-related factor 2(Nrf2) by alkylating kelch-like ECH-associated protein 1(Keap1) to initiate anti-inflammatory and antioxidant responses [24]. Besides,

itaconate could also target on activating transcription factor 3(ATF3)- inhibitor of κB-ζ(IκBζ) pathway to mediate the inflammatory response [25]. Recent studies have also found that itaconate can dissociate NLRP3 from NEK7 through alkylation, thereby inhibiting the formation of inflammasome complexes and reducing the inflammatory progression of crystal-stimulated peritoneal inflammation[26].Itaconate has emerged as a critical determinant and participated in the development and progression of inflammation and immunity. Currently, it is not yet known whether itaconate has an analgesic effect on inflammation-induced pain.

This study explored whether itaconate can regulate the progression of CFA-induced inflammation in the peripheral and central nervous systems. We report that dimethyl itaconate (DI), a derivative of itaconate, alleviates CFA-induced pain in mice. In addition, we found that DI inhibited the activation of peripheral macrophages and central microglia and reduced the progression of peripheral and central nervous inflammation. The possible mechanism is to inhibit the formation of the NLRP3 complex and reduce the release of IL-1β.

Materials And Methods

Animals

Adult (6 to 8 weeks) C57BL/6J male mice weighing 20 to 25 grams obtained from SLAC Laboratory Animal Centre (Shanghai, CN), housed in a specific pathogen-free facility at 22 ℃ and relative humidity of 30% under a constant 12-hours day and night cycle, in the Second Affiliated Hospital of Zhejiang University, School of Medicine. Procedures conducted in this study were approved by the Zhejiang Animal Care and Use Committee and the Second Affiliated Hospital, School of Medicine, Zhejiang University. This research was taken to meet scientific objectives while maintaining high animal welfare standards and minimizing animal use. All experiments were carried with investigators blind to viral content or drug treatment during behavioral testing.

Induction of paw inflammation and Drug treatment

Inflammation was induced using 20 microL of CFA(Sigma) administered subcutaneously into the left hind paw. As an internal control, the mice were treated with the same volume of sterile saline injections. From the second day, DI(Sigma,10mg/d,20mg/d) or PBS was injected intraperitoneally every day. Behavioral measurements of hyperalgesia were assessed on the same animals before and on 2,4,6 hours and days 1, 2, 4,6,8,12 following CFA injection. For the experiment of itaconate pretreatment, three days after DI(20mg/d) was injected intraperitoneally and the CFA model was established on day 4.

Behavioral Tests

Paw withdrawal frequencies (PWF) were defined as mechanical pain tests using nylon von Frey filaments (DanMic Global. Campbell, CA) as a response to physical stimulation. In short, rodents were put on top of a Cartesian mesh plane in Plexiglas chamber individually for 30 min of acclimation. 70 and 400 mg von

Frey filaments were prodded perpendicularly with sufficient force to stimulate the plantar surface of both hind paws for 1-2 s and with a repeated simulation of 10 times. Paw withdrawal responses were scored as a percent response frequency, PWF ((number of paw withdrawals/10 trials) × 100 = % response frequency)

The thermal nociception stimulus for paw withdrawal latencies (PWL) was measured using a Model 336a plantar algesia device (IITC Inc. Life Science Instruments, Woodland Hills, CA). Briefly, the mice were put on a glass plate in a Plexiglas chamber individually for 30 min of acclimation. The withdrawal latency is defined as the period between light striking the hind paw's plantar surface and the paw flick or withdrawal. A maximum stimulation period of 20 s was set to avoid skin injury during the procedure. The mean recorded value between three measurements with 5-min intervals was used as PWL.

H&E staining

On day 6 of CFA administration (the fifth day after DI treatment)，the rodent was sedated with 1% pentobarbital and transcardially perfused with 37°C normal saline conditions and 4% PFA (pH, 7.4; 4°C) in 0.01 M PBS. Then, the subcutaneous tissue at inflammatory sites medial to athe left hind paw was immediately removed and fixed in 4% paraformaldehyde. Tissues were then dehydrated, and embedded in paraffin, and sliced with a thickness of 5-μm. Hematoxylin and eosin were used to stain all slices

Immunohistochemistry

The slides were subsequently deparaffinized and incubated in 3% H2O2 (10 min) washed in PBS (5min, 3 times), and 5% bovine serum albumin (BSA) was used for the half an hour incubation period at 25°C. The sections were stained with primary antibodies, MPO (1:100, Santa Cruz), CD45(1:200, Abcam), CD68(1:200, Proteintech), iNOS(1:200, Abcam),Arg1(1:200,GeneTex) and incubated at 4°C overnight. The sections were washed in PBS (5min, 3 times) and incubated with polymerized horseradish peroxidase-labeled goat anti rabbit IgG (Wuhan Boster Company) at 37°C for 30 min and washed in PBS (5min, 3 times). Sections were detected by DAB(Vector Labs) under the lens and counterstained with hematoxylin. Appropriate positive and negative controls were added for every immunostaining. Sections were dehydrated by graded alcohol，transparented and finally mounted by neutral gum. The staining intensity was evaluated as: 0, no staining; 1, weak, 2, moderate; or 3, strong. A double score system for the fluorescent intensity, the proportion of the positive cells was utilized to evaluate the reactiveness and multiplied to generate a score (IHC score range 0-300) as described.

Immunofluorescence was performed with frozen sections (L4-6 spinal cord, DRG neurons) or paraffin sections (hind paw). Slices were washed in 10 mM Phosphate Buffered Saline (5min, 3 times) and a one-hour incubation with 5% BSA at room temperature. The following antibodies were added and left overnight (4 °C): rabbit anti-ASC (1:200, CST), mouse anti-ASC (1:100, Santa Cruz),mouse anti-Caspase 1 (1:100, Santa Cruz),mouse anti-IL-1b (1:100, Santa Cruz) ,mouse anti-CD68 (1:200,Abcam),rabbit anti-CD206 (1:200,Abcam),rabbit anti-CD86 (1:200,abcam), rabbit anti- Iba1 (1:200; Wako), F4/80 (1:200, Invitrogen). Subsequently, the slices were rinsed in 10 mM Phosphate Buffered Saline (5min, 3 times) and

were incubated with corresponding second antibodies for 1 hour at room temperature. Finally, the slices were mounted with diamidino-phenyl-indole (DAPI, Abcam) to stain for the nucleus. The slides were inspected under fluorescence with Leica DMI4000.

Real-time polymerase chain reaction (RT-PCR)

The subcutaneous tissue medial to the plantar, DRG neurons and spinal cord tissue was used for total RNA isolation with TRIzol reagent (Invitrogen). The cDNA was synthesized from total RNA (1 μg) a Hifair II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, Shanghai). All primers' sequences for qPCR listed in Table 1 were synthesized in Sango Biotech (Shanghai, CN). qPCR was carried out as follows: initial 2 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. qPCR was conducted according to the instructions using the SYBR-Green System (Yeasen, Shanghai). Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, with β-Actin or tublin as the internal control and reference gene. Primer sequences and conditions were shown in Table 1.

Table 1. Specific primers used for qPCR.

	Forward primers	Reverse primers
β-Actin	AGGCATTGTGATGGACTCCG	AGCTCAGTAACAGTCCGCCTA
Tublin	GATGCTGCCAATAACTATGCTC	TTGGACTTCTTTCCGTAATCCA
IL-1β	TCGCAGCAGCACATCAACAAGAG	AGGTCCACGGGAAAGACACAGG
TNF-α	ATGTCTCAGCCTCTTCTCATTC	GCTTGTCACTCGAATTTTGAGA
IL-10	GCCTGCTCTTACTGACTGGC	AGCTCTAGGAGCATGTGGCT

Western blotting

The L4-6 spinal cord, DRG neurons and hind paw protein was isolated on the sixth day after CFA injection (the fifth day after itaconate treatment). The tissues were lysed in Radioimmunoprecipitation Assay buffer (RIPA) which contained 0.05 M Tris (pH 7.4), 0.15 M sodium chloride, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulphate. The supernatants were gathered after being centrifuged for 15 m at 4°C with 1500 RCF and the concentration of protein was measured using a bicinchoninic acid (BCA) assay kit (Beyotime, CN). The samples were boiled at 100°C for 5 min and transferred on a 10% sodium deoxycholate-polyacrylamide gel (Genshare Biology, CN) for electrophoretic separation and immunoblotting before being transferred electrophoretically with a current of 0.25 A onto a PVDF film (Millipore, Burlington, MA, United States) for 90 min. The films were blocked with 5% nonfat milk TBST for 1 h and then incubated at 4 °C overnight with the following antibodies: mouse anti-GAPDH (1:1,000,

Zhongshan Golden Bridge Biotechnology), rabbit anti-ASC antibody (1:1,000, CST), rabbit anti-NLRP3 antibody (1:1,000, CST), mouse anti-Caspase 1 antibody (1:200, Santa Cruz), mouse anti-ASC antibody(1:200,Santa Cruz), mouse anti-IL-1b antibody (1:200, Santa Cruz). The protein was detected by HRP-conjugated anti-mouse or rabbit secondary antibody (1:5,000, Jackson ImmunoResearch). Clarity Western ECL Substrate (EMD Millipore) was utilized for visualization and ChemiDoc XRS with System (Bio-Rad) for exposure. The western blots intensity was determined using Fiji ImageJ.

