Antipruritic effects of electroacupuncture on morphine-induced pruritus model mice through the TLR2/4-MyD88-NF-κB pathway

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Pruritus is one of the common side effects of intrathecal or epidural injection of opioids. The aim of this study was to test the antipruritic effect of acupuncture and its possible mechanism. We used electroacupuncture (EA), toll-like receptor (TLR)2/4 antagonist sparstolonin B (SsnB), and TLR2/4 agonist peptidoglycan (PGN) to precondition female wild-type BALB/c mice, and then prepared a morphine-induced pruritus model. The mRNA and protein expression levels of TLR2, TLR4, MyD88, and NF-κB were detected by RT-PCR and western blotting. The contents of interleukin (IL)-1, IL-6, IL-12, IL-10, and tumor necrosis factor-α in serum were measured by ELISA assays. Flow cytometry was performed to analyze the ratio of M1-phenotype to M2-phenotype macrophages. Our results showed that EA preconditioning improved pruritus; reduced the expressions of TLR2, TLR4, MyD88, and NF-κB both at the mRNA and protein levels ($P < 0.05$); reduced the expression of proinflammatory cytokines IL-1, IL-6, IL-12, and tumor necrosis factor-α; and increased the expression of anti-inflammatory cytokine IL-10 ($P < 0.05$). EA promoted M2-phenotype macrophage differentiation. Moreover, these results showed no significant difference between the SsnB group and the EA + SsnB group ($P > 0.05$), but showed a significant difference between the PGN group and the EA + PGN group ($P < 0.05$). Therefore, we propose that EA may be involved in the remission of pruritus in morphine-induced pruritus model mice through the TLR2/4-MyD88-NF-κB pathway. EA is a potential therapeutic treatment for pruritus. *NeuroReport* **30:331–337**

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Introduction

Opioids are used in the management of moderate to severe pain, and pruritus is a common adverse effect. The incidence of pruritus is reported to be 10–50% following intravenous administration of opioids, and 20–100% following neuraxial administration [1]. Although not life threatening, pruritus is discomforting and may decrease patient satisfaction. Mu opioid receptor antagonists have been the most consistent in terms of attenuating opioid-induced pruritus, but present problems in dose and administration. And other drugs (e.g. mixed opioid receptor agonist-antagonists, serotonin 5-HT3 receptor antagonists) have also been demonstrated to be useful [2]. However, current evidence is still insufficient to clearly mandate prophylactic administration of drugs to prevent opioid-induced pruritus. It has been reported that morphine and other opioids can provoke neuroinflammation by activating the innate immune system, including toll-like receptors (TLRs), but how the compounds turn on TLRs' signaling remains unclear [3,4].

Acupuncture is an ancient Chinese medical technique that is widely used as a form of complementary and alternative medicine in Asia. Acupuncture has been reported to be a relatively safe and well-tolerated therapy with only minor side effects, such as mild pain and local hematoma [5–7]. In clinical settings, reports about acupuncture treatment for pruritus have increased in recent years [8]. In a retrospective case series, acupuncture seemed to be effective in alleviating the distressing symptom of itching in patients presenting with neurogenic pruritus [9]. Pfab *et al.* [10] found that acupuncture at Quchi (LI11) and Xuehai (SP10) produced a significant reduction of clinically relevant type I hypersensitivity itch sensation in patients with atopic dermatitis. However, as a traditional Chinese medicine, the underlying mechanism of acupuncture on pruritus is not clear [11].

Sparstolonin B (SsnB), isolated from the Chinese herb *Scirpus yagara*, is a newly identified polyphenol with structural features of xanthone and isocoumarin [12].

Peptidoglycan (PGN) is the main cell wall component of Gram-positive bacteria [13]. A number of studies have shown that SsnB and PGN are TLR2/4 antagonist and agonist, respectively [12,14–16]. In the current study, we used SsnB to block, and PGN to activate, the TLR2/4-MyD88-NF-κB pathway, to study whether electroacupuncture (EA) plays an antipruritic role through the TLR2/4-MyD88-NF-κB pathway.

Materials and methods

Animals and reagents

Seventy adult female wild-type BALB/c mice were purchased from the Experimental Animal Center of Sun Yat-sen University. The mice were fed in 12-h light/dark cycles, with a light time of 8:00–20:00. In-vivo experiments were conducted at 9:00–12:00. Both the animal care and study protocol were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and followed the guidelines of the Ethics Committee of Sun Yat-sen Memorial Hospital, Sun Yat-sen University. All efforts were made to minimize suffering. SsnB and PGN were purchased from InvivoGen (California, USA). The mice were divided into blank group, model group, EA group, SsnB group, EA + SsnB group, PGN group and EA + PGN group, 10 mice in each group.

