

## Stage-dependent anti-allodynic effects of intrathecal Toll-like receptor 4 antagonists in a rat model of cancer induced bone pain

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**Abstract** It has been reported that Toll-like receptor 4 (TLR4), which plays an important role in glial activation in neuropathic pain, is significantly increased in cancer pain. The present study was designed to assess the role of TLR4 in cancer-induced bone pain (CIBP) by intrathecal administration of TLR4 signaling pathway blocker naloxone or lipopolysaccharide *Rhodobacter sphaeroides* (LPS-RS). The rats developed significant mechanical allodynia from day 8 after intratibial Walker 256 inoculation. Intrathecal injection of naloxone or LPS-RS at day 8 significantly attenuated mechanical allodynia as shown by increased paw withdrawal thresholds. In contrast, the same pharmacological treatment showed no or slight pain relieving effect at day 16. Our findings demonstrate that the spinal TLR4 signaling pathway contributes to the mechanism underlying CIBP in a stage-dependent manner in rats, and it may be an efficacious target at early stage for the treatment in the future.

**Keywords** Cancer-induced bone pain · Toll-like receptor 4 · Spinal cord · Glia

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### Introduction

Cancer-induced bone pain (CIBP), usually caused by primary or secondary bone cancer, is one of the most common and serious pain conditions in cancer patients that severely impairs patients' quality of life [1]. Because of the relative ineffectiveness and the adverse effects of the current available treatments, almost half of cancer patients have inadequate or under-managed pain control [1, 2]. Thus, it is important to find new potential therapeutic targets for cancer pain relief.

It has been reported that, in the rat model of CIBP [3, 4], there is a sustained increase in the expression of spinal Toll-like receptor (TLR) 4. TLR4 is a member of the Toll-like receptors family and can recognize the invariant molecular structures of pathogens (termed pathogen-associated molecular patterns, PAMP) and participate in innate immunity [5]. Further, there is evidence that the TLR4 signaling pathway correlates with the formation of neuropathic pain [6–8]. These reports suggest that TLR4 may play an important role in cancer-induced pain. Intrathecal injection of TLR4 small interfering RNA (siRNA), leads to a pain relieving effect at an early stage (day 9 post-tumor inoculation) of CIBP in the rat model [4, 9]. However, very little literature is available on the role of TLR4 in cancer pain relief by the use of pharmacological agents.

Naloxone is a classic antagonist for opioid receptors. Numerous researchers have reported that naloxone can effectively inhibit microglial activation and production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) through blocking the TLR4 signaling pathway, and it has been widely used as a nonstereoselective TLR4 signaling pathway blocker in the animal research [10–12]. Recently, we reported that intrathecal injection of naloxone had a pain relieving effect at day 8 post-tumor cells inoculation in a rat

model of CIBP induced by intratibial injecting with rat mammary gland carcinoma cells Walker 256 [3]. In Lan's research, the effect of TLR4 siRNA on pain initiation or pain relief were only observed at a relative early stage (day 4 or day 9) in a rat model of CIBP [4]. However, the role of the TLR4 signaling pathway at a relative late stage of CIBP still remains unknown.

Therefore, the present study was designed to investigate the stage-dependent role of the spinal TLR4 signaling pathway in a rat model of CIBP induced by intra-tibial Walker 256 inoculation. As TLR4 can be activated by LPS (lipopolysaccharide, a well-known exogenous ligand for TLR4) and potential endogenous ligands (e.g., members of the heat shock protein family, proteoglycans and host DNA, RNA, etc.), leading to nuclear factor kappa B (NF- $\kappa$ B) activation and subsequent induction of proinflammatory cytokines [13, 14], in the present study both naloxone and LPS-RS (lipopolysaccharide *Rhodobacter sphaeroides*, one kind of analogue of LPS commonly used as antagonist for TLR4) were intrathecally administrated at different stages of CIBP and the pain relieving effects were evaluated.