Statistical analyses

All of the results used the notation of mean ± SEM. Two-way ANOVA followed by Bonferroni's posthoc test was used for withdrawal thresholds the thermal latency between groups. One-way ANOVA with Tukey procedure was utilized to evaluate the biochemical results (GraphPad Prism 8). Significance between different groups was set at $p < 0.05$.

Results

DI reduces inflammatory pain development

To investigate the role of DI in chronic inflammatory pain, the left hind paw was infused with CFA. PWF to 0.07g and 0.4 g von Frey filament stimuli changed with robust increases and ipsilateral PWL to thermal nociception changed with rapid reductions in 2 hours and subsequently reached a steady-state at day 1 (Figure1.A-C). Daily intraperitoneal injection of DI (20mg) attenuated CFA-induced mechanical allodynia as demonstrated by the reduction of PWF to mechanical stimuli from day 4 and improved CFA-induced thermal hyperalgesia as indicated by the increase in PWL to heat stimulation from day 6 to day 12 compared to the CFA and CFA+PBS groups (Figure1.A-C). The low dose of DI (10mg) began to relieve the mechanical and thermal hyperalgesia in mice on the 6th day post CFA injection. Due to the gender difference in pain, we also verified the relief of DI on inflammatory pain in female mice. Same as male mice, DI can relieve mechanical and thermal hyperalgesia in female mice. DI (20mg) relieves mechanical pain on the 8th day and thermal hyperalgesia on the 6th day after CFA injection (Figure1.G-I). The low dose of DI only relieve mechanical pain on the 8th day, but there is no significant difference in thermal hyperalgesia.However, there was no noticeable change in the PWT or PWF of the contralateral plantar (Figure1.D-F,J-L). These results suggested that DI could attenuate pain behavior.

DI pretreatment did not alleviate inflammatory pain development

In order to study the role of DI pretreatment in chronic inflammatory pain, DI (20mg) was pre-administered for 3 days in naive mice and CFA was injected into the left hind paw. As is shown in figure, DI (20mg) pretreatment did not affect baseline thermal pain thresholds and and mechanical pain threshold both in naive mice (Figure2.A-C, G-I). PWF to 0.07g and 0.4 g von Frey filament stimuli changed with robust increases and ipsilateral PWL to thermal nociception changed with rapid reductions at day 1. DI (20mg) pretreatment did not alter CFA-induced mechanical allodynia and thermal hyperalgesia compared to

CFA+PBS groups (Figure2.A-C, G-I).The contralateral plantar has no noticeable change in the PWT or PWF (Figure2.D-F, J-L).These results suggested that DI pretreatment could not attenuate pain behavior.

DI treatment antagonizes CFA-induced local inflammation.

Mice with inflammatory pain are usually accompanied by swelling of the painful site and infiltration of inflammatory cells. After CFA injection, the subcutaneous tissue at inflammatory sites medial to the left hind paw swelled rapidly, and the thickness of the hind paw increased from 2-2.5mm to 3.5-4mm (Figure3.A,B). Moreover, HE staining on the 6th day post CFA injection of the CFA and CFA+PBS hind paw samples displayed numerous inflammatory cells invading the nearby dermal samples (Figure3.C), and immunohistochemical analysis revealed a large number of MPO-positive neutrophils, CD45-positive immune cells (white arrows) infiltration. When injected with DI (20mg), it significantly reduced hind paw swelling (Figure3.A,B and inflammatory cell infiltration compared with CFA and CFA+PBS groups (Figure3.G-I,$p<0.05$).The above data indicated that DI could inhibit local inflammation.

DI inhibits macrophage activation in hind paw and DRG and microglia activation in spinal cord

The duality of peripheral macrophage and central microglia activation are the primary pathways responsible for inflammatory pain's start point and progression [27, 28]. We first examined whether DI administration affected macrophages activation in the ipsilateral hind paw with F4/80 marker immunofluorescence tagging. The numbers of F4-80$^+$ macrophages exhibited profuse activation in the ipsilateral hind paw after CFA injection compared to those in the vehicle group (Figure4.A and B). However, CFA mice treated with DI (20 mg) showed markedly scarcer F4-80$^+$ cells ($p<0.05$). Treatment with DI also significantly decreased IL-1β, TNF-α mRNA expression relative to the CFA group (Figure4.C and D, $p < 0.05$) and promoted anti-inflammatory cytokine IL-10 (Figure4. E, $p < 0.05$). Similar results were also found in the DRG consistent with hind paw. DI also suppressed macrophages activation in the ipsilateral DRG (Figure4. F and G, $p < 0.05$) and inhibited pro-inflammatory factors TNF-α IL-1β (Figure4. H and I, $p < 0.05$) and reversed the reduction of anti-inflammatory factors IL-10 induced by CFA (Figure4.J, $p < 0.05$). Moreover, The numbers of Iba1 positive cells were significantly increased and the expression of these proinflammatory cytokines were also upregulated in CFA and CFA+PBS groups (Figure4.K and L, $p < 0.05$). DI intervention mitigated this expression similar to that of the vehicle group (Figure4, M-O, $p<0.01$).The above data show that DI may inhibit macrophages/microglia activation and the expression of pro-inflammatory factors and promote the activation of anti-inflammatory factors in peripheral and central systems.

DI reduced M1 polarization and facilitated M2 polarization

Different subsets of macrophages in the injured hind paw were characterized. CFA triggered an increase of macrophages in injured hind paw. Moreover, local inflammation was associated with increased iNOS, reduced Arg1 expression (Figure 5.A,B). DI (20 mg) inhibited the expression of iNOS but decreased Arg1 expression compared with CFA and CFA+PBS group ($p<0.05$). There was a reduction of M1 macrophages with marker CD86 (Figure5.C and D) and increased the proportion of M2 macrophages and upregulated

the expression of their marker CD206 (Figure5.E and F, $p < 0.05$).Similarly, there was a reduction of CD86 macrophages (Figure5.G and H) and an increase of CD206 macrophages in DRG treated with DI. (Figure5.I and J , $p < 0.05$). Similar results were also found in the spinal cord dorsal horn consistent with DRG. DI also reversed the reduction of CD206 positive cells induced by CFA. (Figure5.K and L), signifying DI might stimulate macrophage polarization from classically activated pro-inflammatory phenotypes to alternatively activated anti-inflammatory phenotypes.

DI inhibits the activation of the NLRP3 inflammatory complex and reduces the release of IL-1β.

The NLRP3-inflammasome complex is one of the most important regulators of inflammation. Expression of NLRP3, ASC, caspase-1 and IL-1β at the protein level of skin tissues were evaluated. The expressions of NLRP3, ASC were higher in the CFA and CFA+PBS groups than in the vehicle group (Figure6. A and B). Following DI treatment, NLRP3 and ASC were significantly reduced ($p<0.05$). We also tested the expression of NF-κB. Itaconate inhibited the increase of NF-κB caused by CFA (Figure6. A and B).In addition, the caspase-1 p45 and IL-1β release were initiated by CFA injection. DI eliminated caspase-1 p20 and IL-1β p17 release compared with CFA and CFA+PBS groups (Figure6.C and F, $p<0.05$). Immunofluorescence staining showed that positive ASC, IL-1β and Caspase-1in CFA and CFA+PBS groups were less than those in CFA and CFA+PBS groups (Figure6.G-I). We also detected the DI effect on NLRP3 inflammatory complex in DRG. NLRP3 and NF-κB protein was found to increase which was concomitant with high ASC level while DI treatment reversed this activation (Figure6.J,K). We also observed an increase in the levels of caspase-1 p45 and p20 as well as the maturity and release of IL-1β compared to the Vehicle group (Figure6.L-O). DI treatment reversed these results ($p<0.05$). Consistent with plantar tissue and DRG results, DI reduced the formation of the NLRP3 complex and NF-κB in spinal cord and inhibited the IL-1β activated into the section of IL-1β p17(Figure6.P-U). In general, CFA significantly elevated the expression of NLRP3 inflammasome, which was decreased by treatment with DI. These results are indicated that DI may alleviate inflammatory pain induced by CFA by inhibiting the NLRP3 inflammasome.

Discussion

The overall goal of our study was to determine the analgesic effects of DI on inflammatory pain models. Our results found that the enhanced activation of macrophages into paw tissue and the DRG and microglia in the spinal cord contribute to the development of inflammatory pain. The analgesic mechanism of DI is via regulating the balance between pro-inflammatory and anti-inflammatory response and suppressing the assembly of NLRP3 inflammasomes complexes (Figure 7).

