

Inhibition of chemokine CX3CL1 in spinal cord mediates the electroacupuncture-induced suppression of inflammatory pain

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Purpose: Chemokine CX3CL1 and its receptor CX3CR1 in the lumbar spinal cord play crucial roles in pain processing. Electroacupuncture (EA) is recognized as an alternative therapy in pain treatment due to its efficacy and safety. However, the analgesic mechanism of EA remains unclear. The aim of this study was to investigate whether EA suppressed complete Freund's adjuvant (CFA)-induced pain via modulating CX3CL1-CX3CR1 pathway. **Materials and methods:** Inflammatory pain was induced by intraplantar injection of CFA to the left hind paw of Sprague-Dawley rats. EA with 2 Hz for 30 mins was given to bilateral Zusanli acupoints (ST36) on the first and third day after CFA injection. Mechanical allodynia and thermal hyperalgesia were tested with von Frey tests and Hargreaves tests, respectively. The expressions of CX3CL1, CX3CR1 and p38 mitogen-activated protein kinase (MAPK) were quantified with Western blots. The release of IL-1 β , IL-6 and TNF- α were evaluated with ELISA. Recombinant CX3CL1 or control IgG were then injected through intrathecal catheters in the EA-treated CFA model rats. The behavioral tests, p38 MAPK activation and cytokine release were then evaluated.

Results: EA significantly inhibited inflammatory pain induced by CFA for 3 days. Meanwhile, EA downregulated the expression of CX3CL1 but not CX3CR1 in the lumbar spinal cord of the CFA rats. Besides, activation of p38 MAPK and the release of pain-related cytokines (IL-1 β , IL-6 and TNF- α) were inhibited by EA. Intrathecal injection of CX3CL1 largely reversed the analgesic effect of EA treatment and re-activated p38 MAPK signaling, and resulted in pro-inflammatory cytokines increase in acupuncture-treated rats.

Conclusion: Our findings indicate that EA alleviates inflammatory pain via modulating CX3CL1 signaling in lumbar spinal cord, revealing a potential mechanism of anti-nociception of EA in inflammatory pain.

Keywords: inflammatory pain, electroacupuncture, CX3CL1, p38 MAPK, cytokine

Introduction

Acupuncture, introduced and promoted by World Health Organization as early as the 1970s, is recognized to be effective in the treatment of more than 100 kinds of diseases. Accumulating evidence suggests that acupuncture is a powerful approach for alleviating pain.¹ Nowadays, acupuncture has been widely applied in clinic for pain treatment.²⁻⁴ Derived from traditional acupuncture, electroacupuncture (EA) has been proved to be effective in pain management in various studies,⁵ and become popular in clinical practice because of its accuracy and standardization of the stimulating frequency, intensity and duration. Although regulating inflammatory

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reaction could be the important ways through which acupuncture exerts the analgesic effect,^{6–9} the underlying molecular mechanism is largely unknown.

As the only member of chemokine CX3C subfamily, CX3CL1 is reported to be involved in neuroinflammation.¹⁰ CX3CL1 is expressed in neurons, while its receptor CX3CR1 locates at microglia in neural system. Abundant evidences revealed that the interaction of CX3CL1/CX3CR1 in spinal cord is involved in the generation of multiple kinds of pain. Rats developed mechanical allodynia after establishing a pain model of disc herniation, together with an upregulation of CX3CL1 mRNA transcription in the spinal cord;¹¹ the expression of CX3CL1 also increased in the spinal cords of the rats suffered from complete Freund's adjuvant (CFA)-induced inflammatory pain.¹² In the neuropathic pain model of partial nerve ligation, the nociceptive response to the pain was alleviated in the CX3CR1 knockout mice.¹³ However, whether CX3CL1/CX3CR1 signaling in spinal cord could be the regulative target of acupuncture-induced pain suppression remains unclear.

In the present study, we aim to investigate if EA treatment alleviates inflammatory pain via regulating the CX3CL1/CX3CR1 pathway in spinal cord, and also unveil the potential downstream targets and molecular mechanism of CX3CL1/CX3CR1-mediated acupuncture analgesia.

Materials and methods

Animals

Adult male Sprague-Dawley rats weighing 220–240 g (Experimental Animal Center of the Fourth Military Medical University, Xi'an, Shaanxi, People's Republic of China) were used to perform the experiments. Rats were group-housed (four per cage) on a 12-hr light/dark cycle at a temperature of 22–24°C, with free access to food and water. The rats were allowed to acclimate to the housing facilities for at least 3 days before experimental manipulation in case their stress responses affected experiment results. All animal experiments were approved by the Ethics Committee of the Fourth Military Medical University and followed the policies on the use of laboratory animals issued by the International Association for the Study of Pain. All efforts were made to minimize the number of animals used and their suffering.

