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EphrinB-EphB receptor signaling contributes to bone cancer pain via Toll-like receptor and proinflammatory cytokines in rat spinal cord

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ABSTRACT

Treating bone cancer pain poses a major clinical challenge, and the mechanisms underlying bone cancer pain remain elusive. EphrinB-EphB receptor signaling may contribute to bone cancer pain through *N*-methyl-D-aspartate receptor neuronal mechanisms. Here, we report that ephrinB-EphB signaling may also act through a Toll-like receptor 4 (TLR4)-glial cell mechanism in the spinal cord. Bone cancer pain was induced by tibia bone cavity tumor cell implantation (TCI) in rats. TCI increased the expression of TLR4 and the EphB1 receptor, the activation of astrocytes and microglial cells, and increased levels of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). The increased expression of TLR4 and EphB1 were colocalized with each other in astrocytes and microglial cells. Spinal knockdown of TLR4 suppressed TCI-induced behavioral signs of bone cancer pain. The TCI-induced activation of astrocytes and microglial cells, as well as the increased levels of IL-1β and TNF-α, were inhibited by intrathecal administration of TLR4-targeting siRNA2 and the EphB receptor antagonist EphB2-Fc, respectively. The administration of EphB2-Fc suppressed the TCI-induced increase of TLR4 expression but siRNA2 failed to affect TCI-induced EphB1 expression. Intrathecal administration of an exogenous EphB1 receptor activator, ephrinB2-Fc, increased the expression of TLR4 and the levels of IL-1β and TNF-α, activated astrocytes and microglial cells, and induced thermal hypersensitivity. These ephrinB2-Fc-induced alterations were suppressed by spinal knockdown of TLR4. This study suggests that TLR4 may be a potential target for preventing or reversing bone cancer pain and other similar painful processes mediated by ephrinB-EphB receptor signaling.

1. Introduction

Treating bone cancer pain continues to present a major clinical challenge, and the specific cellular and molecular mechanisms underlying bone cancer pain remain elusive [4,54]. Studies have demonstrated that these mechanisms may involve a combination of inflammatory and neuropathic pain with unique characteristics [11,12]. Nociceptors in bone are stimulated via the activation of transient receptor potential vanilloid type 1, endothelin A, and the TrkA receptor. Such activation is directed by an acid microenvironment, endothelin-1, and nerve growth factor, respectively [12]. We have recently demonstrated that ephrinB-EphB receptor signaling, which is critical to the developmental processes of the nervous system and synaptic plasticity of adult nervous systems [22,39,56,60,61], is greatly upregulated in the dorsal root ganglion and spinal cord (SC), and contribute to the development of bone cancer pain [28]. This may open a new avenue to understanding the mechanisms of bone cancer pain and supports the idea that the process of bone cancer may elicit neuronal alterations that recapitulate events during development and are important to the production or persistence of bone cancer pain.

Ephrin (Eph) receptors comprise the largest family of receptor tyrosine kinases, which play vital roles in transmitting external signals to the interior of many types of cells. EphB receptors and their ligands, ephrinBs, are critical in regulating cell shape, adhesion and repulsion, migration and positioning during developmental processes [39,56,60]. Alteration of the mechanisms controlling adhesion and motility has a central role in promoting tumor invasion and angiogenesis [3,10,43]. Altered expression of ephrins and/or Eph receptors has been implicated in tumor progression in a number of human malignancies [18,36,50].

EphrinB-EphB receptor signaling in SC may contribute to bone cancer pain through interaction with *N*-methyl-D-aspartate

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receptors (NMDARs) and the subsequent Ca^{2+}-dependent signals. In addition, blocking ephrinB-EphB receptor signaling can inhibit the activation of spinal astrocytes and microglial cells [28]. Studies have demonstrated that astrocytes and microglial cells, which act as parts of the innate immune system, become active in the status of bone cancer and release various substances, including the proinflammatory cytokines interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), which could evoke hyperalgesia and allodynia [16,40,57,58,62,63]. Inhibiting astrocytes and/or microglial cell activity in SC can attenuate bone cancer pain [40,57,58,62].

Cells of the innate immune system recognize the invariant molecular structures of pathogens through Toll-like receptors (TLRs), most of which are stable and genetically conserved cell-surface receptors [33]. Among these, TLR4 activation is thought to be important in microglial cell activation and the development of bone cancer pain [8,19,24,27,46]. We thus hypothesized that ephrinB-EphB signaling might contribute to the pathogenesis of bone cancer pain through the glial cell activation pathway, in addition to the neuronal NMDAR-mediated pathway. This study reports that ephrinB-EphB receptor signaling activates astrocytes and microglial cells by activating or interacting with TLR4, increases the activity of the proinflammatory cytokines IL-1β and TNF-α, and thus leads to bone cancer pain.

2. Methods

2.1. Animals, anesthesia, drugs, and administration

All animals were used in accordance with the regulations of the ethics committee of the International Association for the Study of Pain and all protocols were approved by the Institutional Animal Care and Use Committees. Four hundred thirty-six adult female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 160–80 g and 6 TLR4-deleted mice (C57BL/10ScNCr; The Jackson Laboratory, Bar Harbor, ME) were used in this study. All surgery was done under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally).

An EphB1 receptor-blocking reagent—EphB2-Fc chimera (E9402, mouse recombinant, Sigma-Aldrich, St. Louis, MO)—was used in vivo to determine the possible roles of the EphB1 receptor in tumor cell implantation (TCI)-induced pain-like behaviors and the associated neurochemical alterations. An exogenous EphB2-Fc chimera can combine with endogenous ephrinB1–B3, which normally combine with their corresponding EphB receptors, including EphB1, and thus endogenous EphB1 is substituted and cleaved. This EphB1 substitution and cleavage results in dysfunction of the EphB1 receptor and the inhibition of EphB1-mediated downstream signals.

An EphB1 receptor activator—ephrinB2-Fc chimera (E0778, mouse recombinant, Sigma-Aldrich)—was also employed in the experiments. EphrinB2-Fc chimera can combine with EphB1–B4 receptors and thus activate the EphB1 receptor and its downstream signals [9,17,23,28,49]. Each of the drugs used was dissolved in phosphate buffered saline (PBS) or dimethylsulfoxide (1%) and then 20 μl were injected intrathecally (i.t.) by means of lumbar puncture at the intervertebral space of L4–5, and L5–6 for multiple injections. The doses of these drugs were: EphB2-Fc, 5 μg; ephrinB2-Fc, 2 μg; and control Fc at 5 μg and 2 μg, respectively, for EphB2-Fc and ephrinB2-Fc.

2.2. Small interfering RNA knockdown of TLR4

Based on the complete genome of TLR4 (GenBank Accession No. NM_019178), we designed 3 small interfering RNA (siRNA) duplexes to target rat TLR4. The sequences were as follows: siRNA1: 5′-CGTACCAACAGAGAGGATATT-3′ and 5′-TATCCTCTCTCTTGGTAC GTT-3′; siRNA2: 5′-GTCTCAGATATCTAGATCTTT-3′ and 5′-AGATCTA GATATCTGAGACTT-3′; siRNA3: 5′-GGGAGCCGGAAAGTTATTGTG-3′ and 5′- CACAATAACTTTCCGGCTCTT-3′. Sequences were subjected to a BLAST search (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that there was no significant homology with other genes. A scrambled sequence was designed as a mismatch control (misiRNA).

The siRNA (siRNA1, siRNA2, or siRNA3) was dissolved in RNase-free water at a concentration of 1 μg/μL as stock solution. The siRNA was mixed with branched polyethyleneimine (PEI; Sigma Co., St. Louis, MO) for 10 minutes at room temperature before in vivo i.t. to increase cell membrane penetration. PEI was dissolved in 5% glucose, and 1 μg of siRNA was mixed with 0.18 μL of PEI. In the rats that received TCI, an siRNA was administered daily for 3 consecutive days on postoperative days 3, 4, and 5 or postoperative days 7, 8, and 9, each at 2 μg. TLR4 knockdown was analyzed by immunoblotting with an antibody to TLR4.

2.3. Model of bone cancer pain

Tumor cells were extracted from the ascitic fluid of rats that had received Walker 256 mammary gland carcinoma cells. TCI was achieved by injecting the tumor cells (1×10^5 cells/μL, 5 μL) into the intramedullary space of the right tibia to induce bone cancer in rats. Sham surgery was done using a similar procedures by injecting boiled cells. The protocol was similar to that previously described [28,32]. Of the 436 Sprague-Dawley rats used in this study, 208 received TCI, 124 received sham surgery, and 104 naïve rats received no surgery. Of the 6 TLR4-deleted mice, 3 received TCI and 3 received sham surgery. The numbers of animals and SC tissues used in each of the experiments are indicated in the corresponding figure captions. Experimenters who performed behavioral tests, drug administration and preparation of SC tissues for Western blot analysis and immunohistochemistry were blinded to this surgery.

2.4. Assessment of bone cancer-related pain behaviors

Mechanical allodynia and thermal hyperalgesia were assessed in rats with bone cancer to evaluate bone cancer-related pain. Mechanical allodynia was indicated by a significant decrease in the threshold of paw withdrawal to mechanical indentation of the plantar surface of each hind paw. The withdrawal was measured using an electronic von Frey meter (Model 1601; IITC Life Science, Woodland Hills, CA) with a protocol similar to that described previously [48,59]. In brief, the animals were placed individually beneath an inverted ventilated cage with a metal-mesh floor. The rigid von Frey filaments with uniform tip diameter were applied perpendicularly to the mid-plantar surface of each hind paw from beneath until the paw was withdrawn, and the result was shown digitally on a display screen. The maximum pressure was set as 30 g. The duration of each stimulus was approximately 1–2 seconds and the interstimulus interval was approximately 10–15 seconds. A total of 5 tests were conducted on each hind paw.

