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ORIGINAL PAPER

Interleukin-1 Receptor Associated Kinase 1 Mediates the Maintenance of Neuropathic Pain after Chronic Constriction Injury in Rats

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Abstract

Neuropathic pain (NP) has complicated pathogenesis as it mainly involves a lesion or dysfunction of the somatosensory nervous system and its clinical treatment remains challenging. Chronic constriction injury (CCI) model is a widely used neuropathic pain model and involved in mechanisms including both nerve inflammatory and injury. Cytokines and their receptors play essential roles in the occurrence and persistence of neuropathic pain, but the underlying mechanisms have not well been understood. Therefore, Interleukin-1 receptor-associated kinase 1 (IRAK1) is chosen to explore the possible mechanisms of NP. In the present study, IRAK1 was found to persistently increase in the dorsal root ganglion (DRG) and spinal cord (SC) during CCI detected by western blot. The staining further confirmed that IRAK1 was mainly co-located in the DRG astrocytes or SC neurons, but less in the DRG microglia or SC astrocytes. Moreover, the region of increased IRAK1 expression was observed in superficial laminae of the spinal dorsal horn, which was the nociceptive neuronal expression domain, suggesting that IRAK1 may mediated CCI-induced pain by nociceptive primary afferent. In addition, intrathecal injection of Toll-like receptor 4 (TLR4) inhibitor or IRAK1 siRNA decreased the expression of IRAK1 accompanied with the alleviation of CCI-induced neuropathic pain. The upregulation of p-NF-κB expression was reversed by IRAK1 siRNA in SC, and intrathecal injection of p-NF-κB inhibitor relieved neuropathic pain. Taking together, targeting IRAK1 may be a potential treatment for chronic neuropathic pain.

Keywords Neuropathic pain (NP) · IRAK1 · P-NF-κB · Chronic constriction injury (CCI)

Introduction

Neuropathic pain, which is characterized by allodynia and hyperalgesia resulting from damage or abnormal function of the nervous system, is one of the most intractable human complaints its clinical treatment effect is still unsatisfied [1]. Chronic sciatic nerve injury is a major cause of sciatica in adults, which is related to complex mechanisms including both local inflammatory and neuropathic pain. For example, the CCL2-CCR2 axis modulate CCI-induced pain hypersensitivity via driving monocyte/macrophage infiltration [2], which means inflammatory mediators (TNF-α, IL-6, et al.) can be synthetized and released in surgery area [3, 4]. The inflammatory mediators work on damage region and further affect neurons and/or glial cells of peripheral and central nervous system, subsequently induce the release of inflammatory mediators as well as pain-related substances [5–7]. Throughout the process, the inflammatory mediators positively feedback between nerve cells, which plays an important role in the

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emergence and maintenance of pain. On the other hand, neural plasticity, synaptic plasticity and neuronal adaptive responses are also regarded as significant features of neuropathic pain. Neuronal and synaptic plasticity, which include ectopic generation of action potentials, facilitation and disinhibition of synaptic transmission, is a state that the morphology of cell bodies as well as the corresponding function change caused by various factors, such as damage and inflammation.

Interleukin-1 receptor-associated kinases (IRAKs) family of serine-threonine kinases, reported to be involved in various biological responses, such as inflammatory response [8], tumor cell migration and proliferation [9], consist of four members including IRAK1 and IRAK4 (active kinases), IRAK2 and IRAKM (inactive kinases). IRAK1 is a multifunctional protein that interacts with many molecules involved in intracellular signaling, such as MyD88. IRAK1 binds the MyD88 and subsequently undergoes phosphorylation by IRAK4 as well as by auto-phosphorylation [10, 11]. The auto-phosphorylation can be triggered by IRAK4 [10] and its phosphorylation increased MyD88s affinity for downstream signaling of IRAK1 [12, 13] to mediate inflammatory response. However, whether IRAK1 takes part in chronic pain, and the expression as well as cell distribution of IRAK1 in the DRG and SC still remains obscure.

Multiple reports show that the toll-like receptor 4 (TLR4) in nerve tissue contributes to neuropathic pain, innate neuroimmunity, arthritis pain, and peripheral neuropathy [14–16]. After stimulating of its ligand LPS, TLRs induces MyD88-dependent signaling through the activation of IRAK4 which acts as the upstream of IRAK1 and phosphorylates this kinase [17], to forming TLR4-MyD88-IRAK4-IRAK1 signaling. And subsequently, several signaling pathways are activated by IRAK1, including NF-κB, MAPKs, and IFN [18, 19]. For example, the head and neck cancer cells also were induced to death by the inhibition of IRAK1 and its downstream signaling such as ERK1/2 was attenuated at the same time [20]. After phosphorylation, IRAK1 and TRAF6 then form a protein complex which cause the activation of NF-κB signaling pathway to mediate inflammatory response [21]. Although the expression of cytokine is independent, IRAK1 also can increase the cytokine production via downstream signaling [19, 22]. Moreover, both relate-downstream pathway (eg, NF-κB, p38) and cytokine (eg, TNF-α, IL-6) have a significant effect caused by IRAK1/4 inhibitor in AMs or PBMCs [18]. In general, IRAK1, activated by TLR4, have been shown to mediate inflammation via the regulation of cytokine. However, whether IRAK1 mediates CCI-induced pain through the above-mentioned mechanism remains to be detected.

In this study, we investigated the expression and distribution of IRAK1 in the dorsal root ganglion (DRG) and spinal cord (SC), and the region of increased IRAK1-positive cells. Then its role was assessed in central and peripheral nervous system of CCI-induced neuropathic pain in rats.

Materials and Methods

Animals and Surgical Procedure

Experiments were conducted using male Adult Sprague Dawley rats (220–250 g) obtained from the Animal Center of Nantong University, which were housed at 22 ± 1 °C under a 12 h/12 h light–dark cycle. They had free access to food and water. All experiments were performed according to the Animal Use and Care Committee for Research and Education of Nantong University. Animal treatments were approved by the Guidelines of the International Association for the Study of Pain [23]. According to Bennett's method, CCI was performed in the rat following the established surgical procedures. After anesthetized by isoflurane, the rats were placed in a prone position. The sciatic nerve was exposed after the separation of biceps femoris muscle at the middle of the femur. Four ligatures were tied loosely around the sciatic nerve above the start of the nerve by 4.0 silk thread. The same procedure was performed in sham group without ligation of the sciatic nerve.

Behavioral Testing

Before the experiments, the animals were allowed to habituate to the testing environment for 2 days. The whole procedure was performed at fixed room temperature and humidity. For testing mechanical allodynia, the rats were placed on a wired mesh inside a plexiglas compartment for 10 min to adapt to the new environment, and then different von-Frey filaments were used to measure the mechanical allodynia. Each filament (1.4–26 g, Stoelting, Wood Dale, IL) was pressed to the rat's plantar for 1 s at intervals of 3 min between the tests. The 50% withdrawal threshold was determined using Dixon's up–down method [24]. For testing heat sensitivity, the rats were placed in the Plantar Test device (Life Science Model 390G; IITC Life Science Inc., Woodland Hills, CA) to acclimate for 10 min. The rats' foot plantar surface was radiated by infrared ray, which was placed under their injured foot. The duration of rats' tolerance in seconds can be record form turning on the device to which rats moved their feet because of burning. The radiant heat was set at 30% intensity during all experiments. The device was cut off when more than 20 s to prevent potential injury.

