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Full-length Article

# STAT1 as a downstream mediator of ERK signaling contributes to bone cancer pain by regulating MHC II expression in spinal microglia

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ABSTRACT

Major histocompatibility class II (MHC II)-specific activation of $CD4^+$ T helper cells generates specific and persistent adaptive immunity against tumors. Emerging evidence demonstrates that MHC II is also involved in basic pain perception; however, little is known regarding its role in the development of cancer-induced bone pain (CIBP). In this study, we demonstrate that MHC II expression was markedly induced on the spinal microglia of CIBP rats in response to STAT1 phosphorylation. Mechanical allodynia was ameliorated by either pharmacological or genetic inhibition of MHC II upregulation, which was also attenuated by the inhibition of pSTAT1 and pERK but was deteriorated by intrathecal injection of IFNγ. Furthermore, inhibition of ERK signaling decreased the phosphorylation of STAT1, as well as the production of MHC II *in vivo* and *in vitro*. These findings suggest that STAT1 contributes to bone cancer pain as a downstream mediator of ERK signaling by regulating MHC II expression in spinal microglia.



## 1. Introduction

In 2016, 1,685,210 new cancer cases and an estimated 595,690 cancer deaths are projected to occur in the United States (Siegel et al., 2016). Malignant tumors such as those in lung, prostate, and breast can undergo skeletal metastases that could result in severe cancer-induced bone pain, which substantially reduces the quality of life in cancer patients (Selvaraj et al., 2015). Unfortunately, current treatments for bone cancer pain (BCP) are insufficient due to a lack of understanding of the underlying mechanisms.

Growing evidence suggests that the neuroimmune response plays a pivotal role in the development and maintenance of chronic pain (Dominguez et al., 2008; Grace et al., 2011; Sato-Takeda et al., 2006; Sweitzer and DeLeo, 2002); however, the precise mechanism is still largely unknown. Microglia are the resident macrophages of the central nervous system (CNS), which continuously survey the microenvironment. Any alteration of neuronal activity can induce specific microglial changes (Nimmerjahn et al., 2005). Under chronic pain or neuroinflammatory conditions, microglial cells are activated and express high levels of major histocompatibility complex class II (MHC II). The most recognized function of MHC II is to be constitutively expressed on the surface of professional antigen-presenting cells loaded with antigenic peptide (Roche and Furuta, 2015). Notably, many studies on the effects of spinal dorsal microglia suggest that MHC II may be a critical molecule in chronic neuropathic pain and autoimmune diseases (Hashizume et al., 2000; Lincoln et al., 2005; Sato-Takeda et al., 2006; Sweitzer et al., 2002). Therefore, modulating the expression of MHC II in spinal microglia to inhibit maladaptive neuroimmune responses could be a potential therapeutic strategy for pain relief.

The Janus kinase/signal transducer and activator of transcription 1 (JAK/STAT1) signaling pathway is a key regulator of MHC II expression by modulating the expression of class II transactivator (CIITA) (Nikodemova et al., 2007; Ting and Trowsdale, 2002). In particular, JAK/STAT1 signaling is not only activated by interferon γ (IFNγ) (Herrera-Molina et al., 2012; Muhlethaler-Mottet et al., 1998; Nikodemova et al., 2007; Zhou et al., 2015) but is also among the five pathways that lead to microglial activation (Graeber, 2010; Hanisch and Kettenmann, 2007; Smith, 2010). To date, no direct evidence links the JAK/STAT1 signaling pathway to tactile allodynia under BCP conditions. We recently reported that ERK signaling is involved in the pathogenesis of BCP (Guan et al., 2015a); moreover,

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the fact that the activation of ERK signaling potentiates STAT1 phosphorylation was reported by others (Herrera-Molina et al., 2012). In the present study, we investigated the role of MHC II in BCP. In particular, we analyzed the relationship between ERK and STAT1 in regulating MHC II expression *in vivo* in a BCP rat model and *in vitro* using primary microglial cultures.

## 2. Materials and methods

### 2.1. Animals

Adult virgin female Sprague Dawley (SD) rats (weighing 200–220 g) used in the present study were purchased from the Experimental Animal Research Center of Hubei Province, Wuhan, China (No. 42000600003611). Twenty-four-hour-old rat pups were used for the preparation of primary microglia. All rats were kept under specific pathogen-free and climate-controlled conditions (temperature 23 ± 1 °C, relative humidity 60 ± 10%) with 12-h light/dark cycles, individually housed in polystyrene cages containing wood shavings and fed standard rodent chow and water *ad libitum*. All experiments were conducted with the approval of the Animal Care and Use Committee of Huazhong University of Science and Technology and were in accordance with the Guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### 2.2. Bone cancer pain model

Rat BCP models were established as *per* the previous report with minor modifications (Guan et al., 2015b,). Briefly, Walker 256 mammary carcinoma cells were inoculated into the abdominal cavity of adult female SD rats and extracted from the ascites after 7 days, washed with D-Hank's solution and centrifuged at 500*g* for 5 min at 4 °C (5 cycles), and then calibrated at a concentration of $4 \times 10^7$ cells/mL and maintained on ice until inoculation. Under anesthesia with pentobarbital sodium (40 mg/kg, ip), rats were laid in the supine position, and the right legs were shaved and disinfected with 7% iodine. A small incision was made parallel to the tibia in order to expose the plateau. A 23-gauge needle was inserted into the tibial medullary canal to make a pathway for injecting carcinoma cells, which was replaced with a 10 μL Hamilton syringe. A 10-μL volume containing approximately $4 \times 10^5$ Walker 256 mammary carcinoma cells was slowly injected in the BCP group, while an equivalent volume of D-Hank's solution was injected into the sham rats. The injection site was sealed with bone wax as soon as the syringe was retracted, and the skin sutured with 4/0 thread (SA83G, Johnson & Johnson Medical (China) Ltd., Shanghai, China). All rats were placed on a warm pad until recovery from anesthesia and then transferred into their individual cages.