## Materials and methods

### Animals

A total of 184 adult female Wistar rats (160–180 g) were housed in temperature- ( $22 \pm 2$  °C) and light-controlled (12-h light/dark cycle) rooms with standard rodent chow and water available ad libitum. All experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the Ethical Issues of the International Association of Study Pain IASP [15]. All efforts were made to minimize the number of animals used and their suffering.

### Cell preparation and implantation

Consistent with previous reports [16–18], the procedure for the induction of cancer-induced bone pain was as follows. Briefly, Walker 256 rat mammary gland carcinoma cells were collected from cancerous ascitic fluid and diluted to a final concentration of  $1 \times 10^8$  cells/ml phosphate-buffered saline (PBS) solution. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for surgery. Four microliters of carcinoma cells ( $4 \times 10^5$ ) or vehicle (PBS solution only) were injected into the bone cavity by using a 10- $\mu$ l microinjection syringe with 23-gauge needle. The syringe was left in place for an additional 2 min to prevent the carcinoma cells from leaking out along the injection track.

### Behavioral tests

Rats were placed individually on an elevated iron mesh in a clear plastic cage and were allowed to adapt to the testing environment for at least 30 min. Calibrated von Frey hairs (0.16, 0.4, 0.6, 1.0, 1.4, 2.0, 4, 6, 8, 10, 15 g) were applied to the plantar aspect of each hind paw. Each stimulus was applied for approximately 1 s with an interstimulus interval of approximately 3 s. Only robust and immediate withdrawal responses from the stimulus were recorded. A positive result was considered when 3 or more withdrawal responses were obtained from 5 consecutive trials with each monofilament. The 15-g hair was selected as the upper cut-off limit for testing [19].

### Drugs and intrathecal administration

Researchers have reported that naloxone could effectively inhibit microglial activation and the production of TNF- $\alpha$  and IL-1 $\beta$  through the TLR signal pathway [12] and it has been widely used as a nonstereoselective TLR4 signaling pathway blocker in the study of the TLR4 [10, 11, 20]. LPS-RS was a TLR4 signaling pathway blocker naturally produced by *Rhodobacter sphaeroides*. Naloxone (Sigma, St Louis, MO, USA) and LPS-RS (Invitrogen, San Diego, CA, USA) were dissolved in endotoxin-free physiological saline with aseptic procedures. Endotoxin-free physiological saline was administered in equal volumes to the drugs as vehicles in the test.

According to the previous report [13], after the rats were anesthetized with gaseous isoflurane, LPS-RS (10, 20 or 40  $\mu$ g) in 20  $\mu$ l, naloxone (60  $\mu$ g) in 20  $\mu$ l or normal saline (NS) was intrathecally injected by using a modified lumbar puncture technique at days 8, 12, and 16 after surgery. Briefly, an 27-gauge needle attached to a 25- $\mu$ l microinjection syringe was inserted through the space between the fifth lumbar vertebra (L5) and L6 at a 45° angle toward the cranioventral direction. When a sudden tail movement was observed, the drug was injected slowly in 60 s and the syringe was held in place for over 10 s to prevent outflow of the drug. The i.t. injection of the drug by this technique shows an average spread of the drug up to the 10th thoracic vertebra (T10)–T11 level, and the success rate of lumbar punctures in our hands is more than 95 %.

### Bone histology

As described previously [16], the tibia bone was fixed in 4 % paraformaldehyde, then immersed in a decalcifying solution, cut into 7- $\mu$ m cross-sections, and stained with hematoxylin and eosin to visualize the extent of tumor infiltration and bone destruction.

## Statistical analysis

All data were presented as the mean  $\pm$  standard error of the mean (SEM) and analyzed by one-way ANOVA.  $P < 0.05$  was considered statistically significant.

## Results

To evaluate the bone destruction induced by Walker 256 cancer cells, sections from the tibial bone were stained with hematoxylin and eosin. As shown in Fig. 1c, d, the bone marrow cavity was full of tumor cells and the bone matrix was destroyed at days 12 (Fig. 1c) and 16 (Fig. 1d) post-inoculation. In cases of severe bone destruction, the tumor destroyed the bone matrix and periosteum and grew outside the bone at the late stage after cancer cell inoculation. However, no bone destruction was observed in the PBS inoculation group in the whole period of the experiment (Fig. 1b).

