Up-regulation of interleukin-23 induces persistent allodynia via CX3CL1 and interleukin-18 signaling in the rat spinal cord after tetanic sciatic stimulation

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Abstract

Tetanic stimulation of the sciatic nerve (TSS) induces sciatic nerve injury and long-lasting pain hypersensitivity in rats, and spinal glial activation and proinflammatory cytokines releases are involved. In the present study, we showed that spinal interleukin (IL)-23 and its receptor, IL-23R, are crucial for the development of mechanical allodynia after TSS. In the spinal dorsal horn, both IL-23 and IL-23R are expressed in astrocytes, and this expression is substantially increased after TSS. Inhibition of IL-23 signaling attenuated TSS-induced allodynia and decreased the induction of glial fibrillary acidic protein (GFAP, an astrocytic marker). Conversely, intrathecally delivered IL-23 induced a persistent allodynia. Similar to IL-23 signaling, an increase in IL-18 and its receptor, IL-18R, as well as CX3CL1 and its receptor, CX3CR1, was simultaneously observed in the spinal dorsal horn after TSS. Interestingly, IL-18 and CX3CR1 were exclusively expressed in microglia, while IL-18R was mainly localized in astrocytes. In contrast, CX3CL1 was predominately expressed in neurons and secondarily in astrocytes. The functional inhibition of CX3CL1 and IL-18 signaling attenuated TSS-induced allodynia and suppressed IL-23 and IL-23R upregulation. Activation of CX3CR1 and IL-18R induced similar behavioral and biochemical changes to those observed after TSS. These results indicate that the interaction among CX3CL1, IL-18 and IL-23 signaling in the spinal cord plays a critical role in the development of allodynia. Thus, interrupting this chemokine-cytokine network might provide a novel therapeutic strategy for neuropathic pain.

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1. Introduction

Tetanic stimulation of the sciatic nerve (TSS) has previously been shown to induce long-term potentiation (LTP) of C- and A-fiber-evoked field potentials in the spinal dorsal horn (Chu et al., 2010; Liu and Sandkuhler, 1997; Zhang et al., 2006). This stimulation is capable of inducing a long-lasting allodynia, a common symptom of neuropathic pain, which is characterized by painful responses to normally innocuous tactile stimuli (Liang et al., 2010; Ying et al., 2006). Our previous studies found that TSS elicited the activation of spinal glia (Chu et al., 2010), which are likely involved in the production and release of proinflammatory cytokines and chemokines, which in turn, lead to sustained changes in the properties of the dorsal horn environment and the pain sensation (Cao and Zhang, 2008; Chu et al., 2012; Tsuda et al., 2005). Increasing evidence shows that the chemokine-cytokine networks in the CNS contribute to the development and maintenance of chronic pain (Austin and Moalem-Taylor, 2010; Gosselin et al., 2010). However, the multiple dialogues between these molecules remain unknown.

Interleukin (IL)-23 is a heterodimeric cytokine composed of the same p40 subunit as IL-12 and a unique p19 subunit (Oppmann et al., 2000). The receptor for IL-23 is also a heterodimeric complex composed of a subunit of the receptor for IL-12 (IL-12Rbeta1) and a subunit of the receptor for IL-23, IL-23R (Parham et al., 2002). Transgenic overexpression of p19 in multiple tissues induced systemic inflammation and high levels of circulating TNF-α and IL-1, indicating that IL-23 is a proinflammatory cytokine (Wiekowski et al., 2001). In brain tissues of multiple sclerosis patients (Li et al., 2007) and experimental autoimmune encephalomyelitis (EAE) mice (Cua et al., 2003), the production of IL-23 by microglia is critical for disease pathogenesis. It has also been reported that stimulated astrocytes produce biologically active IL-23 under inflammatory conditions (Constantinescu et al., 2005). In microglia, IL-23 induces the production of IL-17, an essential proinflammatory cytokine that can increase the expression of IL-6, macrophage inflammatory protein-2 and nitric oxide (Kawanokuchi et al., 2008). Additionally, IFN-gamma-induced production of chemokines (CCL-2, CCL-5 and CCL-10) is promoted
by IL-23 (Sonobe et al., 2008). Given that all of the IL-17 (Kim and Moalem-Taylor, 2010; Meng et al., 2012), IL-6 (Lee et al., 2009; Wei et al., 2012) and CCL-2 (Guo et al., 2012; Van Steenwinckel et al., 2011) are involved in the central mechanisms of pathological pain, it is reasonable to hypothesize that IL-23 may also be involved in the activation of spinal glia and the development of pathological pain.