Western Blotting

On deeply anesthetized rats, the spinal cord of lumbosacral enlargement and dorsal root ganglia of L4–5 were quickly

extracted at naïve groups, sham groups, 1 day to 3 weeks groups after CCI. Tissue samples were homogenized in lysis buffer, which contains Tris, 50 mmol/l; EDTA, 0.5 M; SDS, 10%; NP-40, 1%; sodium deoxycholate, 1%; Triton X-100, 1%; PMSF, 1 mM; aprotinin, 101 g/ml; leupeptin, 11 g/ml. Then, they were centrifuged at 13,000 rpm for 15 min at 4 °C. The protein concentration was estimated according to the Bradford method (Bio-Rad). Then the samples, which were loaded for each lane, separated with SDS-polyacrylamide gel electrophoresis and transferred onto PVDF filter membrane. The membranes were then blocked in 5% non-fat milk for 2 h at room temperature. The membranes was scissored according to molecular weight and were separately incubated overnight at 4 °C with mouse anti-IRAK1 antibody (1:500, Santa Cruz), mouse anti-β-actin (1:500, Santa Cruz), mouse anti-p-NF-κB (1:500; Cell Signaling Technology) primary antibodies. After primary incubation, the membranes were washed for 3 × 5 min with TBST and then were incubated with secondary antibody conjugated with horseradish peroxidase (1:5000, Proteintech) for 2 h. The protein signals were detected using enhanced chemiluminescence (ECL, Millipore, USA).

Immunofluorescent

After being deeply anesthetized, the rats were transcardially perfused with 0.9% sodium chloride (300 ml), and then with 4% paraformaldehyde (500 ml). After that, the spinal cord of lumbosacral enlargement and L4–5 DRG were quickly extracted and fixed in paraformaldehyde overnight. Later, the tissues were removed to 20% sucrose following 30% sucrose to equilibrating. In a cryostat, SC and DRG sections of L4–5 (10 μm) were cut onto the glass slides after embedding in optimum cutting temperature (OCT). These sections were incubated in blocking solution, which consists of 10% normal serum (species the same as the secondary antibody), 3% (w/v) BSA, 0.1% Triton X-100 and 0.05% Tween-20, at room temperature for 2 h, following incubated overnight at 4 °C with the primary antibodies: IRAK1 antibody (anti-mouse, 1:50, Santa Cruz), microtubule-associated protein 2 (MAP2) antibody (anti-rabbit, 1:200; Abcom), glial fibrillary acidic protein (GFAP) antibody (anti-rabbit, 1:200; Sigma), IbA1 antibody (anti-rabbit, 1:200; Wako), Neuron-specific nuclear protein (NeuN) antibody (anti-rabbit, 1:200; Cell Signaling Technology), p-NF-κB (anti-mouse, 1:50; Cell Signaling Technology). After primary incubation, the sections were washed for 3 × 5 min in PBS and were then incubated in fluorescein isothiocyanate (1:800, Jackson lab) and/or CY3-conjugated secondary antibodies (1:800, Jackson lab) for 2 h in dark room. After washed in PBS for 3 × 5 min again, a coverslip was adhered onto each glass slide. The images of the sections were examined using a fluorescence microscope (Leica, DM 5000B; Leica CTR 5000; Germany).

Drugs, IRAK1 siRNA and Lumbar Intrathecal Injection

The rat IRAK1 small interfering RNA (siRNA, targeting the complementary DNA sequence) was designed and synthesized by Genepharma Technology (Shanghai, China) company. The nucleotide sequences were: 5′-CCUUAGAGG AUCAGCUUCATT-3′ (sense), 5′-UGAAGCUGAUCC UCUAAGGTT-3′ (antisense). Negative control siRNA was synthesized by a scrambled sequence of nucleotides as a control siRNA.

TAK242 (an inhibitor of Toll-like receptor 4) and BAY11-7082 (an inhibitor of p-NF-κB) were purchased from MedChem Express (USA), which were dissolved in 40% dimethyl sulfoxide (DMSO). siRNA was dissolved by RNase-free water to a concentration of 0.75 μg/μl and then mixed with polyethyleneimine (PEI, dissolved in 5% glucose, 1 μg of siRNA was mixed with 0.18 μl of PEI) for 10 min. After isoflurane inhales anesthesia, TAK242 (50 μg or 100 μg) or BAY11-7082 (100 μg) or siRNA (20 μl) was slowly injected into the subarachnoid space within 30 s between L5 and L6. When the needle entered into the subarachnoid space, a sudden slight flick of the tail could be observed.

TAK-242 (50 μg or 100 μg) or BAY11-7082 (100 μg) or siRNA was performed intrathecal injection for three continuous days (day 1, 2, 3) after CCI. Western blot analysis was used to confirm the knock-down efficiency at day 3, 6 h after the last injection.

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein samples were prepared in the same way as for Western blot. The levels of TNF-α and IL-6 were detected by ELISA kits (Elabscience Biotechnology, E-EL-R0015c; Beyotime) according to the manufacturer's instructions.

Quantification and Statistics

Data were analyzed by SPSS software and expressed as means ± SEM. The behavioral data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni test as the multiple comparison analysis. The density of specific bands and fluorescence intensity were measured with a computer-assisted imaging analysis system (ImageJ). Differences between two groups were analyzed with Student t-test. A value of $P < 0.05$ was considered statistically significant.

Results

CCI Produced Neuropathic Pain Over Time

To investigate the role of IRAK1 in nerve injury-induced neuropathic pain, we established CCI model of rat to induce neuropathic pain. Compared with the sham groups, rats of CCI groups showed an obvious and persistent thermal hyperalgesia and mechanical allodynia which showed a distinct decrease of paw withdrawal latency (PWL) and paw withdrawal threshold (PWT) in ipsilateral hind limb paw from postoperative day 1 to day 21, partially recovered at 28 days ($P < 0.05$, Fig. 1a, b). However, no differences were observed in PWT and PWL of sham groups compared with their value of baseline ($P > 0.05$, Fig. 1a, b). These results indicated the successful establishment of the CCI models.

The Expression and Cellular Distribution of IRAK1 in DRG Glia Cells in Rats After CCI

We further examined the expression of IRAK1 in the rat DRG of CCI model. The western blot analysis showed that CCI induced the expression of IRAK1 in the DRG to increase gradually, evidently and long-lastingly from post-injury day 1, peaked from day 3 to day 5, and declined at day 10 compared with the naive groups ($P < 0.05$, Fig. 2a, b). These results meant that the sciatic nerve ligature-induced the expression of IRAK1. Then, we characterized the cellular profiles of IRAK1 expression in the DRG to explore its potential role in the nociceptive response. The staining showed that IRAK1 had high expression in the DRG at day 5 post CCI but was with low expression in sham groups (Fig. 2c, d). The result also showed that IRAK1 was mostly co-localized with astrocyte marker GFAP (Fig. 2k–m) and less co-localized with microglia marker IbA1 (Fig. 2h–j), except neurons marker MAP2 (Fig. 2e–g), suggesting that IRAK1 was localized in DRG glia cells to participate in the modulation of NP. In other words, IRAK1 mediated CCI-induced pain through inflammation-related cells in the DRG, and cytokine probably plays an irreplaceable role.

