SPINAL INTERLEUKIN-33 AND ITS RECEPTOR ST2 CONTRIBUTE TO BONE CANCER-INDUCED PAIN IN MICE


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Abstract—Cancer pain, particularly bone cancer pain, affects the quality of life of cancer patients, and current treatments are limited. Interleukin (IL)-33, a new member of the IL-1 super family, has been reported to be involved in the modulation of inflammatory pain. However, studies focused on its role in the modulation of cancer pain have been rare. The present study was designed to investigate whether spinal IL-33/ST2 signaling was involved in bone cancer-induced pain in mice. Bone cancer was induced via intra-femoral inoculation of 4T1 mammary carcinoma cells. The mice inoculated with carcinoma cells showed mechanical allodynia, heat hyperalgesia and a reduction in limb use, whereas phosphate-buffered saline or heat-killed cells-injected mice showed no significant difference compared to non-treated mice. The pain hypersensitive behaviors worsened over time and with bone destruction. Both the mRNA and the protein levels of IL-33 and relative cytokines (IL-1β, IL-6, TNF-a) were significantly increased in the spinal cord after the inoculation of carcinoma cells. Intrathecal administration of ST2 antibody to block IL-33/ST2 signaling alleviated pain behaviors in a dose-dependent manner in bone cancer pain mice compared with vehicle-injected mice. Moreover, the ST2-/- mice showed a significant amelioration of limb use and heat hyperalgesia compared to wild-type mice. Meanwhile, concentrations of spinal IL-1β, IL-6 and TNF-a in the cancer-bearing ST2-/- mice had no significant changes. These data further suggested that IL-33/ST2 signaling played a vital role in cancer pain. Our results provided evidence that IL-33 and its receptor ST2 may be a potential therapeutic target for the treatment of pain in bone cancer patients. © 2013 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: interleukin-33, ST2, bone cancer-induced pain, spinal cord, proinflammatory cytokines.

INTRODUCTION

Bone cancer pain is one of the most frequent symptoms in bone cancer patients and is severely disruptive to a patient’s quality of life (Coleman, 2006). However, relatively little is known regarding the mechanisms that generate and maintain this type of pain. Although opiates, particularly, the opioid receptor agonist morphine, have been used as conventionally prescribed analgesic drugs for the treatment of advanced bone cancer pain, patients often undergo tolerance to analgesics as well as dose limiting side effects (Banning et al., 1991; Hansen et al., 2011), which limit its long-term clinical application. Thus, bone cancer pain remains one of the most challenging symptoms to manage in patients with cancer and represents an urgent need for higher insights into the mechanisms underlying pain development.

Interleukin (IL)-33, a new member of the IL-1 superfamily, has attracted growing interest since its identification in 2003 (Baekkevold et al., 2003; Schmitz et al., 2005). Similar to IL-1x, IL-33 functions both as a traditional cytokine and as a nuclear factor that regulates gene transcription (Ali et al., 2011). As a cytokine, IL-33 is thought to act as an ‘alarm’ when released following cell necrosis to alert the immune system of tissue damage or stress (Carrol and Girard, 2009). In addition, it mediates its biological effects via its interaction with the ST2 (IL-1RL1) receptors and IL-1 receptor accessory protein (IL-1RAcP) (Arend et al., 2008; Smith, 2011a,b), both of which are widely expressed, particularly in T helper (Th) 2 cells in the peripheral nervous system (Liew et al., 2010). When combined with its receptor complex, IL-33 strongly promotes the pathogenesis of Th2-related diseases such as asthma (Prefontaine et al., 2009), splenomegaly (Ohto-Ozaki et al., 2010) and eosinophilia (Dyer et al., 2012). Moreover, IL-33 treatment enhances resistance to trichuris infection, attenuates sepsis and defends against nematode parasites (Liew, 2012; Liew et al., 2010). Thus, the effects of IL-33 are either pro- or anti-inflammatory depending on the disease and model.

Although the IL-33/ST2 signaling pathway has been shown to be involved in a broad range of diseases, investigation of its role in the transmission and modulation of pain has been rare. It has been reported...
that IL-33, similar to other IL-1 cytokines, may induce inflammatory pain in the peripheral nervous system and mediate antigen-induced cutaneous and articular hypernociception in mice (Verri et al., 2008), which suggests that IL-33 plays a pivotal role in the modulation of arthritic pain in the peripheral nervous system.