Thermal hyperalgesia was determined by the significantly shortened latency of foot withdrawal in response to heat stimulation. An analgesia meter (Model 336, Series 8; IITC Life Science) was used to provide a heat source. The protocol was similar to that described previously [48,59]. In brief, each rat was placed in a box ($22 \times 12 \times 12$ cm) containing a smooth, temperature-controlled glass floor. The heat source was focused on a portion of the hind paw, and a radiant thermal stimulus was delivered to that site. The stimulus shut off automatically when the hind paw moved (or after 20 seconds to prevent tissue damage). The intensity of

the heat stimulus was maintained constant throughout all experiments. Thermal stimuli were delivered 4 times to each hind paw at intervals of 5–8 minutes.

Spontaneous and movement-evoked pain-like behaviors were also analyzed [20]. Spontaneous nocifensive behaviors were evaluated by measuring spontaneous guarding and flinching over a 2-minute period of observation. Movement-evoked pain was assessed by measuring the time spent guarding over a 2-minute period of observation after non-noxious palpation, and limb use during spontaneous ambulation, which was scored on a scale of 0 to 4: 0 = normal use; 1 = slightly limping; 2 = clearly limping; 3 = no use of the limbs (partial); and 4 = no use of the limbs.

2.5. Western blot analysis

The whole SC at the L4–L5 segments was quickly removed from deeply anesthetized rats. The tissues were homogenized in radioimmunoprecipitation assay lysis buffer (Bio-Rad Laboratories, Hercules, CA) containing a cocktail of protease inhibitor and phosphatase inhibitors (Sigma). The homogenates were incubated for 20–30 minutes in ice-cold water, vortexed for 10 seconds on the highest setting every 5 minutes, and then centrifuged at 13,000*g* for 10 minutes. The supernatants were collected and centrifuged at 13,000*g* for 10 minutes again. The second supernatants were collected and the protein concentration in supernatants was estimated using the bicinchoninic acid assay.

The samples were boiled at 100°C for 10 minutes with 1× loading buffer (Bio-Rad Laboratories). Equivalent amounts of protein (60–70 μg) were separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis) at 100 V for about 2 hours, and then transferred onto a polyvinylidene fluoride membrane at 300 mA for about 40–70 minutes (depending on the molecular weight of target protein). Membranes were blocked with 3–5% no-fat dry milk (Bio-Rad Laboratories) for 1–3 hours at room temperature or overnight at 4°C, and then incubated with primary antibodies [TLR4 (1:500; Cat#ab13556, rabbit polyclonal antibody, Abcam, Cambridge, UK); ionized calcium-binding adapter molecule 1 (Iba-1; 1:1000; Abcam; glial fibrillary acidic protein (GFAP; 1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:50,000; Sigma)] overnight at 4°C. The primary antibodies were diluted with 3% no-fat dry milk (Bio-Rad Laboratories). After washing with Tris-buffered saline with Tween 20 (TBST) for 3 × 5 minutes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (R&D Systems, Minneapolis, MN) (1:4000∼5000) for 2 hours at room temperature and then washed with TBST for 3 × 5 minutes.

To detect more than one protein on the same membrane, the membrane was stripped with stripping buffer (Thermo Fisher Scientific, Waltham, MA). In brief, after being visualized with chemiluminescence reagents, the membrane was washed with TBST for 3 × 5 minutes and then incubated in the stripping buffer for 20–30 minutes at room temperature. After washing again with TBST for 3 × 5 minutes, the membrane was blocked with 3–5% no-fat dry milk (Bio-Rad Laboratories) for 1 hour at room temperature and then incubated with another primary antibody overnight at 4°C.

To analyze the possible interaction of EphB1 and TLR4 proteins, coimmunoprecipitation (coIP) was used. The coIP protocol was similar to that described previously [38]. In brief, 400 μL of precleared extract were incubated with EphB1 antibody at 4°C overnight. Then 40 μL protein G agarose beads were added and incubated for 3 hours. The beads that contained the immunocomplex were collected by centrifugation, washed 4 times with PBS, and used for subsequent Western blot analysis. The bands were visualized with chemiluminescence reagents provided with the ECL kit (PerkinElmer, Waltham, MA).

Data were analyzed with Quantity One 1-D Analysis Software Version 4.6.5 (Bio-Rad Laboratories). Each of the control (sham) and treatment (TCI and other treatments) groups was first standardized with the control IgG (for EphB1) or GAPDH (for TLR4, GFAP and Iba1). The mean value of the controls (n = 4) was set as "1", which was used for normalization of each of the 4 control groups. For each of the molecules detected (n = 4), the control group (set as "1") was used for normalization of the protein expression in the corresponding groups at each of the data points after different treatments. The results were expressed in the figures as fold changes.

2.6. Immunohistochemistry

Under deep anesthesia, the rats were transcardially perfused with 4% paraformaldehyde. The whole SC at the L4–L5 segments was then dissected out, postfixed overnight at 4°C and cryoprotected in 20% and 30% sucrose at 4°C. The embedded blocks were sectioned (25 μm thick). Sections were placed in a small, heat-resistant basket, immersed in retrieval solution (10 mM sodium citrate buffer, pH 6.0) for 5 minutes, and then stored in PBS at 4°C.

Sections of SC (10–12 sections from each rat, a total of 30–36 sections from 3 rats in each group) were incubated with blocking solution containing 1% BSA, 5% donkey serum (Abcam) and 0.3% Triton X-100 at room temperature for 1.5 hours. The sections were then incubated in the following primary antibodies: rabbit anti-TLR4 polyclonal antibody (1:200; Abcam), mouse anti-EphB1 polyclonal antibody (1:100; #3980; Cell Signaling Technology, Danvers, MA), mouse antineuronal nuclear protein (NeuN; 1:100; FITC conjugated; Chemicon, Temecula, CA), goat anti-GFAP (1:500; sc-6170, Santa Cruz Biotechnology), and goat anti-Iba1 (1:100; ab5076; Abcam). The sections were then washed 3 times with Tris-buffered saline and incubated with the specific secondary antibodies raised in donkey (conjugated to Cy3 or FITC; Abcam) overnight at 4°C. For double and triple staining, sections were incubated in the same blocking solution for a second and the third time and the staining process was performed once or twice again. Following immunostaining procedures, the SC sections were examined using laser scanning confocal microscopy (FluoView FV1000, Olympus Co., Tokyo, Japan).

2.7. Level of IL-1β and TNF-α determination

The whole SC at the L4–L5 segments was rapidly removed from deeply anesthetized rats (n = 4 each group). The tissues were homogenized in ice-cold 100 mM PBS. Protein concentrations were determined by the bicinchoninic acid assay. The levels of IL-1β and TNF-α were measured using an enzyme-linked immunospecific assay (ELISA) (IL-1β kit: RLB00; TNF-α kit: RTA00) (R&D Systems) according to the manufacturer's instructions [15,45].

2.8. Statistical analysis

SPSS Rel. 15 (SPSS Inc., Chicago, IL) was used to conduct all the statistical analyses. Differences in the latency of thermal withdrawal and the threshold of mechanical withdrawal over time were tested with repeated measures analysis of variance (RM ANOVA). After demonstrating that the data were normally distributed using the Shapiro-Wilks *W* test, a two-way RM ANOVA followed by Bonferroni post hoc tests involving the treatment factors and postoperative days were used to test the significance of difference in withdrawal latency and threshold between experimental conditions. Alterations of the concentrations of

cytokines and the expression of the proteins detected among groups were tested with one-way ANOVA with repeated measures followed by Bonferroni post hoc tests. All data are presented as means ± SEM. Statistical results are considered significant if $P < 0.05$.

3. Results

3.1. TCI causes increased expression and colocalization of EphB1 and TLR4 in astrocytes and microglial cells in the dorsal horn

The results confirmed our recent finding that the expression of the EphB1 receptor in SC is significantly increased in a time-related manner following TCI. Meanwhile, the expression of TLR4 in SC was also significantly increased in a pattern similar to that of EphB1. The increased expression of EphB1 and TLR4 started at day 5 after TCI, peaked at day 7–14, and remained at a high level of expression until day 21, the last test day. In addition, TCI caused an increase in the microglial Iba1 and astrocyte GFAP marker in SC, indicating activation of the microglial cells and astrocytes. Iba1 was significantly upregulated at day 5 after TCI, peaked at day 7–14, and remained a high level with a slight decrease on day 21. The increased expression of GFAP started at day 7 after TCI (delayed by 2 days compared with Iba1), peaked at day 14 and remained at a high level until day 21.

Representative Western blotting bands and a summary of the data for the expression of EphB1, TLR4, GFAP, and Iba1 are shown in Fig. 1A. The increased immunoreactivity of EphB1 and TLR4 receptors, as well as that of Iba1 and GFAP, was distributed extensively in the superficial layers of the dorsal horn (DH; Iba1 also in the deep DH), ipsilateral to TCI, an important region in SC processing of nociceptive information (Fig. 1B). Although the specificity of some commercially available rat and mouse TLR4 antibodies has been questioned, our data showed that the rabbit polyclonal TLR4 antibody used in this study successfully detected TLR4 expression in SC. A representative Western blotting band and an immunoreactivity image of TLR4 showing specificity of TLR4 antibody are shown in Fig. 1C and D.