Fig. 2 The expression of IRAK1 in the DRG after CCI. Western blot showed that IRAK1 protein level was upregulated in the CCI groups compared to the naïve groups (**a**, **b**). The distribution of IRAK1 in the DRG after CCI. IRAK1 was constitutively expressed in the sham groups (**c**) and increased at 5 days (**d**) after CCI operation. Double staining showed that IRAK1 was co-localized with astrocyte marker GFAP (**k**–**m**), less co-localized with microglia marker IbA1 (**h**–**j**), except neuron marker MAP2 (**e**–**g**). Scale bars, 50 μm (**c**–**m**). *$P < 0.05$ versus naïve. n = 3 for immunohistochemistry

The Expression and Cellular Distribution of IRAK1 in SC Neurons of Rat After CCI

To investigate whether participated in central pain sensitization, we then checked IRAK1 protein expression and cellular distribution in the SC of rats. The results showed that IRAK1 protein level was observed to increase from post-operation day 1, peaked at day 5, and downgraded at day 14 detected by western blot compared with naïve groups ($P < 0.05$, Fig. 3a, b), which was parallel with the pain course induced by CCI. To investigate the cellular

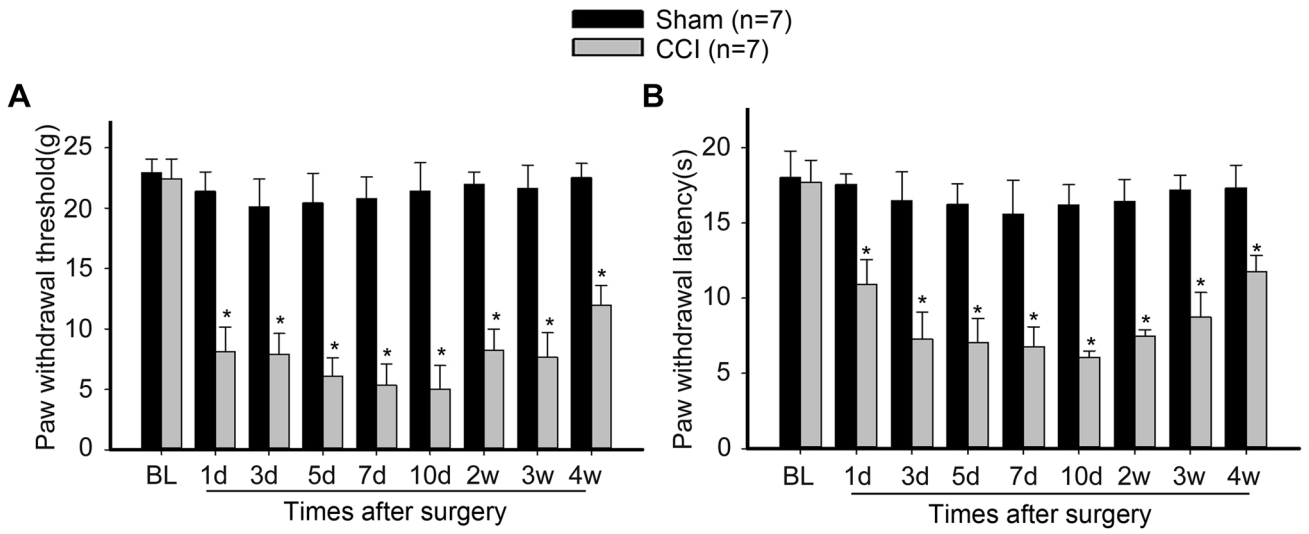
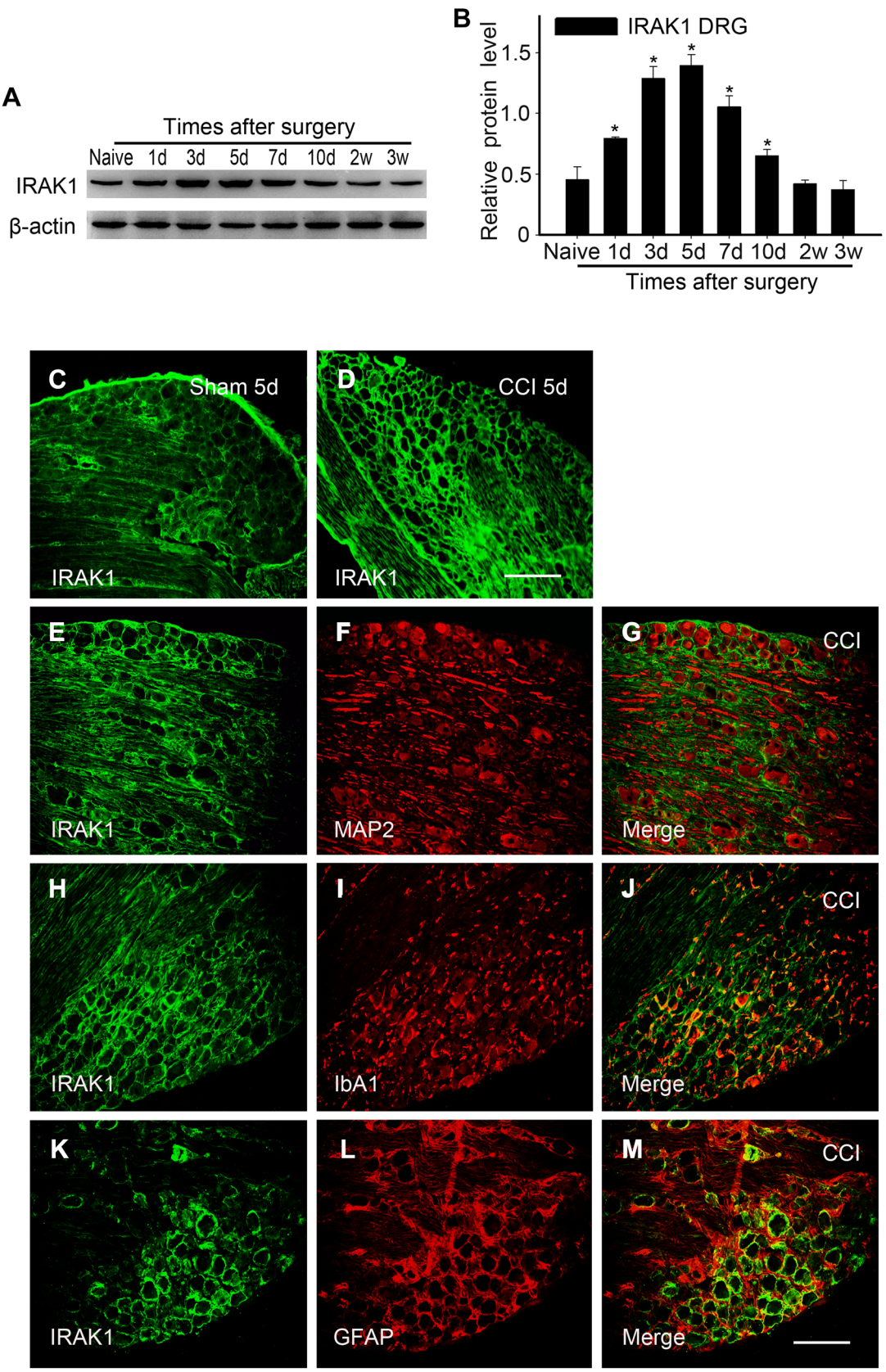