The role of IL-33 in pain modulation in the CNS still remains unclear, although there have been several reports that have shown that it is expressed at a high level in the central nervous system and is localized in astrocytes, which were the main mediators of central sensitization, suggesting that central IL-33 might be associated with pain modulation (Zarpelon et al., 2013). Our recent studies using the classical acute inflammatory formalin pain mouse model, have demonstrated that central IL-33, and its receptor ST2, mediate acute inflammatory pain in mice (Han et al., 2013). On the study of bone cancer pain, accumulating evidence has shown the essential role of robust neuroinflammation in the maintenance of central sensitization at the spinal cord (Honore et al., 2000; Mantyh et al., 2002; Zhang et al., 2008; Wang et al., 2012; Mao-Ying et al., 2012). Thus, we hypothesize that spinal IL-33/ST2 plays an important role in bone cancer-induced pain.

To test our hypothesis, ST2 antibody was intrathecally administered or ST2−/− transgenic mice were used to block the IL-33/ST2 signal, and the subsequent effects were observed in a murine model of bone cancer pain. Our results indicated that IL-33 was upregulated in the bone cancer pain mice, and intrathecal administration of ST2 antibody suppressed both mechanical allodynia and thermal hyperalgesia compared to vehicle-injected mice. These results were further confirmed by comparing ST2−/− mice and wild-type (WT) mice. The present study suggests that IL-33/ST2 contributes to bone cancer pain in mice. Thus, IL-33/ST2 may be a potential therapeutic target for the control of bone cancer pain in patients.

**EXPERIMENTAL PROCEDURES**

**Animals**

The experiments were performed on female BALB/c mice (aged 7–9 weeks, weighing 18–25 g from Shanghai Laboratory Animal Center, China Academy Sciences, Shanghai, China). The ST2−/− BALB/c mice were generated as previously described and were kindly provided by Dr. Andrew McKenzie at the MRC Laboratory of Molecular Biology, Cambridge, UK (Brint et al., 2004). The ST2−/− BALB/c mice were healthy and did not display any overt phenotypic abnormalities. The gene-targeted mice were backcrossed into their respective background for 10 generations. The ST2−/− and its WT littermates of the same background were maintained in the same animal facilities for extended periods. The mice were housed in a vivarium with a 12-h alternating light–dark cycle and had access to food and water ad libitum. The experimental procedures were approved by the Animal Care and Use Committee of the Fudan University, and were consistent with the NIH’s Guide for the Care and Use of Laboratory Animals and the Ethical Issues of the IASP (Zimmermann, 1983).

**Cell cultures**

Bone cancer was induced as previously described (Honore et al., 2000; Schwei et al., 1999) with a few modifications. Briefly, the mice were anesthetized with chloral hydrate (i.p. 400 mg/kg). After their hair was shaved and their skin was disinfected, a superficial incision of approximately 1 cm was made above the knee that overlayed the patella. A light pressure was applied to identify the condyles of the distal femur. A hole was drilled into the right femoral medullary cavity with a 30-gauge needle. The needle was then removed and replaced with a 10-µL microinjection syringe. Four-microliters of PBS containing 1.0 × 10⁶ cancer cells were inoculated into the intramedullary space of the mouse femur. The syringe was left in place for one additional minute to prevent the carcinoma cells from leaking out along the injection track. The hole was closed with an absorbable gelatin sponge, followed by copious irrigation with sterile-filtered saline, and the skin was sutured using a 5-0 Ethicon Vicryl rapid suture. Sham-operated controls underwent the same operation, but were inoculated with 4-µL PBS alone or heat-killed carcinoma cancer cells (1.0 × 10⁶). All of the animals were allowed to recover from the inoculation surgery for 3 days prior to any experimentation.

**Behavioral tests**

**Limb use.** Limb use was determined in an open field that allowed the mouse to move freely in a transparent cage. One 2-min trial was performed per testing period. The gait of the affected limb was assigned a limb use score on a scale from 4 to 0, where 4 = normal use, 3 = minor limping, 2 = substantial limping, 1 = substantial limping and partial lack of use, and 0 = complete lack of use (Minami et al., 2009).