In order to assess the development of mechanical allodynia induced by the intratibial Walker 256 inoculation, the paw withdrawal threshold (PWT) to von Frey hair stimulation was detected. Before Walker 256 inoculation, there were no significant differences in the mean baseline of PWT among the normal group, sham group, and CIBP group. After inoculation, though the rats inoculated with cancer cells and PBS displayed a decrease in PWT as compared with the normal rats, the rats inoculated with cancer cells displayed a profound decrease in PWT to von Frey hair stimulation from 8 days after inoculation as compared with PBS rats ( $P < 0.05$ ) (Fig. 1e). Furthermore, the mechanical allodynia were observed on both sides of the hind paws in Walker 256-inoculated rats though it had been inoculated unilaterally, which was consistent with our previous report [16].

It has been reported that the spinal TLR4 level increased gradually with the growth of tumors and the bone destruction [3, 4]. Our previous work had shown that the spinal TLR4 was significantly increased from day 8 post-Walker 256 inoculation [3]. In order to evaluate the stage-dependent role of TLR4 in CIBP, the TLR4 signaling pathway blocker naloxone or LPS-RS were intrathecally injected and the anti-allodynic effect was measured at day 8 (a relatively early stage of CIBP) and day 16 (a relatively late stage) as well as day 12 (middle stage) post-inoculation in this study. Forty-five minutes after naloxone administration, the PWT in naloxone-treated rats at day 8 post-Walker 256 inoculation was significantly increased in both sides of the hind paws as compared to the normal saline-treated rats (Fig. 2a). However, naloxone showed a slight pain relieving effect on the contralateral hind paw but no effect on the ipsilateral hind paw at day 16 post-Walker 256 inoculation (Fig. 2b).

Similar results were observed when LPS-RS was intrathecally administered in this rat model of CIBP. LPS-RS showed no significant effect on mechanical thresholds at day 16 post-Walker 256 inoculation (Fig. 3c), while it showed significant anti-allodynic effect on CIBP at both day 8 (Fig. 3a) and day 12 (Fig. 3b) post-Walker 256 inoculation. The anti-allodynic effect was also bilateral.