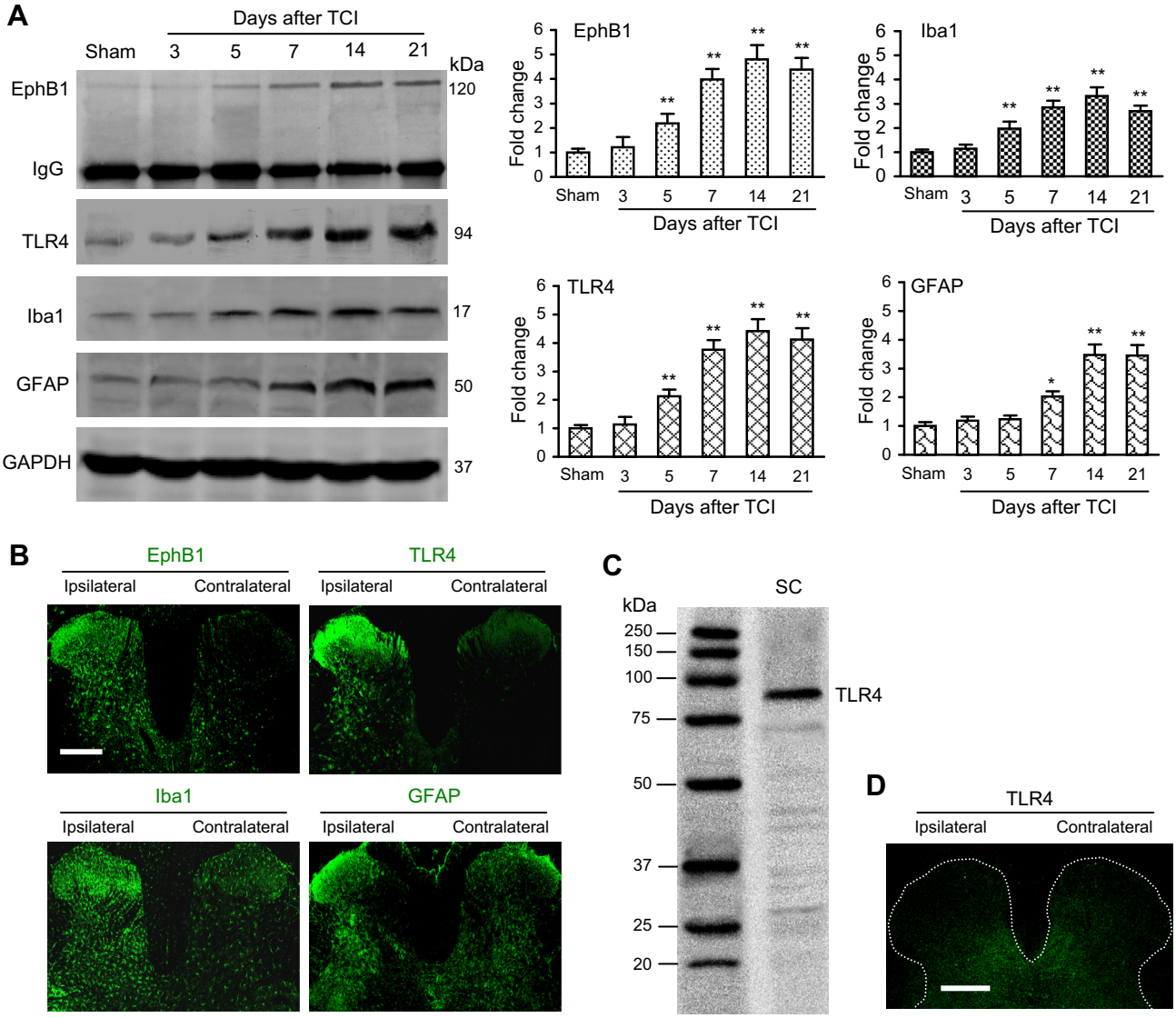

Fig. 1. Upregulation of expression of EphB1 and TLR4 as well as astrocytes (GFAP) and microglial cells (Iba1) in spinal cord (SC) after tumor cell implantation (TCI). (A) Examples of Western blot analysis and data summary (n = 4 in each group) showing a time-dependent increased expression of the molecules. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham. (B) Representative confocal images showing distribution of immunostaining for EphB1 and TLR4 as well as GFAP and Iba1 in the dorsal horn (DH) ipsilateral and contralateral to TCI. (C, D) Examination of specificity of the TLR4 antibody used for detecting TLR4 expression in the Western blot analysis (C) and immunofluorescent staining (confocal image, D). The dotted line in D indicates the outline of the DH. Tissues were collected from Sprague-Dawley rats (A–C) and TLR4-deleted mice (D). All the tissues in B–D were collected on day 14 after TCI. Magnification: 100× for the images in B and D (bar = 400 μm).

Confocal images showed that TCI-induced increased EphB1 immunoreactivity was colocalized with neurons as well as astrocytes and microglial cells in the DH (Fig. 2A). Unlike for EphB1, TCI-induced increased TLR4 immunoreactivity was colocalized to a large extent with GFAP, a small amount with Iba1, and not at all with NeuN in the DH (Fig. 2B). This is consistent with the observation that TLR4 is expressed mostly on glial cells [2,13], although TLR4 is also expressed in trigeminal sensory neurons [6]. Our results also showed that TLR4 was colocalized with EphB1 in the DH (Fig. 2C). We then continued to identify the possible cell type(s) in which TLR4 might be colocalized with EphB1. Since EphB1 was colocalized with NeuN, GFAP, and Iba1 (Fig. 2A), and TLR4 extensively with astrocytes and to a small degree with microglial cells, but not with NeuN (Fig. 2B), we examined the possible colocalization of TLR4 and EphB1 in astrocytes and microglial cells in the DH. Confocal images with triple staining showed that TLR4 and EphB1 were colocalized extensively with astrocytes and occasionally with microglial cells (Fig. 2D). In addition, coimmunoprecipitation analysis (Fig. 2E) showed that EphB1 was colocalized with TLR4 in SC. These results strongly suggest the possibility of

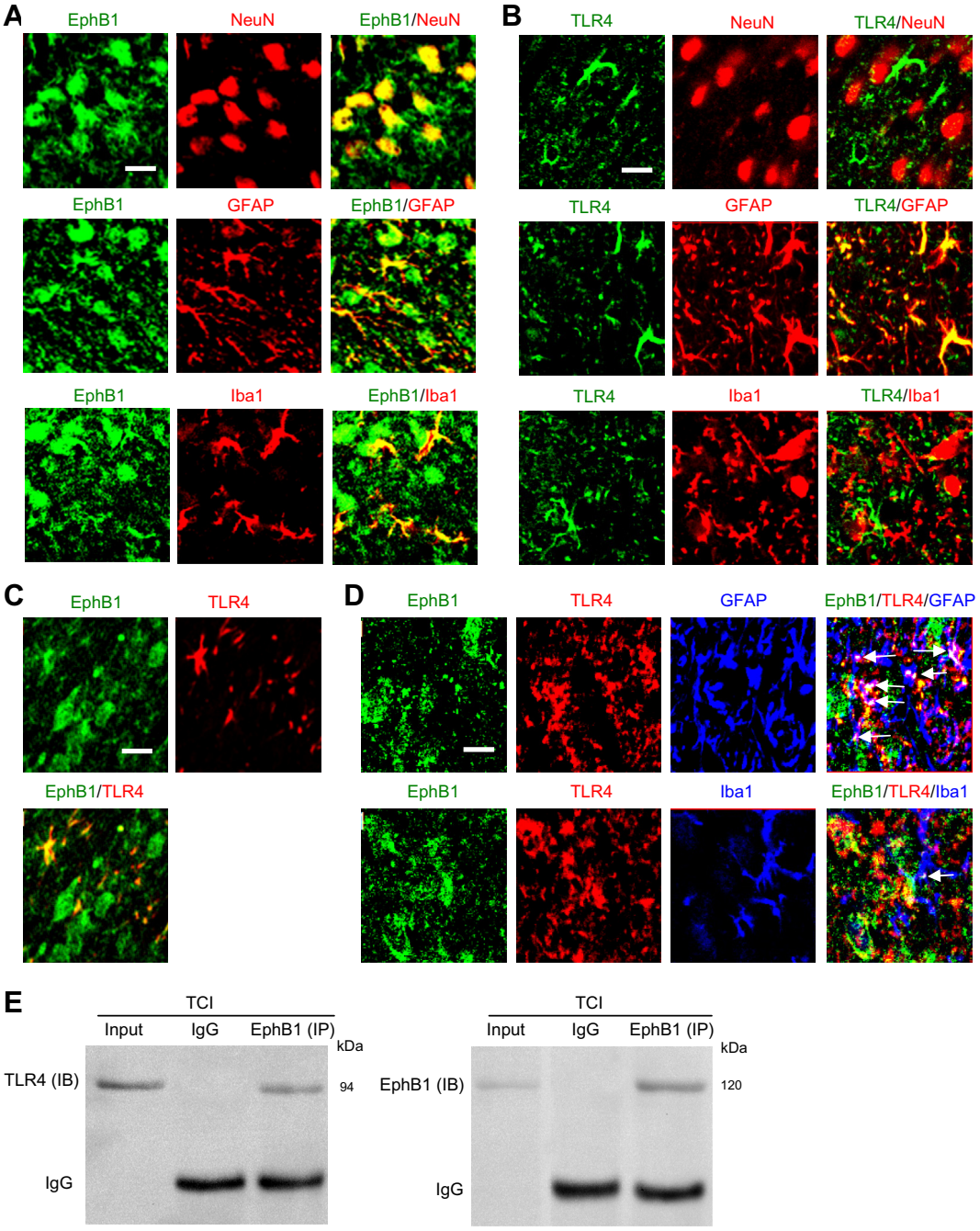

Fig. 2. Expression and colocalization of TLR4 and EphB1 in the superficial dorsal horn (DH) and whole spinal cord (SC) after tumor cell implantation (TCI). (A, B) Representative confocal images showing expression of EphB1 (A, green) and TLR4 (B, green) and their colocalization with neurons (NeuN, red), astrocytes (GFAP, red) and microglial cells (Iba1, red) in the superficial dorsal horn (DH) ipsilateral to TCI. (C) Representative confocal images showing colocalization of EphB1 (green) and TLR4 (red) in the superficial DH ipsilateral to TCI. (D) Representative confocal images (triple staining) showing colocalization of EphB1 (green), TLR4 (red) and GFAP (blue) (top) as well as EphB1 (green), TLR4 (red) and Iba1 (blue) (bottom) in the superficial DH ipsilateral to TCI. The white dots indicated by the arrows represent the merging of the 3 molecules. Top: most of the merge (yellow) of EphB1 (green) and TLR4 (red) also merged (became white) with GFAP (blue). Bottom: a very small amount of merge (yellow) of EphB1 (green) and TLR4 (red) was merged (became white) with Iba1 (blue). Magnification: 1000× for all the images in A–D (bar = 10 μm). (E) Representative Western blots showing coimmunoprecipitation (IP) of EphB1 and TLR4 in the whole SC (repeated in 3 SC samples). All the tissues in A–E were collected on day 14 after TCI.

a functional link and interaction between EphB1 and TLR4 in astrocytes and microglial cells in the DH.