Treatment and preparation of the pruritus model

The bilateral acupoints of Quchi (LI11) and Xuehai (SP10) were selected for EA. Ipsilateral Quchi and Xuehai were connected to the same pair of electrodes. Quchi is located in the proximal side of the radial proximal joint of the mouse, and Xuehai is located in the medial hind-limb femur, medial end of the patella to the pubic symphysis junction under the 1/9. The mice were fixed on a mouse frame and treated with an intensity of 2 mA and a frequency of 2/15 Hz dilatational wave for 30 min. The acupuncture needle (7 mm × 0.20 mm; Huatuo, Suzhou, China) was inserted into the specific point at a depth of 5 mm vertically. EA preconditioning (i.e. preventive approach before itch provocation) was conducted each day for 5 consecutive days before modeling.

The EA mice were randomly divided into an EA group (intraperitoneal injection of the same volume of normal saline), EA + SsnB group, and EA + PGN group. For SsnB/PGN pretreatments, wild-type BALB/c mice were injected intraperitoneally with 5 mg/kg SsnB or 2 mg/kg PGN, respectively, without EA preconditioning (served as SsnB group and PGN group), or with EA preconditioning (served as EA + SsnB group and EA + PGN group) before modeling. The mice of the blank group were only fixed on the mouse frame for 30 min each day for 5 consecutive days before modeling, but without EA preconditioning or SsnB/PGN pretreatment.

After the last EA intervention, the model group and EA mice were used immediately for modeling. In this study, we used intrathecal morphine injection to induce pruritus [17,18]. Each mouse was held firmly by the pelvic girdle in one hand, while a microsyringe (Guangda Co. Ltd, Shanghai, China) was held in the other hand at an angle of about 20° above the vertebral column, and the experimenter inserted it into the tissue to one side of the L5 or L6 spinous process. The needle was then moved carefully forward to the intervertebral space, as the angle of the microsyringe was decreased to about 10°. The tip of the needle was inserted 4 mm into the vertebral column. Five microliters (0.3 nmol) morphine hydrochloride (Northeast Pharmaceutical Group, Shenyang, China) solution was injected for 5 s, and the needle was rotated on withdrawal. The blank group was injected intrathecally with 5 μl of normal saline according to the above procedure.

Behavioral assays

Behavioral assessment was carried out with reference to the literature [15]. Each group of mice was placed in the quiet environment of a behavior observation device without human presence, and their activity was recorded within 30 min after modeling using a camera. Using video playback, three persons blinded to the treatment groups independently counted the number of times the mice scratched. Scratching behavior of a mouse within 1 s was recorded as a scratch.

Sampling methods

Mice were anesthetized with halothane and decapitated. The spinal cord tissue was perfused with normal saline. When the liquid became colorless and transparent, it indicated that the spinal cord had been rinsed. The intumescentia lumbalis of the spinal cord was taken out and frozen in liquid nitrogen. After normal saline perfusion and then reperfusion with prechilled 4% paraformaldehyde, the intumescentia lumbalis of the spinal cord was eviscerated and sliced until all tissues were fully fixed (stretching the limbs with a sense of stiffness).

Total RNA extraction and quantitative RT-PCR

Total RNA was extracted from the spinal cords by using TRIzol reagent (Invitrogen, California, USA). A cDNA Synthesis Kit (Takara, Dalian, China) was used for the synthesis of cDNA according to the manufacturer's instructions. Quantitative RT-PCR was performed to detect the expression levels of mRNA using SYBR Green and a LightCycler 480 detection system (Roche Diagnostics, Indianapolis, Indiana, USA). GAPDH mRNA levels were used for normalization. The quantitative RT-PCR results were analyzed and expressed as relative mRNA levels on the basis of C_t value, which was then converted to fold change.

Western blot assays

Spinal cord tissue frozen by liquid nitrogen was ground, and then the cells were lysed using Lysis Buffer (P0013; Beyotime, Shanghai, China). The total protein concentration of lysates was measured using a Micro BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA).