To our best knowledge, there were no reports describing the application of DI in pain. However, itaconate has been shown to exert significant anti-inflammatory effects under various inflammatory conditions, including sepsis, neuroinflammation, bacterial or virus infection,rheumatoid arthritis and pneumonia in previous studies [23, 24, 29, 30]. So we infer that the anti-inflammatory effect of itaconate has a therapeutic effect on inflammatory pain. In this study, we found that various DI (10 and 20 mg) doses

could effectively reduce mechanical allodynia and thermal allodynia caused by CFA. Since the current research is based on the anti-inflammatory effects of itaconate to speculate its analgesic potential, other types of pain such as neuropathic pain, cancer pain and acute pain still need to be further explored to broaden the application of itaconate in pain. In addition, in view of the gender differences in pain, we also found that itaconate also has analgesic effects in female mice. However,male mice displayed an immediate and significant reverse in paw withdraw frequencies whereas female mice that received the DI injection displayed a delayed analgesic effect. This may be due to gender differences in the prevalence and intensity of chronic inflammation[31]. Recent studies have found that pretreatment with DI offer a protective effect to the macrophages later stimulated with LPS [24, 26]. We speculate whether itaconate pretreatment would prevented the development of CFA-induced inflammatory pain.However, contrary to the prediction, itaconate does not change the threshold of mechanical and thermal pain in naive mice and does not prevent the occurrence of inflammatory pain in mice. The possible reason are that DI does not change the threshold of mechanical pain and thermal pain in mice. Moreover, short-term effect because of rapidly degradation or further metabolization of DI was not enough to release endogenous itaconate[32]. It will quickly produce pharmacological effects in 3-4 hours and is easily excreted from the body within 24 hour[33], which may also explain its low toxicity. Taken together,even three days of pre-administration, mechanical pain and thermal pain caused by CFA are still irreversible.

Meanwhile, we found that DI inhibited plantar skin swelling and inflammatory cell infiltration in plantar tissues. HE staining showed a large amount of inflammatory cells (MPO+ neutrophils, CD45+ immune cells, macrophages) infiltrated around paw muscles. Long-term infiltration of a large number of inflammatory mediators will lead to unresolved inflammation [34-36]. Among these inflammatory cells, macrophages occupy a large proportion. Moreover,peripheral inflammatory processes lead to central sensitization of spinal cord circuits, which may lead to resultant hyperalgesia and allodynia [37, 38]. Our study found that itaconate treatment reduced macrophage over activation both in hind paw and DRG tissues. This may indicate that DI can inhibit the occurrence of peripheral inflammation further to prevent peripheral sensitization of pain. Microglia are resident macrophages in the central nervous system. Results of studies to date indicate that the activation of microglia plays a critical role in the development of central sensitization in the spinal cord in CFA model [28, 39]. Moreover, minocycline (a non-selective microglial inhibitor) or propentofylline (a glia modulating agent) can prevent or reverse mechanical and thermal hyperalgesia in rodents [40]. DI treatment also inhibit the activation of microglia in spinal cord, indicating that itaconate can inhibit the formation of central sensitization. Macrophages usually differentiate into different functional phenotypes to regulate complex inflammatory processes. Inflammatory (M1) macrophages are characterized by high production of inflammatory cytokines and chemokines (IL-1β, TNFα, CCL3, CCL4, and iNOS), hence further intensifying inflammation [41-44]. By contrast, M2 macrophages are characterized by expressing high levels of IL-10 and arginase 1 (Arg1) to counterbalance the extensive inflammatory response and promote the resolution of inflammation [41]. In a recent study, it was found that DI promoted the transition from M1 to M2, reduced the expression of inflammatory mediators in microglia treated with LPS and ATP[45]. In our study, DI promoted Arg1 and CD206 and inhibited the expression of iNOS and CD86 in CFA-treated paw tissues. DI promoted the

transformation of M2 macrophages to counterbalance the extensive inflammatory response and promote the resolution of inflammation. We also found DI treatment could inhibit the activation of peripheral DRG macrophages and excessive activation of DRG macrophages can cause peripheral sensitization. DI treatment also inhibited the expression of CD86 but promoted CD206 in DRG tissues and increased the expression of CD206 in the spinal dorsal horn. The regulation of anti-inflammatory and pro-inflammatory may be an important factor in DI analgesic effect. Moreover, it was also found that DI can reduce the production of TNF-α and promote the expression of anti-inflammatory factors, IL-10. These cytokines help the recruitment of inflammatory cells to tissues in the periphery and central, thereby increasing the inflammatory process and pain[46]

Studies have found that IL-1β produced by macrophages promotes the recruitment of neutrophils and lymphocytes at the inflammation site, eventually leading to an inflammatory cascade. On the other hand, mature IL-1β plays an important role in initiating inflammatory pain hypersensitivity [47]. IL-1β can also sensitize or directly activate nociceptors firing and glial activation to induce persistent pain [48-50]. Blocking IL-1β with neutralizing antibodies can significantly alleviate inflammatory pain [51]. In CFA model, NLRP3 can lead to the activation of caspase-1 and elicit the maturation and secretion of the pro-inflammatory cytokines of IL-1β and IL-18 [52, 53]. Therefore, NLRP3 inflammasome activation and release of mature IL-1β plays a key role in CFA-induced pain and inflammation pathogenesis. Recent studies have also found that itaconate could dissociate NLRP3 from NEK7 by alkylation to inhibit the formation of inflammasome complexes and reduce the inflammatory progression of crystal-stimulated peritoneal inflammatory [26]. In view of the fact that itaconate can inhibit NLRP3, and in our experiments, it was also found that DI reversed the activation of NLRP3 complex and the release of IL-1β caused by CFA. We speculated that the analgesic effects of itaconate may be related to the inhibition of NLRP3. In this study, we concluded that the NLRP3 inflammasome complex is activated in the CFA-induced inflammatory pain model, which is consistent with the findings of Gao et al. [52], and itaconate significantly reduces the expression of NLRP3, caspase-1, and IL-1β level in CFA-induced inflammatory tissues. These results indicate that itaconate may alleviate CFA-induced inflammatory pain by inhibiting the activation of the NLRP3 inflammasome as well as mature IL-1β.

Conclusion

In this study, our experiments demonstrated that itaconate could alleviate the pain-like behavior of CFA mice by inhibiting the infiltration of plantar inflammatory cells and macrophages activation in DRG and microglia in the spinal cord. The analgesic behavior of itaconate was related to the inhibition of NLRP3 inflammasome. Itaconate is expected to be a prospective candidate for the treatment of inflammatory pain management.

Abbreviations

Arg-1 Arginase-1

CFA Complete Freund's adjuvant

CNS Central nervous system

DI Dimethyl itaconate

DRG Dorsal root ganglion

IL-1β Interleukin-1 beta

iNOS Inducible nitric oxide synthase

NLRP3 Nod-like receptor protein 3

NF-κB Nuclear factor kappa-B

PNS Periphera nervous system

TNF-α Tumor necrosis factor alpha

Declarations

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding

None.

Acknowledgements

None.

Author information

Department of Anesthesiology, The Second Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang University, Hangzhou 310009, People's Republic of China

Jiaqi Lin, Jinxuan Ren, Bin Zhu,Yi Dai,Dave Schwinn Gao, Suyun Xia,Zhenzhen Cheng,Yangyuxin Huang, Lina Yu

Corresponding author

Correspondence to Lina Yu.

Authors contributions

Jiaqi Lin contributed to the conception of the study and performed the experiment and wrote the manuscript;Jinxuan Ren contributed significantly to analysis and manuscript preparation;Bin Zhu and Yi Dai performed animal model and the data analyses; Dave Schwinn Gao and Suyun Xia manuscript proof reading and editing;Zhenzhen Cheng and Yangyuxin Huang helped perform the analysis with constructive discussions; Lina Yu supervised and lead the whole research. All authors read and approved the final manuscript.

Ethics declarations

Ethics approval and consent to participate

No human data or tissues were used in this study. All animal experimental protocols and handling procedures were approved by the Zhejiang Animal Care and Use Committee and the Second Affiliated Hospital, School of Medicine, Zhejiang University.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

References

1. Tracey I, Woolf CJ, Andrews NA: **Composite Pain Biomarker Signatures for Objective Assessment and Effective Treatment.** *Neuron* 2019, **101:**783-800.

2. Reid MC, Eccleston C, Pillemer K: **Management of chronic pain in older adults.** *BMJ* 2015, **350:**h532.

3. Gaskin DJ, Richard P: **The economic costs of pain in the United States.** *J Pain* 2012, **13:**715-724.

4. Gupta K, Harvima IT: **Mast cell-neural interactions contribute to pain and itch.** *Immunol Rev* 2018, **282:**168-187.

5. Kamaldin MN, Akhtar MN, Mohamad AS, Lajis N, Perimal EK, Akira A, Ming-Tatt L, Israf DA, Sulaiman MR: **Peripheral antinociception of a chalcone, flavokawin B and possible involvement of the nitric oxide/cyclic guanosine monophosphate/potassium channels pathway.** *Molecules* 2013, **18:**4209-4220.

6. Bannister K, Kucharczyk M, Dickenson AH: **Hopes for the Future of Pain Control.** *Pain Ther* 2017, **6:**117-128.

7. Staats Pires A, Tan VX, Heng B, Guillemin GJ, Latini A: **Kynurenine and Tetrahydrobiopterin Pathways Crosstalk in Pain Hypersensitivity.** *Front Neurosci* 2020, **14:**620.