**Mechanical allodynia.** Mechanical allodynia was measured by the hind paw withdrawal response to von Frey hair stimulation according to the up-down method as previously described (Dixon, 1980; Mao-Ying et al.,...
2006). The mice were placed in transparent cages with a wire mesh floor and allowed to acclimatize for 30 min. The plantar surface of hind paws both ipsi- and contralateral to the tumor-bearing legs were stimulated with a series of von Frey filaments that ranged from 0.07 to 2 g (Stoelting, Wood Dale, Illinois, USA). The filaments were vertically poked against the plantar surface with sufficient force to cause a slight bending against the paw and held for 4–6 s with a 5-min interval between the two stimulations. A brisk withdrawal or paw flinching was considered as positive responses. Whenever a positive response to a stimulus occurred, the next lower von Frey hair was applied, and whenever a negative response occurred, the next higher hair was applied. The testing consisted of five more stimuli following the occurrence of the first change in response, and the paw withdrawal mechanical threshold (PWT) was converted to a 50% von Frey threshold using the method previously described by Dixon (1980).

**Bone radiological detection.** To assess the femur bone destruction by tumor, a femur bone radiograph was performed in this study. Briefly, the mice were placed on a clear plane plexiglass and exposed to an X-ray source under anesthesia with chloralhydrate on day 7, 14 and 21 after tumor implantation. Using Siemens’ AXIOM AristosFX digital radiography System (Germany), the femur radiographs were obtained from both the hind limbs of the normal mice, as well as the 4T1 mammary cancer cells, heat-killed cells, and PBS-treated mice (n = 4 for each group).

**Bone histology.** On day 7, 14 and 21 after tumor implantation, the mice were anesthetized with an overdose of chloralhydrate and transcardially perfused with 150 mL 0.9% normal saline followed with 150 mL 4% paraformaldehyde. The bilateral femur bones were removed and decalcified in decalcifying solution for 24 h. The bones were rinsed, dehydrated, and then embedded in paraffin, cut into 7-µm cross-sections using a rotary microtome (Reichert-Jung 820, Cambridge Instruments GmbH, Nussloch, Germany), and stained with hematoxylin and eosin to visualize the extent of tumor infiltration and bone destruction.

**Drug administration**

Different doses of the mouse ST2 antibody (R&D Systems, Shanghai, China) were intrathecally administered on day 10 after tumor implantation, and the same volume of normal goat IgG (R&D Systems)
was used as a control. The paw withdrawal threshold responses to the von Frey and hot plate test were observed 1 h before and 1, 2, 3, 4, 6, 8 and 24 h after drug injection.

The intrathecal injection was conducted via a lumbar puncture as previously reported (Hylden and Wilcox, 1980). Briefly, the mice were anesthetized with isoflurane. Drugs (5 μL in volume) were injected into the subarachnoid space of the lumbar vertebrae at L5 and L6 with a 10-μL microinjection syringe. The correct subarachnoid positioning of the tip of the needle was verified by a tail-or paw-flick response.

Real-time PCR
The L4–L6 spinal cord segments of the mice were dissected and frozen in liquid nitrogen and stored at −70 °C. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. The amount of RNA was measured using a spectrophotometer. Two μg of total RNA was reverse transcribed into cDNA using the M-MLV reverse transcriptase (Promega) with oligo(dT). PCR reactions were performed using the oligonucleotide primers as shown below:

IL-33 primers (upstream primer, 5'-CCAACCTCAAGATTTCCCCG-3', and downstream primer, 5'-GGATGCTTAATGTACAGACG-3'); ST2 primers (upstream primer, 5'-CATGCATGATAACACGCACAC-3', and downstream primer, 5'-GTAGAGCTTGCATCGGTCC-3'). The real-time reverse transcription-polymerase chain reaction was run on IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Expression of IL-33 and ST2 was normalized to the amount of RNA loaded for each sample using the reference gene β-actin (upstream primer, 5'-GATTACTGCTTGCTCCTAGC-3', and downstream primer, 5'-GACTCGTGCTACTCTGC TTGC-3') as an internal standard. The data were presented as the mean ± SEM.

