MHCII contributes to bone cancer pain via activating the NLRP3 inflammasome in microglia of the medial prefrontal cortex

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Abstract

As one of the causes of bone cancer pain (BCP), central hyperalgesia has more and more supporting evidence recently. The medial prefrontal cortex (mPFC) is implicated in emotional disorders associated with BCP. Major histocompatibility complex II (MHCII) is a key component in regulating anti-inflammatory response and antigen presentation. This study aims to investigate whether MHCII of the mPFC participates in the BCP. The rat model of BCP was established by injecting Walker 256 cells into the tibia. BCP-induced decreased pain threshold, increased the expression of MHCII and the downstream protein CTSS, CD74 and upstream protein CIITA in mPFC, and the upregulation of MHCII was mainly observed in the mPFC microglia. Furthermore, pharmacological inhibition of MHCII in mPFC by intraperitoneal injecting minocycline provides relief from BCP. Importantly, the upregulation of MHCII is accompanied by significant NLRP3 inflammasome activation and inflammatory factors (IL-1 β , IL-18 and TNF- α) released in the mPFC of BCP rats. Taken together, our data suggest that MHCII participates in BCP by activating NLRP3 inflammasome in microglia of the mPFC.

Introduction

The incidence of cancer worldwide has been on a steady rise in the past 10 years (Miller et al. 2020), and advanced cancer metastasis to bone is one of the most common complications (Meuser et al. 2001), inflicting patients with severe and chronic pain. Several causes of bone cancer pain (BCP) have been proposed, including bone destruction and nociceptive hypersensitivity (Mantyh 2013; Zajackowska et al. 2019). Recently, more and more evidence has been accumulated to support the role of central nociceptive hypersensitivity in bone cancer pain (Hou et al. 2020; Wu et al. 2021).

In particular, the medial prefrontal cortex (mPFC) is likely to be critically involved in chronic pain like BCP (Kummer et al. 2020). Firstly, it is now well established that the mPFC undergoes structural and functional plasticity during chronic pain states (Sang et al. 2018; Obermann et al. 2013; Liu et al. 2018; Huang et al. 2020). Secondly, the connections of mPFC and many other brain areas including the cerebral cortex, hippocampus, periaqueductal gray matter (PAG), thalamus, amygdala, and basal nucleus, have been shown to exert analgesic effects (Ong et al. 2019). Lastly, it was reported that microglia played a key role in the mPFC in the model of the peripheral nerve injury that causes pain (Fiore and Austin 2019). That's all suggested the key role of mPFC in BCP and microglia may play an important role in this pathophysiological process.

It is well known that the immune system plays a pivotal role in the development of various chronic pain (Totsch and Sorge 2017). As one of the key players in antigen presentation, major histocompatibility complex II (MHCII) has been shown to participate in the occurrence of chronic pain (Hartlehnert et al. 2017; Liu et al. 2012; Dominguez et al. 2008a; Dominguez et al. 2008b). Sweitzer et al. found that knocking out MHCII in a mouse model of neuropathic pain prevented acute pain from shifting to chronic pain (Sweitzer et al. 2002). Furthermore, after peripheral nerve injury, the pain threshold of MHCII knockout mice was significantly increased (Sweitzer et al. 2002). Notably, a study by Song et al. found

that in the spinal cord, the upregulation of MHCII contributes to the BCP (Song et al. 2017). However, the exact role and mechanism of MHCII in mPFC remains not fully understood.

As previously reported, MHCII is believed to participate in the occurrence of chronic pain through the immune response with T cells in the spinal cord (Zhang et al. 2014; Huo et al. 2019; Ding et al. 2020) or direct regulation of the inflammatory factors in the peripheral blood (Hussen et al. 2020). In particular, NLRP3 (NLR Family Pyrin Domain Containing 3) inflammasome is known to be activated by a variety of ligands and stimuli (Wang et al. 2020; Muñoz-Planillo et al. 2013; An et al. 2019). In addition, increased expression in NLRP3 was found in chronic constriction injury (Xu et al. 2019) and BCP (Chen et al. 2019; Fu et al. 2021). At the same time, inhibition of NLRP3 expression provided pain relief (Fu et al. 2021). Significantly, in a mouse model of experimental autoimmune encephalomyelitis, Larissa et al. found that activation of microglia was related to MHCII and NLRP3 inflammasome in the central nervous system (de Oliveira et al. 2020). And in an adipose tissue inflammation model, T cell activation interacts with MHCII, upregulating NLRP3 inflammasome to promote the release of inflammatory factors and aggravate the inflammatory (Yin et al. 2014). However, it is not known, if upregulation of MHCII is involved in the activation of NLRP3 inflammasome in the mPFC of BCP or not.