8. Saika F, Kiguchi N, Matsuzaki S, Kobayashi D, Kishioka S: **Inflammatory Macrophages in the Sciatic Nerves Facilitate Neuropathic Pain Associated with Type 2 Diabetes Mellitus.** *J Pharmacol Exp Ther* 2019, **368:**535-544.

9. Le Bars D, Gozariu M, Cadden SW: **Animal models of nociception.** *Pharmacol Rev* 2001, **53:**597-652.

10. Willemen HL, Eijkelkamp N, Garza Carbajal A, Wang H, Mack M, Zijlstra J, Heijnen CJ, Kavelaars A: **Monocytes/Macrophages control resolution of transient inflammatory pain.** *J Pain* 2014, **15:**496-506.

11. Hanisch UK, Kettenmann H: **Microglia: active sensor and versatile effector cells in the normal and pathologic brain.** *Nat Neurosci* 2007, **10:**1387-1394.

12. Chen G, Zhang YQ, Qadri YJ, Serhan CN, Ji RR: **Microglia in Pain: Detrimental and Protective Roles in Pathogenesis and Resolution of Pain.** *Neuron* 2018, **100:**1292-1311.

13. Ji RR, Suter MR: **p38 MAPK, microglial signaling, and neuropathic pain.** *Mol Pain* 2007, **3:**33.

14. Grace PM, Strand KA, Galer EL, Urban DJ, Wang X, Baratta MV, Fabisiak TJ, Anderson ND, Cheng K, Greene LI, et al: **Morphine paradoxically prolongs neuropathic pain in rats by amplifying spinal NLRP3 inflammasome activation.** *Proc Natl Acad Sci U S A* 2016, **113:**E3441-3450.

15. Raghavendra V, Tanga F, DeLeo JA: **Inhibition of microglial activation attenuates the development but not existing hypersensitivity in a rat model of neuropathy.** *J Pharmacol Exp Ther* 2003, **306:**624-630.

16. Binshtok AM, Wang H, Zimmermann K, Amaya F, Vardeh D, Shi L, Brenner GJ, Ji RR, Bean BP, Woolf CJ, Samad TA: **Nociceptors are interleukin-1beta sensors.** *J Neurosci* 2008, **28:**14062-14073.

17. Takeda M, Kitagawa J, Takahashi M, Matsumoto S: **Activation of interleukin-1beta receptor suppresses the voltage-gated potassium currents in the small-diameter trigeminal ganglion neurons following peripheral inflammation.** *Pain* 2008, **139:**594-602.

18. Gustafson-Vickers SL, Lu VB, Lai AY, Todd KG, Ballanyi K, Smith PA: **Long-term actions of interleukin-1beta on delay and tonic firing neurons in rat superficial dorsal horn and their relevance to central sensitization.** *Mol Pain* 2008, **4:**63.

19. Ren K, Torres R: **Role of interleukin-1beta during pain and inflammation.** *Brain Res Rev* 2009, **60:**57-64.

20. Chan AH, Schroder K: **Inflammasome signaling and regulation of interleukin-1 family cytokines.** *J Exp Med* 2020, **217**.

21. Davis BK, Wen H, Ting JP: **The inflammasome NLRs in immunity, inflammation, and associated diseases.** *Annu Rev Immunol* 2011, **29:**707-735.

22. Cullen SP, Kearney CJ, Clancy DM, Martin SJ: **Diverse Activators of the NLRP3 Inflammasome Promote IL-1beta Secretion by Triggering Necrosis.** *Cell Rep* 2015, **11:**1535-1548.

23. Lin J, Ren J, Gao DS, Dai Y, Yu L: **The Emerging Application of Itaconate: Promising Molecular Targets and Therapeutic Opportunities.** *Front Chem* 2021, **9:**669308.

24. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, Jedrychowski MP, Costa ASH, Higgins M, Hams E, et al: **Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1.** *Nature* 2018, **556:**113-117.

25. Bambouskova M, Gorvel L, Lampropoulou V, Sergushichev A, Loginicheva E, Johnson K, Korenfeld D, Mathyer ME, Kim H, Huang LH, et al: **Electrophilic properties of itaconate and derivatives regulate the IkappaBzeta-ATF3 inflammatory axis.** *Nature* 2018, **556:**501-504.

26. Hooftman A, Angiari S, Hester S, Corcoran SE, Runtsch MC, Ling C, Ruzek MC, Slivka PF, McGettrick AF, Banahan K, et al: **The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation.** *Cell Metab* 2020, **32:**468-478 e467.

27. Allen BL, Montague-Cardoso K, Simeoli R, Colas RA, Oggero S, Vilar B, McNaughton PA, Dalli J, Perretti M, Sher E, Malcangio M: **Imbalance of proresolving lipid mediators in persistent allodynia dissociated from signs of clinical arthritis.** *Pain* 2020, **161:**2155-2166.

28. Sagar DR, Burston JJ, Hathway GJ, Woodhams SG, Pearson RG, Bennett AJ, Kendall DA, Scammell BE, Chapman V: **The contribution of spinal glial cells to chronic pain behaviour in the monosodium iodoacetate model of osteoarthritic pain.** *Mol Pain* 2011, **7:**88.

29. Kuo PC, Weng WT, Scofield BA, Paraiso HC, Brown DA, Wang PY, Yu IC, Yen JH: **Dimethyl itaconate, an itaconate derivative, exhibits immunomodulatory effects on neuroinflammation in experimental autoimmune encephalomyelitis.** *J Neuroinflammation* 2020, **17:**138.

30. Daly R, Blackburn G, Best C, Goodyear CS, Mudaliar M, Burgess K, Stirling A, Porter D, McInnes IB, Barrett MP, Dale J: **Changes in Plasma Itaconate Elevation in Early Rheumatoid Arthritis Patients Elucidates Disease Activity Associated Macrophage Activation.** *Metabolites* 2020, **10**.

31. Glass N, Segal N, Sluka K, Torner J, Nevitt M, Felson D, Bradley L, Neogi T, Lewis C, Frey-Law L: **Examining sex differences in knee pain: the multicenter osteoarthritis study.** *Osteoarthritis and cartilage* 2014, **22:**1100-1106.

32. Swain A, Bambouskova M, Kim H, Andhey PS, Duncan D, Auclair K, Chubukov V, Simons DM, Roddy TP, Stewart KM, Artyomov MN: **Comparative evaluation of itaconate and its derivatives reveals divergent inflammasome and type I interferon regulation in macrophages.** *Nat Metab* 2020, **2:**594-602.

33. Wang SF, Adler J, Lardy HA: **The pathway of itaconate metabolism by liver mitochondria.** *J Biol Chem* 1961, **236:**26-30.

34. Ricardo SD, van Goor H, Eddy AA: **Macrophage diversity in renal injury and repair.** *J Clin Invest* 2008, **118:**3522-3530.

35. Murray PJ, Wynn TA: **Protective and pathogenic functions of macrophage subsets.** *Nat Rev Immunol* 2011, **11:**723-737.

36. Wynn TA, Chawla A, Pollard JW: **Macrophage biology in development, homeostasis and disease.** *Nature* 2013, **496:**445-455.

37. Yam MF, Loh YC, Tan CS, Khadijah Adam S, Abdul Manan N, Basir R: **General Pathways of Pain Sensation and the Major Neurotransmitters Involved in Pain Regulation.** *Int J Mol Sci* 2018, **19**.

38. Pinho-Ribeiro FA, Baddal B, Haarsma R, O'Seaghdha M, Yang NJ, Blake KJ, Portley M, Verri WA, Dale JB, Wessels MR, Chiu IM: **Blocking Neuronal Signaling to Immune Cells Treats Streptococcal Invasive Infection.** *Cell* 2018, **173:**1083-1097 e1022.

39. Liao HY, Hsieh CL, Huang CP, Lin YW: **Electroacupuncture Attenuates CFA-induced Inflammatory Pain by suppressing Nav1.8 through S100B, TRPV1, Opioid, and Adenosine Pathways in Mice.** *Sci Rep* 2017, **7:**42531.

40. Fernandez-Zafra T, Gao T, Jurczak A, Sandor K, Hore Z, Agalave NM, Su J, Estelius J, Lampa J, Hokfelt T, et al: **Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis.** *Pain* 2019, **160:**224-236.

41. Sica A, Mantovani A: **Macrophage plasticity and polarization: in vivo veritas.** *J Clin Invest* 2012, **122:**787-795.

42. Liu T, van Rooijen N, Tracey DJ: **Depletion of macrophages reduces axonal degeneration and hyperalgesia following nerve injury.** *Pain* 2000, **86:**25-32.

43. Mert T, Gunay I, Ocal I, Guzel AI, Inal TC, Sencar L, Polat S: **Macrophage depletion delays progression of neuropathic pain in diabetic animals.** *Naunyn Schmiedebergs Arch Pharmacol* 2009, **379:**445-452.