The samples were separated on 10% SDS-polyacrylamide gels and then electrophoretically transferred to polyvinylidene difluoride membranes. After incubation in blocking solution (5% non-fat milk) for 1 h, the membranes were incubated with anti-TLR2, anti-MyD88, anti-NF-κB, or GAPDH antibodies (CST, Massachusetts, USA) diluted in blocking solution for 2 h at 37°C. Thereafter, the membranes were washed with 1× TBST solution and treated with horseradish peroxidase-conjugated secondary antibodies for 1 h. Next, the membranes were treated with ECL solution (Millipore, Darmstadt, Germany) according to the manufacturer's instructions and then quantified using a ChemiDoc image analysis system (Bio-Rad Laboratories, California, USA). The mean gray values of each protein band were measured by Photoshop CS5 software (Adobe, San Jose, California, USA). The relative band intensity is the ratio of the gray value of the protein of interest to that of the corresponding GAPDH.

Detection of inflammatory cytokines by ELISA

Phosphate buffer (0.5 mol/l Na_2HPO_4 0.5 ml and 0.5 mol/l NaH_2PO_4 0.5 ml) was added to 100 mg spinal cord tissue and homogenized with a tissue homogenizer in an ice bath. The protein concentration was diluted to 1–5%. After centrifugation, the supernatant was saved. The inflammatory cytokines interleukin (IL)-1β (ab100705; Abcam, Cambridge, USA), IL-6 (ab100713; Abcam), IL-10 (ab108870; Abcam), and tumor necrosis factor (TNF)-α (ab100747; Abcam) were measured using ELISA kits. The procedures were carried out according to the manufacturer's instructions.

Flow cytometric analysis of macrophages

Macrophage separation and flow cytometric analysis are described in a previous report [19]. Fresh spinal cord tissue was ground into a single-cell suspension and filtered through a 45-mm nylon mesh filter. The macrophages were then separated by density gradient centrifugation. Isolated macrophages were collected and washed by PBS, and then incubated with FITC anti-CD86 antibodies (BD Pharmingen; BD Biosciences, San Jose, California, USA) and anti-CD163 antibodies (GeneTex, Irvine, California, USA) for 30 min at room temperature. After being washed by PBS, the cells were analyzed on an FACSCalibur flow cytometer (Accuri C6; BD Biosciences).

Statistical methods

SPSS19.0 statistical analysis software (IBM SPSS, Chicago, Illinois, USA) was utilized for statistical analyses. The significance of the differences was determined by Student's *t*-test or one-way analysis of variance. The least significant difference post-hoc test was used where equal variances were assumed, whereas Dunnett's T3 test was used when equal variances were not assumed. The data are presented as the mean ± SD, and *P* value less than 0.05 indicates that the difference was statistically significant.

Results

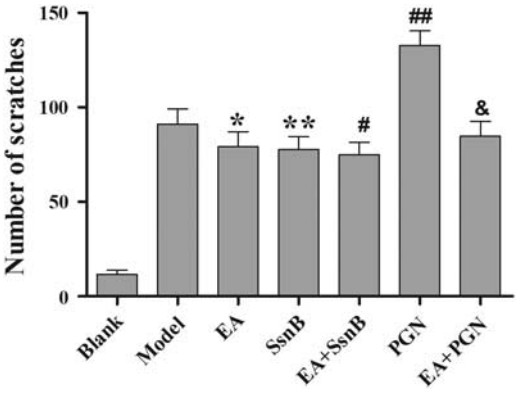
Electroacupuncture improved pruritus through the TLR2/4-MyD88-NF-κB pathway

As shown in Fig. 1, compared with the blank group, the number of scratches in the model group was markedly increased ($P < 0.01$). With EA preconditioning only, the number of scratches was significantly reduced ($P < 0.05$). With EA and TLR2 antagonist SsnB preconditioning, EA + SsnB group did not show stronger antipruritic effect than the EA group or the SsnB group ($P > 0.05$). Preconditioning with TLR2 agonist PGN resulted in significantly increased pruritus in the PGN group compared with the model group ($P < 0.05$). Conversely, together with EA and PGN preconditioning, the EA + PGN group had significantly improved pruritus compared with the PGN group ($P < 0.01$).