In the present study, we attempt to investigate the effect of MHCII and its regulation of NLRP3 inflammasome in the mPFC microglia of BCP rats. We established the model of BCP and detected the increased expression of MHCII in the mPFC. Furthermore, we found this MHCII upregulation was restricted in microglia in the mPFC, accompanied by NLRP3 inflammasome activation. Importantly, we found pharmacological inhibition of MHCII reduced NLRP3 inflammasome activation and provided relief from BCP. In conclusion, our results suggest that MHCII participates in BCP by activating NLRP3 inflammasome in microglia in the mPFC.

Materials And Methods

Bone cancer pain (BCP) animal model

Female Sprague-Dawley (200 to 250 g) and Wistar rats (60–70 g) were purchased from B&K Universal Group Limited (Shanghai, China) and housed in cages at a temperature of $24 \pm 1^\circ\text{C}$ under 12/12h light-dark cycles with free access to food and water. All animal handling and surgical procedures were carried out in accordance with the guidelines of the International Association for the Study of Pain, approved by the Animal Care and Use Committee of Shanghai Chest Hospital, Shanghai Jiao Tong University.

Bone cancer pain (BCP) was induced in rats as previously described (Walker et al. 2002). Briefly, female Wistar rats received an intraperitoneal inoculation of Walker 256 mammary gland carcinoma cells. After one week, cells in the ascites were collected and resuspended in saline to a final concentration of 2×10^7 cells/ml. Next, female Sprague Dawley rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and a 23-gauge needle was inserted into the intramedullary canal of the right tibia at one-third of the length from the lower end, to create a cavity for the injection of saline (10 μl , Sham) or Walker 256 cell

suspension (10 μ l, containing 2×10^5 cells in total, BCP). The cavity was sealed with bone wax and the incision was closed with stitches. Upon waking from anesthesia, the animals were returned to their home cages.

Behavioral tests

Mechanical allodynia was evaluated by measuring the paw withdrawal thresholds (PWT) in response to a series of calibrated von Frey filaments (Remeniuk et al. 2015). In brief, rats were transferred to a chamber with a metal mesh floor and allowed to acclimatize for 30 min. Then, von Frey filaments were applied to the plantar surface of the right hind paw in the ascending order (1, 1.4, 2, 4, 6, 8, 10, and 15 g). Abrupt paw withdrawal, licking, and shaking were defined as positive responses. Once a positive response was observed, the paw was allowed to rest for 5 min. PWT was defined as the lowest force that elicited a positive response and averaged across three repeats of measurement.

Movement-evoked pain was assessed with the limb use score (LUS) (Remeniuk et al. 2015). Rats were permitted to move spontaneously on a smooth plastic table (50 $\times$ 50 cm). The limb use during spontaneous ambulation was scored on a scale of 4 to 0 (4: normal use; 3: slightly limping; 2: clearly limping; 1: no use of the limbs, partial; 0: no use of the limbs, complete).

MHCII inhibitor minocycline was purchased from MedChemExpress (Monmouth Junction, NJ, USA), dissolved in saline (20mg/ml, stored at -20°C for use), and administrated on every other day from one day after surgery (D1, 20mg/kg, i.p.). All behavioral tests were performed by an investigator who was blinded to the experimental design.

RNA interference and transfection

siRNA oligos against MHCII (GenePharma, *RT1-Da* siRNA oligo, which was designed to knockdown MHCII) and negative control siRNA (NC siRNA, which was designed to have no known mRNA targets in the cells being used) were obtained from GenePharma (Shanghai, China).