3.2. Knockdown of TLR4 prevents and suppresses TCI-induced mechanical allodynia and thermal hyperalgesia as well as spontaneous and movement-evoked pain-like behaviors

Rats that received TCI exhibited significant levels of mechanical allodynia and thermal hyperalgesia as well as spontaneous and movement-evoked pain-like behaviors, manifested as guarding, flinching, and reduced limb use. We designed 3 TLR4-targeting siRNAs—siRNA1, siRNA2, and siRNA3—as described in the Methods. TLR4 knockdown by each of these 3 siRNAs was analyzed by immunoblotting with an antibody to TLR4. The results showed that the expression of TLR4 protein in SC from naïve rats was significantly reduced by the in vivo administration of siRNA2 and siRNA3 (each 2 μg i.t. daily for 3 consecutive days), while siRNA1 and the mismatch siRNA (misiRNA) failed to inhibit TLR4 expression (Fig. 3A).

siRNA2 Produced the most effective knockdown of TLR4 and we thus chose siRNA2 in the same protocol. Repeated spinal administration of TLR4-targeting siRNA2 (2 μg i.t., early phase treatment) significantly delayed the onset of mechanical allodynia (Fig. 3B) and thermal hyperalgesia (Fig. 3C) for approximately 1 week following TCI. The same doses of siRNA2 administrated in the late phase produced a significant transient inhibition of mechanical allodynia (Fig. 3D) and thermal hyperalgesia (Fig. 3E). Such inhibition lasted for about 6 days after termination of the last injection.

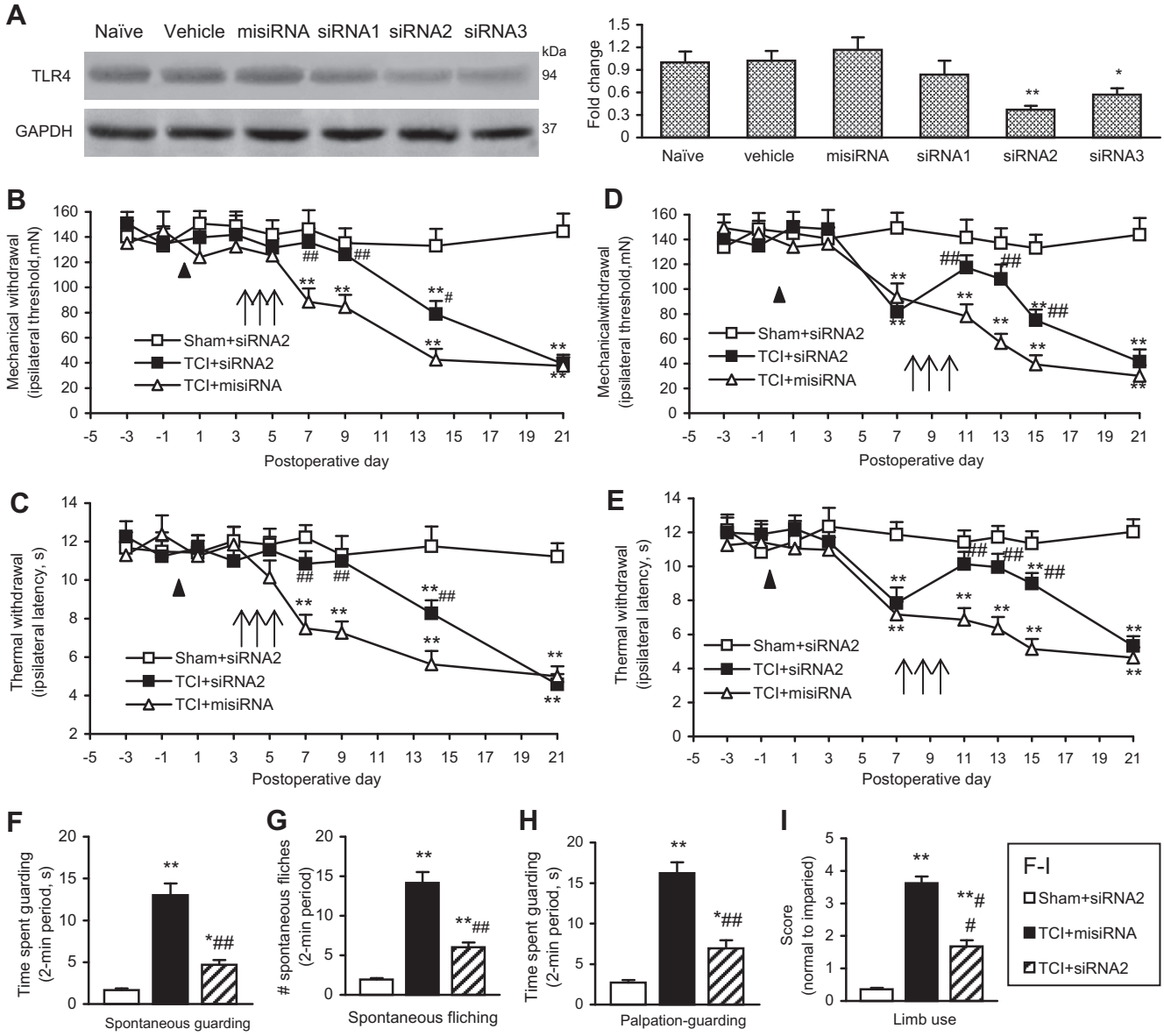

Fig. 3. Knockdown of TLR4 in spinal cord prevents and attenuates mechanical allodynia and thermal hyperalgesia as well as spontaneous and movement-evoked pain-like behaviors in rats after tumor cell implantation (TCI). (A) Effects of intrathecal (i.t.) TLR4-targeting small interfering RNAs (siRNAs) on the expression of TLR4 in naïve rats; vehicle, mismatch siRNA (misiRNA), siRNA1, siRNA2, and siRNA3 (2 μg i.t. daily for 3 consecutive days) were administered to separate groups of rats (n = 4 in each group). Whole spinal cord tissue was collected 2 hours after the last injection. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the control groups (naïve, vehicle and misiRNA). (B–E) Mechanical allodynia (B, D) and thermal hyperalgesia (C, E) in the feet ipsilateral to TCI. TCI was performed on day 0 (▲). $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham + siRNA2. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TCI + siRNA2. (F–I) Spontaneous and movement-evoked pain-like behaviors were suppressed by repeated administration of siRNA (2 μg i.t. daily for 3 consecutive days on postoperative days 7, 8, and 9). Pain behaviors were measured on postoperative day10. $^{*}P < 0.05$, $^{**}P < 0.01$ vs sham + siRNA2. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs TCI + misiRNA. Eight rats were included in each group (B–I).

The siRNA2 control misiRNA administered under the same protocols did not alter allodynia or hyperalgesia (Fig. 3B–E). Normal pain sensation in the sham group was not altered by the siRNA2 administration. Similarly, repeated spinal administration of TLR4-targeting siRNA2, in the early phase protocol of the treatment, significantly reduced spontaneous guarding (Fig. 3F) and flinching (Fig. 3G) as well as palpation guarding (Fig. 3H) and limb use (Fig. 3I). Taken together, these results demonstrate that the activation of TLR4 plays an important role in the production and persistence of bone cancer pain.

3.3. Knockdown of TLR4 suppresses TCI-induced activation of astrocytes and microglial cells as well as increased levels of IL-1β and TNF-α

Given that (1) TCI can activate EphB1 and TLR4; (2) both EphB1 and TLR4 are colocalized with GFAP and Iba1 in the DH; and (3) that knockdown of TLR4 can suppress TCI-induced bone cancer pain, we examined the role of TLR4 in TCI-induced glial cell activation and increased proinflammatory cytokines. Western blot analyses showed that TCI-induced activation of astrocytes and microglial cells was significantly inhibited by TLR4-targeting siRNA2 (2 μg i.t. in early phase and late phase treatment protocols). Because GFAP was not activated until day 7 after TCI (see Fig. 1A), the expression of GFAP at day 6 [GFAP (6d)] was not altered by the early phase administration of siRNA2 (Fig. 4A). These results indicate that activation of TLR4 is required for TCI-induced activation of astrocytes and microglial cells.