Experimental procedures

Two experiments were conducted to investigate CX3CL1's role in EA-induced analgesia. In experiment 1 (Figure 1A),

animals were randomly divided into four groups (n=22 per group): the Control group, in which saline was injected subcutaneously into the left hind paw of rats. The CFA group, in which 50 µL CFA was subcutaneously injected into the left hind paw. The CFA+EA group, in which EA treatment was administered at the first and the third day after CFA modeling. In the CFA+sham EA group, a non-acupoint stimulation (sham EA) was performed after CFA modeling. Thereafter, behavioral tests were conducted to evaluate the allodynia and hyperalgesia 1 day after CFA modeling, once a day, for 3 consecutive days, and spinal cord samples were obtained after EA treatment for Western blots and ELISA to assess the level of CX3CL1, CX3CR1, p38 mitogen-activated protein kinase (MAPK) and cytokines (IL-1β, IL-6, TNF-α). In Experiment 2 (Figure 1B), the rats were given CFA injection, and then randomly divided into four groups (n=8 per group): in the IgG and CX3CL1 group, normal goat IgG or recombinant fractalkine (CX3CL1) domain was injected intrathecally, respectively; rats in the IgG+EA group were treated with EA and intrathecal IgG; the CX3CL1+EA group was administered with EA and CX3CL1 domain. The phosphorylation level of p38 MAPK and release of cytokines in spinal cord were tested after behavioral tests.

Inflammatory pain model

With a brief anesthesia with isoflurane (2% in O₂, Lunan Beite, Linyi, People's Republic of China), inflammatory pain was induced by 50 µL intraplantar injection of CFA (Sigma-Aldrich, St Louis, MO, USA) into the left hind paw of experimental animals. Animals in the Control group underwent the same procedure but saline (Kelun, Chengdu, People's Republic of China) was injected in the left hind paw. Animals were immediately returned to their home cage after modeling.

EA treatment

Given to the adjustability of intensity and frequency of EA, we used EA to carry out the acupoint stimulation. Before EA treatment, rats were immobilized by a fixing device and two stainless steel acupuncture needles (0.1 mm in diameter, Huatuo, Suzhou, People's Republic of China) were inserted into bilateral Zusanli acupoint (ST36, 5 mm lateral to the anterior tubercle of tibia). The needles were connected to Huatuo SDZ-V Nerve and Muscle Stimulator (Suzhou, People's Republic of China), stimulation was given at 2 Hz, 1–2 mA. The treatment was firstly given at the day after CFA modeling, and repeated 2 days

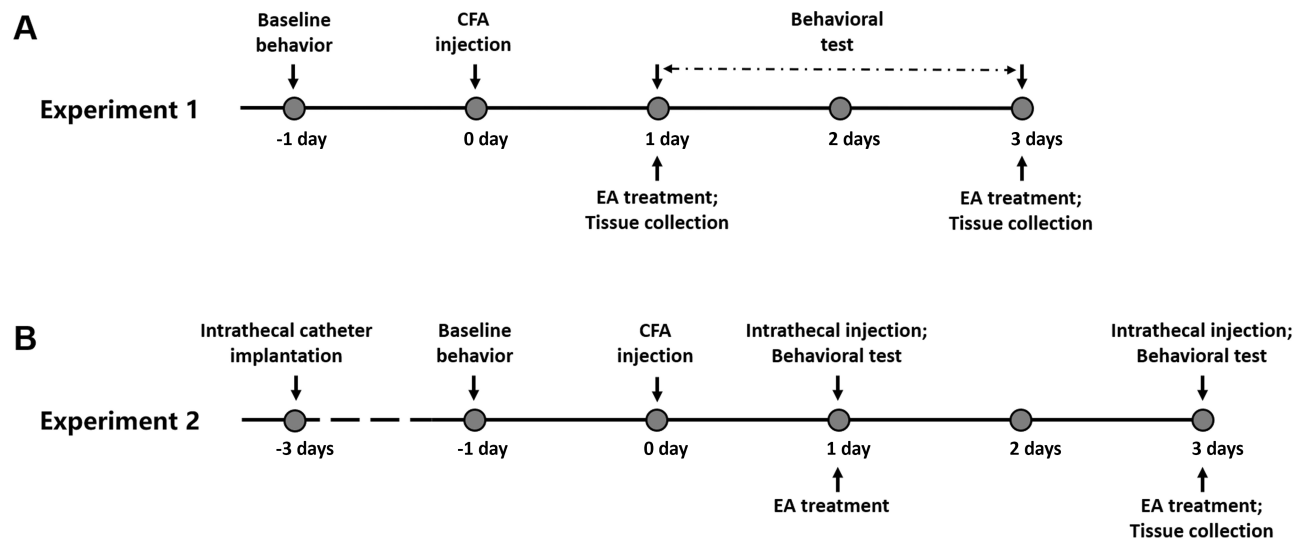


Figure 1 Diagrammatic sketch of the experiment procedures.

Notes: (A) In experiment 1, rats were randomly allocated to Control, CFA, CFA+EA, CFA+sham EA groups and underwent behavioral tests on the day before modeling to obtain the baseline data. In the 3 days after modeling, behavioral tests were repeated once upon a day; EA treatment was given on day 1 and day 3 after modeling, at 1 hr before the behavioral tests. Spinal cord tissue was then collected from six animals in each group after the behavioral tests on day 1 and day 3. (B) In experiment 2, intrathecal catheter was implanted to the lumbar spinal cord 3 days before modeling. IgG and recombinant CX3CL1 were given through intrathecal catheter 30 mins before the EA treatment. The behavioral tests and EA treatments were done in the same way as that in experiment 1.