Fig. 1 The value of mechanical allodynia and heat hyperalgesia in rats after CCI. Compared to sham groups, CCI induced a significant decrease in PWT (**a**) and PWL (**b**). *$P < 0.05$ versus sham. *BL* baseline. Two-way ANOVA followed by Bonferroni test. Seven rats per group


A
Times after surgery
Naive 1d 3d 5d 7d 10d 2w 3w
IRAK1
β-actin
B
IRAK1 DRG
Relative protein level
1.5
1.0
0.5
0
Naive 1d 3d 5d 7d 10d 2w 3w
Times after surgery
C
Sham 5d
IRAK1
D
CCI 5d
IRAK1
E
IRAK1
F
MAP2
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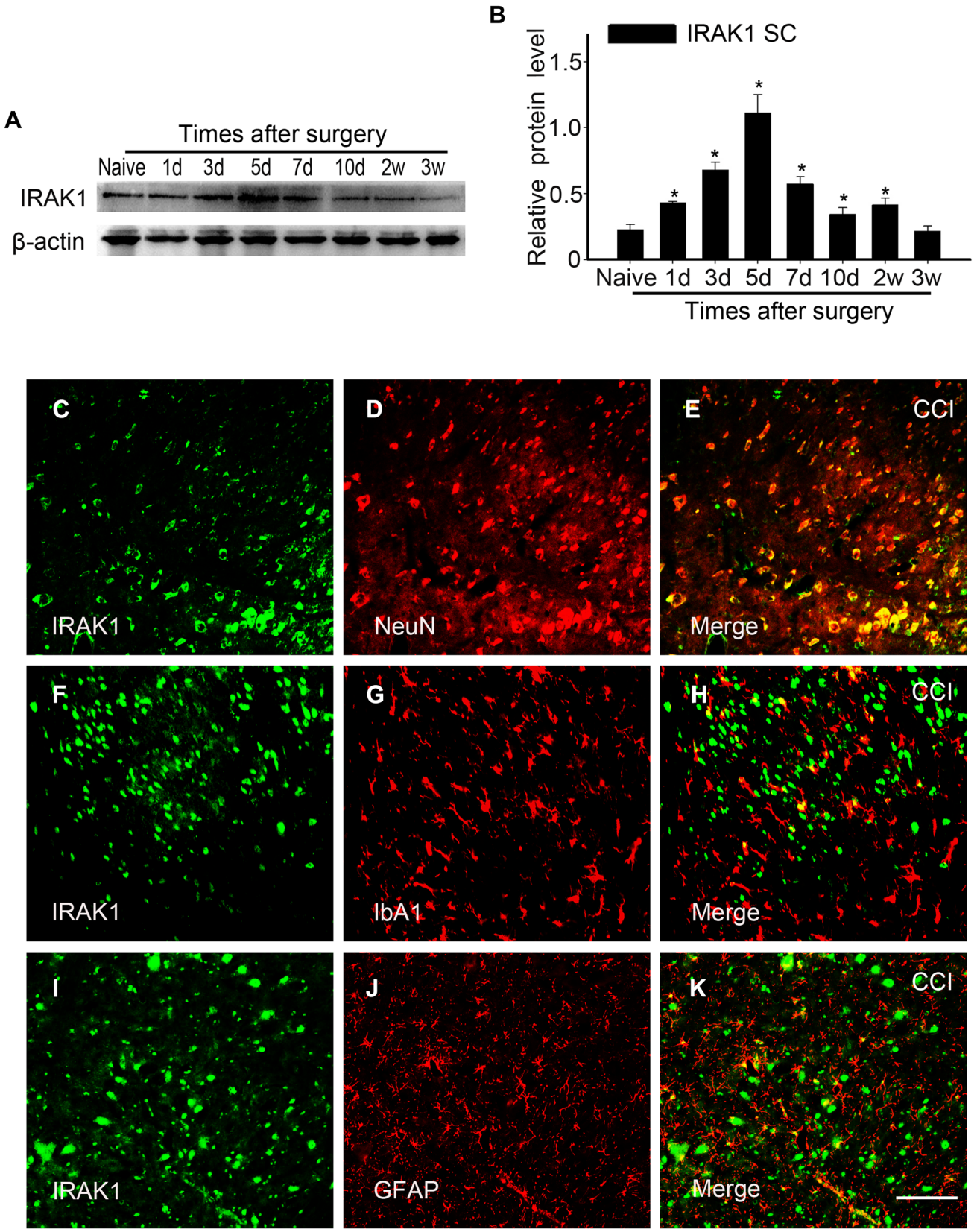
A
Times after surgery
Naive 1d 3d 5d 7d 10d 2w 3w
IRAK1
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Relative protein level
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Naive 1d 3d 5d 7d 10d 2w 3w
Times after surgery
C
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CCI
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F
IRAK1
G
IbA1
H
CCI
Merge
I
IRAK1
J
GFAP
K
CCI
Merge

◂**Fig. 3** The expression and distribution of IRAK1 in the SC after CCI. Western blot was performed to detect IRAK1 expression in the spinal cord (**a**, **b**). Compared to naïve groups, the expression of IRAK1 increased after CCI surgery (**a**, **b**). Double staining showed that IRAK1 co-localized with neuron marker NeuN (**c**–**e**), less co-localized with astrocyte marker GFAP (**i**–**k**), except microglia marker IbA1 (**f**–**h**). Scale bars, 20 μm (**c**–**k**). *$P < 0.05$ versus naïve. Student's t-test. n = 3 for immunohistochemistry