Electroacupuncture affected the expressions of TLR2, TLR4, MyD88 and NF-κB

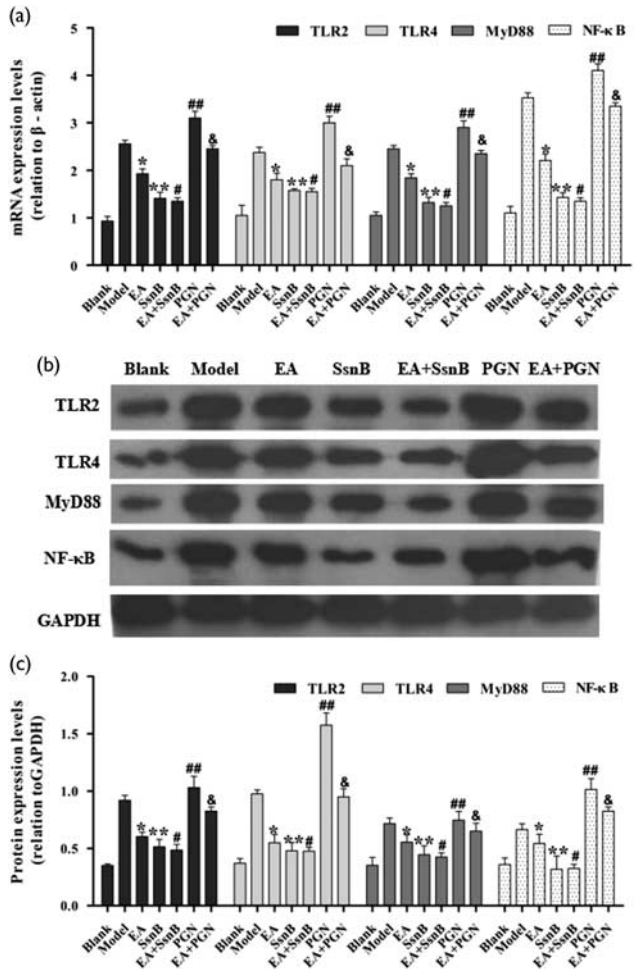
Using qRT-PCR and western blotting (Fig. 2), we found that compared with the blank group, TLR2, TLR4, MyD88 and NF-κB expression was significantly upregulated in the model group ($P < 0.01$), whereas EA preconditioning markedly downregulated their expression levels ($P < 0.05$). Moreover, we also found that the expressions of TLR2, TLR4, MyD88 and NF-κB were significantly downregulated in the SsnB group and the EA + SsnB group compared with the model group ($P < 0.05$), but these results showed no significant difference between the SsnB group and the EA + SsnB

Fig. 1



The statistical results of scratch number in each group. Using video playback, three persons blinded to the treatment groups independently counted the number of times the mice scratched. Scratching behavior of a mouse within 1 s was recorded as a scratch. The data are presented as mean ± SD ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.05$ and $^{\#\#}P < 0.05$ compared with the model group, $^{\#}P > 0.05$ compared with the SsnB group. $^{\&}P < 0.01$ compared with the PGN group. EA, electroacupuncture; PGN, peptidoglycan; SsnB, sparstolonin B.

Fig. 2



mRNA and protein expression levels of TLR2, MyD88 and NF-κB in each group. (a) Relative mRNA expression levels of TLR2, TLR4, MyD88 and NF-κB by RT-PCR. (b) Western blot detected the expression of TLR2, TLR4, MyD88 and NF-κB. (c) Photoshop CS5 software analysis of the protein expression levels (relation to GAPDH). The data are presented as mean ± SD ($n = 10$). Each bar represents the mean of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.05$ and $^{\#\#}P < 0.05$ compared with the model group, $^{\#}P > 0.05$ compared with the SsnB group. $^{\&}P < 0.05$ compared with the PGN group. EA, electroacupuncture; PGN, peptidoglycan; SsnB, sparstolonin B; TLR, toll-like receptor.

group ($P > 0.05$). PGN preconditioning increased TLR2, TLR4, MyD88 and NF-κB expression compared with the model group ($P < 0.05$), whereas EA + PGN treatment reduced TLR2, TLR4, MyD88 and NF-κB expression compared with PGN preconditioning only ($P < 0.05$).