Abbreviations: CFA, complete Freund's adjuvant; EA, electroacupuncture.

later. In each treatment session, stimulation was given for 30 mins. In CFA+sham EA group, needles were inserted in non-acupoints (3 mm under ST36), other parameters were the same as the CFA+EA group.

Behavioral tests

Paw withdrawal threshold (PWT) and latency were obtained, respectively, with von Frey test and Hargreaves test. Before the tests, animals were allowed to acclimate for 30 mins in experimental apparatus. All animals were tested to obtain baseline data on the day before modeling.

In von Frey test, each rat was placed in a chamber (20×10×10 cm) on a platform with 5 mm grids of iron wires throughout the entire area. The up-down method was used to evaluate mechanical allodynia.¹⁴ In brief, the hind PWT was determined using a series of von Frey hairs (Stoelting, Wood Dale, IL, USA) applied to the central region of the plantar surface of the CFA-injected hind paw in ascending order (1, 1.4, 2, 4, 6, 8, 10, 15 and 26 g). Each filament was tested ten times at 10 s intervals. The PWT was defined as the lowest force in grams that produced at least five withdrawal responses in ten consecutive applications.

In Hargreaves test, rats were placed individually into glass chambers on an elevated glass platform, under which a radiant heat source (Fenglan, Xi'an, People's Republic of China) was applied to the glabrous surface of the hind paw

through a 3 mm glass plate. The heat was fixed at a constant intensity, which induced a withdrawal reaction at approximately 10 s in normal rats. The heat source was turned off when the rat lifted the foot, the time during which the heat source is irradiated was defined as the hind paw withdrawal latency (PWL). A 20 s cutoff was used to prevent tissue damage in the absence of a response. In each time, six trials with a 10-min interval were conducted. The average of the six trials was then determined.

Western blotting

The L4-L5 spinal cord was rapidly removed after the rats were sacrificed. The dorsal horn tissues were then homogenized with an ultrasonic tissue homogenizer in strong RIPA buffer containing protease inhibitors cocktail (Sigma-Aldrich) and phosphatase inhibitors cocktail (Sigma-Aldrich). The homogenization was performed in centrifuge tubes wrapped in ice. Protein samples were then obtained from homogenates after centrifugation. After the protein concentrations were measured with the bicinchoninic acid (BCA) method, equal amounts of protein samples from different groups were loaded and separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Cork, IRL). After blocked with 5% bovine (Beyotime, Shanghai, People's Republic of China) in Tris-buffered saline (pH 7.4) with 0.1% Tween-20 for 2 hrs at room

temperature, the membranes were incubated overnight at 4°C with primary antibodies. The primary antibodies were rabbit anti-CX3CL1 (1:1000, Abcam, Cambridge, UK), rabbit anti-CX3CR1 (1:1000, Abcam), rabbit anti-p38 MAPK (1:1000, Cell Signaling, Danvers, MA, USA), rabbit anti-phosph-p38 MAPK (1:500, Cell Signaling) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Cwbiotech, Beijing, People's Republic of China). The blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:8000, Cwbiotech) for 2 hrs. Signals were detected using enhanced chemiluminescent reagent (ECL, Millipore, Billerica, MA, USA), and the bands were analyzed with the ChemiDoc XRS system (Bio-Rad, Hercules, CA, USA). The quantification of band intensity was carried out using Image software (Media Cybernetics, Silver Spring, MD, USA). Band densities were normalized to individual GAPDH internal controls.

ELISA assay

Rat ELISA kits of IL-1 β , IL-6 and TNF- α (Westang, Shanghai, People's Republic of China) were used to assess the content of the downstream cytokines of CX3CL1. Rat recombinant cytokine standards and samples of 100 μ L were run in duplicate according to the manufacturer's instructions. The optical density of each well was read at 450 nm.

Intrathecal injection

According to the published method,¹⁵ 30 ng of endotoxin-free rat recombinant fractalkine (CX3CL1, chemokine domain amino acid residues 22–100, R&D system, Minneapolis, USA) or control drug (normal goat IgG, R&D system) was diluted in 5 μ L PBS containing 0.1% bovine serum albumin (Beyotime) and injected with an intrathecal catheter.

Intrathecal catheter surgery was performed as previously described.¹⁶ In brief, after the rats were anesthetized with 5% sodium pentobarbital (0.2 mL/100 g, intraperitoneally), a PE-10 intrathecal catheter was implanted into the intrathecal space of the spinal cord at L4–L6 level. After filling the catheter with sterile endotoxin-free PBS, rats were individually housed to protect the catheter from gnawing. Three days after surgery, 20 μ L of 2% lidocaine was injected intrathecally, a paralysis of lower limbs occurred within 30 s and recovered within 30 mins indicated successful implantation of the catheter. These rats without signs of spinal cord damage were applied for experimentation. 30 ng

of recombinant fractalkine (CX3CL1) or IgG injected intrathecally 5 mins prior to the EA treatment in a volume of 5 μ L via the catheter within 1 min.

Statistical analysis

Statistical analyses were performed by GraphPad Prism 6.0 (GraphPad Software Inc. La Jolla, CA, USA). The data of mechanical and thermal pain thresholds were expressed as mean \pm SEM, and analyzed by two-way repeated ANOVA followed by Bonferroni's post-hoc analysis to determine individual group differences. The data of Western blot and ELISA assay were expressed as mean \pm SD, and analyzed by one-way ANOVA and Bonferroni's post-hoc analysis. All *P*-values were two-sided, and *P*<0.05 was considered significant.