A rat microglia cell line (GMI-R1) (Sawada et al. 1998) cultured in 89% H-DMEM supplemented with 10% FBS and 1% antibiotics (100 U/ml penicillin and 100 U/ml streptomycin) in a humidified atmosphere of 5% CO₂ at 37°C and passaged every 2–3 d to maintain growth. The GMI-R1 cells were transfected according to the procedure (Liu et al. 2020b). We optimized the siRNA concentration for transfection at a concentration of 40 pM and the time for cell harvest at 48 h. negative control siRNA sense 5'-UUC UCC GAA CGU GUC ACG UTT-3', negative control siRNA antisense 5'-ACG UGA CAC GUU CGG AGA ATT-3'; *RT1-Da-625* siRNA sense 5'-CCU CCU CCC AGA AAC UAA ATT-3', *RT1-Da-625* siRNA antisense 5'-UUU AGU UUC UGG GAG GAG GTT-3'; *RT1-Da-242* siRNA sense 5'-GAG GGU GCA UUG GCU AAU ATT-3', *RT1-Da-242* siRNA antisense 5'-UAU UAG CCA AUG CAC CCU GTT-3'; *RT1-Da-537* siRNA sense 5'-CCU CCA CGG AAG AUU ACU ATT-3', *RT1-Da-537* siRNA antisense 5'-UAG UAA UCU UCC GUG GAG GTT-3'. *RT1-Da-625* siRNA yielded the highest interfering efficiency and therefore used to carry out the experiment (Figure S3)

Rats were anesthetized with sevoflurane (1.5–2%). The skull was exposed and a 1.0 mm-diameter hole was drilled above the target region. The coordinates for the ipsilateral mPFC were as follows: anteroposterior + 0.5 mm, mediolateral $\pm$ 0.5 mm, and dorsoventral – 2.2 mm. 2 μ g *RT1-Da-625* siRNA or negative control RNA in 10 μ L of in vivo-jetPEITM (201-10G, Polyplus-transfection, Illkirch, France) or in vivo-jetPEITM only on days 10 under anesthesia with sevoflurane. The animals were subjected to the von Frey mechanical pain test at 1h, 2h, 4h, 24h, 48h, 96h.

Western blot

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and rapidly sacrificed by cervical dislocation. The mPFC was homogenized in RIPA buffer containing 1 mM PMSF (Beyotime) and centrifuged at 13 000 $\times$ g for 30 min. For cell culture samples, total protein from the cells was extracted in RIPA lysis buffer (Beyotime). The supernatant was collected, and the total protein concentration was titrated using a bicinchoninic acid kit. An equivalent amount of protein (50 μ g) was fractionated on 10% polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes (Millipore) at 300 mA for 90 min. Membranes were blocked with 5% BSA in Tris-buffered saline (TBS, 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 2 h at room temperature and incubated overnight at 4°C with primary antibodies (anti-CD74 at 1:500, sc-6262, SANTA CRUZ; anti-CTSS at 1:1000, ab92780, Abcam; anti-CIITA at 1:1000, sc-13556, SANTA CRUZ; anti-NLRP3 at 1:1000, ab263899 Abcam; anti-ASC at 1:1000, ab180799, Abcam; anti-proCaspase1 at 1:1000, ab179515, Abcam; anti-Caspase1 at 1:500, sc-56036, SANTA CRUZ; anti-Vinculin at 1:1000, A2752, ABclonal; anti-MHCII at 1:1000, sc06-78, invitrogen) in TBS containing 5% BSA. Following three washes with TBS, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit at 1:2000, ab205718, Abcam; goat anti-mouse at 1:2000, ab205719, Abcam) in TBS containing 5% BSA for 2 h at room temperature. Immunoreactive proteins were visualized using the enhanced chemiluminescence western blotting detection system (Santa Cruz). The membranes were analyzed with a computerized image analysis system (ChemiDoc XRS1, Bio-Rad, Hercules, CA), and the intensity of the protein bands was quantified with Image Lab software (ImageJ). All protein expression was normalized to Vinculin.

Flow Cytometry Analysis.

The mPFC was minced by scissors, digested with 0.15% trypsin, and repeatedly blown into a single-cell suspension. The mononuclear cells were isolated by density gradient centrifugation using Percoll. The mononuclear cells were resuspended in PBS and stained with the following antibodies: anti-CD4-APC(BioLegend), anti-CD3-FITC (BioLegend). Cells were then analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with FlowJo software (version vX 0.7).

Bone histomorphometric analysis

The ipsilateral tibia of BCP rats was fixed in 4% paraformaldehyde for 48 h, followed by decalcification in 10% EDTA for 3–4 weeks, and 6- μ m sections were prepared on a rotating microtome. Paraffin-embedded sections were deparaffinized in xylene, rehydrated, and stained with hematoxylin–eosin (H&E; Sigma, St Louis, MO, USA) according to the manufacturer's protocol (Wang et al. 2019).