44. Nadeau S, Filali M, Zhang J, Kerr BJ, Rivest S, Soulet D, Iwakura Y, de Rivero Vaccari JP, Keane RW, Lacroix S: **Functional recovery after peripheral nerve injury is dependent on the pro-inflammatory cytokines IL-1beta and TNF: implications for neuropathic pain.** *J Neurosci* 2011, **31:**12533-12542.

45. Yang S, Zhang X, Zhang H, Lin X, Chen X, Zhang Y, Lin X, Huang L, Zhuge Q: **Dimethyl itaconate inhibits LPS-induced microglia inflammation and inflammasome-mediated pyroptosis via inducing autophagy and regulating the Nrf-2/HO-1 signaling pathway.** *Mol Med Rep* 2021, **24**.

46. Das UN: **Current and emerging strategies for the treatment and management of systemic lupus erythematosus based on molecular signatures of acute and chronic inflammation.** *J Inflamm Res* 2010, **3:**143-170.

47. Opree A, Kress M: **Involvement of the proinflammatory cytokines tumor necrosis factor-alpha, IL-1 beta, and IL-6 but not IL-8 in the development of heat hyperalgesia: effects on heat-evoked calcitonin gene-related peptide release from rat skin.** *J Neurosci* 2000, **20:**6289-6293.

48. Pinho-Ribeiro FA, Verri WA, Jr., Chiu IM: **Nociceptor Sensory Neuron-Immune Interactions in Pain and Inflammation.** *Trends Immunol* 2017, **38:**5-19.

49. Kawasaki Y, Zhang L, Cheng JK, Ji RR: **Cytokine mechanisms of central sensitization: distinct and overlapping role of interleukin-1beta, interleukin-6, and tumor necrosis factor-alpha in regulating synaptic and neuronal activity in the superficial spinal cord.** *J Neurosci* 2008, **28:**5189-5194.

50. Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ: **Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia.** *Br J Pharmacol* 1995, **115:**1265-1275.

51. Torres R, Macdonald L, Croll SD, Reinhardt J, Dore A, Stevens S, Hylton DM, Rudge JS, Liu-Bryan R, Terkeltaub RA, et al: **Hyperalgesia, synovitis and multiple biomarkers of inflammation are suppressed by interleukin 1 inhibition in a novel animal model of gouty arthritis.** *Ann Rheum Dis* 2009, **68:**1602-1608.

52. Gao F, Xiang HC, Li HP, Jia M, Pan XL, Pan HL, Li M: **Electroacupuncture inhibits NLRP3 inflammasome activation through CB2 receptors in inflammatory pain.** *Brain Behav Immun* 2018, **67:**91-100.

53. Latz E, Xiao TS, Stutz A: **Activation and regulation of the inflammasomes.** *Nat Rev Immunol* 2013, **13:**397-411.

Figures

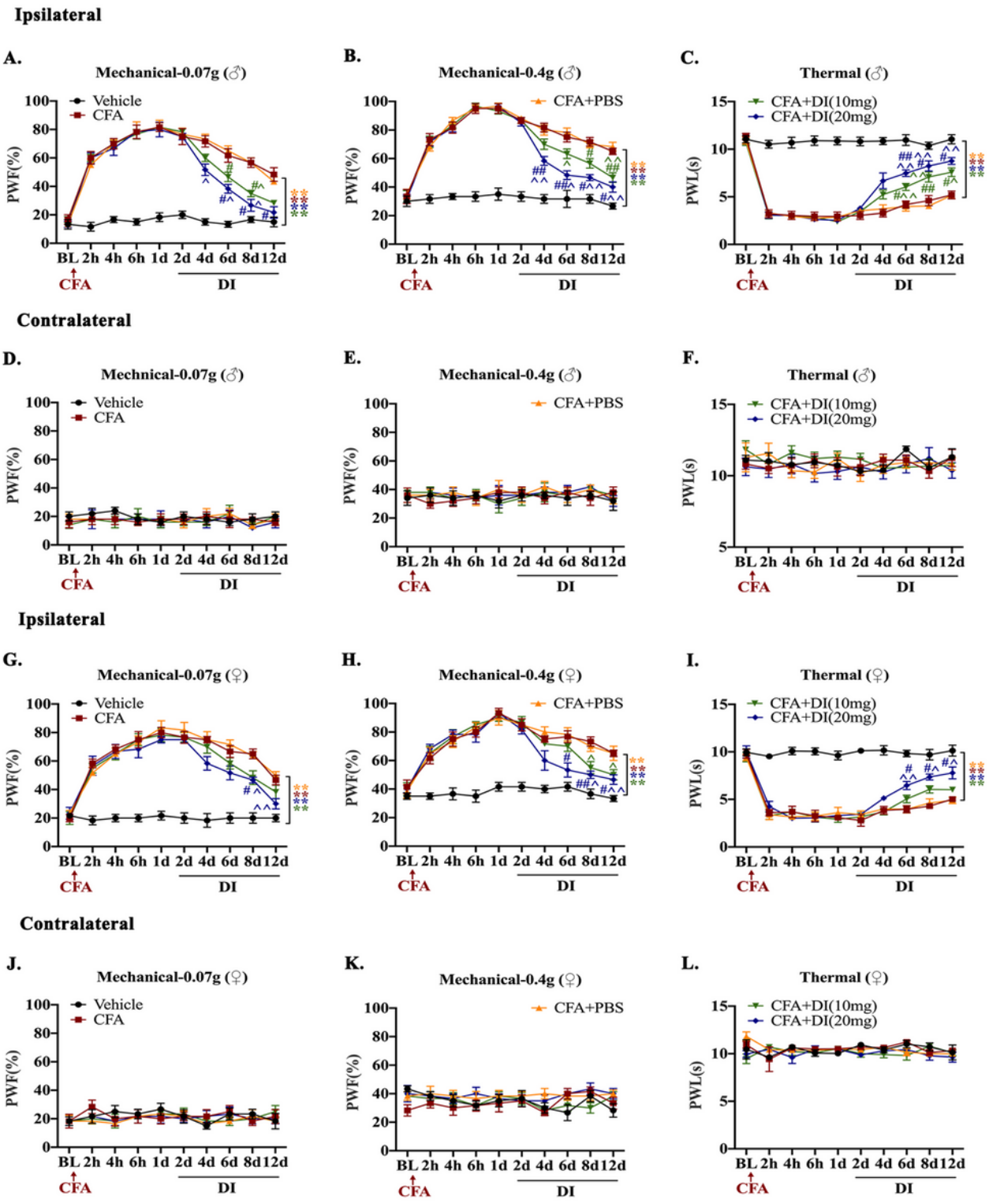

Figure 1

Effects of DI on CFA-induced mice inflammatory pain model. (A-B) The effect on mechanical allodynia (0.07g or 0.4g) of the ipsilateral hind paw in male mice. (C) The effect on thermal hyperalgesia ipsilateral hind paw in male mice. (D-F) The effect of mechanical allodynia and hyperalgesia of the contralateral hind paw. (G-H) The effect on mechanical allodynia (0.07g or 0.4g) of the ipsilateral hind paw in female mice. (I) The effect on thermal hyperalgesia ipsilateral hind paw in female mice. (J-L) The effect of

mechanical allodynia and hyperalgesia of the contralateral hind paw. Data values are expressed as mean ± S.E.M. N = 6 mice/group. **p < 0.01 versus the vehicle group; #p < 0.05, ##p < 0.01 versus the CFA group; ^p < 0.05, ^^p < 0.01 versus the CFA+PBS group.