Results

EA treatment attenuated CFA-induced allodynia and hyperalgesia

As shown in Figure 2A and B, both the PWT and PWL were dramatically decreased after CFA modeling (*P*<0.01 vs Control). EA treatment at bilateral ST36 significantly increased PWT and PWL, compared with CFA group (*P*<0.01), and this pain-suppression effect lasted for more than 24 hrs, as the PWT and PWL of the EA group at day 2 remained higher than those of CFA group (*P*<0.01). Meanwhile, sham EA at non-acupoints did not show any statistical difference in PWT and PWL in comparison with those in CFA group, but were significantly lower than CFA +EA group.

EA treatment suppressed the cleavage of CX3CL1, but not the expression of CX3CR1

In order to transmit biological signals, CX3CL1 combines with its receptor CX3CR1 after cleaving from neuronal membrane into a soluble form. We used Western blot to analyze the effects of CFA and EA on CX3CL1 cleavage. On the first day after the modeling, CFA led to a rapid upregulation of CX3CL1 content in lumbar spinal cord (*P*<0.05 vs Control), while both EA and sham EA reversed this effect (Figure 3A). Three days after modeling, CX3CL1 expression in CFA group further increased (*P*<0.01 vs Control); while EA treatment still kept the level of CX3CL1 as low as Control (*P*<0.01 vs CFA), sham EA no longer suppressed CX3CL1 expression (*P*<0.01 vs CFA +EA, Figure 3B). The expression of CX3CR1, however, was not affected by either CFA injection or EA treatment at

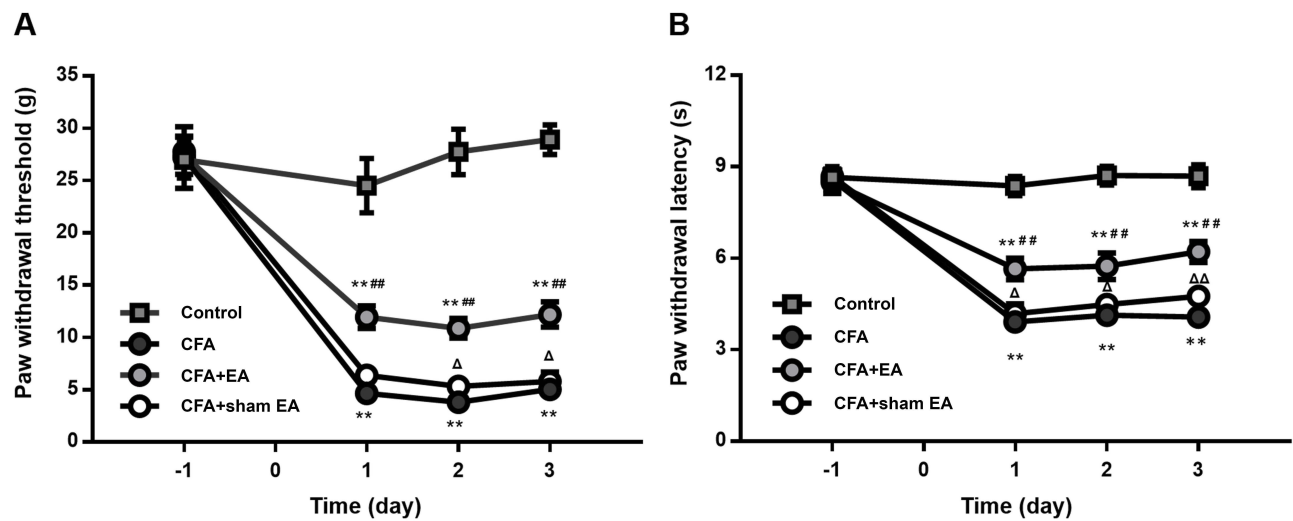


Figure 2 Analgesic effects of EA on pain hypersensitivity in CFA model rats.

Notes: CFA modeling induced inflammatory pain in CFA, CFA+EA, CFA+sham EA group rats. **(A)** CFA-treated rats showed the decreased paw withdrawal threshold, while sham EA did not suppress the abnormal sensation, EA treatment alleviated such allodynia. **(B)** The paw withdrawal latency decreased after CFA modeling, EA treatment prolonged the latency time, sham EA did not show the same effect. $^{**}P < 0.01$ vs Control; $^{###}P < 0.01$ vs CFA; $^{\Delta}P < 0.05$ vs CFA+EA, $^{\Delta\Delta}P < 0.01$ vs CFA+EA; $n = 10$ in each group. **Abbreviations:** CFA, complete Freund's adjuvant; EA, electroacupuncture.