Enzyme-linked immunosorbent assay

ELISA was used to determine the inflammatory cytokines (IL-1 β , TNF- α , and IL-18) in the mPFC. The samples were weighed and then homogenized completely in phosphate-buffered solution (PBS). After centrifugation (1,000 $\times$ g, 10 min) of the homogenates at 4°C, supernatants were collected and stored at -80°C. The concentrations of TNF- α (ml002859-C), IL-1 β (ml037361-C), IL-18 (ml002816-C), Caspase-1 (ml037348-C), proCaspase-1 (ml501627-C) were detected following the instruction of ELISA kits (Shanghai Enzyme-linked Biotechnology Co., Ltd.).

Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from mPFC using a total RNA Kit (Vazyme Biotech Co., Ltd, China), and complementary DNA (cDNA) was generated using a cDNA Synthesis Kit (DBI® Bioscience Co., Ltd, Germany), according to instructions provided by the manufacturers. RT-qPCR) was carried out using a SYBR Green assay (DBI® Bioscience) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Thermo Fisher Scientific, Waltham, MA, USA), and the relative gene expression was determined using the comparative Ct ($\Delta\Delta$ Ct) method. GAPDH was used as a housekeeping gene. The primer sequences are as follows: *RT1-Da* forward 5'-TGGGCTATCAGAGAGGAGCA-3', *RT1-Da* reverse 5'-

TGGTGACAGGTTGTCCGTTC-3'; *RT1-Bb* forward 5'-AAGCGTCAGAAAGGACCTCG-3', *RT1-Bb* reverse 5'-TTGTCTGTTCCCAAGGCCAG-3'; *CD74* forward 5'-TGATGCACCTGCTTACGAAGT-3', *CD74* reverse 5'-ATCACACTTGGGACGGAACG-3'; *CTSS* forward 5'-GGGGCCTTCACTTTGGTGAT-3', *CTSS* reverse 5'-TTAGACGACAGGGTGCAAGC-3'; *GAPDH* forward 5'-CTAAGCAACCAGGTGACCAG-3', *GAPDH* reverse 5'-GGTGGACCTCATGGCCTACA-3'.

Immunofluorescence

Animals were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and underwent sternotomy, followed by intracardiac perfusion with 200 ml saline and 200 ml 4% ice-cold paraformaldehyde in 0.1 M phosphate-buffered saline. The brain was removed, postfixed in 4% paraformaldehyde for 4 h, and subsequently allowed to equilibrate in 30% sucrose in phosphate-buffered saline overnight at 4°C. Brain samples were embedded in Tissue-Tek O.C.T., cut at a thickness of 20 μ m using a cryostat (Leica CM1850-1-1) and mounted on slides. All Brain samples were thoroughly rinsed in PBS to remove any residual OCT. The samples were then blocked in 0.3% Triton-X 100 and 5% normal donkey serum (NDS) in PBS for 1 hour, and incubated overnight with anti-MHCII (1:50, MCA46GA, BIO-RAD), anti-CIITA (1:100, sc-13556, SANTA CEUZ), anti-IBA-1 (1:100, ab178847, Abcam), anti-GFAP (1:100, ab254082, Abcam), anti-NEUN (1:100, ab177487, Abcam). On the next day, the samples were rinsed in PBS and incubated for 1 hour in PBS containing appropriate secondary antibody (1:500) from Jackson ImmunoResearch (catalog nos. 711-545-152 or 715-585-150) or Cell Signaling Technology (catalog nos. 8889 or 4408). The samples were then rinsed in PBS and coverslipped with Antifade Mounting Medium with DAPI (Beyotime, Nanjing, China). The imaging and subsequent analysis were performed using a Confocal Microscope (TCS SP8, Leica Microsystems, Mannheim, Germany).

Statistics

Data are reported as the mean $\pm$ standard deviation. Data were normally distributed as tested using the D'Agostino and Pearson omnibus normality test ($P > 0.05$). The data were analyzed using GraphPad Prism 8.0 for Windows (San Diego, CA, USA). Results from behavioral testing were analyzed using ANOVA for repeated measures. All other measures between 2 groups, the Student's t-test was used for the comparisons of variables with normal distribution from independent samples, For comparisons among > 2 groups, a one-way ANOVA with Turkey's multiple comparisons test was applied. Two-way ANOVA followed by Turkey's multiple comparisons test with repeated measurements were used to determine the interaction between time and group between two groups. Fisher's least significant difference test for pairwise comparisons, all statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