Western blotting analyses
The lumbar spinal cord segments (L4–L6) were homogenized at 4 °C in RIPA (Beyotime) supplemented with 1 mM phenylmethyl sulfonylfluoride (Beyotime), and the protein concentration was determined using the

**Fig. 2.** Radiographs (left and middle) and histology (right) (Hematoxylin–eosin stain) of the femur bone inoculated with 4T1 mammary carcinoma cells (1 × 10⁶ cells) before (P0) and 7, 14 days after inoculation (P7, P14). Seven days after surgery, the femur bone showed signs of radiolucent lesion in the proximal epiphysis, close to the injection site, and the cancer cells were clearly visible in the bone marrow. By day 14 after inoculation (P14), further deterioration was detected with medullary bone loss, and the cancer cells had nearly filled the entire femoral bone marrow cavity. BM designates normal bone marrow cells and TC designates tumor cells.
BCA method according to the kit’s instructions (Bio-RAD). Equivalent amounts of protein (40 μg) were separated by 10% SDS–PAGE and transferred (100 V for 1.5 h) onto polyvinylidene difluoride membranes (Millipore, Boston, MA, USA). The membranes were blocked with 5% nonfat milk at room temperature for 1.5 h and then incubated overnight at 4°C with a primary antibody (goat anti-IL-33 1:1000, Santa Cruz; goat anti-ST2 1:1000, R&D) or horseradish peroxidase (HRP)-conjugated antibody (anti-TUBULIN, anti-GAPDH). HRP-conjugated donkey anti-goat IgG was used as a secondary antibody at a dilution of 1:5000. The chemiluminescence was detected using the ECL system (Millipore, USA). Densitometric analysis of the IL-33 and ST2 bands were performed using ImageJ software. The same size square was drawn around each band to measure the density and to subtract the background near that band. The IL-33 and ST2 levels were normalized against TUBULIN and GAPDH, respectively.

Bio-Plex assay
Bio-Rad Bio-Plex kits were used for the detection of cytokine concentrations. The protein was extracted in the same way as described in Western blotting analyses. All samples were run in duplicate and were assayed for murine IL-1β, IL-6, and TNF-α using multiplex (# X6000000X1) bead-based kits, the cytokine reagent kit (Cat.# 171-304070) and cell lysis kit (Cat.# 171-304011). The standard curve determination and samples assays were run according to manufactures recommended procedures.

Immunohistochemistry
For the immunofluorescence experiments, four mice from each group were used. The L4–L6 spinal segments of the mice were isolated, post-fixed, frozen and then sectioned on a freezing microtome (Leica Microsystems, Wetzlar, Germany) at a 30-μm thickness. The sections were washed three times and blocked with 4% horse serum in 0.3% Triton X-100 for 1 h at 37 °C. The sections were then incubated with primary antibodies at 4 °C overnight and with secondary antibodies at room temperature for 1 h. The primary antibodies used included rabbit anti-glia fibrillary acidic protein (GFAP) (astrocyte marker, 1:2000, Dako, Copenhagen, Denmark), mouse anti-NeuN (neuronal marker, 1:500, Millipore, USA), rat...
anti-CD11b (microglia marker, 1:400, Serotec, USA) and goat anti-IL-33 (1:500, R&D, USA). The secondary antibodies used included Alexa Fluor 594-labeled donkey anti-goat (1:1000, Invitrogen, USA), Alexa Fluor 488-labeled donkey anti-mouse (1:1000, Invitrogen, USA), Alexa Fluor 488-labeled donkey anti-rat (1:1000, Invitrogen, USA) and Dylight 488-conjugated AffiniPure donkey anti-rabbit (1:500, Jackson, USA). The images were obtained using a confocal laser-scanning microscope in the sequential scan mode to avoid channel crosstalk (TCS SP2 Leica Microsystems) and analyzed using the Optimas 6.2 software.

Statistical analysis

The data were presented as the mean ± SEM, and all of the statistical analyses were performed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Differences between groups were performed using one-way ANOVA followed by the Dunnett’s test. A Student t-test was used when only two testing groups were analyzed. In all of the statistical analysis, P < 0.05 was considered statistically significant.

RESULTS

Time course of bone cancer-induced pain behaviors in mice

Body weight was measured systematically in normal, PBS, heat-killed and bone cancer-induced mice (Fig. 1A). No statistical significance was observed between any of the normal and model groups in terms of the average weight gained at any of the time points.