IL-18 is a member of IL-1 family and was first isolated in 1995 as a co-factor that, in synergism with IL-12, stimulated the production of gamma interferon (INF-γ) in TH1 cells (Okamura et al., 1995). The IL-18 receptor (IL-18R) contains two subunits, IL-18Ralpha and IL-18Rbeta (Alboni et al., 2010). IL-18 was demonstrated to mediate microglia and astrocytes interaction in the spinal cord and to contribute to neuropathic pain (Miyoshi et al., 2008).

CX3CL1, which is a chemokine and also named as fractalkine, is highly expressed in the central nervous system. Its sole receptor, CX3CR1, a G protein-coordinated receptor, is mainly expressed in microglia. It is believed that CX3CL1 and CX3CR1 are involved in signaling between neurons and microglia (Harrison et al., 1998) and mediate aberrant signaling in pathological pain states (Liu et al., 2013; Zhu et al., 2012; Zhuang et al., 2007). In addition, the production of IL-23 is regulated by CX3CL1 (Mizutani et al., 2007; Tarrant et al., 2011).

In the current study, we set out to investigate whether IL-23/IL-23R, IL-18/IL-18R and CX3CL1/CX3CR1 are induced in the spinal cord and participate in TSS-induced persistent hypersensitivity. Here, we provide first evidence for the involvement of spinal IL-23 in neuropathic pain through CX3CL1 and IL-18 signaling.

2. Materials and methods

2.1. Animals

Male adult Sprague Dawley rats (200–300 g) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences and were housed in a 12 h light/dark cycle with a room temperature of 22 ± 1 °C and received food and water ad libitum. All experimental protocols and animal handling procedures were approved by the Shanghai Animal Care and Use Committee and were consistent with the policies issued by the International Association for the Study of Pain.

All of the following behavioral testing and quantification of Western blot experiments were performed blind with respect to treatments.

2.2. Tetanic stimulation of the sciatic nerve

Under sodium pentobarbital (80 mg/kg, i.p.) anesthesia and standard antiseptic procedures, the left sciatic nerve was carefully exposed at the mid-thigh level and dissociated from the adhering tissue. The sciatic nerve was moderately hung on a pair of silver electrode hooks that were coated with insulant except the points that were in contact with the sciatic nerve. The tetanic stimulation consisted of 10 two-sec trains with 10-s intervals and 100-Hz 0.5-ms rectangular pulses at 40 V. After stimulation, the muscle and skin were sutured in layers. The sciatic nerves of the sham-operated rats were identically exposed and manipulated but were not stimulated.

2.3. Von Frey test for mechanical allodynia

The Von Frey test protocol is consistent with our previous study (Sun et al., 2007). Briefly, rats were allowed to acclimate for at least 30 min before testing in a chamber (20 x 10 x 20 cm) with a customized platform made of iron wires, which make up a 10-mm grid throughout the entire area. A series of calibrated Von Frey filaments (Stoelting, Wood Dale, IL) was applied to the central region of the plantar surface of one hind paw in ascending order. For one trial, a Von Frey filament was applied five times at 15-s intervals. When the hind paw withdrawal was induced in three of the five consecutive applications of a particular filament, the value of that filament in grams was considered to be the paw withdrawal threshold (PWT). The investigator was blinded to the group being tested.

2.4. Drug administration

Lumbar puncture (LP) injection was performed as described previously (Chu et al., 2010). Under isoflurane anesthesia, the rat was placed on a plexiglass tube for widening intervertebral spaces. Drugs were delivered into the spinal space with a 30-gauge needle between the L5 and L6 vertebrae. Endotoxin-free goat anti-IL-23 P19 (Santa Cruz Biotechnology, USA), rabbit anti-CX3CR1 (Torrey Pines Biolabs, USA), recombinant mouse IL-18 BP (R&D Systems, USA), recombinant rat IL-23 (R&D Systems, USA), recombinant rat IL-18 (R&D Systems, USA), recombinant rat CX3CL1 (R&D Systems, USA), normal rabbit IgG (R&D Systems, USA), goat anti-Cx3c (Santa Cruz Biotechnology, USA), fluorocitrate (Sigma–Aldrich, USA) or 0.01 M PBS was injected over a period of 4 min at a volume of 20 μl.