Glial cell activation results in an increased level and robust release of proinflammatory cytokines, including IL-1β and TNF-α, leading to pain and hyperalgesia [26,27,31]. Our ELISA measurements showed that TCI significantly increased levels of IL-1β and TNF-α in SC, exhibiting a progressive increase with time during the period from 3 to 21 days after TCI. IL-1β increased progressively and significantly at day 5, the earliest detected time, peaked at day 7–14, and remained at a high level with a slight decrease at day 21 after TCI. Meanwhile, TNF-α was also increased significantly at day 3, then increased progressively and peaked at day 14–21 (Fig. 4B). This TCI-induced increase in IL-1β and TNF-α was inhibited by spinal knockdown of TLR4. Repeated administration of TLR4-targeting siRNA2 (2 μg i.t. in the early and late phase treatment protocols) significantly reduced the TCI-induced increase of IL-1β and TNF-α by approximately 70% (Fig. 4C). These results indicate that the TCI-induced increased activity of these proinflammatory cytokines may be mediated through TLR4 activation. Thus, inhibiting TLR4 activation can reduce the TCI-induced

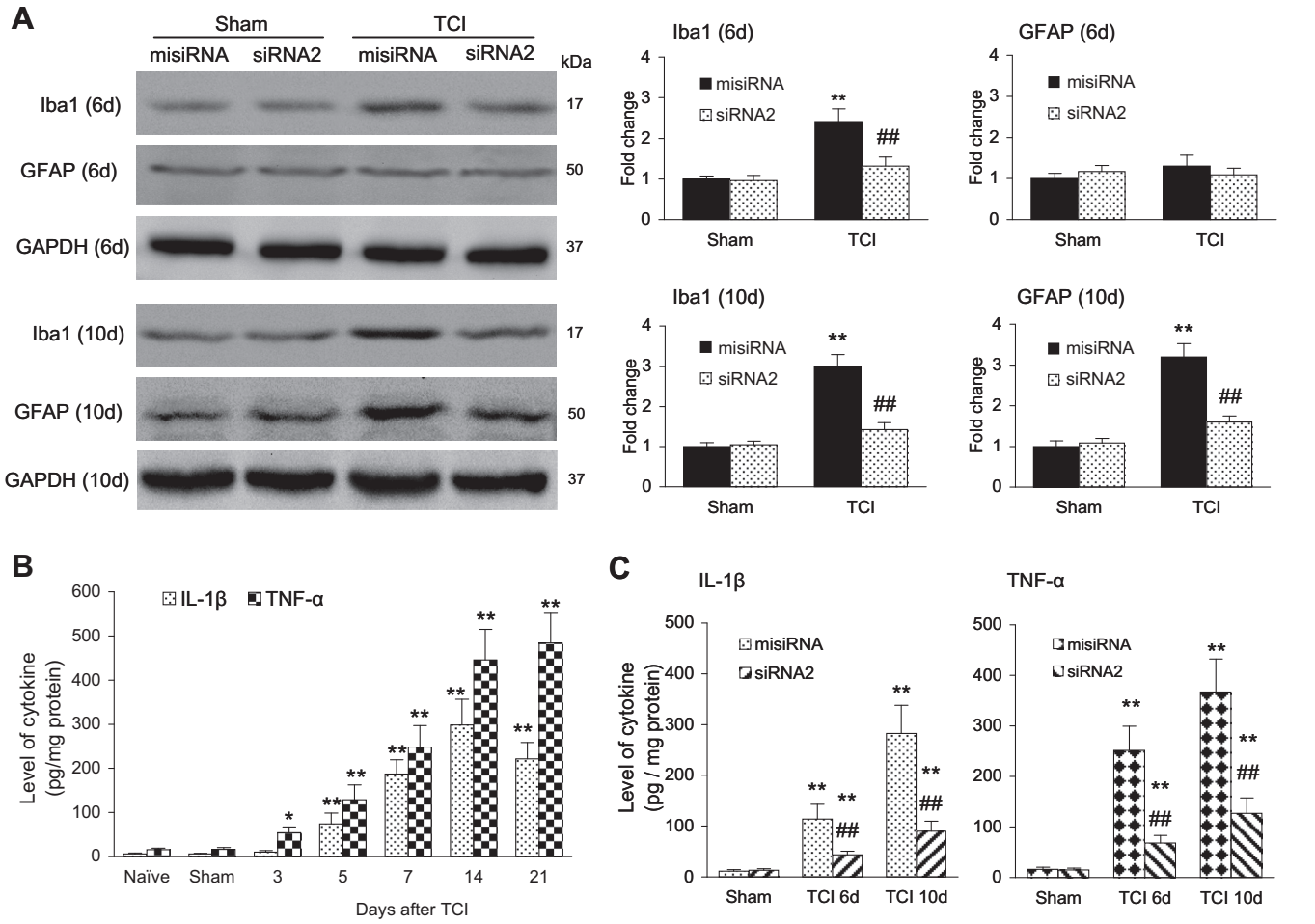

Fig. 4. TLR4 knockdown suppresses the activation of astrocytes and microglial cells and the increased levels of IL-1β and TNF-α in spinal cord after tumor cell implantation (TCI). (A) Knockdown of TLR4 suppresses TCI-induced activation of astrocytes and microglial cells. Small interfering RNA (siRNA2) (2 μg intrathecally) was administrated on postoperative days 3, 4, and 5 (early phase) and postoperative days 7, 8, and 9 (late phase) after TCI. Tissues were collected 1 day after the last injection, ie, day 6 for the early phase treatment and day 10 for the late phase treatment. (B) Time course of changes in IL-1β and TNF-α. (C) Intrathecal administration of siRNA2 inhibited the TCI-induced increase of IL-1β and TNF-α. Four samples were included in each group (A–C). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the corresponding control group of sham + mismatch RNA (misiRNA) or sham + siRNA (A), naïve and sham (B), or sham + misiRNA (C). $^{\#\#}P < 0.01$ vs the corresponding group of TCI + misiRNA (A), TCI (6d) + misiRNA or TCI (10d) + misiRNA (C).

increased activity of IL-1β and TNF-α, in addition to reducing the activation of astrocytes and microglial cells as well as bone cancer-related pain behaviors.

3.4. Blocking EphB receptor activation suppresses TCI-induced activation of astrocytes and microglial cells as well as increased levels of IL-1β and TNF-α

We continued to examine whether the activation of astrocytes and microglial cells, and the activity of the cytokines IL-1β and TNF-α, during bone cancer pain could be regulated by EphB1 activation. Western blot analyses showed that TCI-induced activation of astrocytes and microglial cells was significantly inhibited by repeated spinal administration of the EphB1 receptor inhibitor EphB2-Fc (5 μg i.t.) in the early and the late phase treatment protocols (Fig. 5A). As shown in Fig. 5B, TCI induced increased levels and robust release of IL-1β and TNF-α in SC. These increases were inhibited by the spinal blocking of EphB1. Repeated administration of an EphB1 receptor antagonist, EphB2-Fc (5 μg i.t. in the early and the late phase treatment protocols) significantly reduced the TCI-induced increases of IL-1β and TNF-α by approximately 70–80% (Fig. 5B). These results indicate that the TCI-induced increased activity of these proinflammatory cytokines may be mediated through the activation of EphB1. Thus, blocking EphB1 activation can reduce TCI-induced increased activity of IL-1β and TNF-α.

3.5. Knockdown of TLR4 inhibits ephrinB2-Fc-induced activation of astrocytes and microglial cells, increased activity of IL-1β and TNF-α, as well as thermal hyperalgesia

Given that the activation of both TLR4 and EphB1 is important for astrocyte and microglial cell activation as well as IL-1β and TNF-α activity in bone cancer pain, we examined the possible interaction of EphB1 and TLR4 in SC after TCI. EphB2-Fc administation (5 μg i.t. in the early and the late phase treatment protocols), which inhibited EphB1 receptor activation through combining with EphB1 ligands [28], significantly inhibited TCI-induced activation of TLR4 (Fig. 6A). By contrast, knockdown of TLR4 by siRNA2 (in both the early and late phase treatment protocols), which inhibited upregulation of the expression of TLR4 in naïve and TCI rats, did not alter TCI-induced activation of EphB1 (Fig. 6B). These results indicate the possibility that activation of EphB1 receptor may be upstream of TLR4 activation.