Results

Induction of bone cancer pain (BCP) in rats

To induce BCP in rats, we injected Walker 256 mammary gland carcinoma cells in the intramedullary canal of the right tibia. We performed behavioral tests 30 min before the operation and on D4, D7, D11, D14, and D17 (Fig. 1a). BCP rats were found to exhibit significantly decrease of PWT and LUS when compared to the Sham rats (D7, PWT: from 10.74 ± 2.04 to 6.44 ± 0.81 , $p < 0.0001$; LUS: from 3.67 ± 0.42 to 2.17 ± 0.28 , $p < 0.0001$; D17, PWT: from 13.04 ± 4.27 to 2.78 ± 0.78 , $p < 0.0001$; LUS: from 3.94 ± 0.14 to 1.62 ± 0.14 , $p < 0.0001$; Fig. 1b and 1c), consistent with previously reported values in the literature (Remeniuk et al. 2015). Next, we sacrificed animals on D17 and examined pathological changes. Firstly, in gross images, we found the surface of the tibia of Sham rats was smooth while that of BCP rats was swollen and uneven (Fig. 1d). Secondly, we found bone destruction in BCP rats, in contrast to uniform bone quality in Sham rats with tibia Micro-CT (Fig. 1e). Lastly, we performed hematoxylin and eosin staining of the tibia, we found tumor cell proliferation and invasion of the bone cortex in BCP rats, but not in Sham rats (Fig. 1f). Taken together, these behavioral and pathological results strongly suggested that BCP was successfully induced in rats.

Increased expression of MHCII in the medial prefrontal cortex (mPFC) of rats with BCP

As the key role of MHCII in chronic pain, we investigated the expression of MHCII in the mPFC after BCP, targeting *RT1-Da* and *RT1-Bb*, two subunits of MHCII, and performed qRT-PCR. We found both subunits of MHCII were strongly upregulated at the mRNA level in BCP rats when compared to Sham rats (Fig. 2a). Consistently, both western blot and immunostaining of MHCII confirmed its increased expression in the mPFC of BCP rats at the protein level (Fig. 2b-e).

Next, in order to identify specific cell types of MHCII upregulation in the mPFC of BCP rats, we performed double immunostaining and colocalization analysis. Astrocytes and microglia were significantly activated in the mPFC of BCP rats (Fig. 3a, b). Intriguingly, we found that MHCII was localized mainly in

microglia, not in astrocytes or neurons (Fig. 3a-c), suggesting a critical role of MHCII in the mPFC microglia in the BCP.

Given the critical roles of MHCII in the immune response to tumors by recruiting CD4 + T cells (Alspach et al. 2019), we first evaluate the expression of CD3 and CD4 in the mPFC and found no significant difference in their expression between Sham and BCP rats (Fig. S2). Remarkably, the expression of CD74 and CTSS, two binding partners of MHCII in downstream antigen presentation (Fig. 4a, b), and the class II trans-activator (CIITA) (LeibundGut-Landmann et al. 2004), a key transcription factor for MHCII, were all significantly increased in BCP rats when compared to Sham rats (Fig. 4c-e). Taken together, these data suggest that MHCII and its signaling partners may participate in BCP, either through the recruitment of CD4 + T cells.

To investigate the role of MHCII in BCP physiologically, we injected minocycline (MC), an inhibitor of MHCII (Nikodemova et al. 2007), intraperitoneally in BCP rats (20 mg/kg, every other day from D1), and performed behavior tests to assess possible changes of pain perception (Fig. 5a). Rats were randomly divided into three groups, Sham, BCP + saline, and BCP + MC (n = 6 for each group). Consistent with our validation of BCP induction described above, both PWT and LUS were significantly reduced in the BCP + Saline group when compared to the Sham group (Fig. 5b). Importantly, this reduction was reversed in the BCP + MC group, suggesting a pivotal role of MHCII in the occurrence of BCP. At the same time, we also found the reversal of upregulation in MHCII (Fig. 5c-f) and its downstream binding partners (CD74 and CTSS) and key transcription factor (CIITA) (Fig. 5g-j) as well in the BCP + MC group. Thus, we proposed that inhibiting the expression of MHCII in mPFC microglia may relieve BCP.

Moreover, we stereotaxic microinjected siRNA targeting *RT1-Da* at D10 in the mPFC in BCP rats and performed behavior tests to assess possible changes in pain perception (Fig. S4a). Consistent with the effect of minocycline, *RT1-Da* siRNA could reverse the reduction of PWT in BCP rats when compared to the effect of negative control (Fig. S4b). Thus, we proposed that MHCII in the mPFC plays a critical role in BCP.