To evaluate ambulation-induced pain behavior, limb use was scored within 2 min of free motion (Fig. 1B). At day 5 after inoculation, limb use was affected in tumor-bearing animals, and the score was significantly reduced compared to non-treated animals (P < 0.05). Thereafter, the limb use showed a tendency to gradually decrease. After 14 days post-cell injection, the animals generally showed a partial to substantial non-use of the tumor-bearing limb and were more affected by day 21 (P < 0.01). The PBS group injected with PBS alone or heat-killed group injected with dead cells, demonstrated no reduction in limb use throughout the observation period.
Next, the von Frey test and hot plate test were used to demonstrate the presence of mechanical allodynia and thermal hyperalgesia in the bone cancer pain model mice. The baseline of the PWT (Fig. 1C) and paw withdrawal thermal latency (PWL) (Fig. 1D) prior to surgery was similar in all groups of mice, and there was no statistical difference between the left and right hind limb. Compared to the non-treated mice, the tumor-bearing mice displayed a profound decrease in both the ipsilateral PWT and PWL, with a lowest level on the 14th day post-inoculation ($P < 0.01$). Compared to the non-treated mice, the PWT in the PBS- and heat-killed groups were decreased on the 3rd day, but recovered to normal levels by day 5. In addition, the contralateral limb of all groups of mice showed no change in pain behaviors (data not shown), and no statistical differences were observed in the PWT and between the PBS- and heat-killed groups.

Radiological and histological evaluation of tumor invasion and bone destruction

Bone destruction was monitored using radiological and histological methods. No radiological change was found in the non-treated mice as well as the mice treated with PBS or heat-killed cells (data not shown). However, 7 days after injection with $1 \times 10^4$ 4T1 mammary cancer cells, the femur bone showed signs of a radiolucent lesion in the proximal epiphysis, close to the injection site. At day 14 after inoculation, further deterioration was detected with medullary bone loss. Representative examples are shown in Fig. 2 (left and middle).

Sections obtained from the proximal end of the femur at 7 and 14 days after cancer cell injection were stained with hematoxylin and eosin (Fig. 2 right). Tumor growth and various degrees of bone destruction were observed in the animals that received live 4T1 mammary cancer cells ($1 \times 10^4$ cells). Data were expressed as the mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$ vs. Model + IgG (400 ng). (C and D) Changes in the limb use score and thermal hyperalgesia on the ipsilateral hind paws after ablating the ST2 gene. Model, mice were inoculated with intra-femoral live 4T1 mammary carcinoma cells ($1 \times 10^4$ cells). Data were expressed as the mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$ vs. ST2+/− + PBS group. $^{#}P < 0.05$, $^{##}P < 0.01$ vs. WT+ model group.

Up-regulation of spinal IL-33 expression in bone cancer pain mice

The expression of IL-33 and ST2 in the cancer-induced pain model mice was investigated using RT-PCR and western blotting analyses. The representative original immunoblotting bands detected in the spinal dorsal horn were obtained from five groups of mice ($n = 4$ for each group) including the non-treated, PBS, day 7, day 10 and day 14 after injection of carcinoma cells. As shown in Fig. 3, both the mRNA level and the protein level of IL-33 were significantly increased in the spinal cord on day 7, 10 and 14 after injection of carcinoma cells.
However, no significant change in the mRNA or protein level of spinal ST2 was found among the five groups.

Distribution of spinal IL-33

To define the cellular distribution of IL-33, double immunofluorescence staining of IL-33 (Fig. 4) was performed with different cell markers including NeuN (neurons), GFAP (astrocytes) and CD11b (microglia). IL-33-immunoreactivity (IL-33IR) was observed to be primarily colocalized with GFAP IR (Fig. 4A–D), but not with NeuN or CD11b IR (Fig. 4E–L). These results suggested that IL-33 in the spinal cord is mainly expressed in astrocytes.

A function-neutralizing antibody against ST2 suppresses pain behaviors in bone cancer pain mice

ST2-neutralizing antibody (100, 200, 400 ng, i.t.) was provided on the 10th day after cancer cell injection. Paw withdrawal thresholds (Fig. 5A) were tested at 1 h before intrathecal drug injection and at different time points after injection. Administration of the ST2 antibody at doses of 200 and 400 ng had remarkably alleviated the mechanical allodynia induced by bone cancer. This effect appeared at 1 h, peaked at 3–4 h and disappeared at 6 h after injection. 100 ng ST2 antibody only increased the paw withdrawal thresholds at 1 h after drug injection.