### 2.3. Bone radiological and histological detection

To analyze tibia bone destruction by Walker 256 carcinoma cells, bone radiography and histological staining were performed. Rats were placed on a clear plane plexiglass and exposed to an X-ray source under sodium pentobarbital anesthesia for 10 s at 25 $kV_p$. Lateral radiographs of the right tibia were acquired using a digital radiographer system (DR-F, GE, Atlanta, GA, USA). Based on the radiological results, the right tibias were then collected from euthanized rats for bone histological investigation. The bones were post-fixed with 4% paraformaldehyde (PFA) for 72 h, decalcified in 10% ethylenediaminetetraacetic acid (EDTA, pH = 7.4) for 3 weeks, and decalcified in decalcifying solution for another 24 h. The tibias were washed, dehydrated, and embedded in paraffin, then cut into 5-μm sections and stained with hematoxylin and eosin to investigate carcinoma cell invasion and bone destruction.

### 2.4. Mechanical paw withdraw threshold test

As in previous reports (Dominguez et al., 2008; Guan et al., 2015b), mechanical allodynia was examined using the blind method. To avoid stress resulting from the test conditions, all rats were placed in a quiet test room for 5 d before basal measurements. Mechanical paw withdraw threshold (PWT) was measured using a series of calibrated von Frey filaments at 9:00 am. Rats were placed in plexiglass chambers with a wire net floor and were habituated for 30 min. A range of von Frey filaments (1-, 1.4-, 2-, 4-, 6-, 8-, 10-, and 15-g bending force; Stoelting, Wood Dale, IL, USA), starting with 1 g and ending with 15 g in ascending order, were applied to determine the mechanical PWT. The duration of each stimulus was maintained for approximately 1 s. Quick withdrawal or paw flinching was considered a positive response. Each monofilament was applied 5 times with a 30 s interval between applications, and the mechanical PWT was determined as the bending force of the filament for which at least 60% of the applications elicited a response.

### 2.5. Intrathecal catheter implantation, drug administration

Intrathecal cannula operation was performed as *per* previous reports. Briefly, rats were anesthetized with 2% isoflurane in 60% oxygen and intrathecally implanted with a polyethylene (PE)-10 catheter (inner diameter 0.3 mm, outer diameter 0.6 mm, PE-0503, Anilab Software & Instruments, Ningbo, China) through a gap in the vertebrae between L5 and L6 and extended to the subarachnoid space. Animals were allowed to recover for 3 days and then intrathecally injected with 1% lidocaine (10 μL) to confirm the catheter position. If any sign of nerve injury was observed, the rat was eliminated. The reagents minocycline (S4226), AG490 (S1143), Fludarabine (S1491), and U0126 (S1102) were purchased from Selleck Chemicals (Houston, U.S), and recombinant rat IFNγ (rrIFNγ) was obtained from PeproTech (400-20, Rocky Hill, NJ, USA). The dose we used here was based on previous reports (Dominguez et al., 2008; Guan et al., 2015a; Tsuda et al., 2009; Xu et al., 2015) and our preliminary results (Song et al., 2016). All reagents were freshly prepared in accordance with their respective instructions prior to each administration.

### 2.6. Lentivirus production and infection and spinal microinjection

Recombinant lentiviral vector expressing shRNA-CIITA to knockdown CIITA expression was obtained commercially from Genechem, Shanghai, China. The sense oligonucleotide for CIITA was 5′-TCAGGAGAGAAGCCTCAGA-3′, and the antisense was 5′-TCTGAGGCTTCTCTCCTGA-3′; the two oligos were separated by a loop and inserted downstream of the U6 promoter in the lentiviral vector GV248. Lentiviruses were acquired from triple-infected 293T cells with approximately 80% confluence. The lentiviral vector backbone was hU6-MCS-Ubiquitin-EGFP-IRES-puromycin; in contrast, the same vector backbone without the shRNAs carrying eGFP was used as a negative control lentivirus (NC-LV). The viral titer for the stock was $6.0 \times 10^8$ TU/mL.

Lentiviral vectors were microinjected into the lumber spinal cord as *per* previous reports with minor modifications (Ke et al., 2016, 2013). Briefly, the rat's spine was stabilized with two individual bars fixed around the L3 vertebra under deep isoflurane anesthesia (2%). Under an operation microscope (Yihua Optical Apparatus Co. Ltd., Zhenjiang, China), the laminectomy of the thoracic T13 vertebra was gently and precisely performed to disclose the right side of the lumbar spinal cord. The intact dura mater