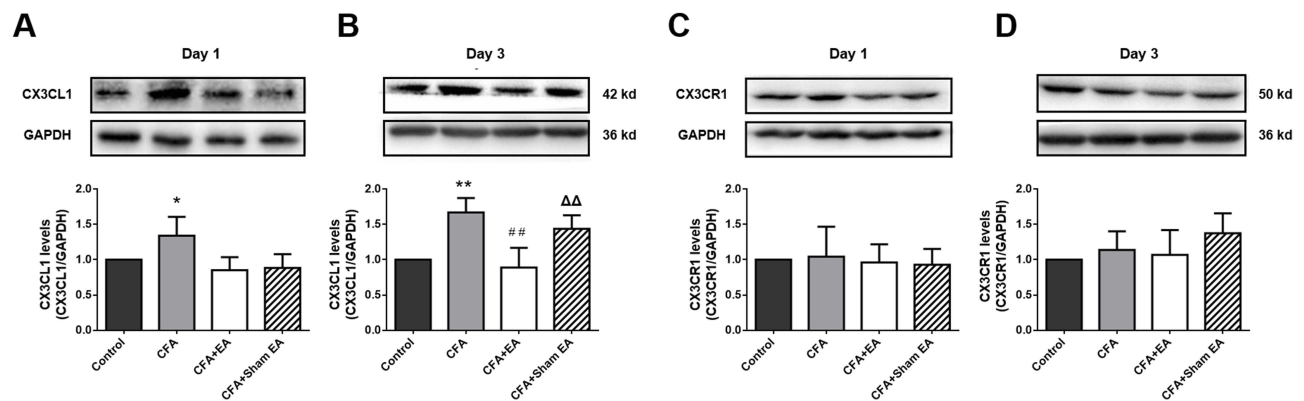


Figure 3 Expression of CX3CL1 and CX3CR1 in the lumbar spinal cord.

Notes: **(A)** The expression of CX3CL1 in CFA group increased 1 day after modeling. **(B)** The CX3CL1 level in CFA group was upregulated significantly, but decreased to be as low as the Control in CFA+EA group at day 3 after modeling, sham EA did not prevent upregulation of CX3CL1 at day 3. **(C and D)** The expression of CX3CL1 receptor CX3CR1 was not affected by CFA or EA treatment. $^{*}P < 0.05$ vs Control, $^{**}P < 0.01$ vs Control; $^{###}P < 0.01$ vs CFA; $^{\Delta\Delta}P < 0.01$ vs CFA+EA; $n = 6$ in each group.

Abbreviations: CFA, complete Freund's adjuvant; EA, electroacupuncture; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

two time points (Figure 3C and D), indicating that CX3CL1 instead of CX3CR1 could be the regulative target for EA treatment in CFA-induced pain model.

EA treatment reversed the CFA-increased p38 MAPK phosphorylation and cytokines release

p38 MAPK and cytokines are crucial at the downstream of CX3CL1/CX3CR1 signaling pathway and play important roles in pain modulation. As the Western blot analyses illustrated, the total amounts of p38 MAPK did not show

any difference among four groups at 24 and 72 hrs after CFA modeling (Figure 4A). However, as seen in Figure 4B, the phosphorylation of p38 MAPK was significantly elevated in CFA group at 72 hrs after modeling ($P < 0.01$ vs Control), but went back to the control level after EA treatment at 72 hrs ($P < 0.01$ vs CFA). Unlike EA treatment, sham EA did not prevent p38 MAPK from activating, the expression of phosph-p38 MAPK markedly increased in CFA+sham EA group ($P < 0.01$ vs CFA+EA).

The ELISA assay further demonstrated that at 3 days after modeling, CFA led to an increased release of IL-1 β , IL-6, TNF- α in the spinal cord ($P < 0.01$ vs Control,