Electroacupuncture reduced inflammatory response

We used ELISA assays to explore the effects of EA on the expression of inflammatory cytokines in serum. ELISA results (Table 1) showed that EA significantly reduced the contents of IL-1, IL-6, IL-12, and TNF-α and increased the concentration of IL-10 compared with the pruritus model mice ($P < 0.05$). Furthermore, we found that the concentrations of IL-1, IL-6, IL-12, and TNF-α were significantly downregulated, and IL-10 upregulated, in the SsnB group and the EA + SsnB group compared with the model group ($P < 0.01$), but these results have no significant difference between the SsnB group and the EA + SsnB group ($P > 0.05$). PGN preconditioning increased the concentrations of IL-1, IL-6, IL-12 and TNF-α, and reduced the concentration of IL-10 compared with the model group ($P < 0.05$). However, EA + PGN treatment significantly reduced the concentrations of IL-1, IL-6, IL-12 and TNF-α, and increased the concentration of IL-10 compared with PGN preconditioning only ($P < 0.05$).

Electroacupuncture regulated macrophage differentiation

We evaluated the M1-phenotype to M2-phenotype ratio using flow cytometry (Fig. 3). In morphine-induced pruritus model mice, the M1 macrophage ratio (CD86-FITC positive) was 65.2 ± 3.45%, whereas the proportion of M2 macrophages (CD163-PE positive) was only 34.7 ± 3.40%. EA preconditioning markedly downregulated the proportion of M1 macrophages and upregulated the proportion of M2 macrophages compared with the model group ($P < 0.05$). In addition, the ratio of M1/M2 macrophages showed no significant difference between the SsnB group and the EA + SsnB group ($P > 0.05$), but showed significant difference between the PGN group and the EA + PGN group ($P < 0.05$).

Discussion

In long-term practice, acupuncturists have gradually found effective combinations of anti-itching points-Quchi (LI11) and Xuehai (SP10). Wang *et al.* [20] found that EA at LI11 and SP10 significantly inhibits itching reactions in mice induced by compound 48/80, which might be an effective method for treatment of pruritus. Meng *et al.* [21] discovered that acupuncture at LI11 and SP10 relieves itching as well as having an antihistamine and antianaphylaxis effect. A further study comparing electrical stimulation of acupoints LI11 and SP10 to cetirizine showed significant itch reduction after verum acupuncture or cetirizine treatment compared with respective placebos and no treatment in atopic dermatitis patients [22]. In the present study, we found that EA improved morphine-induced pruritus. This is consistent with the above previous studies suggesting that EA does indeed inhibit pruritus. Specific acupoint selection (LI11 and SP10) and manipulation (electrical stimulation method, needle insertion depth, and time) may play an important role in antipruritic effect. Weak acupoint stimulation might not generate an antipruritic effect with application of intrathecal morphine [23].

The TLR2/4-MyD88-NF-κB pathway is associated with immune response; its activation is key to the development of many diseases, and NF-κB is a pivotal

Table 1 **The levels of inflammatory cytokines in each group ($n = 10$)**

	IL-1	IL-6	IL-12	IL-10	TNF-α
Blank	24.12 ± 4.68	16.42 ± 3.78	70.25 ± 8.95	80.22 ± 10.42	175.68 ± 20.27
Model	612.75 ± 49.45	90.75 ± 10.26	281.4 ± 52.42	20.26 ± 5.21	504.71 ± 36.69
EA	198.42 ± 32.75*	40.16 ± 8.76*	167.44 ± 35.31*	42.16 ± 10.23*	314.59 ± 32.65*
SsnB	100.24 ± 37.16**	24.68 ± 6.30**	102.36 ± 33.58**	63.57 ± 9.27**	241.33 ± 41.11**
EA + SsnB	95.32 ± 26.34#	20.68 ± 5.46#	98.36 ± 26.13#	65.34 ± 12.47#	248.67 ± 36.98#
PGN	756.68 ± 52.28##	134.16 ± 12.52##	346.42 ± 48.87##	30.45 ± 6.69##	621.44 ± 47.15##
EA + PGN	606.37 ± 38.98&	83.24 ± 11.48&	271.26 ± 42.22&	21.56 ± 8.64&	481.28 ± 36.52&

Data are presented as mean ± SD of three dependent experiments.
EA, electroacupuncture; PGN, peptidoglycan; SsnB, sparstolonin B.
*$P < 0.05$.
**$P < 0.05$.
##$P < 0.05$ compared with the model group.
#$P > 0.05$ compared with the SsnB group.
&$P < 0.05$ compared with the PGN group.

downstream signaling molecule in the TLR2/4-MyD88 pathway [16,24]. MyD88, the first identified member of the TLR family, is universally used by all TLRs, except TLR3, and activates the transcription factor NF-κB to induce inflammatory cytokines [25]. The subfamily of TLR2 receptors can activate NF-κB and AP-1-regulated genes, including the inflammatory cytokines TNF-α and IL-6 [26]. The inflammatory cytokines include IL-1, IL-4, IL-5, IL-10, IL-12, IL-13, IL-17, interferon-γ, and TNF-α. In this study, the mRNA and proteins of TLR2, TLR4, MyD88 and NF-κB increased in the morphine-induced pruritus model mice, and EA decreased the expression of all four. Moreover, EA decreased the concentrations of proinflammatory cytokines IL-1, IL-6, IL-12, and TNF-α and increased that of the anti-inflammatory cytokine IL-10. These results showed that EA can regulate the immune response.