2.5. Western blots

After defined survival periods, rats were killed with an overdose of urethane and the L4–L6 spinal cord was rapidly removed. The spinal segment was cut into a left and right half relative to the ventral midline, and the left half was further split into the dorsal and ventral horn at the level of the central canal. The dorsal horn tissues were homogenized with an ultrasonic cell processor in an SDS sample buffer that contained a mixture of proteinase inhibitors and PMSF. Equal amounts of protein samples were loaded and separated on an 10% Tris–Tricine SDS–PAGE gel and transferred to PVDF membranes (Millipore). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (pH 7.5) with 0.1% Tween-20 for 2 h at room temperature (RT) and then incubated overnight at 4 °C with primary antibodies. The blots were then incubated with HRP-conjugated secondary antibodies (1:1000, Pierce) for 2 h at RT. Signals were finally detected using enhanced chemiluminescence (ECL, Thermo, USA), and the bands were visualized with the ChemiDoc XRS system (Bio-Rad, USA). All Western blot analysis was performed at least three times, and consistent results were obtained. The concentrations of the main primary antibodies were as follows: goat anti-IL-23 P19 (1:300, Santa Cruz Biotechnology, USA), goat anti-IL-23R (1:300, Santa Cruz Biotechnology, USA), goat anti-CX3CL1 (1:500, R&D Systems, USA), rabbit anti-CX3CR1 (1:2000, Torrey Pines Biolabs, USA), goat anti-IL-18 (1:500, R&D Systems, USA), goat anti-IL-18 (1:500, R&D Systems, USA), goat anti-IL-18R (1:500, R&D Systems, USA), rabbit anti-TNF-alpha (1:500, Peprotech, USA), goat anti-Iba1 (1:500, Abcam, Hong Kong), and mouse anti-GFAP (1:2000, Cell Signal Technology, USA).

2.6. Immunohistochemical staining

Animals were terminally anesthetized with urethane and perfused intracardially with warm saline followed by 4% cold paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After perfusion, the L4–L6 segments of the spinal cord were removed and postfixed in the same fixative for 2–4 h, then replaced with 10–30% gradient sucrose in PB for 24–48 h at 4 °C. The spinal cord tissues were sectioned into 35 μm sections using a freezing microtome (Leica, Germany). The sections were blocked with 10% donkey serum in 0.3% Triton X-100 for 2 h at RT, and then incubated for 24–72 h at 4 °C with a mixture of rabbit anti-IL-23 P19 (1:50, Abcam, Hong Kong),
goat anti-IL-23R (1:100, Santa Cruz Biotechnology, USA), goat anti-CX3CL1 (1:500, R&D Systems, USA), rabbit anti-CX3CR1 (1:2000, Torrey Pines Biolabs, USA), goat anti-IL-18 (1:500, R&D Systems, USA) or goat-anti-IL-18R (1:500, R&D Systems, USA) and mouse anti-GFAP (1:2000, Cell Signal Technology, USA), mouse anti-NeuN (1:2000, Chemicon, USA), mouse anti-CGRP (1:1000, Sigma–Aldrich, USA), goat anti-iBA-1 (1:500, Abcam, Hong Kong) or rabbit anti-iBa-1 (1:500, Wako, Japan). The sections were then incubated with a mixture of rhodamine- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:200; Jackson Immuno-research, West Grove, PA, USA) for 2 h at RT. The stained sections were examined by a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan). The specificity of immunostaining was verified by omitting the primary antibodies. The specificity of the primary antibodies was verified in the preabsorption experiment. Sections were first incubated with a mixture of primary antibody and the corresponding blocking peptide for 24 h, followed by secondary antibody incubation. Immunostaining signal was abolished after absorption.

2.7. Data analysis

Data were expressed as the means ± SEM. Student’s t-test (for comparisons of two groups) or one- (or two-) way ANOVA (for multiple group comparisons) followed by post hoc Student-Newmann-Keuls test was used to identify significant differences. In all cases, p < 0.05 was considered statistically significant.