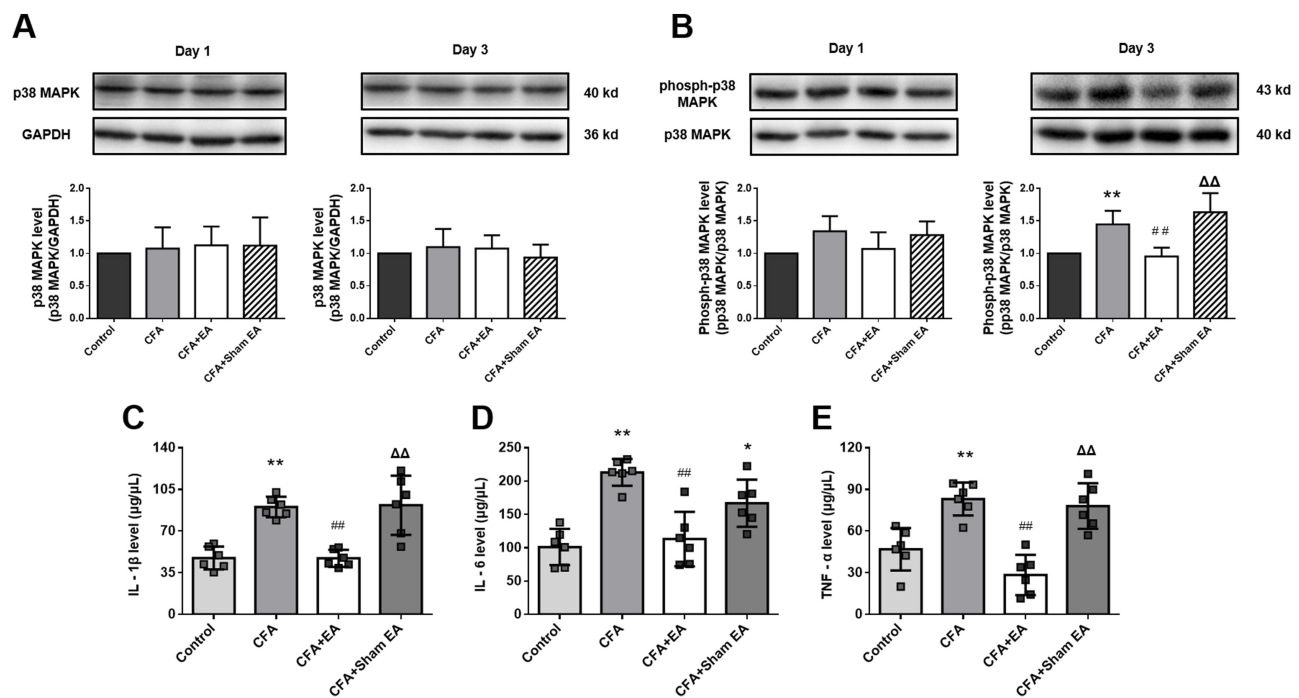


Figure 4 EA blocks the activation of p38 MAPK and the release of cytokines in spinal cord.

Notes: (A) The total amount of spinal cord p38 MAPK was not changed by either CFA modeling or real/sham EA treatments. (B) CFA activated the phosphorylation of p38 MAPK on the first and third day after modeling, EA inhibited the activation of p38 MAPK at day 3 after modeling, sham EA failed to suppress p38 MAPK activation at day 3. (C–E) EA suppressed the release of pro-inflammatory cytokines (IL-1 β , IL-6, TNF- α) on the day 3 after CFA injection, sham EA exerts no effect on suppressing cytokine release. * P <0.05 vs Control, ** P <0.01 vs Control; ### P <0.01 vs CFA; $\Delta\Delta$ P <0.01 vs CFA+EA; n =6 in each group.

Abbreviations: p38 MAPK, p38 mitogen-activated protein kinase; phosph-p38 MAPK, phosphorylated p38 MAPK; CFA, complete Freund's adjuvant; EA, electroacupuncture.

respectively), but these cytokines in CFA+EA group were constrained to be at the normal level; sham EA treatment, however, failed to suppress releasing of these cytokines. The level of IL-1 β and TNF- α were significantly increased compared with the CFA+EA group (P <0.01); even though the level of IL-6 was not significantly higher than CFA+EA group, it still increased when comparing to the Control group (P <0.05) (Figure 4C–E).

EA treatment produced analgesic effect was CX3CL1 inhibition dependent

To clarify if EA treatment alleviates CFA-induced pain through the CX3CL1-p38 MAPK-cytokine pathway, we intrathecally administrated recombinant fractalkine (CX3CL1) or normal IgG as control into the CFA-injected rats. In CX3CL1 group, CX3CL1 intrathecal administration did not aggravate the pain caused by CFA, since neither PWT nor PWL of CX3CL1 group was significantly reduced compared with the IgG group (P >0.05). However, while IgG itself did not change the threshold of pain, CX3CL1 intrathecal administration partially inhibited the analgesic effect of EA treatment (Figure 5A and B).

Specifically, in von Frey test, the PWT of CX3CL1+EA group was decreased in comparison with IgG+EA group (P <0.05, Figure 5A). In Hargreaves test, as it is shown in Figure 5B, the PWL of CX3CL1+EA group was shorter than IgG+EA group (P <0.01). Spinal cord tissues were then collected at day 3 after modeling when the last behavioral tests were completed. The expression of p38 MAPK in lumbar spinal cord was not affected by CFA modeling, EA treatment or intrathecal administration (Figure 5C). Western blot results also demonstrated that intrathecal administration of CX3CL1 partially activated p38 MAPK in the spinal cords of EA-treated CX3CL1+EA group rats (P <0.01 vs IgG+EA), but the level of phosph-p38 MAPK was lower than IgG group (P <0.01, Figure 5D); in addition, compared to IgG group, CX3CL1 administration did not further activate p38 MAPK in CX3CL1 group. The ELISA assays of IL-1 β , IL-6 and TNF- α revealed CX3CL1 significantly reversed cytokine inhibition induced by EA treatment (P <0.05, P <0.01, P <0.01 vs IgG+EA, respectively, Figure 5E–G), but in CX3CL1 group, cytokine release was not further increased compared with the IgG group (P >0.05).