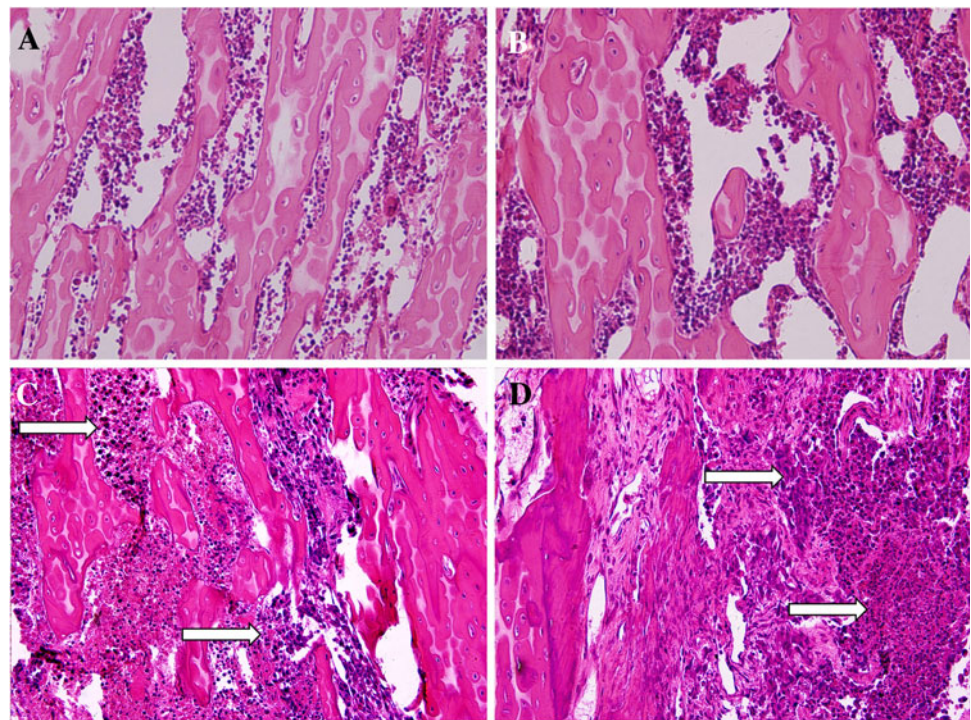
## Discussion

In the present study, the stage-dependent anti-allodynic effects of naloxone and LPS-RS were observed in a rat model of CIBP. Intrathecal administration of naloxone or LPS-RS significantly attenuated bilateral mechanical allodynia induced by unilateral bone cancer at days 8 or 12 after tumor inoculation—the relatively early and middle stages of CIBP, which is consistent with the reported anti-allodynic effects of siRNA against the spinal TLR4 signaling pathway in the similar rat model of CIBP [4]. However, LPS-RS showed no effects while naloxone showed a slight effect on the contralateral hind paw at day 16 (relatively late stage) after tumor inoculation in the present study. Our results indicated that the anti-allodynic effect by naloxone and LPS-RS on CIBP was stage-dependent.

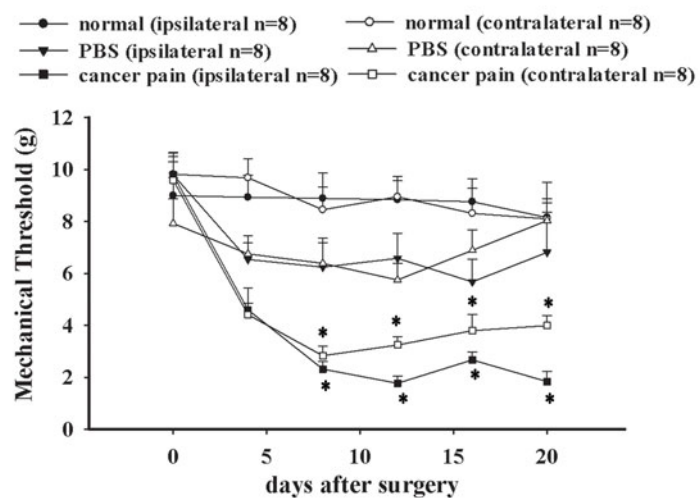
Following previous reports [10, 11, 20, 21], naloxone and LPS-RS were used as TLR4 signaling pathway blockers in the present study. In the present rat model of CIBP induced by intratibial inoculation of Walker 256 carcinoma cells, both Lan's and our previous work had shown that the spinal TLR4 mRNA and protein level were significantly increased at an early stage of CIBP [3, 4]. Previous *in vivo* and *in vitro* studies have established that TLR4 is almost exclusively expressed by microglia in the central nervous system (CNS) [22–24]. The TLR4 signaling pathway contributes to behavioral hypersensitivity in chronic pain by producing proinflammatory cytokines through initiating microglial activation [25, 26]. Mice lacking TLR4 showed markedly reduced microglia activation and reduced pain hypersensitivity after nerve injury. Furthermore, it has been reported that naloxone can effectively inhibit microglial activation and the production of TNF- $\alpha$  and IL-1 $\beta$  through the TLR signaling pathway, consequently relieving pain behavior [10–12]. Our work also demonstrated that naloxone can prevent the increasing expression of spinal TNF- $\alpha$  and IL-1 $\beta$  induced by tibial bone cancer [3]. Based on these results, we propose that the anti-allodynic effect of naloxone and LPS-RS on CIBP may be through inhibition of microglia activation and the induction of inflammatory cytokines via spinal TLR4.