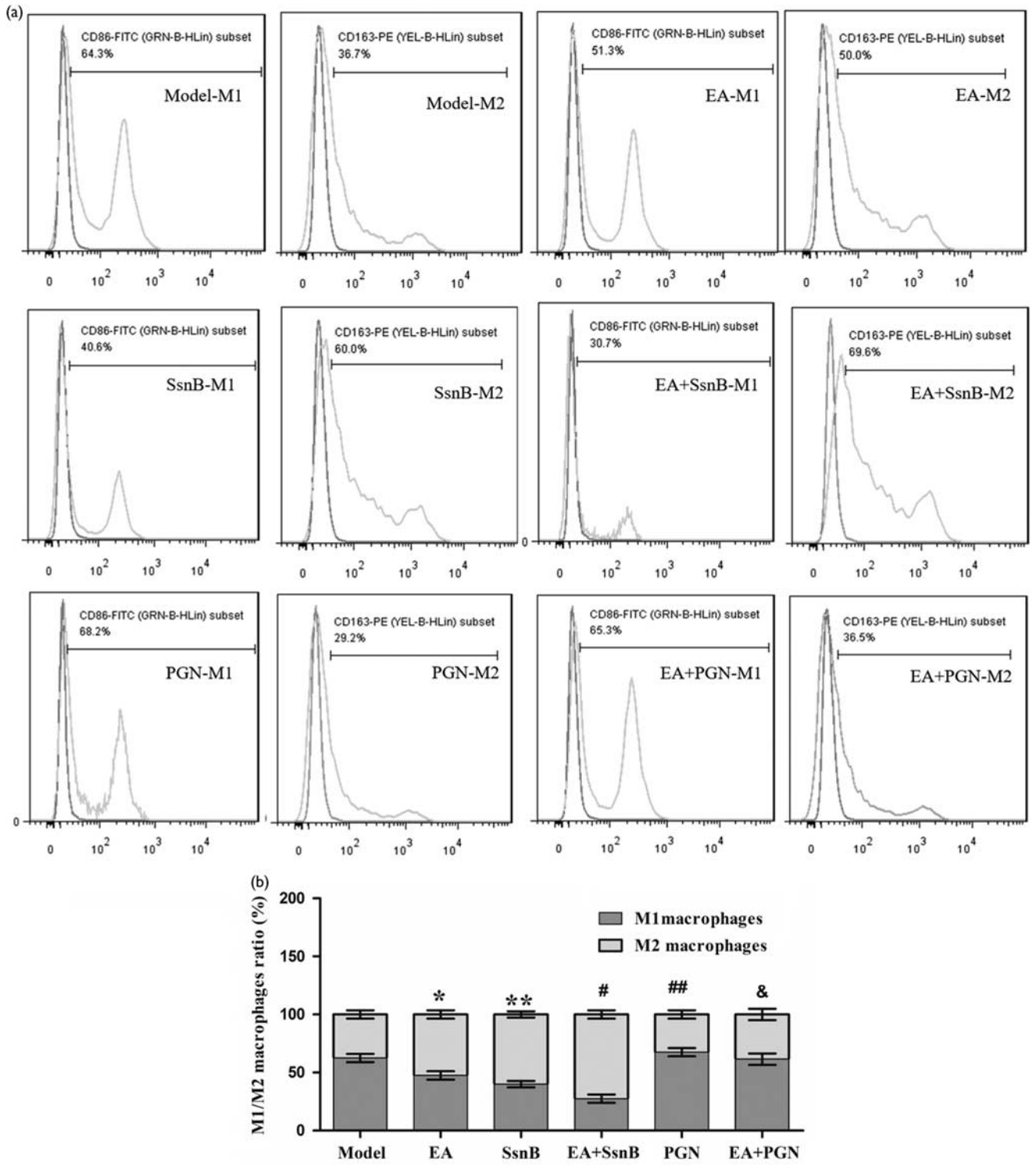
Macrophages have functions of damage and repair, and these different effects may be administered by different macrophage subsets: the 'classic activation' of proinflammatory (M1) macrophages and 'alternative activation' of anti-inflammatory (M2) macrophages [27]. Phenotypes associated with M1 and M2 macrophages can be distinguished from the direct effects of microbial stimuli, such as lipopolysaccharide, which induce innate macrophage activation, often through TLRs. Innate stimuli are able to synergize with M1 macrophages to achieve full expression of macrophage effector pathways [28]. In the presence of cytokines, the activating macrophages increase their 'alternatively activated' M2-phenotype, which promotes matrix remodeling and angiogenesis while suppressing destructive immunity [29]. In this study, the M1 macrophages were activated in the morphine-induced pruritus model mice, and EA inhibited M1 macrophage activation. In addition, after EA treatment, the inflammatory cytokine levels decreased, and these effects play a useful role in attenuating pruritus.