In addition, the PWL decreasing (Fig. 5B) of the tumor-bearing mice was also ameliorated by intrathecal administration of the ST2 antibody. Two hours after administration, the drug showed a clear effectiveness, and the effects lasted for at least 8 h after injection. There were no statistical differences in the PWL in the IgG vehicle group compared with the baselines.

ST2 gene knockout alleviated bone cancer pain and reversed the upregulation of the expression of spinal IL-1β, IL-6 and TNF-α

To further confirm the role of ST2 in bone cancer pain, ST2 gene knockout (ST2 KO) mice were employed. ST2 KO mice also developed pain-related behaviors...
after the induction of bone cancer. However, the cancer-bearing ST2 KO mice did not exhibit a significant decrease in their limb use score until day 7 post-inoculation compared to the PBS-treated mice, whereas the cancer-bearing WT mice exhibited a significant decrease in their limb use score as early as day 3 after surgery (Fig. 5C). Moreover, the ST2 KO mice injected with cancer cells exhibited a significantly higher paw withdrawal threshold on the hot plate test compared to the cancer-bearing WT mice ($P < 0.01$) (Fig. 5D). These effects appeared on day 3 after inoculation with carcinoma cells and lasted until day 14. These findings demonstrated that in the cancer-bearing ST2 KO mice, the significant decrease in limb use score exhibited a later onset and had suppressed thermal hyperalgesia compared to the cancer-bearing WT mice.

The changes of concentration of spinal proinflammatory cytokines including IL-$\beta$, IL-6 and TNF-$\alpha$ were detected using Bio-Plex Pro Assays. 14 days after cancer cells injection, WT mice showed significantly increased protein concentration of IL-$\beta$ (Fig. 6A, $P < 0.01$), IL-6 (Fig. 6B, $P < 0.05$) as well as TNF-$\alpha$ (Fig. 6C, $P < 0.05$) compared with the normal mice. However, cancer-bearing ST2 KO mice showed no significant change in any of the three cytokines.

**DISCUSSION**

Bone cancer pain, which is one of the most severe cancer pains, is commonly induced by primary bone cancer or secondary bone metastasis from a variety of cancers, including breast, prostate, and lung, among others (Smith, 2011a,b). Several models of bone cancer pain have been developed in mice (Jimenez-Andrade et al., 2010; Pacharinsak and Beitz, 2008; Ren et al., 2012) and rats (Mantyh and Hunt, 2004; Mao-Ying et al., 2006; Zhang and Lao, 2012). Inoculation of different cancer cells into the intramedullary cavity of the femur or tibia in syngenic animals produces a series of behavioral, cellular, and neurochemical changes that are correlated with cancer growth and bone destruction (Jimenez-Andrade et al., 2010; Nakanishi et al., 2011; Wakabayashi et al., 2006). These models, which may parallel well with clinical bone cancer pain, promoted further study of the mechanisms underlying cancer pain.

In our study, we modified the bone cancer pain model that was first described by Schwei et al. (1999). Prior to cancer cells injection, a superficial incision was made above the knee of the mouse, which overlaid the patella. Rather than performing arthrotomy as previously described by Schwei et al. or a patellar ligament cut as reported by Moueedden et al., a light pressure was applied to identify the condyles of the distal femur. Next, a hole was drilled between the condyles into the marrow cavity over the knee. All of the modifications employed were performed to minimize the amount of damage to the knee joint. Moreover, the syringe was left in place for one additional minute prior to being brought out, and the hole was closed using an absorbable gelatin sponge to prevent the carcinoma cells from leaking out along the injection track. This technique is easy to operate and the rate of success ranges from 95% to 100%. No significant difference was found in the pain behaviors between the non-treated, PBS and pain model mice even during the early days after inoculation, which indicated that the function of the knee joints were kept intact.

Bone cancer pain is characterized by pathological symptoms, such as mechanical and thermal hyperalgesia, as well as spontaneous pain. In the present study, bone cancer developed from an intrafemoral 4T1 mammary carcinoma cells injection, which induced a significant limp by the injected hind limb, and severely reduced the paw withdrawal threshold in the von Frey test and hot plate test within days following the injection. In addition, bone histology and radiological analysis indicated more severe bone destruction over time. Taken together, these results were consistent with a previously reported bone cancer pain model and demonstrated that this modified model could mimic the key features of human bone cancer pain (Takahashi, 2012).