3. Results

3.1. Tetanic stimulation of the sciatic nerve induces IL-23 and IL-23R upregulation in the spinal dorsal horn

To examine the distribution and cell types that express the IL-23 and IL-23R in the spinal dorsal horn, we performed double immunostaining of the IL-23 or IL-23R with NeuN (neuronal marker), iBa-1 (microglial marker) and GFAP (astrogial marker) on sections of the L4–L6 spinal cord. IL-23-immunoreactive (IR) cells were detected sparsely in the superficial lamina of naive rats. Low to moderate IL-23R diffused in all the layers of the dorsal horn. IL-23 and IL-23R were both colocalized with GFAP, which indicated that they were induced in astrocytes (Fig. 1 A and B). Tetanic stimulation of the sciatic nerve (TSS) induced a robust increase in the number and intensity of IL-23-IR and IL-23R-IR cells in the ipsilateral spinal cord when examined 3 days after TSS, and almost all IL-23-IR and IL-23R-IR cells expressed GFAP, which indicated that they were induced in astrocytes (Fig. 1 C and D). With a few IL-23-IR terminal expressing calcitonin gene-related peptide (CGRP) in the primary afferent termination of the superficial spinal cord (Fig. 1E).

Consistent with our previous studies (Liang et al., 2010), following TSS, a robust mechanical allodynia was observed from day 1 after TSS and lasted at least for 7 days (Two-way ANOVA, treatments: F1, 14 = 16.944, p < 0.01; treatment × time: F8, 84 = 2.766, p < 0.01) (Fig. 2A). Western blot analysis showed that either IL-23 or its receptor, IL-23R, was significantly upregulated in the ipsilateral spinal dorsal horn at all examined time points after the TSS (One-way ANOVA, IL-23: F2, 20 = 8.833, p < 0.01; IL-23R: F2, 20 = 8.796, p < 0.01) (Fig. 2B and C). In addition, the activation of astrocytes was observed in the spinal dorsal horn, with statistically increased GFAP levels on days 3 and 7 post-TSS (One-way ANOVA, F3, 20 = 5.969, p < 0.01) (Fig. 2D).

3.2. IL-23 contributes to TSS-induced mechanical allodynia

To address whether spinal IL-23 is involved in the development of neuropathic pain, we examined the influence of blocking IL-23 signaling on TSS-induced allodynia. When anti-IL-23 antibody (0.4 μg and 4.0 μg) was intrathecally delivered 2 h before and 24 h after TSS by lumbar puncture (LP) injection, TSS-induced allodynia was markedly alleviated only at the dose of 4.0 μg compared with control IgG (Two-way RM ANOVA, treatments: F2, 20 = 6.751, p < 0.01; treatment × time: F10, 100 = 2.525, p < 0.01) (Fig. 3A). Accordingly, the TSS-induced upregulation of GFAP in the spinal dorsal horn was also partially suppressed by the anti-IL-23 antibody (One-way ANOVA, F3, 20 = 7.553, p < 0.01) (Fig. 3B). To further assess the effect of IL-23 on pain behaviors, the exogenous IL-23 was injected into the spinal subarachnoid space of naïve rats. A single LP injection of IL-23 directly produced mechanical allodynia in a dose-dependent manner (Two-way RM ANOVA, treatments: F2, 20 = 23.129, p < 0.01; treatment × time: F18, 180 = 4.232, p < 0.01). At a dose of 2.0 μg, IL-23-induced allodynia occurred at 1 h and persisted for 5 days (Fig. 3C). To address whether astrocytic activation is involved in IL-23-mediated hypersensitivity, we examined the effects of blocking glial function on IL-23-induced allodynia. As shown in Fig. 3D, the intrathecal application of fluorocitrate (FC, 20 nmol), a reversible glial metabolic inhibitor, completely blocked IL-23-induced allodynia (Two-way RM ANOVA, treatments: F2, 17 = 10.376, p < 0.01; treatment × time: F18, 153 = 6.625, p < 0.01). Moreover, we found that following intrathecal injection of IL-23, the expression of tumor necrosis factor alpha (TNF-alpha), one of the astrocytic products (Abdelmoaty et al., 2013; Liu et al., 2013; Omran et al., 2013), was increased in the spinal dorsal horn (Fig. 3E). The upregulation of trimer (52 kDa), dimmer (34 kDa) and transmembrane (26 kDa) TNF-alpha was statistically significant (Student t-test, trimer: t = 3.291, p < 0.01; dimmer: t = 4.137, p < 0.01; transmembrane: t = 4.063, p < 0.01).