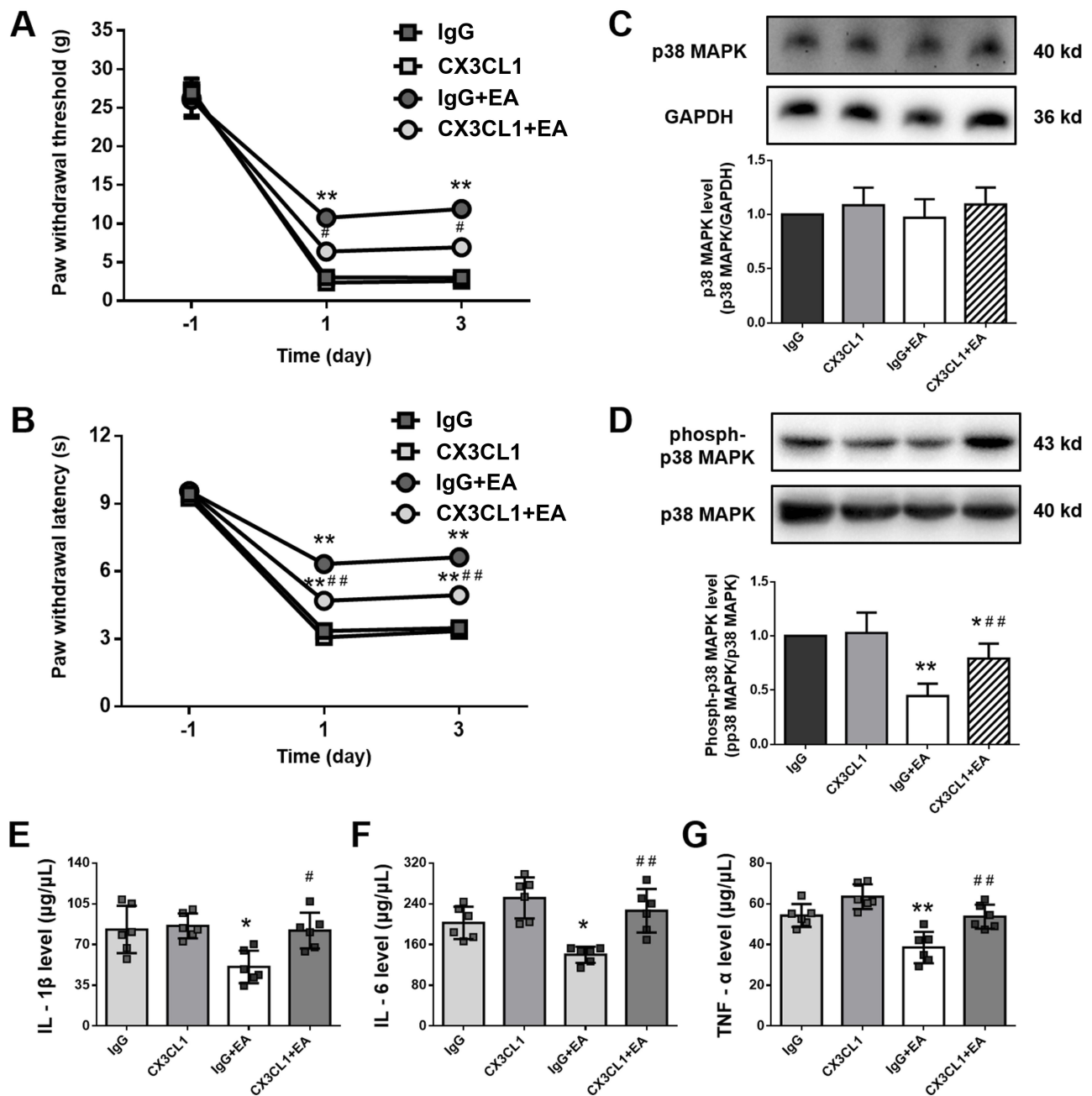


Figure 5 Intrathecal injection of CX3CL1 blocked the analgesic effect of EA treatment.

Notes: (A) Intrathecal injection of CX3CL1 partially decreased the PWT improved by EA treatment, but did not aggravate the pain in CX3CL1 group. (B) The PWL of the CX3CL1+EA group was lower than it of the EA treatment group (IgG+EA group), but higher than IgG group, the PWL of CX3CL1 group was not affected by drug administration. (C) The amount of p38 MAPK kept a similar level among four groups. (D) The p38 MAPK signaling suppressed by EA treatment was reactivated by CX3CL1 administration in CX3CL1+EA group; but CX3CL1 did not further activate p38 MAPK signaling in CX3CL1 group. (E–G) Intrathecal injection of CX3CL1 led to a higher level of pro-inflammatory cytokine release in the spinal cord of the EA-treated rats. * $P < 0.05$ vs IgG, ** $P < 0.01$ vs IgG, # $P < 0.05$ vs IgG+EA, ### $P < 0.01$ vs IgG+EA; n=8 in each group in behavioral tests, n=6 in each group in Western blots and ELISA assay.

Abbreviations: PWT, paw withdrawal threshold; PWL, paw withdrawal latency; p38 MAPK, p38 mitogen-activated protein kinase; phosph-p38 MAPK, phosphorylated p38 MAPK; CFA, complete Freund's adjuvant; EA, electroacupuncture; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

EA has been widely used for analgesia in clinical practice, but the mechanism remains unclear. The present study revealed the involvement of CX3CL1-p38 MAPK-cytokine axis in EA analgesia. Specifically, EA treatment at

day 1 and day 3 after CFA modeling showed a significant efficacy on pain relief. EA treatment also inhibited the expression of CX3CL1 in the spinal cord, inhibited the activation of downstream p38 MAPK, lead to a decreased release of pro-inflammatory cytokines. Intrathecal

administration of recombinant CX3CL1 attenuated the analgesic effect of EA in CFA model, and activated p38 MAPK and increased the level of cytokines as well in EA group. These findings provided new evidence on the analgesic mechanism of acupuncture.