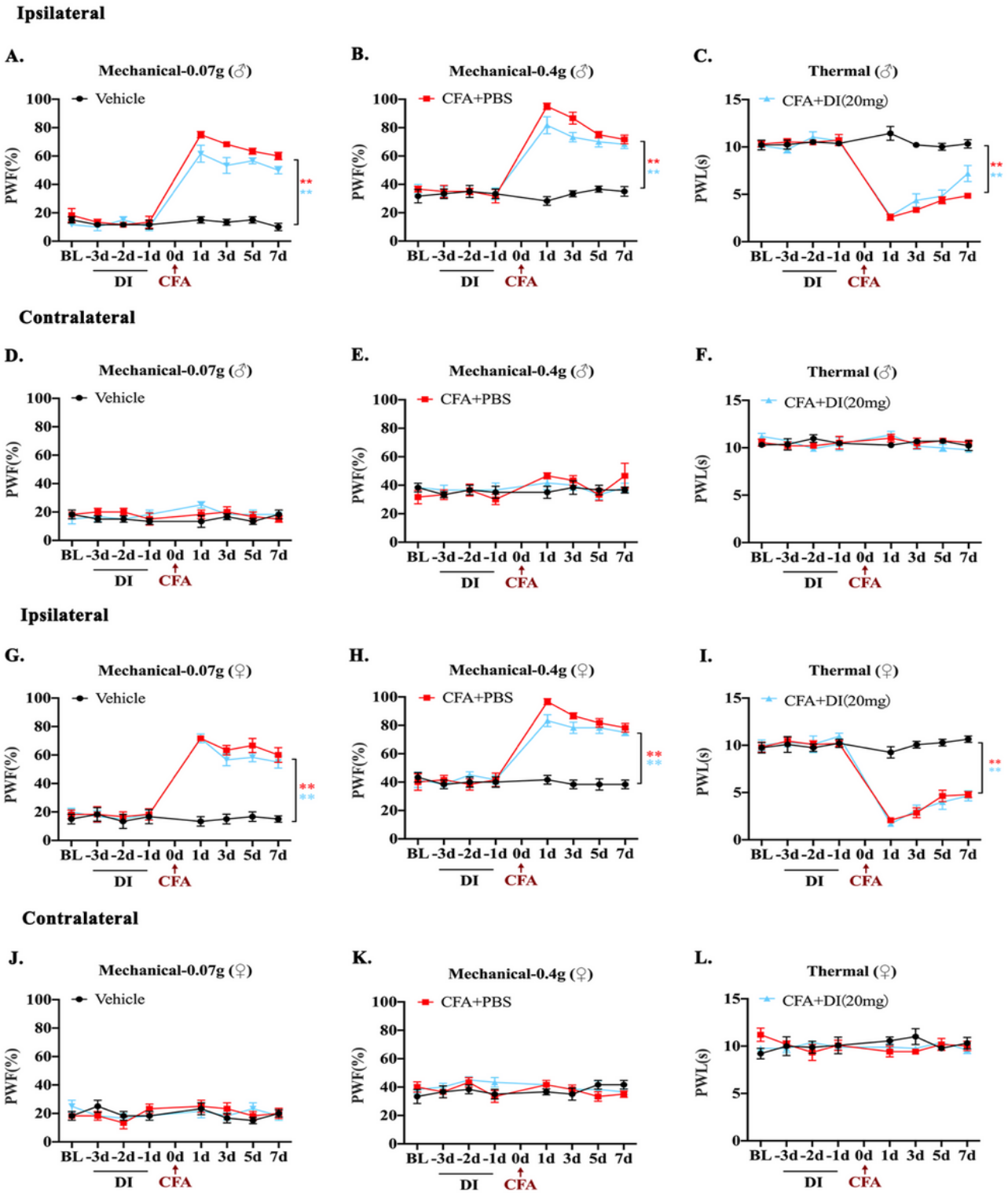

Figure 2

Effects of DI pretreatment on CFA-induced mice inflammatory pain model. (A-B) The effect on mechanical allodynia (0.07g or 0.4g) of the ipsilateral hind paw in male mice. (C) The effect on thermal

hyperalgesia ipsilateral hind paw in male mice. (D-F) The effect of mechanical allodynia and hyperalgesia of the contralateral hind paw. (G-H) The effect on mechanical allodynia (0.07g or 0.4g) of the ipsilateral hind paw in female mice. (I) The effect on thermal hyperalgesia ipsilateral hind paw in female mice. (J-L) The effect of mechanical allodynia and hyperalgesia of the contralateral hind paw. Data values are expressed as mean ± S.E.M. N = 6 mice/group. **p < 0.01 versus the vehicle group.

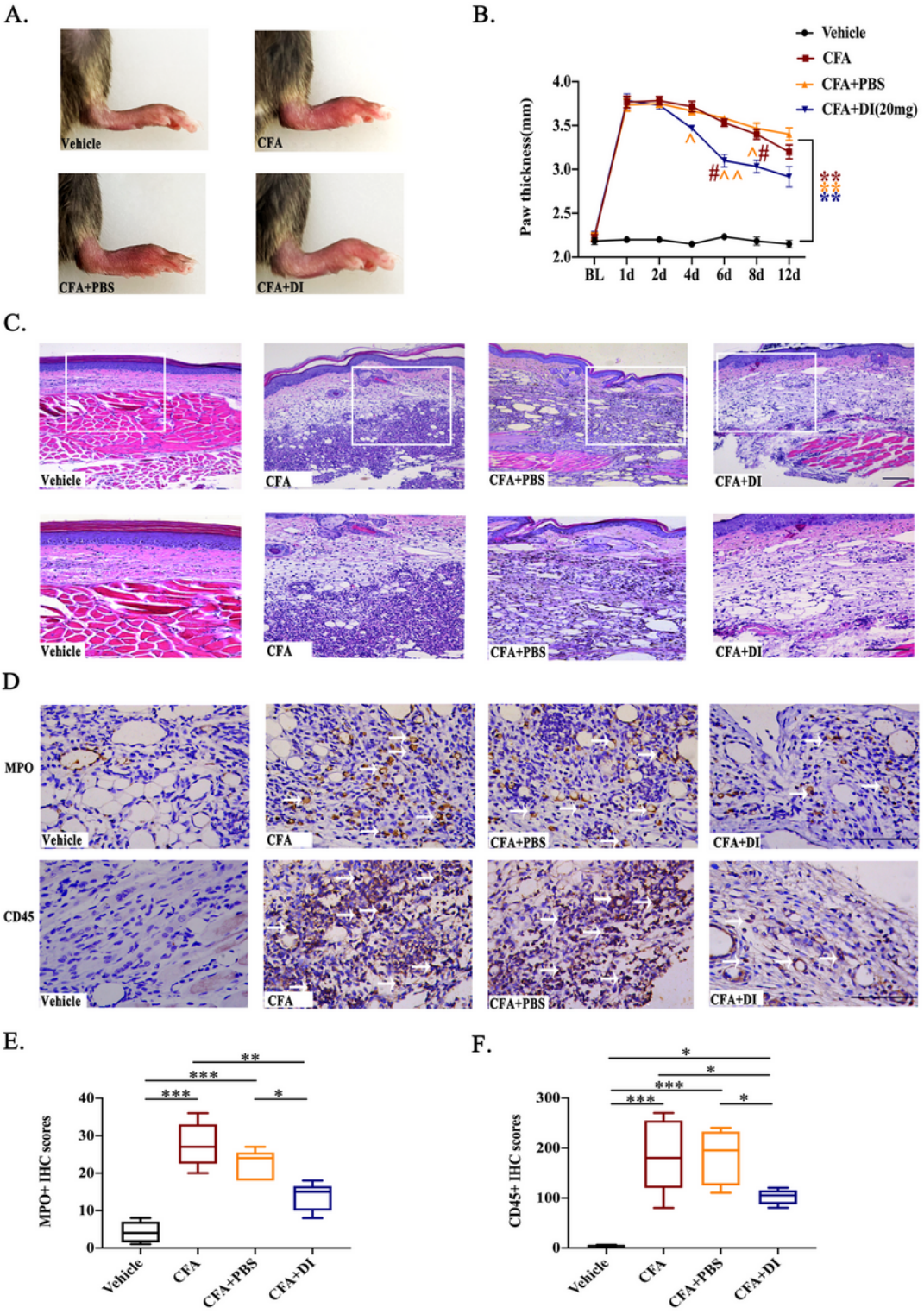

Figure 3

Effect of DI on paw thickness and Inflammatory cell infiltration in CFA model. (A) Macroscopic images of hind paw derived from vehicle, CFA and CFA+PBS and CFA+DI-treated mice. (B) Hind paw thickness (mm) as a function of time (days) was measured in each group. (n=6 mice/group) (C) H&E staining. Inflammation with abundant lymphocytes and sparse neutrophilic granulocytes in an CFA mouse with hypodermic injection and low-grade inflammation in an CFA mouse with hypodermic injection of DI (20mg). This experiment was repeated independently 3 times and similar results were obtained. Scale bar represents 200 μm and 100μm. (D) Photomicrographs representing MPO and CD45 immunoreactivity in each group. Arrowheads indicate positive cells. (E-F) MPO score and CD45 score. Scale bar represents 50 μm. This experiment was repeated independently 3 times and similar results were obtained. *p < 0.05, **p < 0.01and ***p < 0.001 versus the vehicle group; #p < 0.05 versus the CFA group; ^p < 0.05, ^^p < 0.01 versus the CFA+PBS group.