3.3. IL-18 mediates TSS-induced mechanical allodynia by IL-23 signaling

The previous studies showed that IL-23 p19 expression could be regulated by NF-kappa-B (Mise-Omata et al., 2007), whereas the phosphorylation of NF-kappa B in spinal astrocytes was regulated by IL-18 (Miyoshi et al., 2008). We therefore predicted that TSS-induced allodynia and upregulation of IL-23 and IL-23R in the spinal dorsal horn should be mediated by IL-18 signaling. To test this, we first examined the expression of IL-18 and IL-18R in the spinal dorsal horn. Consistent with previous reports from our laboratory and other laboratories (Chen et al., 2012; Miyoshi et al., 2008), spinal IL-18 and IL-18R were predominately expressed in microglia and astrocytes in naïve rats, respectively (Fig. 4A and B). Following TSS, numerous IL-18-IR and IL-18R-IR cells were found in the spinal dorsal horn. These IL-18-IR cells were exclusively microglia, and IL-18R-IR cells were astrocytes (Fig. 4 C and D). Western blot analysis showed a similar upregulation of IL-18 (24 kDa, corresponds to the size of pro-IL-18) and IL-18R to IL-23 and IL-23R after TSS (One-way ANOVA, IL-18: F4, 25 = 7.555, p < 0.01; IL-18R: F4, 25 = 6.020, p < 0.01) (Fig. 5A and B).

The role of spinal IL-18 signaling in TSS-induced allodynia was also observed. TSS-induced mechanical allodynia was clearly alleviated by LP injection of IL-18 BP (0.3 and 3.0 μg), a naturally occurring inhibitor of IL-18 activity with higher affinity for mature IL-18 (Alboni et al., 2010) (Two-way RM ANOVA, treatments: F2, 19 = 4.867, p < 0.05) (Fig. 5C). In addition, LP injection of exogenous IL-18 (3 μg) induced persistent allodynia for 3 days (Two-way ANOVA, treatments: F1, 13 = 20.814, p < 0.01; treatment × time: F10, 104 = 6.868, p < 0.01) (Fig. 5D).

To address whether blockade of IL-18 signaling in the spinal cord suppresses TSS-induced upregulation of IL-23, IL23R and GFAP, IL-18BP was intrathecally delivered 2 h before and 24 h after TSS. As shown in Fig. 5E-G, IL-18BP caused a decrease in IL-23, IL-23R and GFAP in the spinal dorsal horn by day 3 after TSS, although
this decrease in IL-23 did not reach statistical significance (One-way ANOVA, IL-23: $F_{3, 20} = 3.146, p < 0.05$; IL-23R: $F_{3, 20} = 12.763, p < 0.01$; GFAP: $F_{3, 20} = 16.158, p < 0.01$). Furthermore, we found that a single lumbar puncture injection of exogenous IL-18 produced a robust increase in both IL-23 and IL-23R by 24 h after injection (Student t-test, IL-23: $t = 3.242, p < 0.01$; IL-23R: $t = 4.565, p < 0.01$) (Fig. 5H). Consistently, IL-18-induced allodynia was remarkably inhibited by pre-administration of anti-IL-23 antibody 40 min prior to IL-18 injection (Two-way RM ANOVA, treatments: $F_{2, 18} = 14.147, p < 0.01$; treatment × time: $F_{16, 144} = 5.653, p < 0.01$). Lumbar puncture injection of anti-IL-23 antibody per se had no effect on PWTs (Fig. 5I).