In clinical experience, various acupoints could have the similar analgesic effects on one experimental model.¹⁷ For example, acupuncture at Zusanli (ST36), Yanglingquan (GB34)¹⁸ and Qihai (BL24)¹⁹ were reported to be effective in the treatment of neuropathic pain. The therapy on Zusanli, Kunlun (BL60),²⁰ Sanyinjiao (SP6),²¹ Huantiao (GB30) and Yanglingquan²² could suppress the inflammatory pain. In the present study, we chose ST36 as the target acupoint, mainly because its analgesic effect in CFA model was previously verified.²³ Furthermore, EA was given with 2 Hz to the animals in the current research, since previous studies suggested that low-frequency EA could stimulate the A δ afferent, formulate synapses between afferent nerve fibers and gelatinous neurons in the spinal cord to induce long-term depression, which helped achieving better analgesic effects.²⁴ In order to avoid the tolerance of EA analgesia, we undertook EA treatment with an interval of 2 days, as it has been proved that application of EA for consecutive days leads to the decrease of analgesic effect.²⁵

The increasing evidence demonstrated that cytokines and signaling molecules are involved in acupuncture analgesia in the spinal cord.^{26,27} The neuronal chemokine CX3CL1 and its microglial receptor CX3CR1 play an important role both in pain initiation and inflammatory response.²⁸ In particular, Cathepsin S from activated microglia in the spinal cord results in the release of soluble CX3CL1, which acts on CX3CR1 of microglia and resulted in the production of pro-inflammatory cytokines. Our results revealed that EA treatment inhibited the elevation of CX3CL1 after CFA modeling in the lumbar spinal cord; and interestingly, the expression of CX3CL1 can be suppressed by sham EA momentarily after CFA modeling, it is possible that the potential anti-inflammatory effect of electric physical stimulation leads to the delayed activation of CX3CL1.²⁹ Our study also revealed that neither CFA nor EA affected the expression level of CX3CR1. These results suggest that EA produces analgesic effects by regulating the expression of CX3CL1 in the lumbar spinal cord other than CX3CR1. Similarly, a previous study used neck incision model reported EA at upper-limb acupoints inhibited the expression of CX3CL1 in cervical spinal cord via regulating upstream ATP and purinergic P2X7 receptor.³⁰

Activated by cellular stress and proinflammatory cytokines, p38 MAPK is an important signal transduction molecule and plays an important role in inflammatory response as well as nociception.³¹ p38 MAPK has been reported to participate in the inflammatory pain induced by CFA.³² In the present study, EA treatment did not inhibit the expression of p38 MAPK, but regulated the pathological process of inflammatory pain by inhibiting its activation (phosphorylation). Intrathecal injection of CX3CL1 partially reversed the inactivation of p38 MAPK induced by EA, suggesting the p38 MAPK activation after EA treatment may be CX3CL1 related but not completely dependent. It was reported previously that transcriptional factors ATF-2 are involved in the modulation of p38 MAPK by EA.³³ We also found that in CFA rats without EA treatments, p38 MAPK was not further activated and the downstream cytokines were not increased by CX3CL1 intrathecal injection, this result suggests there is a ceiling effect on CX3CL1's potential of activating p38 MAPK, and CFA modeling may have maximally activated the CX3CL1-p38 MAPK signaling.

As the most significant indicator of inflammation, the cytokines of IL-1 β , IL-6 and TNF- α in lumbar spinal cord were all increased at the third day after CFA modeling, and decreased by EA treatment. It has been illustrated that the hyper-nociceptive effect elicited by CX3CL1 injection could be blocked by neutralizing IL-1 β and TNF- α ,³⁴ which indirectly confirmed that the regulation of CX3CL1 has a significant effect on the release of cytokines. However, the present study revealed that intrathecal injection of CX3CL1 increased the cytokine release in the spinal cord of EA-treated rats. This suggested the inhibition of CX3CL1 by EA is a direct cause of cytokine reduction in the spinal cord. As previous study has shown, the cytokine release exaggeration induced by CFA could be reversed by nociceptin/orphanin FQ (N/OFQ)³⁵ and EA could elicit the release of N/OFQ in the spinal cord,³⁶ the inhibition of CX3CL1 expression may not be the only pathway for EA to reduce the cytokine release, or there may be a contact between CX3CL1 and N/OFQ, but the specific ways are not clear at present.

There are some limitations in the present study. In order to investigate the analgesic effect of EA, we only gave EA treatment twice after the CFA injection, and tested the pain thresholds for 3 days. Although there is a report that acupuncture was effective only in the short-term management of pain,³⁷ a recent meta-analysis concluded that the effects of acupuncture with a course of treatment did not decrease for over 12 months in clinical practice.³⁸ Since previous

studies have demonstrated the changes of CX3CL1 in inflammatory reaction occur in the acute phase (no more than 4 days),^{13,38} we designed a 3-day experiment to explore the mechanism of the role of CX3CL1 in acupuncture-induced analgesia. However, expanding the observation window of EA-induced analgesic effect in CFA model as well as the regulation of CX3CL1-CX3CR1-p38 MAPK axis may help to provide more evidences for understanding the long-term effect of EA analgesia.

Conclusion

The current study demonstrated that EA treatment exerts analgesic effect through suppressing the signal transduction of CX3CL1-p38 MAPK-cytokine axis and the subsequent cytokine releasing, exogenous CX3CL1 could attenuate the analgesic effect of EA in CFA model. These findings provided new evidence on the analgesic mechanism of acupuncture.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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