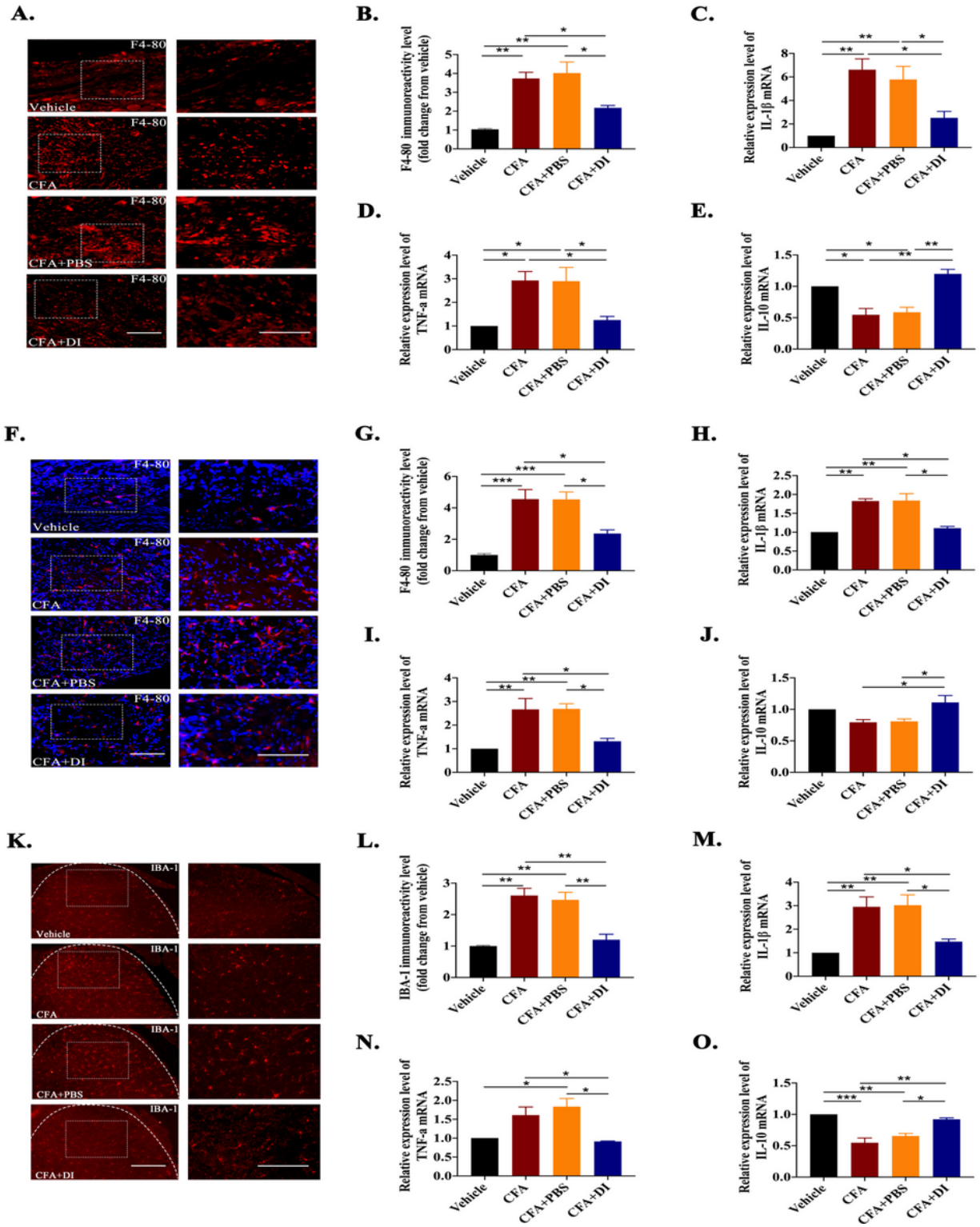

Figure 4

Effect of DI on macrophage and microglia activation. (A) Immunostaining of F4-80 (red) showing macrophages activation in paw tissue. Scale bar represents 100 μm (left) and 50 μm (right). (B) Representative immunofluorescence staining level for F4-80 , fold change versus the vehicle group. Real-time PCR were used to detect pro-inflammatory factors IL-1β (C), TNF-α (D) and anti-inflammatory factor IL-10 (E). (F) Immunostaining of F4-80 (red) and DAPI (blue) showing macrophages activation in DRG.

Scale bar represents 100 μm (left) and 50 μm (right). (G) Representative immunofluorescence staining level for F4-80 , fold change versus the vehicle group. Real-time PCR were used to detect pro-inflammatory factors IL-1β (H), TNF-α (I) and anti-inflammatory factor IL-10 (J). (K) Immunostaining of IBA-1(red) showing microglia activation in spinal cord. Scale bar represents 200 μm (left) and 100 μm (right) .(L) Representative immunofluorescence staining level for IBA-1, fold change versus the vehicle group. Real-time PCR analysis of IL-1β (M), TNF-α (N) and IL-10 (O) expression relative to vehicle levels in spinal cord. This experiment was repeated independently 3 times and similar results were obtained. *p < 0.05, **p < 0.01 and ***p < 0.001.

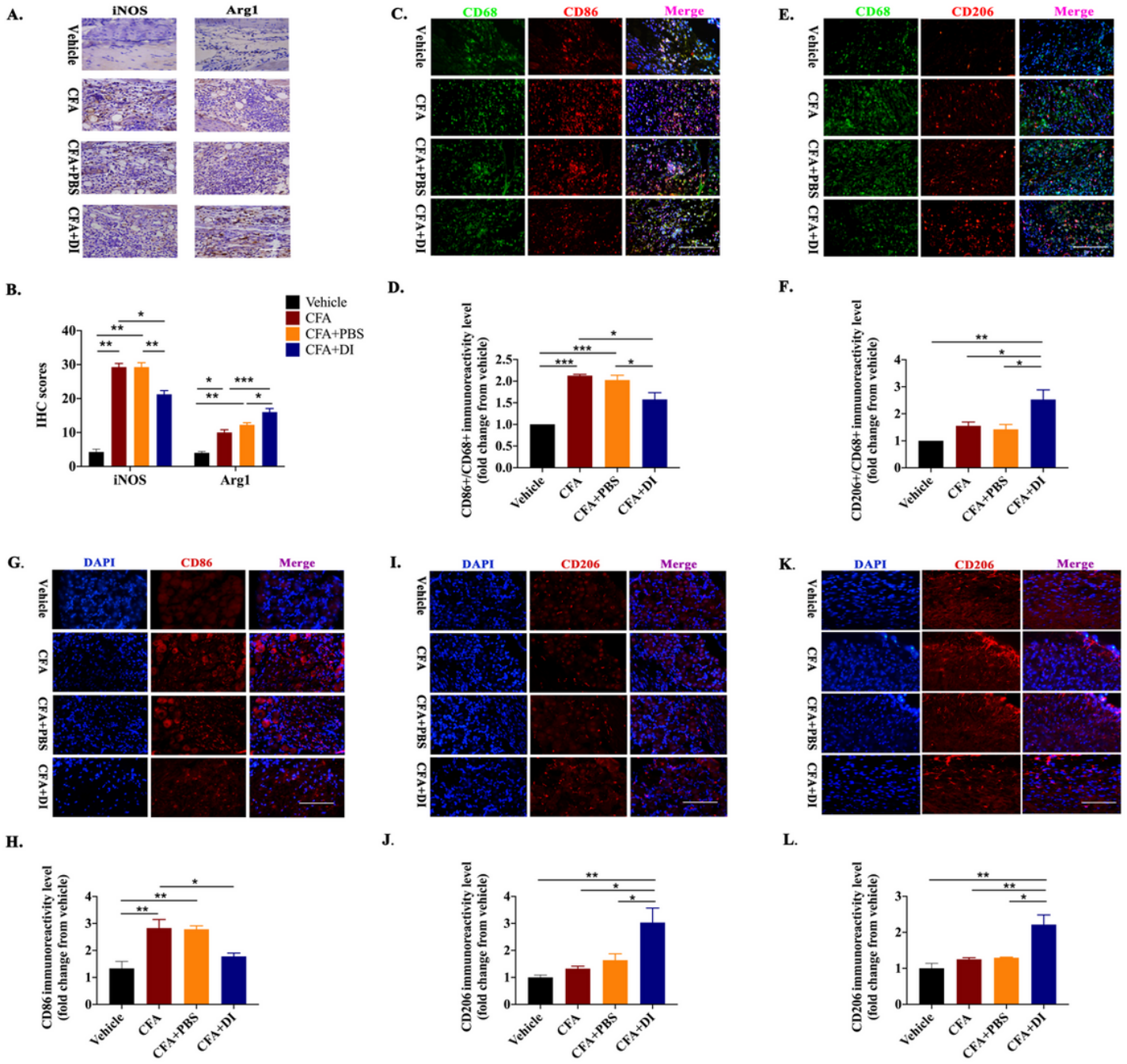

Figure 5

Effect of DI on macrophage/microglia polarization. (A) Photomicrographs representing iNOS and Arg1 immunoreactivity in each group. Arrowheads indicate positive cells. (B) iNOS score and Arg1 score were detected by the way same as above. Scale bar represents 50 μm.(C,E) Immunofluorescent staining for CD68 (Green), CD86 (red) and CD206 (red) , and nuclei were counterstained using DAPI (blue). Scale bar represents 50 μm. Representative immunofluorescence staining level for CD86+/CD68+ (D) and CD206+/CD68+ (F), fold change versus the vehicle group.Immunostaining of CD86 (G) and CD206 (I) in DRG. Scale bar represents 50 μm. Representative immunofluorescence staining level for CD86 (H) and CD206 (J), fold change versus the vehicle group. (K) Immunostaining of CD206 in spinal cord. Scale bar represents 50 μm. (L) Representative immunofluorescence staining level for CD206, fold change versus the vehicle group. This experiment was repeated independently 3 times and similar results were obtained. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