A previous study showed that SsnB is a selective TLR2 and TLR4 antagonist that can block TLR2-triggered and TLR4-triggered inflammatory signaling by inhibiting the recruitment of MyD88 to the TIR domains of TLR2 and TLR4 [12,14]. A number of studies have shown that PGN activates cells through TLR2, which results in the activation of NF-κB [15,16]. It is believed that TLR2 agonists mainly induce the production of inflammatory cytokines [30], while, conversely, TLR2 antagonists inhibit the production of inflammatory cytokines. In the present study, compared with the model group, EA, SsnB and the combination of EA and SsnB preconditioning, respectively, improved pruritus; decreased the expression of TLR2, TLR4, MyD88 and NF-κB; decreased the concentrations of proinflammatory cytokines IL-1, IL-6, IL-12, TNF-α; increased the concentration of the anti-inflammatory cytokine IL-10; and inhibited M1 macrophage increase. EA and SsnB did not have mutual enhancement or counteraction with each other but had similar effects on antipruritus and inflammatory response. PGN preconditioning strengthened pruritus. The expressions of TLR2, TLR4, MyD88, NF-κB and related inflammatory response in PGN group had stronger increased effects than those in model group. However, a combination of EA and PGN preconditioning affected the above results markedly compared with PGN preconditioning only. These results indicate that EA exerts its antipruritic effects through the TLR2/4-MyD88-NF-κB pathway.

Limitations

Our study has several limitations. First, we did not use gene knockout mice to confirm the specific role of TLR2 or TLR4 related to the mechanisms of acupuncture-related antipruritic effect. Second, besides TLR2/4-MyD88-NF-κB pathway, there are many other mediators and neuronal pathways in the relieving process of itch, the potential effects of which we cannot exclude. Third, there was no acupuncture control in this study. Therefore, the changes in this research might not be induced by acupuncture or acupoint specificity but rather by mechanical and electrical stimulation of acupuncture. Hence, further observations with stricter control of these relevant variables are inevitable to undrape the antipruritic mechanism of acupuncture.

Fig. 3



Flow cytometric analysis of M1-phenotype and M2-phenotype. (a) M1-phenotype and M2-phenotype macrophages measured using flow cytometry. (b) The M1-phenotype to M2-phenotype macrophage ratio in each group. CD86 used for M1-phenotype macrophage-specific marker and CD163 used for M2-phenotype macrophage-specific biomarker. The purple line in each image represents the blank group, and the orange line represents the corresponding group. Each bar represents the mean of three independent experiments performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.05$ and $^{\#\#}P < 0.05$ compared with the model group, $^{\#}P > 0.05$ compared with the SsnB group. $^{\&}P < 0.05$ compared with the PGN group. EA, electroacupuncture; PGN, peptidoglycan; SsnB, sparstolonin B.

Conclusion

EA was involved in the remission of pruritus in morphine-induced pruritus model mice through the TLR2/4-MyD88-NF-κB pathway, and EA may be a potential therapeutic treatment for pruritus. Further research is needed to assess the effect of acupuncture on morphine-induced pruritus in human patients and to explore other related molecular events.

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Yu Shan Ye and Ai Zhen Pan had the same contribution to the paper. Yan Zhen, Meng Ru Kang and Bin Zhang participated in the research. Wei Min Yi wrote the final paper.

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Conflicts of interest

There are no conflicts of interest.

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