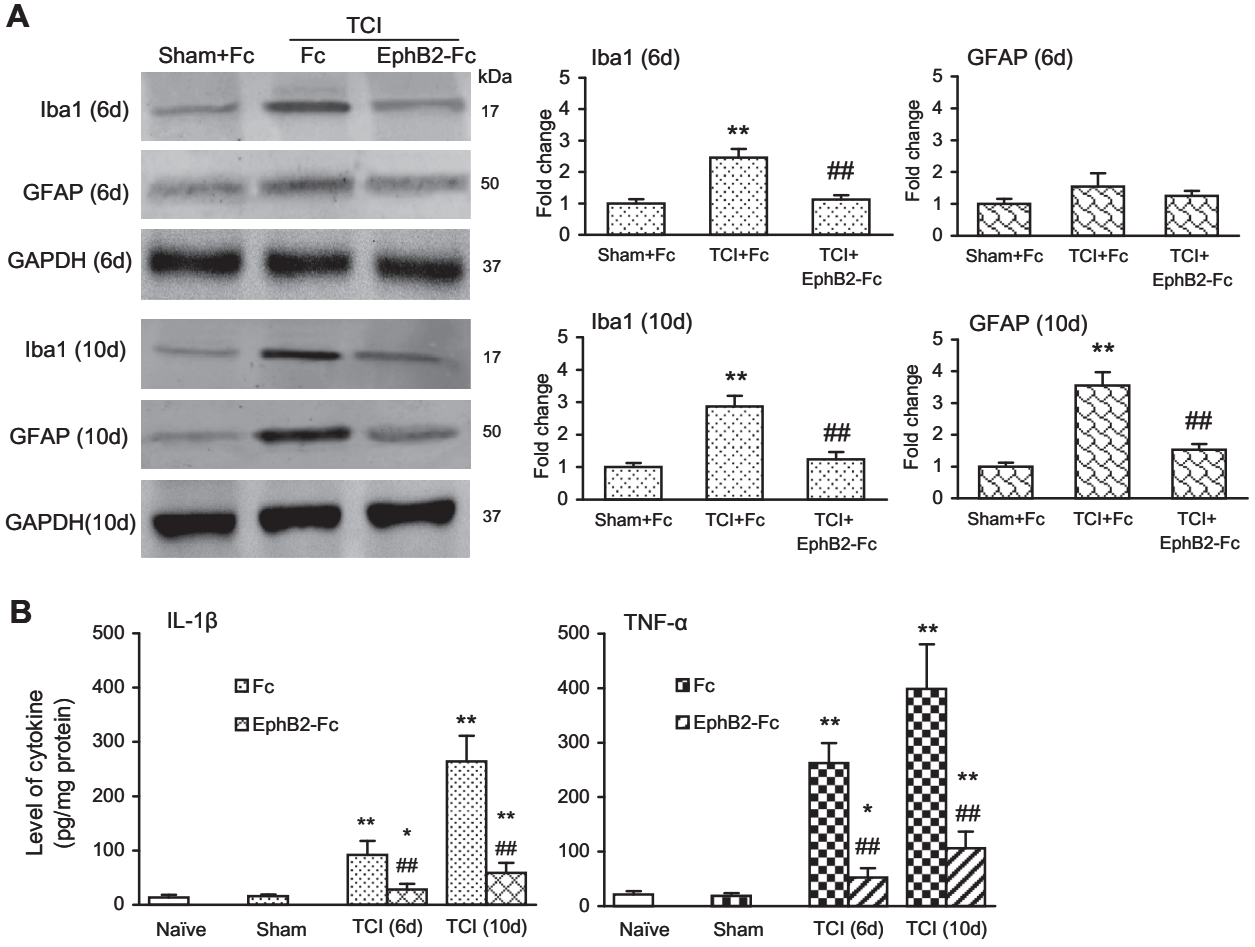

Fig. 5. Intrathecal administration of EphB2-Fc suppresses tumor cell implantation (TCI)-induced activation of astrocytes and microglial cells as well as the increased level of IL-1β and TNF-α in spinal cord. (A) EphB2-Fc suppresses the TCI-induced activation of astrocytes and microglial cells. (B) EphB2-Fc inhibited the TCI-induced increase in IL-1β (left) and TNF-α (right). EphB2-Fc (5 μg intrathecally) was administrated on postoperative days 3, 4, and 5 (early phase) and postoperative days 7, 8, and 9 (late phase) after TCI. The whole spinal cord tissue was collected 1 day after the last injection, ie, day 6 for the early phase treatment and day 10 for the late phase treatment. Four samples were included in each group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the corresponding control group of sham + Fc (A), sham and sham + Fc (B). $^{\#\#}P < 0.01$ vs the corresponding group of TCI + Fc (A), TCI + Fc (6d), or TCI + Fc (10d)(B).

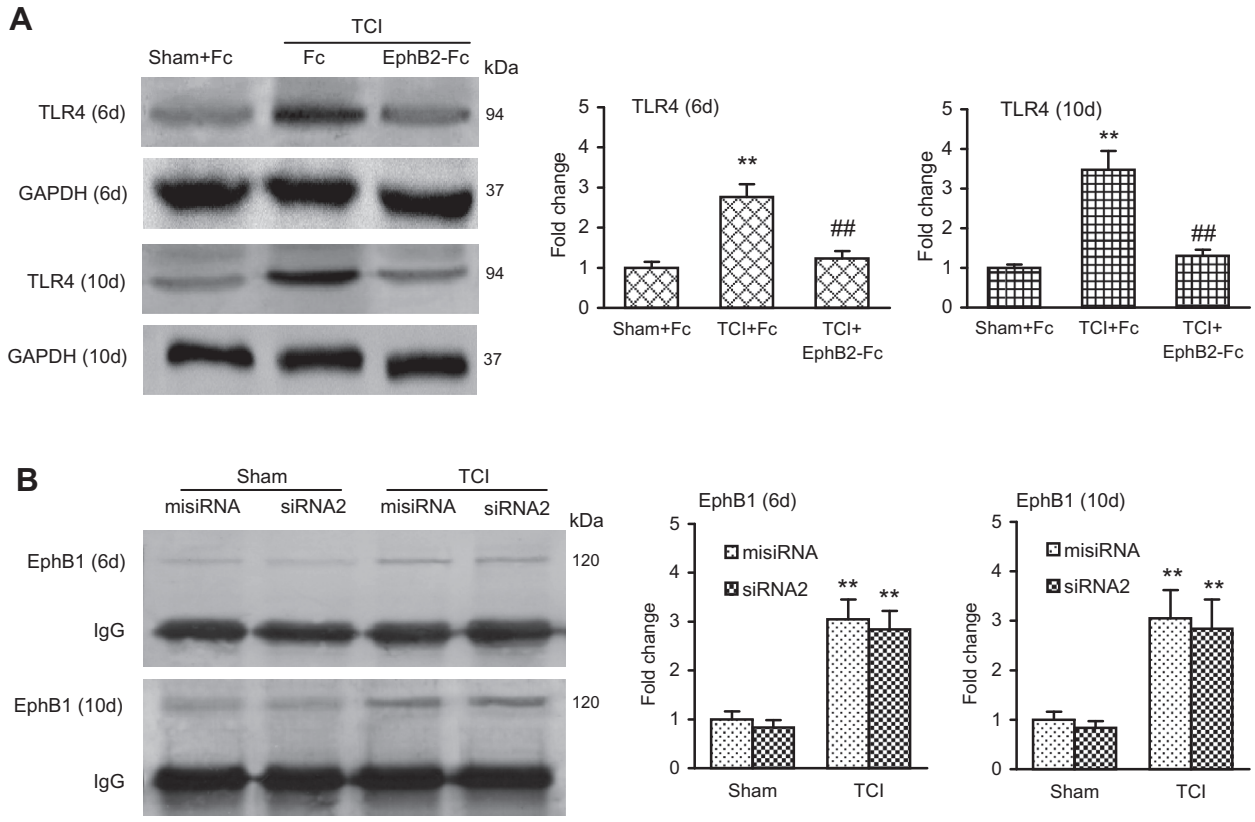

Fig. 6. Relationship of activation of EphB1 and TLR4 in spinal cord after tumor cell implantation (TCI). (A) Spinal administration of EphB2-Fc inhibited TCI-induced upregulation of expression of TLR4. (B) Knockdown of TLR4 failed to inhibit TCI-induced upregulation of expression of EphB1. EphB2-Fc (5 μg intrathecally) (A) and small interfering RNA (siRNA2) (2 μg intrathecally) (B) was administrated on postoperative days 3, 4, and 5 (early phase) and postoperative days 7, 8, and 9 (late phase) after TCI. The whole spinal cord tissue was collected 1 day after the last injection, ie, day 6 for the early phase treatment and day 10 for the late phase treatment. Four samples were included in each group. $^{**}P < 0.01$ vs the corresponding control group of sham + Fc (A), sham + misiRNA, or sham + siRNA (B). $^{\#\#}P < 0.01$ vs the corresponding group of TCI + Fc (A).

We therefore performed further experiments to test this possibility. A single spinal injection of an exogenous EphB1 receptor activator, ephrinB2-Fc (2 μg i.t.), induced activation of TLR4, astrocytes, and microglial cells in naïve rats (Fig. 7A). These alterations were successfully inhibited by knockdown of TLR4 by pretreatment with siRNA2 (each 2 μg i.t., 30 minutes prior to ephrinB2-Fc administration) (Fig. 7B). The same treatment of ephrinB-Fc markedly increased levels of IL-1β and TNF-α, and both were greatly reduced by pretreatment with siRNA2 (Fig. 7C). Thermal hypersensitivity induced by spinal administration of ephrinB2-Fc (2 μg) [17] was significantly reduced by repeated pretreatment with TLR4-targeting siRNA2 (2 μg i.t., once a day for 3 consecutive days with the 3rd dose administered 30 minutes before i.t. ephrinB2-Fc) (Fig. 7D). These results strongly support the idea that EphB1 receptor-mediated activation of astrocytes and microglial cells increases the activity of IL-1β and TNF-α in SC, and that pain-related behaviors may be mediated, at least partly, by TLR4 activation.

3.6. Blocking EphB receptor activation suppresses increased immunofluorescent staining of NR2B in astrocytes and microglial cells after tumor cell implantation

We have recently demonstrated that ephrinB-EphB receptor signaling may contribute to bone cancer pain through the neuronal NMDAR pathway. However, glial cells also express NMDAR. We wondered whether blocking ephrinB-EphB receptor signaling could downregulate glial NMDAR. Our results showed that the immunofluorescent staining of NMDAR subtype NR2B was greatly increased in GFAP- and Iba1-positive cells in the DH after TCI. Spinal blocking of EphB receptor activation by repeated administration of EphB2-Fc (each 5 μg i.t., daily for 3 consecutive days, in the early phase treatment protocol) greatly suppressed the increased immunofluorescent staining of NR2B in astrocytes and microglial cells (Fig. 8A). However, the NMDAR subtype NR1 was barely expressed in GFAP and Iba1 in the DH in both sham-operated and TCI rats. After TCI, NR1 was not found in glial cells (neither in GFAP nor in Iba1) (Fig. 8B). These results suggest that spinal blocking of EphB receptor signaling may downregulate NB2B in astrocytes and microglial cells in the DH.