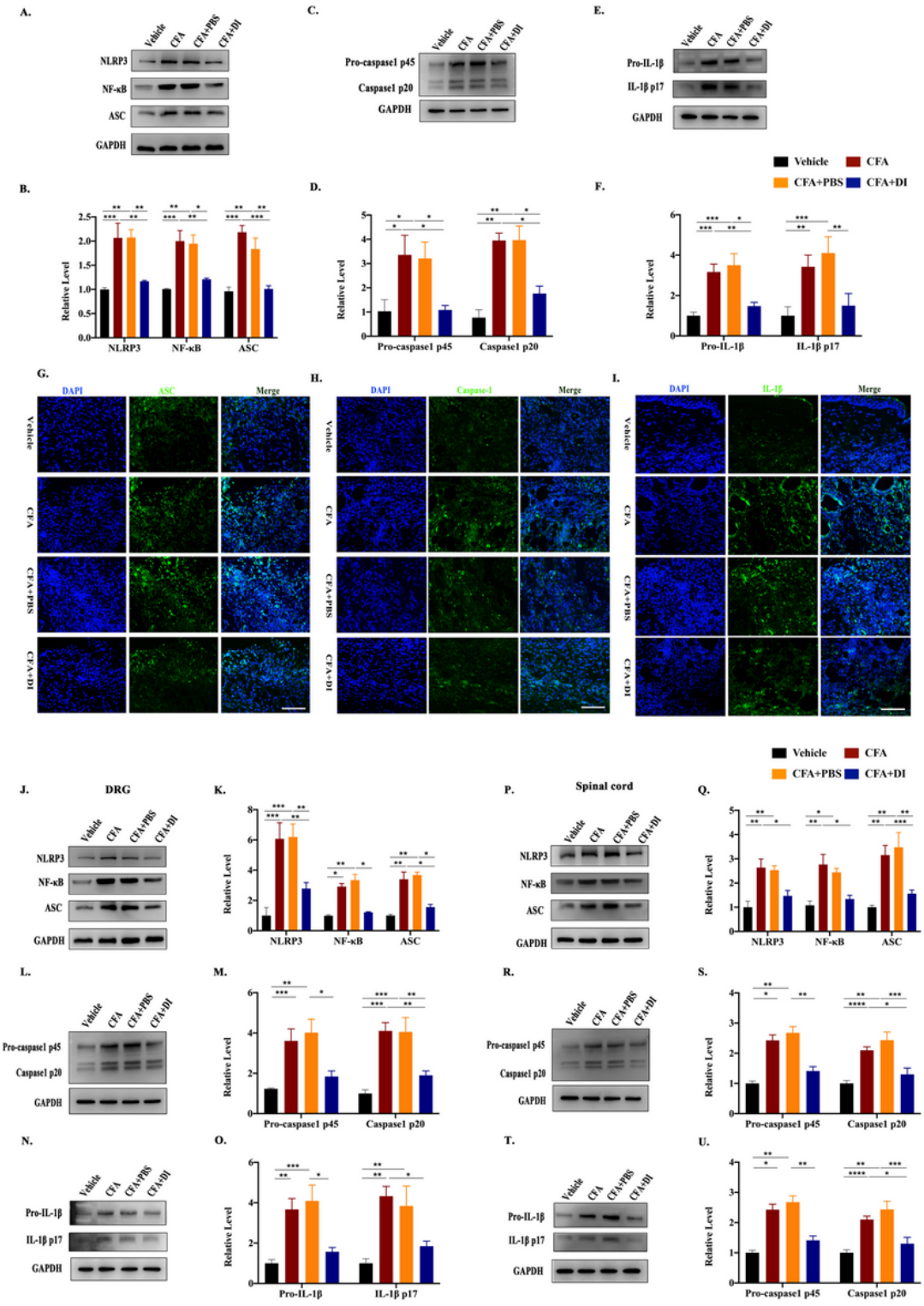

Figure 6

DI inhibited NLRP3 inflammasome activation and IL-1β secretion. (A,B) Western blotting bands and analysis of NLRP3, ASC, NF-κB in plantar tissue. (C,D) Western blotting bands and analysis of Caspase1 p45 and p20 in plantar tissue. (E,F) Western blotting bands and analysis of pro- IL-1β and IL-1β p17 in plantar tissue. Immunofluorescent staining for ASC (green) (G), Caspase-1(green) (H), IL-1β(green) (I), the nuclei were stained blue with DAPI. Scale bar indicates 50 μm. (J,K) Western blotting bands and analysis

of NLRP3, ASC, NF-κB in DRG. (L,M) Western blotting bands and analysis of Caspase1 p45 and p20 in DRG. (N,O) Western blotting bands and analysis of pro- IL-1β and IL-1β p17 in DRG. (P,Q) Western blotting bands and analysis of NLRP3, ASC, NF-κB in spinal cord. (R,S) Western blotting bands and analysis of Caspase1 p45 and p20 in spinal cord. (T,U) Western blotting bands and analysis of pro- IL-1β and IL-1β p17 in spinal cord. N=3-5 biological repeats, 2 mice/group/repeat. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

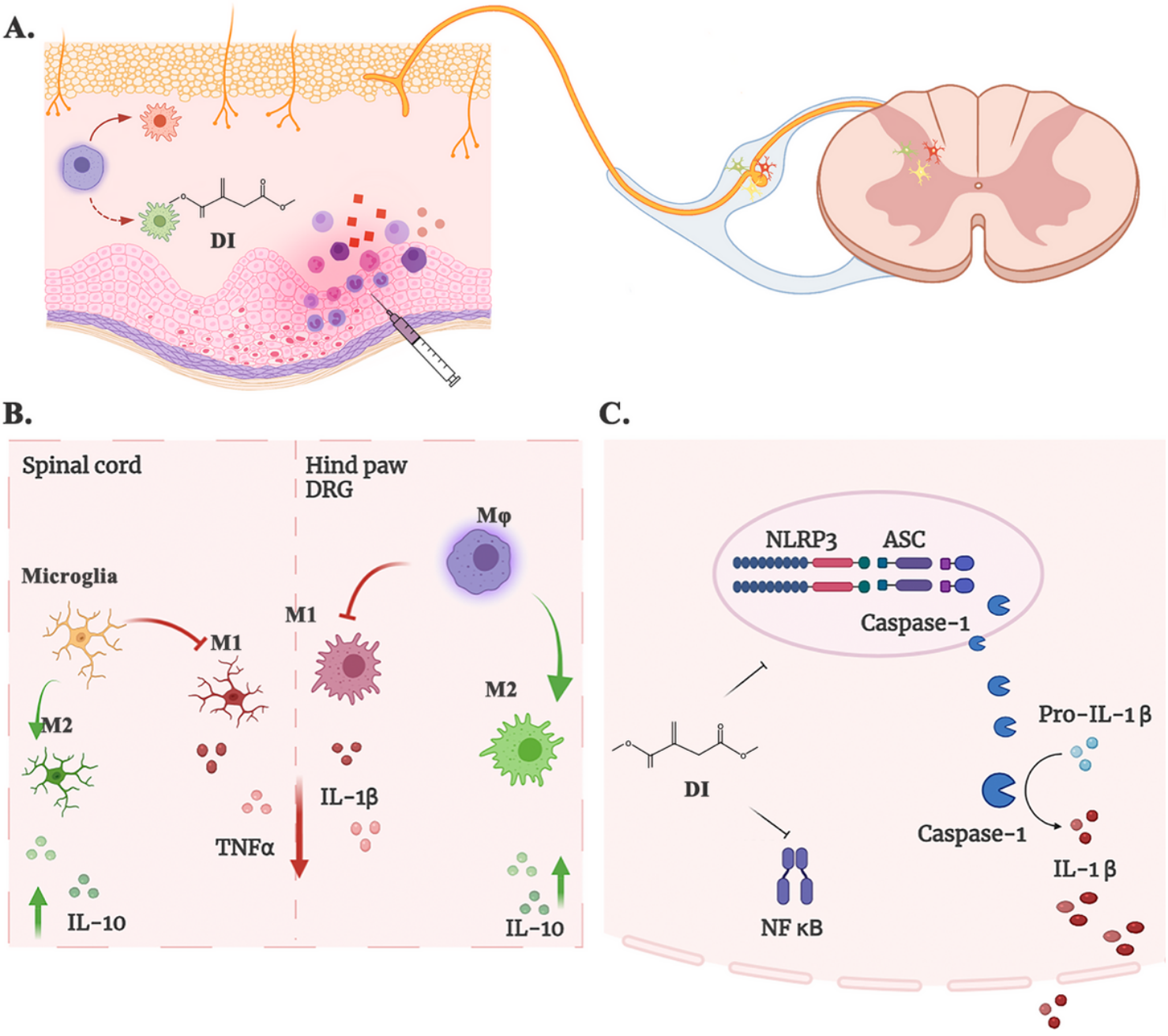

Figure 7

(A)The enhanced microglia proliferation in the spinal cord and the infiltration of macrophages into the DRG and paw tissue of the CFA mice contribute to the development of inflammatory pain. (B) DI regulated and inhibited the imbalance between pro-inflammatory and anti-inflammatory response.(C) DI

suppressed the assembly of NLRP3 inflammasomes complexes and NF-κB thereby inhibited the secretion of IL-1β.