Activation of the inflammasome in microglia of the mPFC in rats after BCP

It has been shown repetitively that neuroinflammation in the central nervous system is involved in the occurrence of pain (Davis 2018). We, therefore, hypothesized that the upregulation of MHCII in microglia of the mPFC in BCP is likely to activate inflammasome, releasing inflammatory factors that contribute to the occurrence of BCP. Indeed, with double immunostaining and colocalization analysis, we found an increased expression of NLRP3 in microglia of the mPFC in rats with BCP (Fig. 6a). In animal models with chronic pain, the activation of NLRP3 will recruit apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and proCaspase-1 to form inflammasome complex, which in turn activates Caspase-1 and further promotes maturation and release of inflammatory factors such as IL-1 β , IL-18, and TNF- α (Chen et al. 2021). Consistently, western blot analysis revealed an increase of not only NLRP3 but also its downstream co-activators, including ASC, proCaspase-1, and Caspase-1 (Fig. 6b, c).

Furthermore, ELISA analysis confirmed the increased expression of proCaspase-1 and Caspase-1 in the mPFC in rats with BCP (Fig. 6d) and revealed the co-upregulation of key inflammatory factors (Fig. 6e). In conclusion, these data suggest that the NLRP3 inflammasome was activated in the microglia in the mPFC.

Lastly, we set out to investigate if MHCII can regulate the activation of the NLRP3 inflammasome, and we found the increased expression of NLRP3 in the mPFC of rats with BCP was reversed by MHCII blocker (Fig. 7a, b). Furthermore, similar results were observed with not only NLRP3 co-activators ASC, proCaspase-1, and Caspase-1, but also with related inflammatory factors IL-1 β , IL-18, and TNF- α (Fig. 7a-d). Finally, we tested if MHCII inhibition by siRNA could also affect the expression of NLRP3 inflammasome. Knocking down the expression of *RT1-Da*, in rat microglia GMI-R1 cells, we found indeed that knocking down MHCII reduced the expression of not only NLRP3 but also its co-activators ASC, proCaspase-1, and Caspase-1 (Fig. 7e, f), further supporting our finding that inflammasome activation may be the downstream mechanism for MHCII in BCP in mPFC.

Discussion

In this study, we demonstrated, to our knowledge for the first time, that MHCII in the medial prefrontal cortex plays a pivotal role in the progression of bone cancer pain. Firstly, we found that the expression of MHCII was increased in microglia in the mPFC of BCP rats, and the pain was relieved after inhibiting the expression of MHCII. Secondly, we found that the increased expression of MHCII in microglia of the mPFC in rats with BCP was accompanied by the activation of NLRP3 inflammasome and that an MHCII blocker can reverse it.

A wealth of evidence has suggested that BCP is a complex clinical syndrome, that shares a number of features with inflammatory and neuropathic pain (Falk and Dickenson 2014), and often comes with concurrent depression, cognitive impairment, and other diseases (Feller et al. 2019; Liu et al. 2021). It has been reported that the differences between the sexes were minor and did not affect the overall progression of the pain state (Falk et al. 2013). To study the bone metastasis of breast cancer in the clinical more closely, we have established the bone cancer pain model by inoculating Walker 256 cells into female SD rats.

MHCII is important for antigen presentation, and secretion of pro-inflammatory mediators, thereby contributing to chronic pain. Our result showed that the expression of MHCII was significantly upregulated in multiple brain regions (Figure S1), including mPFC which is a central hub for the comorbidity of chronic pain, depression, and anxiety (Ong et al. 2019; Huang et al. 2019). At the same time, mPFC has been reported to participate in pain by connecting with PAG (Yin et al. 2020). Further microglia activation and subsequent pro-inflammatory responses in mPFC play a crucial role in the development of neuropathic pain (Fiore and Austin 2019; Kosek et al. 2016). In this study, focusing on mPFC, we found that microglia were activated during the progression of BCP, and the expression of MHCII was upregulated mainly in microglia, but not in astrocytes or neurons. Consistently with our study, Li et al