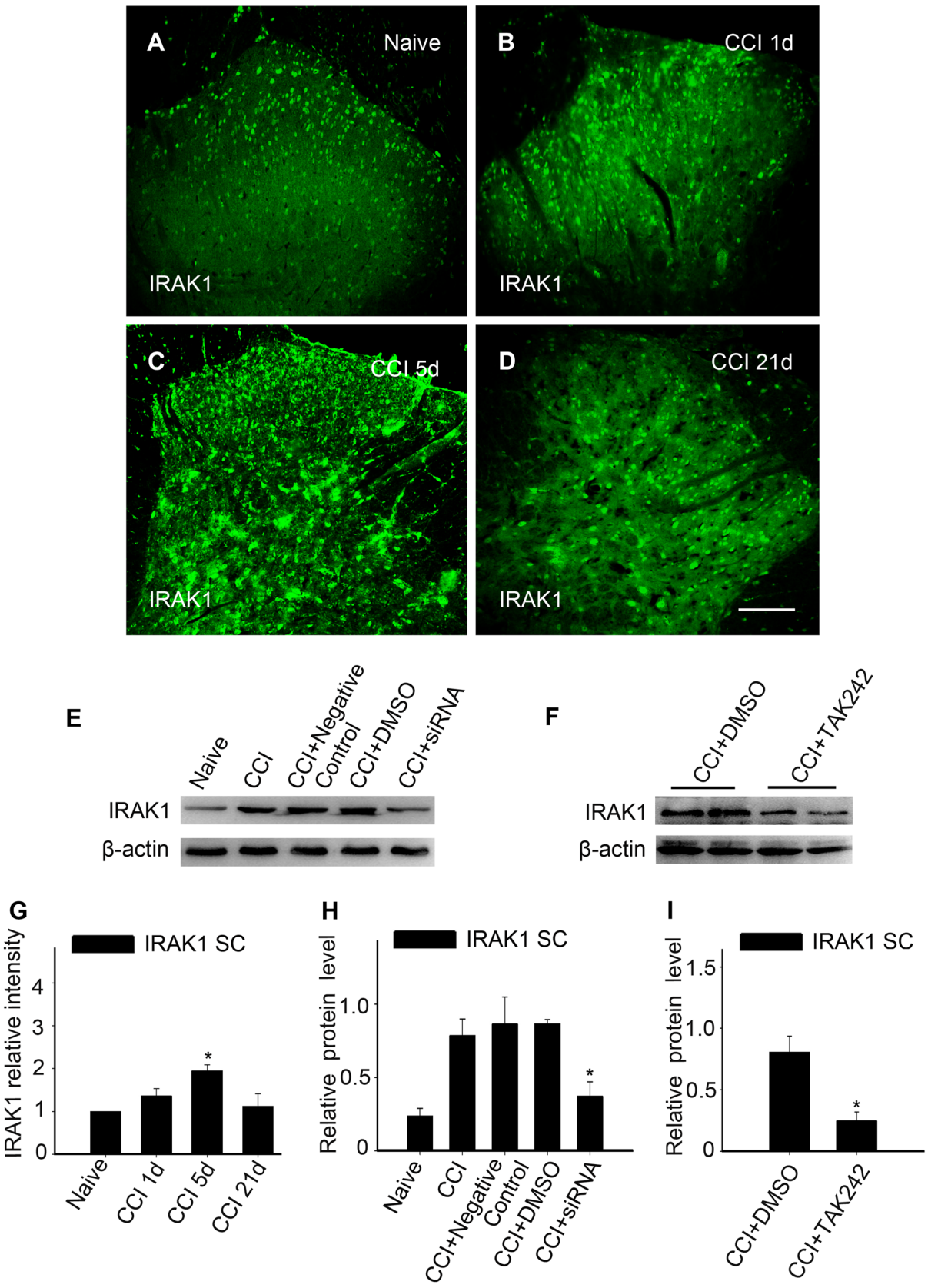
distribution of IRAK1 in the SC, the stain of immunofluorescence was performed for further detection. It was shown that IRAK1 was mostly co-localized with neurons marker NeuN (Fig. 3c–e), and less co-localized with astrocyte marker GFAP (Fig. 3i–k), except microglia marker IbA1 (Fig. 3f–h), suggesting that IRAK1 was mainly localized in SC neurons to participate in central pain sensitization, except through almost inflammation related-cells.

Immunofluorescence Analysis of IRAK1 at Different Time Points After CCI and the Upregulation of IRAK1 Expression be Reversed by TAK242 or IRAK1 siRNA

The spinal cord is the primary center for the transmission and integration of pain information. And the spinal dorsal horn (SDH), which contains multiple layers and a variety of sensory fibers, is an important channel for nociceptive stimulation afferent that is an important position in pain regulation. In our study, IRAK1 was observed to predominantly express in the superficial laminae of the SDH, which was the region of nociceptive neuronal expression (Fig. 4a–d). These results suggested that IRAK1 might mediate CCI-induced pain by the nociceptive primary afferent. Moreover, compared with naïve, CCI day 1 or CCI day 21 groups, a marked increase of IRAK1 was induced in the ipsilateral side at post-operation day 5 of CCI rats (Fig. 4a–d, g). And then western blot analysis was performed to exam the expression of IRAK1 in the spinal dorsal of CCI after intrathecal injection of different medicines. CCI + negative control groups were with similar high expression of IRAK1 compared with CCI groups ($P > 0.05$, Fig. 4e, h), which indicated that the small control interference had no extra effect on the expression of IRAK1. However, the CCI + IRAK1 siRNA groups showed an evidently lower expression of IRAK1 compared with the CCI groups ($P < 0.05$, Fig. 4e, h), suggesting that IRAK1 siRNA significantly reduced IRAK1 expression in the spinal cord. In comparison with the rats of CCI groups, no difference was found of the IRAK1 expression with the CCI + DMSO groups ($P > 0.05$, Fig. 4e, h), which indicated that the intrathecal injection of DMSO did not change the expression of IRAK1. However, there was a completely different result that IRAK1 expression was much lower in CCI + TAK242 groups than that in CCI + DMSO groups ($P < 0.05$, Fig. 4f, i), suggesting that TLR4 antagonist TAK242 significantly reduced the expression of IRAK1 in the spinal cord. TLR4 worked as an upstream membrane protein to mediate the function and expression of IRAK1 in the spinal cord.

IRAK1 siRNA or TAK242 Inhibited the Expression of TNF-α, IL-6 or p-NF-κB Signaling Induced by CCI

As we know, cytokines (TNF-α, IL-6, etc.), which are mainly synthesized and secreted by inflammatory cells such as astrocytes and microglia, play an essential role in neuropathic pain. In the current study, IRAK1 was mostly co-localized with astrocytes and less co-localized with microglia (Fig. 2h–m) in the dorsal root ganglion. Therefore, ELISA was performed to exam the expression of TNF-α or IL-6 in the DRG to explore the relationship between cytokines and IRAK1. The statistical results showed that when compared with the CCI groups, similar expression of TNF-α or IL-6 were observed in the CCI + negative control groups ($P > 0.05$, Fig. 5a, b), which indicated that the small control interference was with no significant influence on TNF-α or IL-6 expression of DRG. By contrast, CCI-induced TNF-α or IL-6 expression were found to decrease apparently in the CCI + IRAK1 siRNA groups compared with CCI groups ($P < 0.05$, Fig. 5a, b), suggesting that IRAK1 siRNA significantly reduced the expression of TNF-α or IL-6 in the dorsal root ganglion. IRAK1 could be used as an upstream regulatory protein of inflammatory factors to mediating CCI-induced pain in the peripheral nervous system. On the other hand, the same as IRAK1 was expressed in neurons (Fig. 3c–e), double staining showed that p-NF-κB was partially expressed in NeuN-positive neurons of SC (Fig. 5e–g), suggest they may take part in neuropathic pain in the same cells. We then checked p-NF-κB protein change in the SC after intrathecal injection of TAK242 or IRAK1 siRNA to investigate whether IRAK1 participated in central sensitization by p-NF-κB signaling. Subsequently, our western blotting results found that there was a similar trend of p-NF-κB expression between CCI groups and CCI + DMSO groups ($P > 0.05$, Fig. 5c, d), which indicated that the intrathecal injection of DMSO did not change the expression of p-NF-κB. However, there was a statistical difference of p-NF-κB expression not only between the CCI groups and the CCI + TAK242 groups, but also between the CCI groups and the CCI + siRNA groups ($P < 0.05$, Fig. 5c, d), suggesting that TLR4 antagonist TAK242 or IRAK1 siRNA significantly reduced the expression of p-NF-κB in the spinal cord. TLR4 or IRAK1 could be used as an upstream regulatory protein of p-NF-κB in the spinal cord.