### 3.4. CX3CL1 signaling regulates expression of IL-18/IL-18R and IL-23/IL-23R

As shown above, IL-18 was predominately expressed in spinal microglia, and the upregulation of this cytokine in the spinal dorsal horn is likely to have an important role in mechanical allodynia after TSS. CX3CR1, a unique receptor for chemokine CX3CL1, was found to be mainly expressed on OX-42- and CD4-positive microglia in the spinal dorsal horn (Zhuang et al., 2007). Thus, we assessed whether IL-18 and subsequent IL-23 are modulated by CX3CL1 signaling. As shown in Fig. 6, TSS-induced increase in the levels of IL-18, IL-23 and their receptors, IL-18R and IL-23R, in the spinal dorsal horn were significantly reduced by anti-CX3CR1 antibody (20 μg) by day 3 after TSS (One-way ANOVA, IL-18: $F_{3, 20} = 6.020, p < 0.01$; IL-18R: $F_{3, 20} = 10.070, p < 0.01$; IL-23: $F_{3, 20} = 3.145, p < 0.05$; IL-23R: $F_{3, 20} = 4.499, p < 0.05$) (Fig. 6A–D). Furthermore, LP injection of exogenous CX3CL1 (2.0 μg) markedly elevated IL-18, IL-18R, IL-23 and IL-23R in the spinal dorsal horn by day 2 after injection (Student t-test, IL-18: $t = 2.777, p < 0.05$; IL-18R: $t = 3.197, p < 0.01$; IL-23: $t = 6.594, p < 0.01$; IL-23R: $t = 3.915, p < 0.01$) (Fig. 6E and F). Consistently, a robust allodynia was also evoked by intrathecal CX3CL1 (Two-way RM ANOVA, treatments: $F_{2, 21} = 16.644, p < 0.01$; treatment × time: $F_{18, 189} = 2.363, p < 0.01$).
which could be partially relieved by IL-18BP (Two-way RM ANOVA, treatments: $F_{2, 20} = 18.344$, $p < 0.01$; treatment × time: $F_{18,180} = 5.732$, $p < 0.01$) (Fig. 6G and H).

The relevance of CX3CL1 signaling and IL-18 was confirmed by double immunofluorescence. As shown in Fig. 7, either in naïve (Fig. 7A, B, D and E) or TSS-treated rats (Fig. 7C and F), CX3CL1 was detected mostly in neurons and low expression was detected in astrocytes in the spinal dorsal horn, while its receptor CX3CR1 was exclusively expressed in spinal microglia and colocalized with IL-18.

3.5. CX3CL1 signaling is involved in TSS-induced mechanical allodynia

The above data revealed that both IL-18/IL-18R and IL-23/IL-23R were regulated by CX3CL1 signaling. Next, we verified the role of CX3CL1 signaling in TSS-induced neuropathic pain. Western blot analysis revealed that either CX3CL1 or its receptor, CX3CR1 was significantly upregulated in the spinal dorsal horn at all time points examined after the TSS (One-way ANOVA, CX3CL1: $F_{4, 25} = 4.465$, $p < 0.01$; CX3CR1: $F_{4, 15} = 13.24$, $p < 0.01$) (Fig. 8A and B). Additionally, the activation of microglia was observed in the spinal dorsal horn, with marked increase in Iba-1 on days 3 and 7 post-TSS (One-way ANOVA, Iba1: $F_{3, 20} = 10.471$, $p < 0.01$; GFAP: $F_{3, 20} = 7.321$, $p < 0.01$) (Fig. 8E–G).

4. Discussion

Tetanic stimulation of the sciatic nerve (TSS) induced upregulation of IL-23 and IL-23R and the activation of astrocytes in spinal dorsal horn. (A) TSS induced a robust mechanical allodynia in hindpaw ipsilateral to TSS. (B–D), Time course of IL-23 (B), IL-23R (C) and GFAP (D) upregulation in the ipsilateral spinal dorsal horn after TSS. *$p < 0.05$, **$p < 0.01$, vs. sham.
Fig. 3. Involvement of IL-23 signaling in the development of TSS-induced neuropathic pain. (A) TSS-induced mechanical allodynia was dose-dependently reversed by lumbar puncture (LP) injection of anti-IL-23 neutralizing antibody (IL-23AB). IL-23AB was delivered 2 h before and 24 h after TSS. \(^* p < 0.05, \quad \ast \ast p < 0.01, \quad \text{vs. control (IgG)}; \quad \# p < 0.05, \quad \# \# p < 0.01, \quad \text{vs. 0.4 l g IL-23AB. (B) TSS-induced upregulation of GFAP in the spinal dorsal horn was partly inhibited by IL-23AB on day 3 after TSS. IL-23AB was delivered by LP 2 h before and 24 h after TSS. \(^\ast \ast p < 0.01, \quad \text{vs. sham}; \quad \# p < 0.05, \quad \text{vs. IgG control. (C) LP delivery of exogenous IL-23 induced mechanical allodynia in a dose-dependent manner. \(^\ast p < 0.01 \quad \text{vs. vehicle control (PBS)}; \quad \# p < 0.05, \quad \# \# p < 0.01 \quad \text{vs. 0.2 l g IL-23. (D) IL-23-induced allodynia in naïve rats was blocked by fluorocitrate (FC, 20 nmol). Fluorocitrate was delivered 1 h prior to IL-23 injection. \(^\ast p < 0.05, \quad \text{vs. FC alone}; \quad \# p < 0.05, \quad \# \# p < 0.01, \quad \text{vs. IL-23 alone. (E) Western blot analysis revealed that the antibody directed to TNF-alpha recognized four bands at approximately 52, 34, 26 and 17 kDa in rat spinal dorsal horn, corresponding to trimer, dimer, transmembrane precursor and monomer TNF-alpha, respectively. LP delivery of IL-23 (2 l g) produced significant upregulation of the trimer, dimer and precursor of TNF-alpha at 1 day after IL-23 injection. \(^\ast p < 0.01 \quad \text{vs. vehicle (PBS).}}\)