reported that MHCII is not constitutively expressed in resting microglia but can be potently upregulated once microglia are activated in chronic neuropathic pain rats (Hamo et al. 2007; Li et al. 2016). Although, MHCII in microglia contributed to BCP has been reported in the spinal cord (Song et al. 2017), the role of MHCII in mPFC is still unclear. In this present study, our results showed an increase of MHCII in mPFC after BCP. Ever since it is known that minocycline is an inhibitor of CIITA and MHCII (Nikodemova et al. 2007; Nikodemova et al. 2006), Li et al. found that intraperitoneal injection of minocycline attenuated neuronal apoptosis in the PFC at a high dose of 50 mg/kg (Li et al. 2019). We found that intraperitoneal injection of minocycline at a dose of 20 mg/kg every other day from one day after BCP surgery inhibited the upregulated expression of MHCII in the mPFC and provided significant relief from BCP. In the meantime, we stereotaxic microinjected *RT1-Da* siRNA in mPFC at D10 to suppress MHCII expression, which could attenuate mechanical hyperalgesia of BCP rats (Fig. S4). Based on these findings, we concluded that MHCII in mPFC may contribute to BCP.

Previously it was reported that MHCII-positive microglia in the dorsal root ganglion and the lumbar dorsal roots activated T helper cells, contributing to the transition from acute to chronic mechanical allodynia in SNI rats (Sweitzer et al. 2002). And Song et al proposed that infiltrated CD4 + T lymphocytes might interact with MHC II-presented antigen and contributes to BCP in the spinal cord (Song et al. 2017). While, in our result, we found that the infiltration of CD4 + T cells was not increased in the mPFC of BCP models, which was consistent with the similar results found in neuropathic pain (Xu et al. 2020). It is suggested that MHCII in mPFC plays a role in BCP, but it may not be through antigen presentation. In the meantime, previous studies suggested that MHCII and NLRP3 inflammasome contribute to microglia activation (Yin et al. 2014). Moreover, it has been reported that the activation of NLRP3 inflammasome (Liu et al. 2020a; Gao et al. 2018) and the release of pro-inflammatory cytokines including IL-1 β , IL-18, and TNF- α were contributed to the development of chronic pain in the spinal cord (Lu et al. 2015; Chen et al. 2019). Furthermore, in mPFC, the activation of NLRP3 inflammasome was also reported in morphine-induced-analgesic tolerance (Liu et al. 2020b). Therefore, NLRP3 inflammasome in the mPFC could represent a new target for chronic pain treatment. As expected, we found the increased expression of NLRP3 inflammasome in the mPFC of BCP rats, together with increased expression of pro-inflammatory cytokines, including IL-1 β , IL-18, and TNF- α . Further, the activation of NLRP3 inflammasome was reverted after the pharmacological inhibition of MHCII. Whereas minocycline has a wide range of drug effects, knockdown of MHCII expression in rat mPFC and microglia GMI-R1 by *RT1-Da* siRNA reverted the expression of NLRP3 inflammasome, strengthening a potential mechanistic link between the central inflammation and pain.

In summary, we took an interdisciplinary approach combining molecular, behavioral, and pharmacological techniques, to demonstrate the pivotal role of MHCII-mediated central hyperalgesia in the medial prefrontal cortex in bone cancer pain. Furthermore, we found that MHCII upregulated in the medial prefrontal cortex in bone cancer pain is restricted in microglia, accompanied by elevated expression of NLRP3 inflammasome. These findings shed new light on the mechanisms of bone cancer pain and open a new venue for designing treatment plans to alleviate bone cancer pain in patients with advanced cancer.

Declarations

Ethics approval and consent

The study protocol was approved by the Animal Care and Use Committee of Shanghai Chest Hospital, Shanghai Jiao Tong University [permission no. KS (Y)20186]

Consent for publication

Not applicable

Availability of data and material

The datasets generated for this study are available on request to the corresponding author.

Competing Interests

The authors declare that they have no competing interests

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Authors' contributions

J.W and X.Y. designed the study. X.L., W.W., X.Z., Z.G., M.T., and Y.Z. performed the experiments. Z.G., X.Z., M.T., and X.Y. analyzed the data. X.L. and W.W. wrote the manuscript, which all authors reviewed and approved.

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Figures

Figure 1

Induction of bone cancer pain (BCP) in rats. a. Experimental timeline showing the dates of surgery (D0) and behavioral tests (-30min, D4, D7, D11, D14 and D17). On D0, saline (Sham) or Walker 256 mammary gland carcinoma cells (BCP) were injected into the tibia in rats and pain sensation was assessed before and after through behavioral tests. b and c. Induction of significant hyperalgesia in the tumor-bearing hind paw, as assessed through the paw withdrawal threshold (PWT, b) and the limb use score (LUS, c).