A
Naive
IRAK1
B
CCI 1d
IRAK1
C
CCI 5d
IRAK1
D
CCI 21d
IRAK1
E
Naive
CCI
CCI+Negative Control
CCI+DMSO
CCI+siRNA
IRAK1
β-actin
F
CCI+DMSO
CCI+TAK242
IRAK1
β-actin
G
IRAK1 SC
IRAK1 relative intensity
Naive
CCI 1d
CCI 5d
CCI 21d
H
IRAK1 SC
Relative protein level
Naive
CCI
CCI+Negative Control
CCI+DMSO
CCI+siRNA
I
IRAK1 SC
Relative protein level
CCI+DMSO
CCI+TAK242

◂**Fig. 4** Immunofluorescence analysis of IRAK1 at different time points after CCI and the expression of IRAK1 in the SC after intrathecal injection of different medicine. The staining showed that the expression of IRAK1 was increased at day 5 compared to the naïve (**a**–**d**, **g**). The expression of IRAK1 following intrathecal injection of siRNA, negative control siRNA, DMSO, TAK242 (**e**–**f**, **h**–**i**). Scale bars, 50 μm (**a**–**d**); $*P < 0.05$ versus Naive groups (**g**), $*P < 0.05$ versus CCI groups (**h**), $*P < 0.05$ versus CCI + DMSO groups (**i**). Student's t-test. n = 3 for immunohistochemistry

BAY11-7082, TAK242, Knockdown of IRAK1 Attenuated CCI-Induced Mechanical Allodynia or Thermal Hyperalgesia

According to the above results, we speculated that IRAK1 might be involved in CCI-induced neuropathic pain by TLR4/p-NF-κB signaling. To prove the hypothesis, we detected the pain behaviors after intrathecal injection of TLR4 inhibitor TAK242, p-NF-κB inhibitor BAY11-7082, or IRAK1 siRNA. Two-way ANOVA followed by Bonferroni test showed that compared with the vehicle groups, the PWT of BAY11-7082 groups was increased significantly at 2 days, 3 days, 5 days ($P < 0.05$, Fig. 6a), suggesting that p-NF-κB was knockdown by BAY11-7082 reversed the value of PWT. Moreover, intrathecal injection of 100 μg of TAK242 increased PWT at 2 days, 3 days, 5 days ($P < 0.05$, Fig. 6b) when compared with the vehicle groups, suggesting TLR4 reversed the value of PWT. The result also showed that injection of negative siRNA did not affect either PWT or PWL at all the time points ($P > 0.05$, Fig. 6c, d) when compared with the vehicle groups. However, intrathecal injection of IRAK1 siRNA reversed the threshold value of PWT or PWL ($P < 0.05$, Fig. 6c, d). Above all results, we speculated that IRAK1 might be involved in CCI-induced neuropathic pain by TLR4/p-NF-κB signaling.

Discussion

In the present study, neuropathic pain behaviours including mechanical allodynia and heat hyperalgesia were successfully induced in the CCI groups compared with the sham-operated rats. Then we characterised in detail of the cellular profiles of IRAK1 expression after CCI. In the nerve-lesioned rats, IRAK1 expression was induced to increase conspicuously correlated with the time, while seldom expression was observed in the naive groups. Regarding distribution, IRAK1 could express in both IbA1-positive microglia and GFAP-positive astrocytes in the DRG, but mainly be induced in neurons-positive cells in the SC. The diverse cells of IRAK1 between DRG and SC revealed a spatial difference between the two types of CCI-related pain mechanisms, which might be the potential basis of allodynia and hyperalgesia between different cells. Moreover, the region of increased IRAK1 expression was observed in superficial laminae of the spinal dorsal horn, which was the nociceptive neuronal expression domain, suggesting that IRAK1 might mediate CCI-induced pain by nociceptive primary afferent. Then, we demonstrated that IRAK1 mediated pain hypersensitivity via driving monocyte/macrophage infiltration and promoting the production of cytokines, which were synthetized and released from inflammatory cells (astrocytes, microglia, etc.) at the level of the peripheral nervous system (DRG). Different from DRG, we mainly found IRAK1 took part in neuropathic pain by TLR4/p-NF-κB signaling through nociceptive primary afferent related neuronal bodies at the level of the central nervous system (SC).

Interleukin-1 receptor-associated kinase 1 (IRAK1), belonging to the IRAKs family, is a ubiquitously expressed serine/threonine kinase functioning in multiple physiological and pathological processes including cancer [9], microglia activation [25] and disease-associated inflammation. For instance, after being treated by MiR-146b, the down-regulating IRAK1 expression could promote the migration and proliferation of papillary thyroid carcinoma cells [9]. IRAK1 had been reduced by p16 to play an anti-inflammatory effect via inhibiting inflammatory cytokine production in macrophages in a different way [26]. In dendritic cells, knockdown of IRAK1 gene expression significantly decreased the release of the cytokines IL-12 and TNF-α, and significantly reduced the proliferation of T cells to mediate the inflammatory response [27]. Though inflammatory response has been demonstrated to be involved in the mechanism of neuropathic pain, whether IRAK1 takes part in maintaining neuropathic pain after chronic constriction injury is still unknown. This is the first study to evaluate IRAK1 protein expression after CCI surgery, and we found the pain threshold tended to increase due to the intrathecal injection of IRAK1 siRNA, while at the same time the induction of cytokine was observed to decrease in the DRG. This might be attributable to the fact that the IRAK1 was involved in mediating the expression of the cytokine to participate in pain, leading to the observation of regulating the relation between IRAK1 and cytokine. However, the mechanism of the cytokine to mediated pain needs further expounding. There is a theory that cytokines can not only sensitize primary afferents and spinal dorsal horn neurons, but also activate glial cells and induce the release of various pain-modulating substances, such as proinflammatory cytokines and chemokines, and further contribute to the development of neuropathic pain [2]. What's more, after surgery, cytokines can be released in the local tissue or never injury region, which could also enter the central nervous system through the blood and neurochemical pathway to forming glial–neuronal, glial–glial, neuronal–neuronal interaction [2, 5, 28]. These interactions maintain a long time of pain response including pain