However, naloxone has been proved as a non-selective opioid receptor antagonist, and the opioid signaling

**Fig. 1** Development of Walker 256 carcinoma cells induced bone pain. **a–d** Histology of tibia bone destruction stained by H&E. **a** Bone from normal rats; **b** bone injected with PBS; **c**, **d** showing tumor growth and bone destruction after Walker 256 injection at day 12 (**c**) and day 16 (**d**) (tumor cells are marked by *hollow arrows*). **e** Showing the development of mechanical allodynia to von Frey hair stimulation in normal, Walker 256 or PBS-inoculated rats. Data are expressed as means  $\pm$  SEM. \* $P < 0.05$  versus PBS inoculated rats



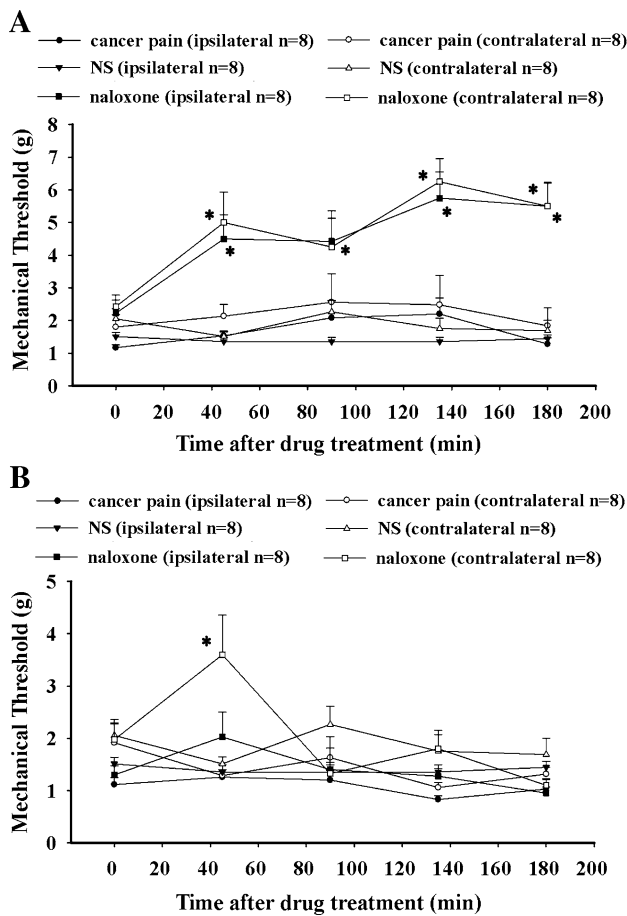
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pathway plays an important role in pain modulation. Recent reports have shown that opioids may non-stereoselectively influence TLR4 signaling and have behavioral consequences resulting, in part, via TLR4 signaling [20, 27]. Naloxone also blocked morphine non-stereoselectively induced TLR4 signaling in vitro. Pharmacological blockade of TLR4 signaling in vivo potentiated acute intrathecal morphine analgesia, and attenuated development of analgesic tolerance, hyperalgesia, and opioid withdrawal behaviors [20, 27]. These further suggested that naloxone can be used as a non-selective TLR4 antagonist and also demonstrated that TLR4 may play a role in cancer-induced

pain. But whether naloxone exerts its analgesic effect through inhibiting the opioid signaling pathway or consequently blocking the TLR4 signaling pathway needs to be further investigated.