Data are presented as mean $\pm$ SD, **** means $p < 0.0001$. d. Photograph of the tibia from a BCP rat on D17 showing obvious tumor growth and bone mass destruction (right) when compared to the one from a Sham rat (left). e. Representative micro-CT images showing the bone microstructure of two tibias from a Sham rat (left) and a BCP rat (right) on D17. Note the reduction in medullary bone and the increase in lesions in cortical bone with the tibia from the BCP rat. f. Hematoxylin and eosin-stained histological sections showing obvious medullary bone loss and bone destruction in the tibia of a BCP rat on D17 (right) when compared to the one from a Sham rat (left). Scale bars, 50 μ m.

Figure 2

Increased expression of MHCII in the medial prefrontal cortex (mPFC) in rats with BCP. a. Increased expression of MHCII at the mRNA level. The mRNA expression of two MHCII subunits, *RT1-Da* and *RT1-Bb*, was determined through RT-qPCR and normalized to that of GAPDH. b. and c. Increased expression of MHCII at the protein level, revealed in both western blot analyses (b and c). d. Schematic diagram of prefrontal cortex slice. e. immunofluorescent staining confirmed the increased expression of MHCII in the mPFC of rats with BCP on D17.

Figure 3

Increased expression of MHCII in the mPFC of rats with BCP was restricted in microglia. Double immunofluorescent staining of MHCII and cell type-specific markers revealed that the increased expression of MHCII in the PFC of rats with BCP occurred mainly in microglia (a), but not in astrocytes (b) or neurons (c). Scale bars, 50 μm.

Figure 4

Co-regulation of MHCII-interacting proteins in the mPFC of rats with BCP. a and b. Western blot analysis confirmed the increased expression of CD74 (a) and CTSS (b) at the protein level in the mPFC of rats with BCP on D17. c. and d. Western blot analysis revealed a significant increase in the expression of CIITA. e. Immunofluorescent staining confirmed the increased expression of CIITA in the mPFC of rats with BCP on D17. Scale bars, 50 μ m.

Figure 5

MHCII inhibition relieved BCP and reduced MHCII expression in the mPFC of rats with BCP. a. Experimental timeline showing injection of MHCII inhibitor minocycline (20 mg/kg, i.p.) every other day from D1. b. BCP was elevated by minocycline, as indicated by both behavioral tests of PWT (left) and LUS (right). c, d and e. f. The increased expression of MHCII in the PFC of rats with BCP was significantly reduced by MHCII inhibition, at both the mRNA level (c, RT-qPCR) and the protein level (d and e, western

blot analysis, and f, immunofluorescent staining). g-j. the increased expression of CD74, CTSS and CIITA in the mPFC of rats with BCP was also significantly reduced by MHCII inhibition (g, RT-qPCR, h and j western blot analysis). Scale bars, 50 μ m.

Figure 6

Activation of the inflammasome in microglia of the mPFC in rats with BCP. a. Double immunofluorescent staining of inflammasome marker NLRP3 and microglia marker IBA-1 revealed an increased expression of the inflammasome in microglia of the mPFC in rats with BCP. b and c. Western blot analysis showing co-upregulation of NLRP3 with ASC (b), proCaspase-1 and Caspase-1 (c). d. Elisa analysis confirmed the increased expression of proCaspase-1 and Caspase-1 in the PFC of rats with BCP. e. Elisa analysis showed the increased expression of inflammatory factors IL-1 β , IL-18 and TNF- α in the mPFC in rats with BCP.

Figure 7

MHCII inhibition reduced activation of inflammasome in the mPFC of rats with BCP. a and b. Western blot analysis showed the increased expression of NLRP3, ASC, proCaspase-1 and Caspase-1 were reduced when MHCII was inhibited by minocycline. c. Elisa analysis confirmed the reduced expression of proCaspase-1 and Caspase-1 by minocycline in the PFC of rats with BCP. d. Elisa analysis showed that the expression of inflammatory factors IL-1 β , IL-18 and TNF- α was reduced by minocycline in the mPFC of rats with BCP. e and f. Western blot analysis showed that the expression of NLRP3, ASC, proCaspase-1 and Caspase-1 in GMI-R1 cells was reduced when the expression of MHCII was knocked down by *RT-Do*-siRNA.

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