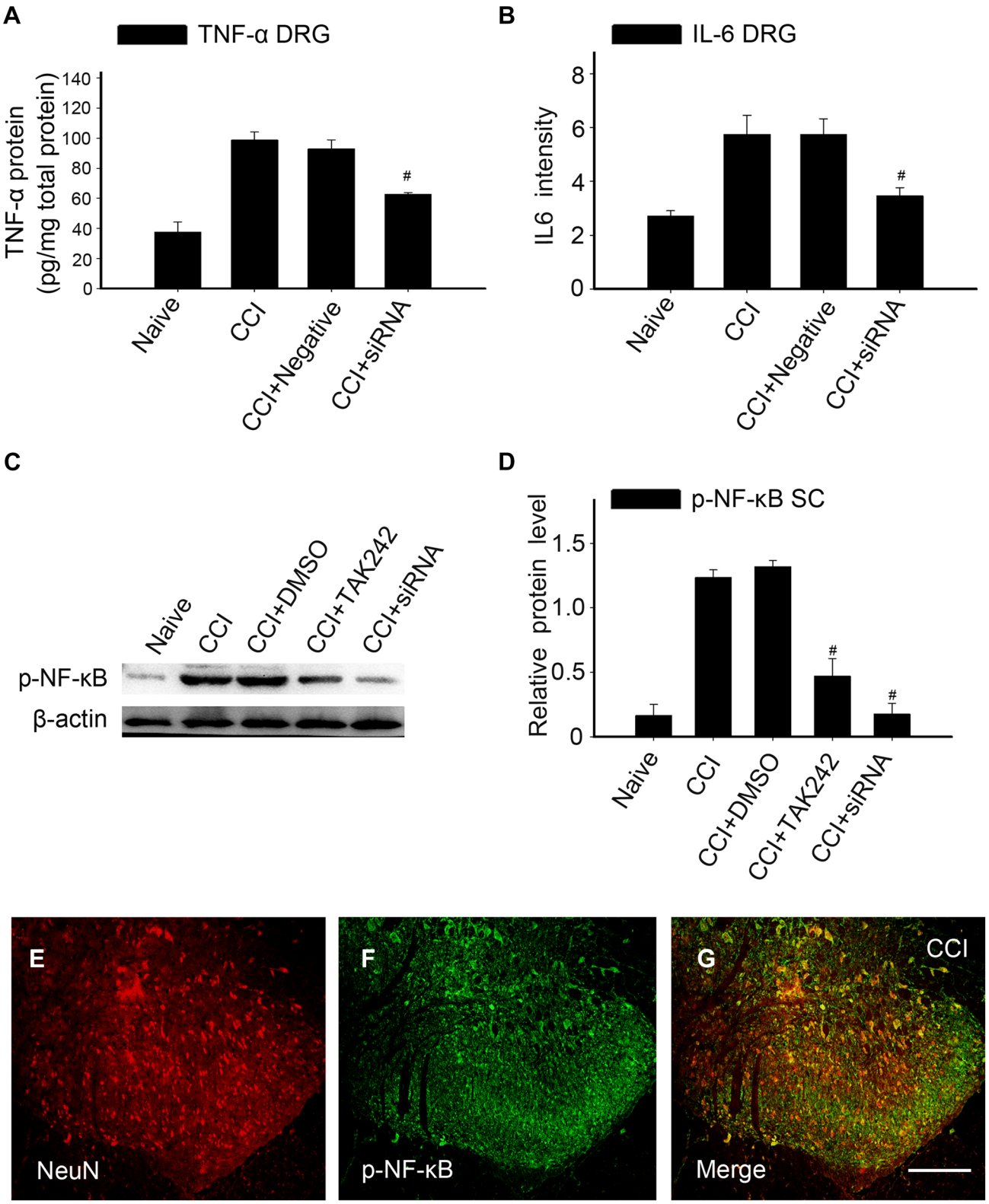


Fig. 5 IRAK1 siRNA or TAK242 inhibited the expression of TNF-α, IL-6 or p-NF-κB signaling induced by CCI. The increasing of TNF-α or IL-6 was significantly inhibited by IRAK1 siRNA compared with CCI groups by ELISA (**a**, **b**). In the SC, p-NF-κB production was also inhibited by IRAK1 siRNA or TAK242 (**c**, **d**). Double staining showed that p-NF-κB co-localized with neuron marker NeuN (**e**–**g**). $^{\#}P < 0.05$ versus CCI groups (**a**, **b**, **d**). Scale bars, 50 μm (**e**–**g**). Student's t-test. n = 3 for immunohistochemistry