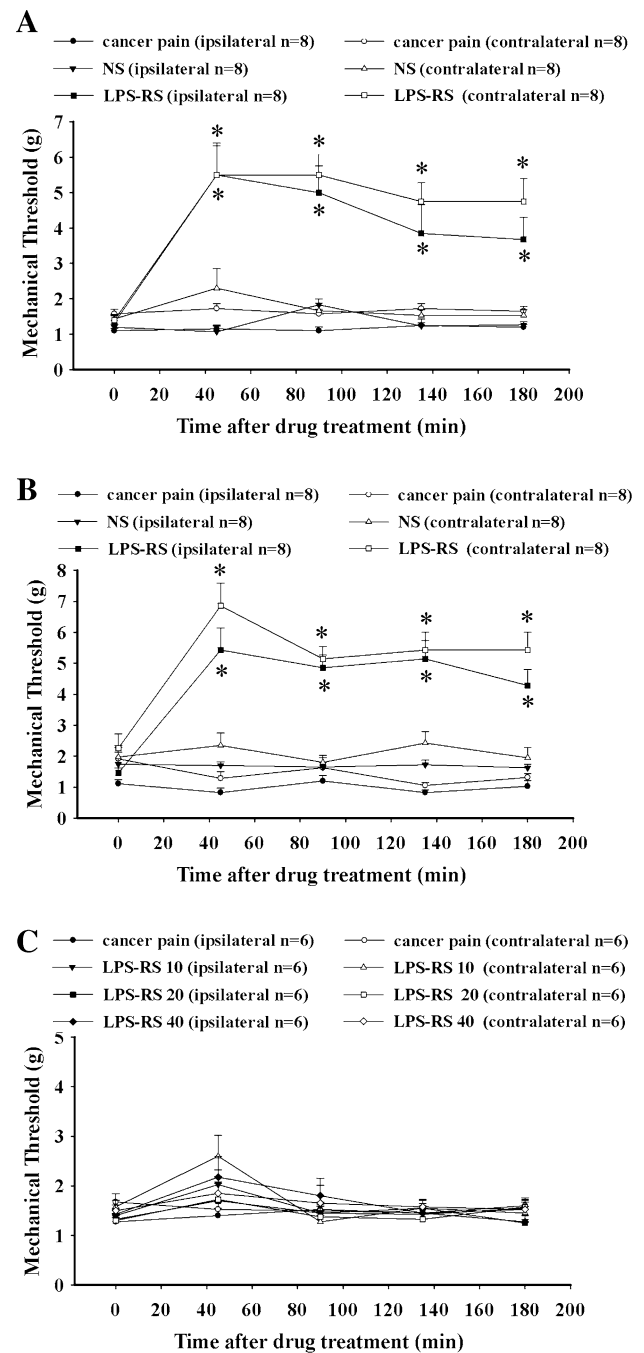
Notably, although a long-lasting elevation of spinal TLR4 mRNA and protein expression were observed after cancer cell inoculation [3, 4], no or only very weak anti-allodynic effects of naloxone and LPS-RS were observed at day 16—the relatively late stage of CIBP in the present study. Our results further suggested the complex underlying mechanisms of cancer-induced pain. It has been reported that, in addition to the neuropathic component,



**Fig. 2** The effects of naloxone on cancer induce pain. Naloxone (60  $\mu$ g, i.t.) increased PWT significantly at day 8 after surgery (a), while increased PWT only at 45 min after i.t. injection in the contralateral paw at day 16 (b). \* $P < 0.05$  versus NS-treated rats

tumor-induced inflammation is considered to be another important ingredient in the development of cancer-induced pain [28–30]. The complexity of neurochemical changes in the central nervous system may give rise to the complex cancer-induced pain [16, 31]. Hence, we strongly believe that there may be some other neurochemical factors rather than the TLR4 signaling pathway contributing to cancer-induced pain, since both LPS-RS and naloxone have no or slight effect at day 16 but have an analgesic effect at day 8. This is further supported by Lan’s report that only a partly pain relieving effect is observed by intrathecal injecting of TLR4 siRNA at day 9. Further studies at the relative stage of CIBP are expected to achieve great insights into the complex mechanisms underlying CIBP and to lead to new approaches for cancer pain management.

Taken together, our present results further confirmed that the spinal TLR4 signaling pathway is important for the development of CIBP. And, to the best of our knowledge, we report for the first time that both LPS-RS and naloxone



**Fig. 3** The effects of LPS-RS on cancer-induced bone pain. I.t. administration of LPS-RS (20  $\mu$ g) increased PWTs significantly at day 8 (a) and day 12 (b) after surgery while LPS-RS (10, 20, 40  $\mu$ g) showed no effect at day16 (c). \* $P < 0.05$  versus NS-treated rats

(the TLR4 signaling blocker) could attenuate mechanical allodynia in the early, but not late, stages of CIBP, indicating a stage-dependent role of the spinal TLR4 signaling pathway in the mechanism underlying CIBP.

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**Conflict of interest** The authors declare no conflict of interest.

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