Fig. 4. Expression of IL-18 and IL-18R in the spinal dorsal horn. (A & B) Double immunofluorescence reveals that IL-18 was expressed in Iba1-labeled microglia (A), while IL-18R was expressed in GFAP-labeled astrocytes in naïve rats (B). (C&D) Three days after TSS, increased IL-18R-IR (C) and IL-18R-IR (D) were found in spinal microglia and astrocytes, respectively.
Disruption of astrocytic functions prevented IL-23-induced allodynia. These results suggest that spinal IL-23 signaling may be involved in astrocytic activation and thus increased pain hypersensitivity. It is noteworthy that after TSS, an increased IL-23R-IR signal was detected in CGRP-positive primary afferent termination in the superficial spinal cord, suggesting the involvement of presynaptic IL-23R in TSS-induced neuropathic pain as well.

In contrast to our present study, IL-23 was reported to be expressed either in microglia (Li et al., 2003; Li et al., 2007; Lv et al., 2011) or in astrocytes (Constantinescu et al., 2005) from brain tissues of humans, rats and mice. IL-23R was also observed in microglia of mice brain (Sonobe et al., 2008). However, to date, there is no direct evidence for IL-23 and IL-23R expression in the spinal cord. Our present results show that spinal astrocytes produce IL-23 and express IL-23R, which suggests that IL-23 signaling may function in an autocrine manner to facilitate astrocytic activation. Despite the focus on CNS IL-23 in the pathogenesis of multiple sclerosis (Oppmann et al., 2000) (Li et al., 2007; Niimi et al., 2012; Thakker et al., 2007), the evidence for the involvement of IL-23 in pathological pain is still limited. In a mouse CCI (chronic constriction injury) model, upregulated levels of IL-23 mRNA were observed in injured sciatic nerve (Kleinschnitz et al., 2006). In support and expansion of this, in the current study, we provide direct evidence for the contribution of spinal IL-23 to neuropathic pain.

Another important finding in this study is that spinal IL-18 and IL-18R presented similar upregulation to IL-23 and IL-23R after TSS, and IL-18R and IL-23 were both expressed in the spinal astrocytes, suggesting that IL-23 might be regulated by IL-18 signaling. The previous studies showed that NF-kappa-B could regulate IL-23.

Schafers et al., 2004; Zhang et al., 2010).
expression by the NF-kappa-B binding sites in the p19 subunit gene promoter of IL-23 (Mise-Omata et al., 2007; Utsugi et al., 2006). In both IL-1β-stimulated expression of IL-23 p19 in human periodontal ligament fibroblasts (Zhu et al., 2012) and CCR7-mediated IL-23 production in dendritic cells (Kuwabara et al., 2012), the NF-kappa-B signaling pathway was demonstrated to play a critical role. It was also found that NF-kappa-B could be activated in IL-18R-expressing astrocytes in the spinal dorsal horn after nerve injury, and intrathecal injection of an NF-kappa-B inhibitor, SN50, dose-dependently blocked the IL-18-induced allodynia,
suggesting that nerve injury induces NF-kappa-B activation in the spinal astrocytes via the IL-18R (Miyoshi et al., 2008). In the present study, we demonstrated that blocking IL-18 signaling suppressed TSS-induced upregulation of IL-23 and IL-23R, whereas activating IL-18R increased IL-23 and IL-23R; furthermore, IL-18-induced allodynia was significantly attenuated by blockade of IL-23 signaling. However, whether NF-kappa-B mediates transcriptional regulation of IL-18R on IL-23 in spinal astrocytes will need to be examined in further studies.