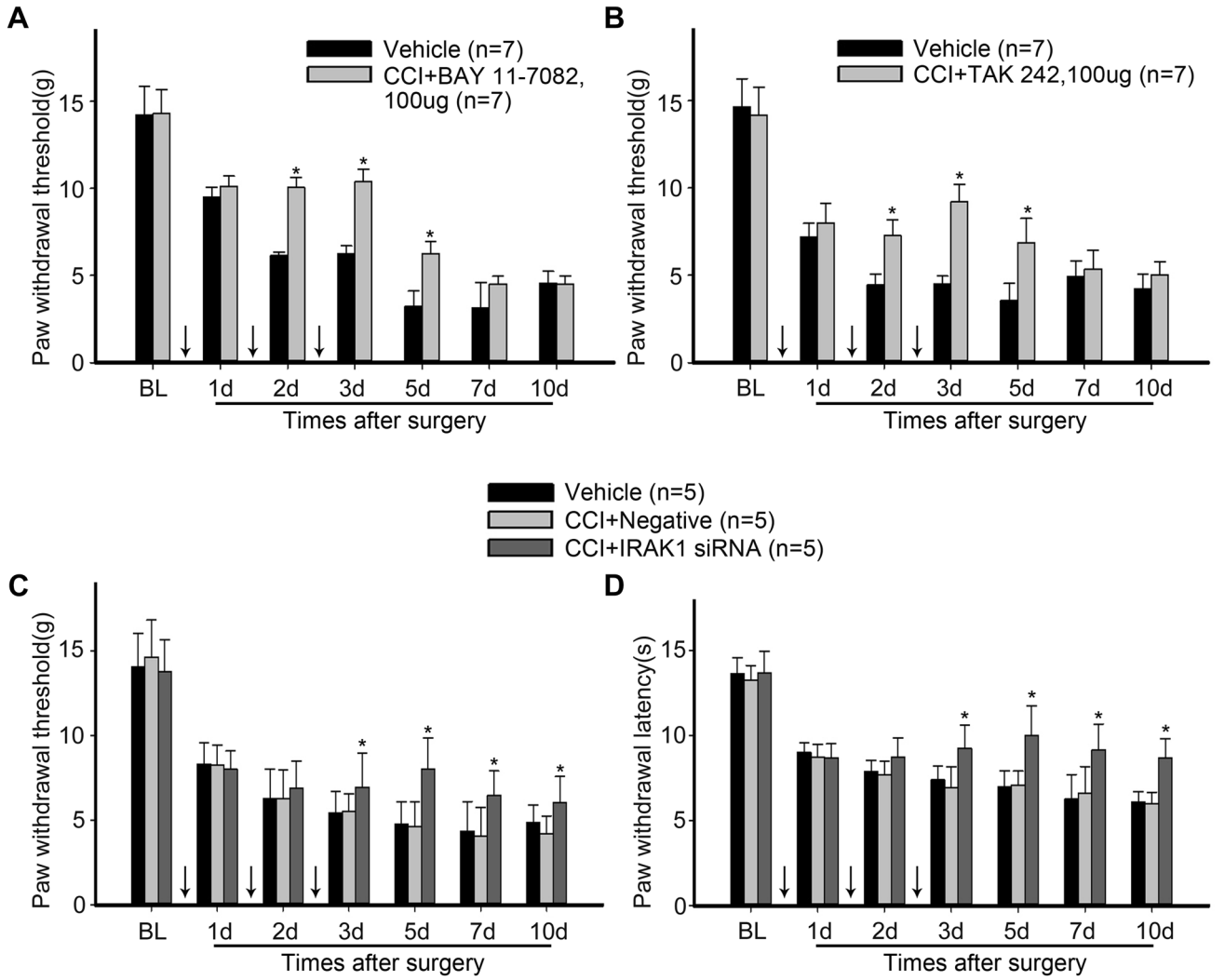


Fig. 6 Intrathecal injection of BAY11-7082, TAK242, or IRAK1 siRNA attenuated CCI-induced mechanical allodynia or thermal hyperalgesia. Intrathecal injection of p-NF-κB antagonist BAY11-7082 increase the value of PWT and attenuate CCI-induced pain hypersensitivity (**a**). Intrathecal injection of TLR4 antagonist TAK242 raises the value of PWT (**b**). Intrathecal injection of IRAK1 siRNA enhances the value of PWT and PWL compared with vehicle groups or negative siRNA groups (**c**, **d**). $^{*}P<0.05$ versus vehicle. *BL* baseline. Two-way ANOVA followed by Bonferroni test. n = 5 or 7 rats per group

message intercellular diffusion and upward conduction by the positive feedback, which cytokines synthesized and released by nerve corpuscles and acts on themselves or other nerve corpuscles [2, 5, 28]. These above opinions were the important mechanisms between the cytokine and pain. Besides, intrathecal injection is regarded as only to affect spinal cells. However, there have a positive effect both on the spinal cord and DRG cells in our results, and the expression of TNF-α or IL-6 in the DRG were evaluated to explore the relationship between cytokines and IRAK1. The results showed that IRAK1 could be expressed in both IbA1-positive microglia and GFAP-positive astrocytes in the DRG, and the induction of cytokines (TNF-α, IL-6, etc.) was observed to decrease by intrathecal injection of IRAK1 siRNA. As we know, cytokines (TNF-α, IL-6, etc.), which are mainly secreted by inflammatory cells such as astrocytes and microglia, play a key role in neuropathic pain. Therefore, we speculate that IRAK1 could induct and maintain allodynia and hyperalgesia through releasing cytokines (TNF-α, IL-6, etc.) by astrocytes or microglia and mainly involved the above internal mechanisms in peripheral nervous system after CCI surgery.

Toll-like receptors (TLRs) are a family of pathogen-recognition receptors that play an important role in many physiological processes including inflammation, itch, cancer and so on by triggering pro-inflammatory pathways in response to microbial pathogens. TLR4 especially has been reported to contribute to initiating the occurrence and development

of neuropathic pain by mediating p62 autophagic impairment [29], whose activation was also enhanced after nerve injury. Inducible lentivirus-mediated siRNA against TLR4 reduces nociception in a rat model of bone cancer pain by inhibiting the mRNA and protein expression [30]. Moreover, after stimulating of its ligand LPS, TLR4 induces MyD88-dependent signaling through the activation of IRAK4, which acts as the upstream of IRAK1 and phosphorylates this kinase [17], to forming TLR4-MyD88-IRAK4-IRAK1 signaling. Subsequently, several signaling pathways can be activated by IRAK1, including NF-κB, MAPKs, and IFN [18, 19]. The transcription factor nuclear factor-kappa B (NF-κB) is a pleiotropic transcriptional factor, which plays a crucial role in regulating the expression of pain related-cytokines to mediating allodynia and hyperalgesia [31]. As we know, toll-like receptors and downstream signaling take part in neuroinflammation, including TLR4-MyD88-IRAK1-NF-κB axis. Meanwhile, neuropathic pain and chronic inflammation were associated with the TRAF6/NF-κB pathway, which indicated that they share some common mechanisms [32, 33]. Above all, we speculate whether IRAK1 influence CCI-induced pain in forming the TLR4-IRAK1-NF-κB axis, which is similar to inflammation response. In our results, the intrathecal injection of Toll-like receptor 4 (TLR4) inhibitor or IRAK1 siRNA decreased the expression of IRAK1, and also alleviated CCI-induced mechanical and thermal hyperalgesia. The upregulation of p-NF-κB expression was reversed by IRAK1 siRNA in SC, and intrathecal injection of p-NF-κB inhibitor also relieved mechanical and thermal hyperalgesia. The final result was the same as the former hypothesis and no distortion about one of the mechanisms about IRAK1 contributing to pain in the SC. These conclusions were also supported by several in vivo studies. For instance, IRAK1s, which is one of the IRAK1's three alternatively spliced forms in both humans and mice including 1s, 1b, and 1c [34, 35], activates the NF-kB and JNK pathway [36] to participate in antibacterial and antiviral immunity [37]. IRAK1b also can combine with the Tollip and TRAF6 and activate the transcription NF-kB, but its levels remain constant after IL-1 induction [38]. In addition, IRAK1 knockout diminished the part of cytokine production (eg. IL-1, IL-18), which induced by activation of TLR [22].

The characteristic of neuropathic pain is peripheral and central sensitization, including ectopic generation of action potentials, facilitation, and disinhibition of synaptic transmission [39]. There is a report that calcium/CaMKII interacts with IRAK1 and is critical for LMP1-induced p65 phosphorylation and NF-κB activation [40]. Down-regulation of CaMKII activity or expression significantly reduced LMP1-induced p65 phosphorylation and NF-κB activation [40]. Calcium plays an essential role in the central mechanism of pathological pain by interacting with T-type Cav3 channels [41]. What's more, the activation of the nuclear factor-κB (NF-κB) pathway was evaluated to gain insight into the mechanisms related to the contribution of IL-33/ST2 signaling to radicular pain [42]. According to these data, we theoretically speculate in the central nervous system, IRAK1 can contribute to multiple types of pain through various sources including ion channel-related plasticity changes, ion transfer-related local potential, different types of pathways. Our results also further confirm these conjectures that IRAK1 worked as an upstream membrane protein to mediate the function and expression of p-NF-κB to mediating neuropathic pain, which both IRAK1 and p-NF-κB expressed in the spinal cord neuron. In addition, IRAK1-positive cells were also mainly located in the superficial layers (laminae I–III) of the dorsal horn, which was labeled by the location of CGRP and IB4. Spinal lamina I–III neurons integrate nociceptive information from the primary afferents and are involved in neuronal excitation, potential conduction, action potential, and the activation of the relevant ion channel to formatting central sensitization. This may be another possible mechanism that IRAK1 mediate electrophysiological and micro-morphological changes to participate in pain.

In summary, the results indicate that IRAK1 may be involved in neuropathic pain by forming TLR4-IRAK1-NF-κB axis and central sensitization in the spinal cord. But, in the dorsal root ganglion, IRAK1 take part in CCI-induced pain by promoting the production of cytokines from inflammatory cells (astrocytes, microglia, etc.), driving monocyte/macrophage infiltration, and peripheral sensitization.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Nantong University.

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