4. Discussion

This study reveals a critical role for ephrinB-EphB signaling in bone cancer pain. It supports the idea that TCI-induced activation of ephrinB-EphB signaling in the DH may contribute to bone cancer pain by activating TLR4 and the proinflammatory cytokines in astrocytes and microglial cells, in addition to interacting with NMDARs in the neuronal synapses and the subsequent activation of Ca^{2+}-dependent signals demonstrated in our recent study [28].

The principal findings of the present study are: (1) TCI increases the expression of TLR4 and EphB1 receptors, activates both astrocytes and microglial cells, and increases the levels of IL-1β and TNF-α in rat SC; (2) spinal blocking of EphB1 receptor activation prevents and suppresses increased expression of TLR4, activation of astrocytes and microglial cells, and increased activity of IL-1β and TNF-α, in addition to inhibiting bone can-

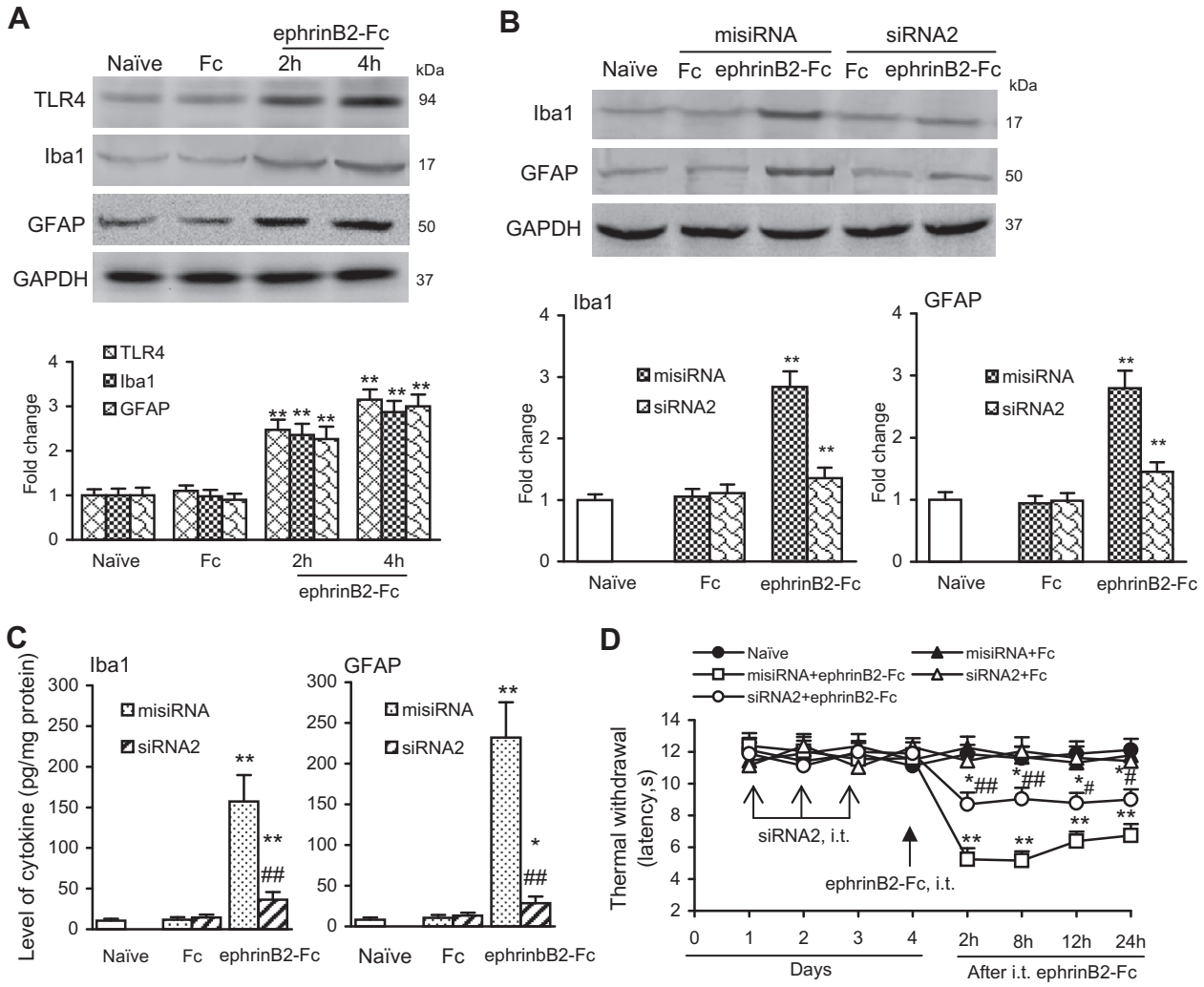

Fig. 7. Knockdown of TLR4 inhibits ephrinB2-Fc-induced activation of astrocytes and microglial cells as well as increased activity of IL-1β and TNF-α in spinal cord (SC), and thermal hyperalgesia. (A) Intrathecal administration of the EphB receptor agonist ephrinB2-Fc increased the expression of TLR4, the astrocytes (GFAP), and microglial cells (Iba1). (B, C) Administration of TLR4-targeting small interfering RNA (siRNA2) suppressed ephrinB2-Fc-induced activation of GFAP and Iba1 (B) as well as ephrinB2-Fc-induced increases in the level of IL-1β and TNF-α (C). The whole SC tissue was collected 4 hours after the last treatment (B, C). Four SC samples were included in each group (A–C). (D) Repeated preadministration of siRNA2 reduced ephrinB2-Fc-induced thermal hypersensitivity. Eight rats were included in each group. Doses: ephrinB2-Fc and its Fc control (each 2 μg intrathecally) (A); mismatch RNA (misiRNA) and siRNA2 (each 2 μg intrathecally, daily for 3 consecutive days) (B–D). EphrinB2-Fc was injected 30 minutes after the last dose of siRNA2 or misiRNA (B and C). $^{*}P < 0.05$, $^{**}P < 0.01$ vs the corresponding control groups of naïve and Fc (A–C) or naïve (D). $^{\#\#}P < 0.01$ vs the corresponding control group of misiRNA + ephrinB2-Fc (B–D).

cer-related pain behaviors following TCI; (3) spinal blocking of TLR4 activation or knockdown of TLR4 suppresses TCI-induced activation of astrocytes and microglial cells, increased activity of IL-1β and TNF-α, as well as bone cancer-related pain behaviors; (4) in naïve rats, spinal administration of an exogenous EphB1 activator, ephrinB2-Fc, increases the expression of TLR4 in SC; knockdown of TLR4 suppresses ephrinB2-Fc-induced activation of astrocytes and microglial cells, increased activity of IL-1β and TNF-α, as well as thermal hypersensitivity; and (5) blocking EphB1 receptor activation prevents TCI-induced increased expression of TLR4, but knockdown of TLR4 fails to prevent TCI-induced upregulation of EphB1.

These findings demonstrate that TLR4 activation in spinal glial cells is an important mechanism underlying the contribution of ephrinB-EphB receptor signaling to the development of bone cancer pain in rats with TCI or thermal hypersensitivity in naïve rats. This study suggests that TLR4 may be a potential target for preventing and reversing bone cancer pain and other similar painful processes mediated by ephrinB-EphB receptor signaling.

4.1. EphrinB-EphB receptor signaling contributes to bone cancer pain by activating glial cells and increasing proinflammatory cytokine activity in SC

EphB receptors and their ligands, ephrinBs, are critical cues in regulating cell shape, adhesion and repulsion, migration, and positioning during developmental processes [39,56,60]. Alteration of the mechanisms controlling adherence and motility has a central role in promoting tumor invasion and angiogenesis [3,10,43]. We have recently demonstrated that TCI-induced activation of the EphB1 receptor and its ligand ephrinB2 in dorsal root ganglion and DH neurons is critical to the development of bone cancer pain [28] and other pain states after sciatic nerve injury [17,47,49] or morphine withdrawal [29,30], in which EphB receptors may regulate synaptic plasticity through interaction with NMDARs. NMDARs have a well-developed role in neuronal plasticity and various pain states. Here, we show that activation of ephrinB-EphB receptor signaling may contribute to bone cancer pain in TCI-treated rats and, in naïve rats, to sensory hypersensitivity by the