As mentioned above, IL-18 and IL-18R are considered to be mediators of the interaction between microglia and astrocytes in the spinal cord because IL-18 is produced in microglia and its receptor IL-18R is expressed in astrocytes (Miyoshi et al., 2008). IL-18 was mainly expressed in p38 positive cells in the spinal dorsal horn of L5 spinal nerve injury rats, and increased IL-18 expression was abolished by inhibiting p38 MAPK activation (Miyoshi et al., 2008), suggesting the p38 MAPK activation regulates IL-18 expression. Serving as a key signaling molecule in microglia by integrating various input to microglia (Ji et al., 2013), p38 MAPK is phosphorylated following the activation of multiple receptors, such as ATP receptors (P2X4, P2X7) (Trang et al., 2007), TRP-like channels (TRL4) (Miyoshi et al., 2008) and chemokine receptors (CX3CR1) (Zhuang et al., 2007). A study by Miyoshi et al. suggests that nerve injury induces enhanced expression of IL-18 through the TRL4/p38 MAPK signaling pathway (Miyoshi et al., 2008). In the present study, we further showed that spinal CX3CL1 and CX3CR1 regulated the expression of IL-18 and IL-18R. Activation of CX3CR1 elevated IL-18 and IL-18R level, blockade of CX3CR1 suppressed TSS-induced upregulation of IL-18 and IL-18R. Based on previous studies and our findings, we hypothesize that following TSS, increased CX3CL1 triggers microglia to synthesize IL-18 through CX3CR1/p38 signaling pathway; The IL-18 stimulates IL-18R on neighboring astrocytes in a paracrine manner to regulate IL-23 by the NF-kappa B signaling pathway; IL-23 produced by astrocytes then stimulates IL-23R in an autocrine manner to facilitate astrocytic activation.

An unexpected finding in the present study is that all of the CX3CL1/CX3CR1, IL-18/IL-18R and IL-23/IL-23R in the spinal dorsal horn were increased at the same time-points (1, 3 and 7 days after TSS), suggesting that functional activation (i.e., increased release of cytokines and chemokines) of microglia and astrocytes may occur simultaneously after TSS. This finding seems to be inconsistent with previously reported activation sequences of the microglia and astrocytes in L5 spinal nerve cryoneurolysis (SPCN) and chronic constriction injury (CCI) neuropathic pain models (Colburn et al., 1997). It is generally believed that nerve injury-induced signaling molecules from primary afferent central terminals trigger microglial activation (Ji et al., 2013). Actually, neuronal signals are also important for the activation of astrocytes. It is estimated that a single astrocyte can envelop 140,000 synapses and 4–6 neuronal somata, and can contact 300–600 neuronal dendrites in rodents (Gao and Ji, 2010). A close contact with neurons and synapses makes it possible for astrocytes not only to support and nourish neurons but also to reactive neuronal activity in physiological and pathological conditions. A link between neuronal activity and astrocytic activation is supported by the findings that inflammatory pain-induced astroglialosis was abrogated by a nerve blockade with lidocaine (Guo et al., 2007). Furthermore, the study by Hald et al. suggested that astrocytic activation does not always depend on microglial activation in some other chronic pain models (Hald et al., 2009). Thus, proinflammatory cytokines and chemokines produced by neurons, microglia and astrocytes may together form a chemokine-cytokine network to sustain perseverant release of inflammatory mediators in a positive feedback manner, facilitating the development of neuronal hypersensitivity, which leads to exaggerated pain.

In conclusion, proinflammatory cytokine IL-23 and its receptor IL-23R are involved in the development of mechanical allodynia by TSS. TSS-induced upregulation of IL-23 and IL-23R was significantly attenuated by blockade of CX3CR1 and inhibition of IL-18 signaling. These results suggest that multiple interactions among CX3CL1/CX3CR1, IL-18/IL-18R and IL-23/IL-23R play important roles in TSS-induced persistent allodynia.
Disclosure of conflicts of interest

No conflicts of interest were disclosed.

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References