Previous reports had demonstrated that IL-33 could induce inflammatory pain in the peripheral nervous system (Verri et al., 2008; Zarpelon et al., 2013). Using the classical formalin-induced acute inflammatory pain model, a previous study performed in our laboratory illustrated a pivotal role of both peripheral and spinal IL-33 in pain modulation. Performed in parallel with this study, our present results showed that both the mRNA and protein levels of IL-33 were significantly increased in the spinal cord after injection of carcinoma cells. Intrathecal administration of ST2 antibody could also dose-dependently relieve the cancer-induced pain behavior, and ST2 knockout mice developed alleviated thermal hyperalgesia in the hot plate test compared to WT mice. Taken together, these results suggested the involvement of IL-33 in the modulation of bone cancer pain. Interestingly, the paw withdrawal threshold to the hot plate test was more susceptible in mice administered with ST2 antibody and ST2 knockout mice compared with the von Frey test, which might be explained by the differences in the distribution of nociceptive receptors and disparate signaling pathways.

IL-33 has been previously reported to be expressed in brain astrocytes, but not in cortical neurons or microglia (Hudson et al., 2008; Yasuoka et al., 2011). Consistent with these reports, our results showed that IL-33-like immunoreactivity was localized in spinal astrocytes, which are critical participants in central sensitization in the pathological pain condition. Moreover, a recent study showed that IL-33 was co-expressed in both astrocytes and neurons in the spinal cord (Jiang et al., 2012), providing conflicting findings regarding the true distribution of IL-33. Similar to IL-33, the expression of ST2 in the CNS is controversial. Some reports have shown that ST2 was expressed in astrocytes, whereas others have demonstrated its expression in both astrocytes and microglia (Yasuoka et al., 2011) or in neurons in the murine spinal cord (Jiang et al., 2012). Our preliminary results showed ST2 distributed in the astrocytes and neurons in the spinal cord in mice.

ILLUSTRATION
(data not shown). Although these results are conflicting, it is clear that both IL-33 and ST2 could be expressed in the CNS.

Lines of evidence suggest that spinal astrocytes play important roles in the initiation and maintenance of cancer-induced bone pain. In the central nervous system, astrocytes showed remarkable and sustained activation induced by tumor cell inoculation. Activated astrocytes released numerous cytokine-to-glia signals proposed for central sensitization, including neurotransmitters, neuromodulators and pro-inflammatory chemokines (Schwei et al., 1999). Our study showed elevated levels of cytokines including IL-1β, TNF-α and IL-6 in the WT cancer-bearing mice, and knocking out of the ST2 gene could reverse the effects. Taken together, these results suggested that the IL-33/ST2 pathway play an essential role in bone cancer pain and its actions might be associated with inducing spinal upregulation of proinflammatory cytokines in astrocytes.

IL-33 has been proposed to combine with ST2L at the cell membrane in the peripheral system, resulting in the recruitment of the adaptor protein MyD88 and activation of transcription factors, such as NF-κB via TRAF6, IRAK-1/4, and MAP kinases as well as the production of inflammatory mediators (Miller, 2011). Previous reports have indicated that spinal extracellular signal-regulated kinase (ERK) activation in glial cells was required for central sensitization during the development of hyperalgesia as well as allodynia and played a crucial role in bone cancer pain (Wang et al., 2012). In addition, an increase in both phosphorylated-p38MAPK (p-p38MAPK) and proinflammatory cytokines (IL-1β) and TNF-α was shown in the spinal cord after cancer cell inoculation, and inhibition of p38 phosphorylation could suppress both tactile allodynia and spontaneous pain in bone cancer pain rats. However, whether the role of IL-33 in mediating bone cancer pain is via the activation of MAPKs requires further investigation.

Taken together, inoculation with 4T1 mammary carcinoma cells into the femurs of mice induced reduced limb use, mechanical allodynia as well as thermal hyperalgesia. Moreover, the bone cancer-induced pain was attenuated by administration of the ST2 antibody as well as by ablating the ST2 gene, indicating that IL-33 and its receptor ST2 contribute to bone cancer pain in mice. Targeting of the IL-33/ST2 signaling pathways might be a promising strategy for the treatment of metastatic bone cancer pain.

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