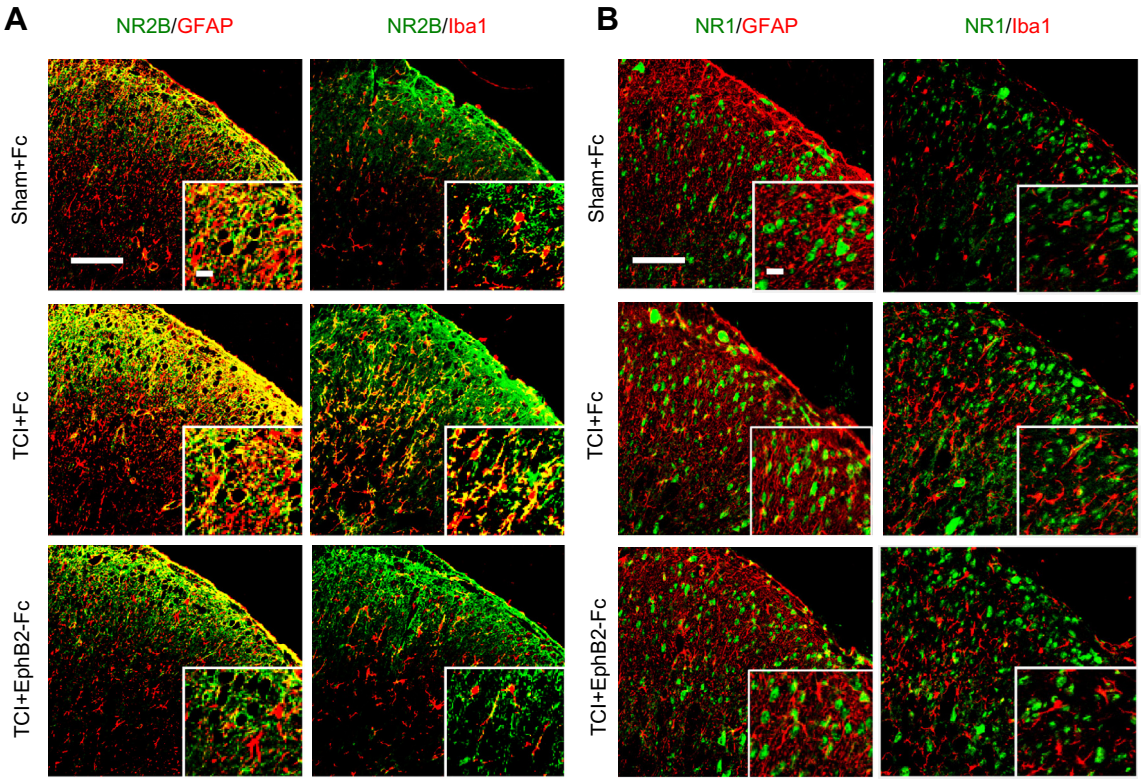

Fig. 8. Representative confocal images showing the distribution and colocalization of immunofluorescent staining of NMDAR subtypes NR2B (A) and NR1 (B) in the dorsal horn with the astrocytes and microglial cells in sham-operated and tumor cell implanted (TCI) rats. EphB2-Fc and its control Fc (each 5 μg intrathecally) were administered on postoperative days 3, 4, and 5 (early phase) after TCI. Magnification: 200× for all the large images showing the dorsal horn ipsilateral to TCI (bar = 100 μm); 400× for the inserted images (bar = 20 μm).

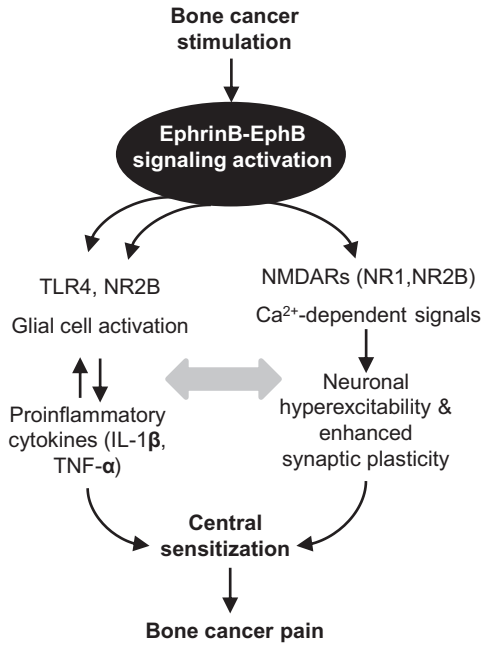

Fig. 9. Schematic representation of the mechanisms of spinal ephrinB-EphB receptor signaling in bone cancer pain. The flowchart illustrates the possible pathways for ephrinB-EphB signaling-induced spinal central sensitization and bone cancer pain. These pathways may include the glial cell-TLR4 and glial cell-NR2B pathways demonstrated in this study and the neuronal-NMDAR pathway demonstrated in our most recent publication [28].

activation of astrocytes and microglial cells and by increasing the activity of the proinflammatory cytokines IL-1β and TNF-α in SC.

Central nervous system (CNS) glia, which act as immune effector cells in both normal and pathological conditions, play a vital role in the initiation of processes promoting persistent pain states [14,35,44]. Studies have shown that the early CNS glial response to peripheral nerve injury is predominantly the activation of spinal microglia, and that astrocytes subsequently undergo activation and proliferation [42,53]. Activation of the astrocytes and microglial cells leads to robust release of proinflammatory cytokines such as IL-1β and TNF-α, as well as other proinflammatory factors such as nitric oxide and prostaglandin E2 [16,34].

The cytokines have been proved to be important factors contributing to the establishment of central sensitization [21]. IL-1β may increase NMDAR phosphorylation and NMDAR-mediated intracellular calcium release in sensory neurons [55]. TNF-α can increase neuronal excitability by stimulating neuronal ion channels [7,41]. These proinflammatory cytokines may also activate the glial cells, resulting in amplification of glia-mediated pain-related cascades. The activation of astrocytes and microglial cells and the increased activity of the proinflammatory cytokines in SC are considered to be common underlying mechanisms that lead to pathological pain in a number of pain syndromes with widely different etiologies, such as peripheral nerve injury, spinal inflammation and bone cancer neuropathy [14,16,35,40,44,57,58,62,63].

Our study provides evidence supporting the idea that ephrinB-EphB receptor signaling may contribute to the development of bone cancer pain by activating astrocytes and microglial cells and increasing the activity of proinflammatory cytokines in SC. In rats with bone cancer pain induced by TCI in this study, ephrinB-EphB receptor signaling was greatly activated in the DH. The

increased expression of EphB1 was largely colocalized with astrocytes and microglial cells, in addition to neurons. Blocking EphB1 receptor activation significantly inhibited the TCI-induced activation of astrocytes and microglial cells, the increased activity of IL-1β and TNF-α, and the bone cancer pain-like behaviors. In naïve rats, spinal administration of an exogenous EphB1 receptor activator, ephrinB2-Fc, activated astrocytes and microglial cells, increased the activity of IL-1β and TNF-α in SC, and induced thermal hypersensitivity.

4.2. EphrinB-EphB receptor signaling activates glial cells and increases activity of the proinflammatory cytokines through TLR4

Both astrocytes and microglial cells express a wide range of pattern recognition receptors. Among the variety of TLR receptors, TLR4, a transmembrane receptor protein with extracellular leucine-rich repeat domains and a cytoplasmic signaling domain [33], is an efficient initiator and mediator of glial activation and neuropathic pain [1,25,26,34,52]. The involvement of TLR4 in CNS innate immune processes and pain processes is well established [1,25,26,31,34,52]. TLR4 activation plays key roles in glial activation and the development of neuropathic pain after peripheral nerve injury [1,52]. Here, we have provided evidence supporting the idea that the TLR4 pathway may mediate ephrinB-EphB receptor signaling-induced glial cell activation, the increased activity of proinflammatory cytokines, and bone cancer pain. The evidence is 3-fold: (1) bone cancer-inducing TCI produces a rapid-onset and long-lasting expression of TLR4 protein in the DH, a region that is the first central relay station and critical for processing nociceptive information. This increased expression of TLR4 is correlated with EphB1 in the astrocytes and microglial cells, suggested by the coimmunoprecipitation analysis and confocal images. The expression of TLR4 is also strongly correlated with the timing pattern of the increased expression of EphB1. (2) Spinal administration of an exogenous EphB1 receptor activator, ephrinB2-Fc, induces upregulation of TLR4, as well as activation of astrocytes and microglial cells, increased levels of proinflammatory cytokines, and thermal hypersensitivity. These alterations can be suppressed by spinal blocking or knockdown of TLR4. (3) Spinal blocking of EphB1 activation prevents TCI-induced TLR4 activation, while TLR4-targeting siRNA2 does not affect TCI-induced increased expression of EphB1, indicating that TLR4 is downstream of ephrinB-EphB signaling.

4.3. Model of ephrinB-EphB receptor signaling contribution to development of bone cancer pain

EphB receptors can regulate the development of glutamatergic synapses and their plasticity in the adult nervous system [22,39,56,60,61], as well as during development of the nervous system by interaction with NMDARs [5,37,51]. We have recently demonstrated that after bone cancer-inducing TCI, ephrinB-EphB receptor signaling is aggressively activated in the dorsal root ganglion and the DH neurons and contributes to the development of increased synaptic plasticity of the DH neurons and behaviorally expressed bone cancer pain. These effects of ephrinB-EphB receptor signaling are realized by interaction with the postsynaptic NMDARs and the subsequent activation of various Ca^{2+}-dependent signals [28]. This is also true in nerve injury-induced neuropathic pain [17,47,49] and morphine withdrawal-induced pain enhancement [29,30].

In this study, our results demonstrate that activation of ephrinB-EphB receptor signaling may activate TLR4 and increase the activity of proinflammatory cytokines. Taken together, we hypothesized that the activation of ephrinB-EphB receptor signaling in SC may contribute to the development of bone cancer pain through ephrinB-EphB/NMDAR-neuron and ephrinB-EphB/TLR4-glial cell pathways. We noticed that the NMDAR subtype NR2B, but not NR1, was also expressed and increased in astrocytes and microglial cells in SC after TCI. Spinal blocking of EphB receptors greatly suppressed NR2B expression in astrocytes. These findings suggest that NR2B, in addition to TLR4, in the DH glia may also contribute directly or indirectly to ephrinB-EphB receptor signaling-mediated spinal central sensitization and bone cancer pain. Thus, a third pathway could be the ephrinB-EphB/NR2B-glial cell pathway. Possible pathways by which ephrinB-EphB receptor signaling in SC may contribute to bone cancer pain are illustrated in Fig. 9. Since ephrinB-EphB receptor signaling may contribute to the development of bone cancer pain by acting on neuronal and glial cell pathways, which are critical to the development of bone cancer pain, blocking ephrinB-EphB receptor signaling may result in stronger analgesia.

Conflict of interest statement

There are no conflicts of interest to declare associated with this study.

Acknowledgements

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